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Acceleration of the Autooxidation of Human Oxyhemoglobin by Aniline and Its Relation to Hemoglobin-catalyzed Aniline Hydroxylation*

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Changes in the ultraviolet/visible spectrum of human oxyferrohemoglobin upon addition of aniline were indicative of a concentration-dependent interaction of aniline with hemoglobin, resulting in accelerated autooxidation of the hemoprotein. Oxygen was found to markedly inhibit this interaction of aniline with oxyhemoglobin. The dependence of the rate of autooxidation on aniline concentration followed saturation kinetics and showed a half-maximal response at 8 mM aniline. This value is equal to the value of K_m for aniline as substrate for the O_2-dependent, hemoglobin-catalyzed hydroxylation reaction which yields *p*-aminophenol (Mieyal, J. J., Ackerman, R. S., Blumer, J. L., and Freeman, L. S. (1976) *J. Biol. Chem.* **251,** 3436-3441). Thus, an aniline-oxyhemoglobin complex is implicated in the overall catalytic reaction. No detectable *p*-aminophenol was formed when aniline was combined with oxyhemoglobin in the absence of an electron donor, but hydroxylation of aniline does occur when NADPH, NADPH plus P-450 reductase, or $Na_2S_2O_4$ are also added.

Almost a century ago (2), the relationship was recognized between the ingestion or absorption of aniline by man and the conversion of the oxyhemoglobin of the blood to ferrihemoglobin (methemoglobinemia). Many common drugs are structurally related to aniline (*e.g.* phenacetin, acetaminophen, lidocaine) and also cause a mild degree of methemoglobinemia in most individuals. This effect may be more serious for certain diseased individuals whose blood oxygen capacity is already compromised. Many mechanisms have been proposed for the autooxidation of oxyferrohemoglobin and for the acceleration of this process *in vivo* and *in vitro* by drugs and other agents (3-6). In a recent comprehensive treatise on the subject, Kiese (6) concluded that aniline itself does not interact with oxyhemoglobin nor does it directly cause methemoglobin formation; rather the agents responsible are hydroxylated metabolites of aniline which are formed in the liver. However, on the basis of our previous studies (7, 8), we were led to suspect that aniline might interact directly with HbO_2.[1] We found that hemoglobin can act as a hydroxylase enzyme in converting aniline to *p*-aminophenol; the saturation kinetics that were observed reflected that aniline binds to some form of hemoglobin during the reaction (7). Since a catalytically significant complex with ferrihemoglobin was found to be an unlikely possibility (8), we sought evidence for aniline interaction with Hb^{2+} or HbO_2, or both.

In this article we report spectral evidence for the interaction of aniline with HbO_2 resulting in an accelerated rate of autooxidation of the hemoglobin. This process was saturable, with half-maximal response at 8 mM aniline, and it was inhibited by oxygen. These results are pertinent to delineating mechanisms both for the autooxidation of hemoglobin and for the hemoglobin-catalyzed hydroxylation reaction for which K_m was also found to be 8 mM aniline (7).

EXPERIMENTAL PROCEDURE

The experimental procedure including materials, preparative and purification procedures, assay methods, and specialized equipment is described in the miniprint supplement following this article.[2]

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[1] The abbreviations used are: HbO_2, human oxyferrohemoglobin; Hb^{2+}, deoxyferrohemoglobin; Hb^{3+}, ferrihemoglobin; P-450, cytochrome P-450, liver microsomal or adrenal mitochondrial; $P\text{-}450_{cam}$, camphor-induced cytochrome P-450 of *Pseudomonas putida;* $P\text{-}450^{3+}$, ferric form of P-450; $P\text{-}450^{2+}$, ferrous form of P-450; $P\text{-}450^{2+}\text{-}O_2$, oxyferro-P-450; MbO_2, sperm whale oxyferromyoglobin; Hb-CO, human carbon monoxyferrohemoglobin; 2,3-DPG, 2,3-disphosphoglycerate.

[2] Supplementary data and discussion for this article and the preceding article, besides being printed in reduced form following this article, are also available as JBC Document number 75M-1261 in the form of 12 pages of photocopy. Figures in the parent articles are designated 1, 2, etc.; figures in the supplement are 1S, 2S etc. Orders for supplementary material should specify the title, author(s) and

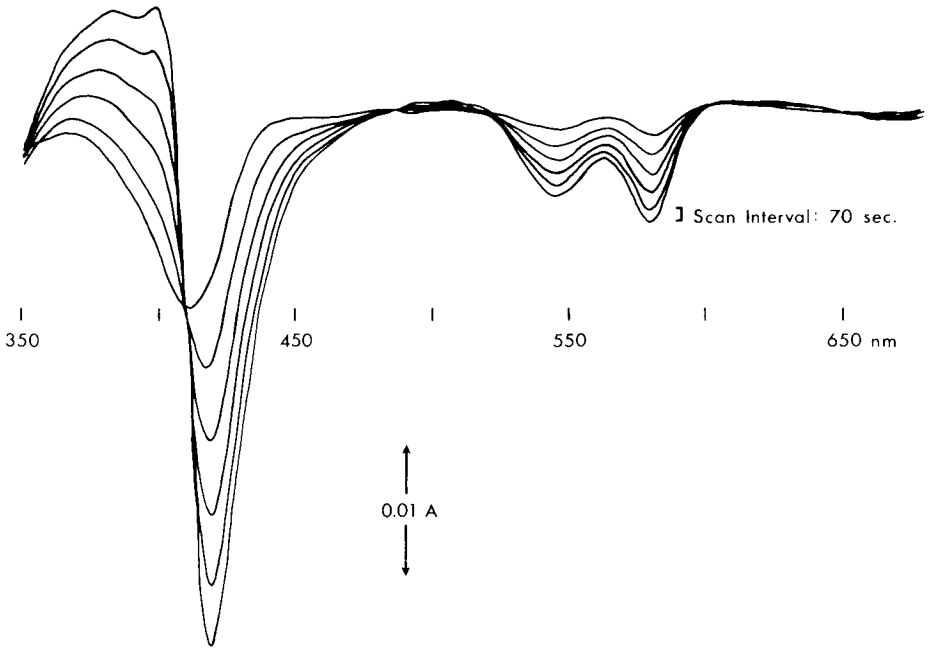

FIG. 1. Aniline interaction with oxyhemoglobin: time course of spectral changes. An Aminco DW-2 dual beam spectrophotometer operating at 38° in the split beam and baseline correction modes was employed for these experiments. Mixing cuvettes (Pyrocell) having two chambers of 4.5 mm path length were used as the sample and reference cuvettes. One milliliter of 2 μM HbO_2 in 20 mM potassium phosphate buffer, pH 6.8 was added to one chamber of both the sample and reference cuvette. Aniline solution (1 ml) of appropriate concentration (Fig. 2*A*), in this case 80 mM, was added to the other chamber of each cuvette. The cuvettes were placed in the light beam and a flat baseline was set after the solutions had equilibrated to 38°. The sample cuvette was inverted at zero time to allow mixing of the HbO_2 and aniline solutions, and it was replaced in the spectrophotometer.

RESULTS

Ultraviolet difference spectroscopic experiments in which aniline at various concentrations was mixed with Hb^{2+} (1 μM final concentration) under N_2 in an anaerobic mixing cuvette at 38° gave variable results. In each of several cases, little (if any) spectral change was detected with aniline concentrations as high as 150 mM. In other cases, large changes did occur, but neither the magnitude nor the rate of development of these changes were correlated with aniline concentration. Since such changes could be reproduced and magnified by momentarily opening the stopcock of the cuvette to introduce O_2, it was concluded that the spectral changes that did occur were most likely attributable to autooxidation of the Hb (see additional discussion in the supplement[2]). It follows either that aniline does not interact with Hb^{2+}, or that if an interaction occurs, it does not affect the heme moiety sufficiently to be manifested in a reproducible, concentration-dependent spectral change.

In contrast, the interactions of aniline with Hb^{3+} (8) and with HbO_2 (Fig. 1) were readily demonstrable. Fig. 1 shows the time-dependent spectral changes elicited when 40 mM aniline was combined with 1 μM HbO_2. Diminution in absorbance occurred at the wavelengths associated with the spectral maxima for HbO_2, while increases occurred at spectral regions associated with the maxima for Hb^{3+} (Fig. 1S[2]). Analogous spectral changes were observed when aniline was combined with MbO_2 or Hb-CO. In all cases, the initial rate of change of absorbance and the projected extent of change were related to the amount of aniline added. The relationship between initial rates and aniline concentration for the HbO_2 case is indicative of aniline binding (Fig. 2*A*). The double reciprocal plot (Fig. 2*B*) yields an interaction constant for aniline, K = 8 mM. This value is identical with K_m for aniline determined from the kinetics of the hemoglobin-catalyzed formation of *p*-aminophenol (7).

The development of the aniline-induced spectral changes was inhibited if the oxygen concentration of the solution was increased, but enhanced if the oxygen content was diminished by flushing with N_2 (Fig. 3). Oxygen appears to be an effective inhibitor of the interaction of aniline with HbO_2, and the inhibition pattern approaches a competitive relationship.

DISCUSSION

On the Nature of Interaction of Aniline with HbO_2—Several different interpretations of the data are conceivable. The mechanism we favor is discussed below; the other possibilities (*a* to *c*), listed here as unlikely, are discussed in the accompanying supplement[2] under the same heading. It seems unlikely (*a*) that aniline acts as a nucleophile to directly displace $O_2^{\cdot -}$ from ($Hb^{3+}-O_2^{\cdot -} \rightleftarrows Hb^{2+}-O_2$); (*b*) that aniline directly donates an electron to bound O_2, thereby accelerating donation of a second electron by the ferrous heme iron atom to generate Hb^{3+} and peroxide; or (*c*) that aniline interacts directly with the ferrous heme iron atom, either displacing O_2 directly or drawing the equilibrium toward aniline-Hb^{2+}. Possibility *c* was rendered unlikely by our inability to demonstrate a consistent concentration-dependent effect of aniline on the spectrum of Hb^{2+} (see above).

It seems most likely that aniline may interact at a site other than the heme iron atom with HbO_2 or Hb^{2+} or both, thus diminishing the affinity of hemoglobin for O_2 and concomitantly increasing its susceptibility to autooxidation. As cited earlier, Kiese (6) has stated that aniline does not accelerate autooxidation of HbO_2 directly; rather hydroxylated metabolites such as phenylhydroxylamine and *p*-aminophenol are responsible for the *in vivo* process. It is clear that the latter agents are more potent than aniline (*i.e.* at <1 mM concentration they can effect complete oxidation of >25 μM HbO_2 (6)), but as electron donors they probably operate via a different mechanism than what we propose for aniline. In contrast to the hydroxylated metabolites, aniline apparently does not cause complete conversion of the HbO_2; rather, the time courses and extrapolated extents of the spectral changes induced by aniline were indicative of the establishment of a new equilibrium in

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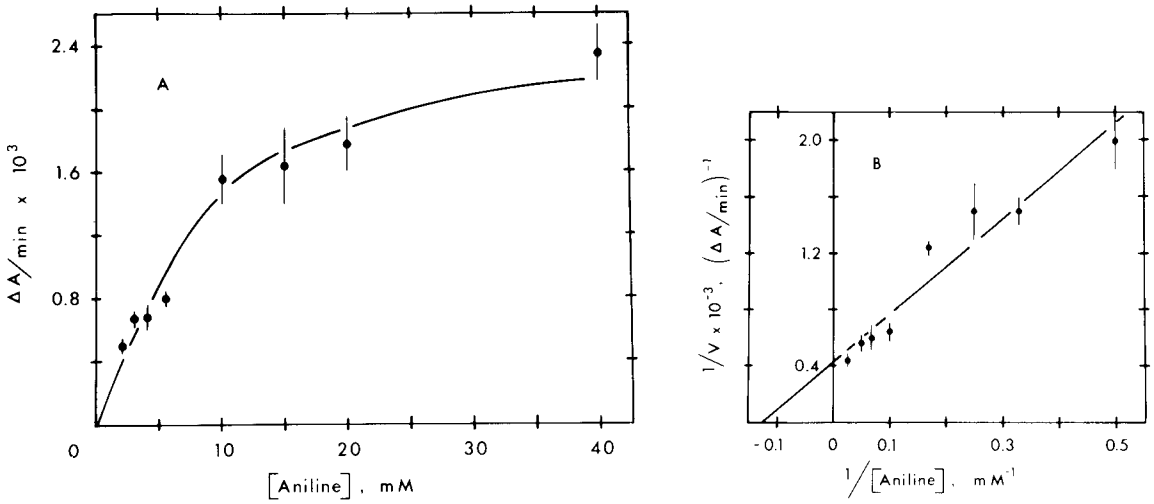

FIG. 2. Dependence of rate of oxyhemoglobin spectral change on aniline concentration. *A*, experiments were set up as described under Fig. 1, except that the aniline concentration was varied as indicated. The absorbance difference ($A_{400\ nm} - A_{420\ nm}$, Fig. 2) was measured at various times, and then plotted as a function of time. The rate of change of absorbance (*i.e.* the tangent to the initial linear portion (~20 min) of each such curve) was determined for each aniline concentration; these rates were then replotted as a function of aniline concentration. *B*, double reciprocal replot of rate of spectral change *versus* aniline concentration. Data are from *A*.

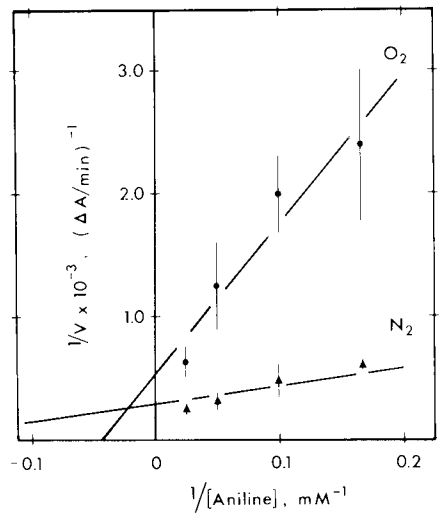

FIG. 3. Inhibition of aniline-induced spectral changes. Experiments analogous to those described under Figs. 1 and 2*A* were carried out, except that special mixing cuvettes (see supplement for details[2]) were used which had been fitted with extended hollow plugs and ground glass stopcocks at the top. This allowed the atmosphere above the HbO_2 and aniline solutions to be replaced by either N_2 or O_2 where appropriate, before mixing.

each case, instead of complete displacement of oxygen and oxidation of hemoglobin.

It is difficult to predict how many different forms of substituted hemoglobin may exist at this new dynamic equilibrium,[3] but the ability of hemoglobin to catalyze transfer of activated oxygen to aniline would favor the existence of some form(s) of hemoglobin to which both aniline and O_2 were bound simultaneously (see below). This model for interaction of aniline with oxyferrohemoglobin is analogous to the case for 2,3-disphosphoglycerate interaction with hemoglobin (9). 2,3-DPG does not bind to the heme iron atoms, but its binding lowers the affinity of hemoglobin for oxygen and concomitantly accelerates autooxidation (10). We have shown that addition of as little as 1 mM 2,3-DPG to 1 μM HbO_2 causes spectral changes identical to Fig. 1. It has been concluded that 2,3-DPG binds more tightly to Hb^{2+} than to HbO_2 and thus competes with O_2 by stabilizing the form of hemoglobin for which O_2 has lower affinity (9). A strict analogy need not be drawn for aniline, since the condition for aniline-O_2 competition (Fig. 3) would be satisfied by any conformational equilibrium between forms of hemoglobin discriminatory to O_2 binding which is induced by the presence of aniline.

A case perhaps closely related to aniline was the observation that the drug propranolol causes a decrease in the oxygen affinity of blood in which it is contained (11, 12). The authors concluded that propranolol alters the permeability of the red cell membrane with a concomitant increase of the H^+ activity inside; thus the decreased oxygen affinity was attributed to the Bohr effect. In contrast, we have found that propranolol interacts directly with HbO_2 in a manner analogous to the effect of aniline, (*i.e.* spectral change identical to Fig. 1), and therefore may elicit part of its effect on hemoglobin oxygen affinity via this mechanism. If aniline and related drugs produce methemoglobinemia directly via this mechanism (in addition to other indirect routes (6)), then the amount of methemoglobin formed may be a minor consequence compared to the correlated decrease in oxygen affinity of the ferrohemoglobin.

Relationship to Enzymic Hydroxylation Mechanisms—It has been reported that aniline causes displacement of CO from liver microsomal cytochrome P-450^{2+}-CO, and this was interpreted as a direct interaction of aniline with the heme Fe^{2+}. As a result, the analogy was drawn that aniline might also directly displace O_2 from P-450^{2+}-O_2, and this was judged to be abortive with regard to catalysis of *p*-aminophenol formation (14). If the competitive nature of the interaction of $CO(O_2)$ and aniline with P-450 were similar to that proposed for Hb, there

[3] For example, it is conceivable that the following forms might be present to some extent: Hb^{3+}, AN-Hb^{3+}, $Hb^{2+}(O_2)_4$, AN~Hb^{2+}~$(O_2)_4$, AN~Hb^{2+}~$(O_2)_{0-3}$, AN~Hb^{2+}, AN~Hb~$(Fe^{2+} - O_2)_{0-4}$ $(Fe^{3+})_{0-4}$, etc.; also, more than 1 mol of AN might be bound. Whether or not aniline binds to Hb^{2+} at all in the absence of oxygen, as well as the number of molecules bound, could be determined subsequently from a direct study such as equilibrium dialysis using radioactive aniline.

would not be a problem with catalysis. We found that whereas oxygen inhibits aniline-accelerated autoxidation of hemoglobin, it enhances the overall rate of Hb-catalyzed hydroxylation of aniline (7). This relationship may indicate that in the overall catalytic system, addition of aniline and O_2 to Hb^{2+} results in a ternary $AN{\sim}Hb^{2+}{\sim}(O_2)_{1\text{-}4}$ complex(es). This possibility could be tested via a detailed two-substrate kinetic study. However, since we were unable to demonstrate aniline-Hb^{2+} complex formation at present, we have proposed the reaction scheme in an order fashion: $Hb^{2+} + O_2 \rightarrow HbO_2 \xrightarrow{AN} AN\text{-}Hb{\sim}O_2$, where the ~ represents an altered form of O_2 binding to Hb (7). According to the Franck-Condon principle, the more closely related the geometry before and after electron transfer, the faster the transfer will occur (15). This principle may explain how aniline may convert the inert oxygen carrier HbO_2 to an oxygen-activating enzyme. Thus, aniline binding to HbO_2 may sufficiently distort the $Hb{\sim}O_2$ bond so that total electron transfer from Fe^{2+} to O_2 would be facilitated. The above order of addition might also pertain to the *in vivo* situation, where hemoglobin is maintained as HbO_2 (16). By analogy we speculate that cytochrome P-450 of liver might also be maintained in the $P\text{-}450^{2+}\text{-}O_2$ state *in vivo*. Thus, a drug entering the liver via the portal circulation might convert an inert form of $P\text{-}450\text{-}O_2$ to an activated substrate-$P\text{-}450{\sim}O_2$ complex which would generate the hydroxylated metabolite. It has been shown both for $P\text{-}450_{cam}$ (17) and for adrenal mitochondrial P-450 (18) that ternary substrate-$P\text{-}450\text{-}O_2$ complexes are formed which do not yield products unless the specific electron donor (putidaredoxin or adrenodoxin, respectively) is added. Analogously, combination of aniline with HbO_2 does not yield *p*-aminophenol unless NADPH, NADPH + P-450 reductase, or dithionite are also added to supply an electron (7).

The remarkable similarities between various cytochrome P-450 oxygenase systems and the catalytic system utilizing hemoglobin as the enzyme indicate that a careful examination of the extent to which such reactions may be catalyzed by the hemoglobin system *in vivo* is warranted.

REFERENCES

1. Mieyal, J. J., Ackerman, R. S., Blumer, J. L., and Wilson, L. S. (1975) *Pharmacologist* **17,** 230
2. Muller, F. (1887) *Dtsch. Med. Wochenschr.* **13,** 27–28
3. Kikuchi, G., Shukuya, R., Suzuki, M., and Nakamura, C. (1955) *J. Biochem. (Japan)* **42,** 267–284
4. Misra, H. P., and Fridovich, I. (1972) *J. Biol. Chem.* **247,** 6960–6962
5. Wallace, W. J., and Caughey, W. S. (1975) *Biochem. Biophys. Res. Commun.* **62,** 561–567
6. Kiese, M. (1974) *Methemoglobinemia: A Comprehensive Treatise,* CRC Press, Inc., Cleveland
7. Mieyal, J. J., Ackerman, R. S., Blumer, J. L., and Freeman, L. S., (1976) *J. Biol. Chem.* **251,** 3436–3441
8. Mieyal, J. J., and Freeman, L. S. (1976) *Biochem. Biophys. Res. Commun.* **69,** 143–148
9. Kilmartin, J. V., and Rossi-Bernardi, L. (1973) *Physiol. Rev.* **53,** 836–890
10. Mansouri, A., and Winterhalter, K. H. (1974) *Biochemistry* **13,** 3311–3314
11. Agostoni, A., Berfasconi, C., Gerli, G. C., Luzzana, M., and Rossi-Bernardi, L. (1973) *Science* **182,** 300–301
12. Lichtman, M. A., Cohen, J., Murphy, M. S., Kearney, E. A., and Whitbeck, A. A. (1974) *Circulation* **49,** 881–886
13. Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967) *Mol. Pharmacol.* **3,** 113–123
14. Mannering, G. J. (1971) in *Drugs and Cell Regulation*, pp. 197–225, Academic Press, New York
15. Hughes, M. N. (1975) *The Inorganic Chemistry of Biological Processes*, pp. 52, 142, John Wiley and Sons, New York
16. Hultquist, D. E., and Passon, P. G. (1971) *Nature New Biol.* **229,** 252–254
17. Gunsalus, I. C., Tyson, C. A., and Lipscomb, J. D. (1973), in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., eds) Vol. 2, pp. 583–603, University Park Press, Baltimore
18. Schleyer, H., Cooper, D. Y., and Rosenthal, O. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., eds) Vol. 2, pp. 469–491, University Park Press, Baltimore

Supplementary Material

to

Characterization of Enzyme-like Activity of Human Hemoglobin
I. Properties of a Hemoglobin: P450 Reductase Coupled Aniline Hydroxylase System

John J. Mieyal, Rollie S. Ackerman, Jeffrey L. Blumer and Linda S. Freeman

and to

Acceleration of Autoxidation of Human Oxyferrohemoglobin by Aniline and Its Relation to Hemoglobin-Catalyzed Aniline Hydroxylation

John J. Mieyal and Jeffrey L. Blumer

Preface

This is a supplement to both of the adjacent articles (1,2). Details of the preparation of the various forms of hemoglobin (or myoglobin), the preparation of solubilized cytochrome P450 reductase, and the determination of optimal conditions for hemoglobin: P450 reductase coupled hydroxylation of aniline are described in this addendum along with the various assay and identification procedures. The reference numbers cited refer to a list at the end of this addendum. Figures in this supplement are identified by "S" (e.g. Fig. 1S); figures in the preceding and the following full-sized texts are not succeeded by a letter (e.g. Fig. 1).

Materials

NADPH, dithiothreitol, trishydroxymethylaminomethane (Trizma Base), sodium cholate, crystalline bovine serum albumin, catalase, horse heart ferricytochrome c, human hemoglobin, bovine hemoglobin and sperm whale myoglobin were obtained from Sigma Chemical Company. The latter three hemoproteins were all purchased as 2x recrystallized, lyophilized products, and they were converted to either the pure ferric or oxyferrous forms as described below. Sodium deoxycholate was obtained from Schwartz Mann. DEAE cellulose was purchased from Reeve Angel, Inc. or from Sigma; fines were removed and it was cycled through acid and base before equilibration with the appropriate buffer. Sephadex G-25 was obtained from Pharmacia Fine Chemicals Co. Dilaurylphosphatidylcholine was purchased from Calbiochem. Bovine erythrocyte superoxide dismutase (erythrocuprein) was purchased from Miles Laboratories or from Sigma. d-Benzphetamine was kindly donated by Drs. Donald DuCharme and Paul O'Connell of the Upjohn Co. Aniline, potassium phosphate, potassium ferricyanide, sodium dithionite, glycerol, trichloroacetic acid (20%) and other common chemicals were reagent grade.

Preparation of Purified Forms of the Hemoproteins

A. Ferric Hemoproteins: Each crystalline hemoprotein (human hemoglobin, bovine hemoglobin or sperm whale myoglobin) was dissolved in dilute K phosphate buffer (20 mM, pH 6.8) in the cold to a concentration ~0.2 to 0.5 mM. The solution was purged with N_2 before a 3–5 fold excess of $K_3Fe(CN)_6$ was added to ensure total conversion of the hemoprotein to the ferric form. The solution was stirred (under N_2, 4°) overnight before chromatographing it twice on Sephadex G-25 (each column volume ≥ 5x solution volume) in order to remove excess oxidant and other small molecules. The purified ferrihemoproteins were examined by UV/visible spectroscopy (e.g. see Fig. 1S). Solutions of the ferrihemoproteins were stored in the refrigerator. The concentrations of these solutions were assayed as described below under Assays.

B. Deoxy-, Carbon monoxy- and Oxyferroushemoproteins: Either crystalline hemoglobin or myoglobin was dissolved to a concentration ~0.2 to 0.5 mM in K phosphate buffer, 20 mM, pH 6.8, in the cold. While the solution was being bubbled with N_2, an excess of $Na_2S_2O_4$ was added to effect rapid and complete reduction to the ferrous form (this was confirmed by UV/visible spectroscopy (see Figure 1S)). At this stage, the solution of ferrohemoprotein containing excess $Na_2S_2O_4$ was further treated in one of two ways to obtain either purified, liganded (O_2 or CO) ferrohemoprotein in the absence of $Na_2S_2O_4$. 1. Carbon monoxy- or oxyferrohemoproteins: the ferrohemoprotein was converted to the carbon monoxyferrous or oxyferrous form by bubbling pure CO or pure O_2 into the solution. The resulting liganded ferrohemoprotein was then separated from excess reductant and stripped of other small molecules by chromatographing it twice on Sephadex G-25 (columns equilibrated and eluted with 20 mM K phosphate, pH 6.8; column volume ≥ 5x solution volume). 2. Deoxyferrohemoprotein: an aliquot of the solution of deoxyferrohemoprotein was diluted with 20 mM K phosphate buffer to a final concentration of ~8 mM with respect to heme in a total volume ≤ 10 ml. This solution was transferred to a dialysis bag containing an oxygen-scavenging system: 1 ml containing glucose (0.1 M), glucose oxidase (10 μg) and catalase (25 μg). The bag was closed and placed into a dialysis medium (2L) containing 20 mM K phosphate buffer and 0.1 M glucose. The medium was in a large filter flask fitted with a one-hole stopper through which a bubbler was placed and immersed in the dialysis medium. The medium was bubbled with N_2 (scrubbed by passing through a solution of chromous chloride in dilute HCl over amalgamated zinc and then through dilute NaOH) for several hours before the dialysis bag was transferred to it. The bubbling was continued throughout the dialysis (overnight) and the medium was stirred magnetically. The entire apparatus was enclosed in a plastic glove bag which was purged, and then inflated with N_2 either when the dialysis bag was transferred to fresh dialysis fluid or when it was punctured in order to transfer the contents to a Thunberg tube containing 1 ml of the oxygen-scavenging system described above. The transfer to the Thunberg tube was made after 2x 2L dialysis. Aliquots from the Thunberg tube were transferred under nitrogen to one side of each of two specially fabricated anaerobic mixing cuvettes for each experiment for studies of possible binding of aniline to deoxyferrohemoglobin (see following article (2)), aliquots of a prebubbled solution of aniline (also containing the O_2 scavenging system) were transferred to the other side of the cuvette. The cuvettes were closed under N_2, opened under high vacuum and repurged with N_2 before UV difference spectroscopic experiments were performed. The high affinity of "stripped" (i.e. no bound organic phosphates) deoxyferrohemoglobin for O_2 made it quite difficult to prepare it free of bound O_2 in the absence of dithionite. Any bound O_2, however, was readily detectable in spectra of samples of deoxyferrohemoglobin. Thus, the Soret band was broadened and shifted toward the blue; the 555 nm band was either broadened or developed a distinct shoulder at 577 nm depending upon the amount of O_2 bound (see Fig. 1S). It was found necessary to remove all of the $Na_2S_2O_4$ before conducting any experiments to test aniline binding, because even a 2-fold excess of dithionite over the hemoglobin concentration apparently caused degradation of the heme. This was observed as an artifactural difference spectrum (i.e. uniform diminution of absorbance throughout the spectrum) upon mixing the sample cuvette. This result also indicated that even after evacuation of the cuvette (vacuum pump) and repurging with N_2, traces of O_2 were still existent in the atmosphere above the solutions. Variation in the amount of O_2 in that atmosphere caused variable results in the tests for interaction of aniline with deoxyferrohemoglobin (see adjacent article (2)), even when these were performed with the oxygen scavenging system (described above) in the absence of dithionite. Anaerobic mixing cuvettes were made by joining a 10/30 female Vycor glass joint to the top of each of two quartz Pyrocell mixing cuvettes. These could then be stoppered with a hollow male ground glass extension which had been fitted with a ground glass stopcock. The mixing cuvettes themselves had two chambers with a path length of 4.5 mm. The UV/visible spectra of typical preparations of human ferrihemoglobin, deoxyferrohemoglobin and oxyferrohemoglobin which were used in subsequent experiments are shown in Figure 1S. The spectral maxima and extinction coefficients agree with those which are considered standard (3).

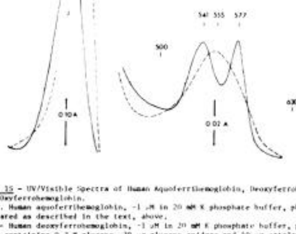

Fig. 1S – UV/Visible Spectra of Human Aquoferrihemoglobin, Deoxyferrohemoglobin and Oxyferrohemoglobin.
..... Human aquoferrihemoglobin, ~1 μM in 20 mM K phosphate buffer, pH 6.8, 38°; prepared as described in the text, above.
– – – Human deoxyferrohemoglobin, ~1 μM in 20 mM K phosphate buffer, pH 6.8, 38°; also containing 0.2 M glucose, 20 μg glucose oxidase and 50 μg catalase; prepared as described in the text, above.
——— Human oxyferrohemoglobin, ~1 μM in 20 mM K phosphate buffer, pH 6.8, 38°; prepared as described in the text, above.

PREPARATION OF MICROSOMES AND P450 COMPONENTS

Male Holtzman rats (80-100 g) were injected intraperitoneally with 75 mg/kg doses of phenobarbital for 3 consecutive days and then starved for 18 hours prior to decapitation and removal of the livers. The following operations were conducted at 4°. The liver homogenate (3 ml of 50 mM Tris HCl, pH 7.7, 1.15% KCl per g wet weight of liver: Waring blender, full speed, 1 min) was centrifuged for 25 min at 10,000 x g; the supernatant was recentrifuged for 25 min at 10,000 x g, and the second supernatant was filtered through cheese cloth then centrifuged at 105,000 x g for 90 min. The microsomal pellets so obtained were resuspended in the same Tris-KCl buffer and recentrifuged for 90 min at 105,000 x g. The microsomes were finally suspended in 0.25 M sucrose at a protein concentration ~50 mg/ml. Aliquots of the microsomal suspension were purged with N_2, frozen, then stored at -20° for use in the preparations described below.
Cytochrome P450 Reductase: Solubilized, partially purified cytochrome P450 reductase from rat liver was prepared from acetone-extracted, sodium deoxycholate-treated microsomes, essentially according to the procedure of Lu et al. (4). The resultant reductase preparation had a specific activity with respect to NADPH-dependent reduction of cytochrome c of ~0.5 μmoles/min/mg (i.e. ~0.5 units/mg) and represented an ~5-fold purification of this activity relative to the microsomes. The ratio of cytochrome c reductase activity to P450 content was increased ~20-fold. All of the cytochrome P450 originally in the microsomes was converted to cytochrome P420 as a result of the purification procedure, and the specific content of P420 (nmoles/mg) was diminished by 75% in the final reductase solution relative to its specific content in the microsomes. The reductase alone could not support hydroxylation of aniline (see Table I of the preceding article (1) and Figure 6S, below). The activity of this enzyme preparation as a cytochrome P450 reductase was confirmed in a reconstituted P450 system, where it was shown to support the demethylation of benzphetamine. The system in 1 ml consisted of solubilized cytochrome P450 (.1 nmole), NADPH (1 mM) dilaurylphosphatidylcholine (50 μg) (lipid component required for full activity (5)), benzphetamine (1 mM) and the P450 reductase. The rate of demethylation of benzphetamine was determined both by monitoring oxidation of NADPH (decrease in $A_{340\ nm}$, ε = 6.22 $mM^{-1}cm^{-1}$) and formation of formaldehyde (quantitated as dihydrolutidine, λmax = 412 nm, ΔA = 3.5 for 1 mM HCHO) by the procedure developed by Nash, using 2x Nash reagent (6,7). The specific activity of the reductase preparation expressed as the benzphetamine-dependent oxidation of NADPH was ~11 nmoles/min/mg of reductase. Aliquots of the reductase enzyme solution were transferred to small tubes, purged with N_2, frozen and stored at -75°.
Cytochrome P450: Solubilized, partially purified cytochrome P450 from rat liver was prepared by a stepwise ammonium sulfate extraction of sodium cholate-treated microsomes, essentially according to the procedure of Autor et al. (8). The resultant cytochrome P450 preparation (~2.3 nmoles/mg protein) was free of cytochrome P420 and represented an ~3-fold increase in specific P450 content relative to that of the microsomes. Aliquots of the solution of P450 were purged with N_2, frozen and stored at -75°. The only use of the solubilized P450 in this study was to document the activity of the cytochrome P450 reductase, as described above.

ASSAYS

All protein determinations were carried out according to a procedure adapted from that of Lowry (9), using bovine serum albumin as the standard. Cytochrome P450 content of microsomal suspensions and of solutions of solubilized P450 components was measured by UV difference spectroscopy according to the method of Omura and Sato (10), using an Aminco DW-2 spectrophotometer operating in the split beam mode. The difference in absorbance between 450 nm and 490 nm for spectra of $P450^{2+}$-CO was used to quantitate the P450 content, ε = 91 $mM^{-1}cm^{-1}$. Cytochrome P420 (the inactivated form of P450) was quantitated in an analogous manner, ε$(A_{420} - A_{490})$ = 111 $mM^{-1}cm^{-1}$.

Cytochrome c reductase activity was measured at 30° by observing the rate of change of absorbance at 550 nm (λmax of Cytochrome c^{2+}, ε = 21.4 $mM^{-1}cm^{-1}$), resulting from the addition of aliquots of microsomal suspension or solutions of cytochrome P450 reductase to solution of 1 ml total volume which contained 0.16 ml of 0.5 mM horse heart cytochrome c^{3+}, 0.02 ml of 10 mM NADPH and sufficient volume of 50 mM K phosphate, pH 7.7, 0.2 mM EDTA to 1 ml. $\Delta A_{550\ nm}$ was monitored at regular time intervals with a Beckman DU-Gilford recording spectrophotometer.

The concentration of hemoglobin in solutions of ferri-, carbon monoxyferro- or oxyferrohemoglobin was determined according to the assay of Van Kampen and Zijlstra (11), whereby the hemoglobin is converted to the cyanoferric form by the action of $K_3Fe(CN)_6$ and KCN and the absorbance is measured at 541 nm, ε per heme = 11 $mM^{-1}cm^{-1}$ (internationally accepted value) (i.e. 44/hemoglobin tetramer). The concentrations of myoglobin solutions were estimated analogously, ignoring the small difference in extinction coefficient.

The concentration of p-aminophenol formed as a result of aniline hydroxylation was quantitiated according to a modification of the procedure reported by Brodie and Axelrod (12), whereby p-aminophenol is converted to the indophenol derivative which absorbs at 630 nm. Each reaction was terminated by adding 0.3 ml of 20% trichloracetic acid to the 1 ml reaction mixture containing p-aminophenol. The resultant suspension was centrifuged to remove precipitated protein and 1 ml of the supernatant was transferred and combined with 0.1 ml of 5% phenol in 2.5 N NaOH (w/v); after mixing, 0.2 ml of 2.5 N Na_2CO_3 was added. The resultant mixture was allowed to stand at room temperature for at least 30 min. One nmole of p-aminophenol in the original 1 ml of reaction mixture gives rise to a $\Delta A_{630\ nm}$ value of 0.015. This method has been shown to be specific for p-aminophenol, i.e. o- and m-aminophenol do not give rise to the same blue color as does p-aminophenol (13); we confirmed those observations and also showed that o,p-dihydroxyaniline does not react like p-aminophenol. In addition, p-aminophenol was confirmed as the predominant product in our reaction mixtures by combination gas chromatographic/mass spectroscopic analysis. Thus, putative p-aminophenol extracted from the reaction mixture into benzene:$CHCl_3$ (4:1) and converted to the methylated derivative gave the same retention time upon gas chromatography on chromasorb (SE30 - OV17, 6:1), column temperature 88°, and gave an identical mass spectrum to authentic p-aminophenol treated in the same way. We thank Dr. John M. Strong of the Clinical Pharmacology Unit, Northwestern University Medical School, for the analyses and the interpretation of the mass spectra.

OPTIMAL CONDITIONS FOR HEMOGLOBIN: P450 REDUCTASE COUPLED HYDROXYLATION OF ANILINE

As with any characterization of optimal conditions for an enzymic system, the process is iterative and requires reconfirmation of the optimum for each parameter after the optima for other parameters have been tested until an overall optimum is established. The following results therefore illustrate the final specific optima under conditions which were maximal for all other variable parameters, and the sequence of presentation is arbitrary. Since reactivity was observed in the absence of the reductase enzyme, most experiments were set up as two series, i.e. in the presence and absence of reductase (see for example Fig. 4S); but the data are generally presented for the complete system (except Fig. 6S). The complete catalytic system for formation of p-aminophenol consisted of human hemoglobin added to the reaction mixture in the purified oxidized state, i.e. ferrihemoglobin, vide infra (except for Fig. 3S), NADPH added in excess, solubilized liver microsomal cytochrome P450 reductase, aniline and atmospheric O_2 (see Table I, adjacent article (1)). p-Aminophenol was confirmed as the predominant product as described above. Fig. 2S shows that the initial rate of aniline hydroxylation by this system is linear for at least 30 minutes at 38°.

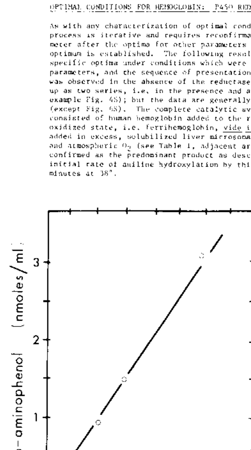

Fig. 2S - Linearity of p-Aminophenol Formation with respect to Time (Ferrihemoglobin added)
Reaction mixtures were 1 ml total volume and contained the following at the concentrations or quantities indicated: 1 mM NADPH, 1 μM human aquo-ferrihemoglobin, 12 μg (.006 units) P450 reductase, 40 mM aniline and 20 mM K phosphate buffer, pH 6.8. The mixtures were prepared and preincubated for 2-3 minutes at 38° before adding NADPH to initiate the reaction. The mixtures were then incubated at 38° for the period indicated. At the appropriate time, 0.3 ml of 20% trichloroacetic acid was added to terminate each reaction. Zero-time controls were prepared by adding the trichloroacetic acid before the NADPH. The amounts of p-aminophenol formed in each case was assayed as described under Assays. The individual points on the graph represent the average of duplicate determinations. This particular graph represents a single experiment performed in duplicate, but the linearity of the reaction with respect to time was re-confirmed frequently.

The temperature of 38° was chosen to be compatible with the physiological state. The rate of the reaction is also directly proportional to temperature and measurements over the range 30° - 40° yielded a typical Q_{10} value of 1.9. For all other experiments with this system, a 15 minute reaction period was employed to ensure measurements of initial rates. Hemoglobin was added as ferrihemoglobin for most of the experiments, because that is the initial form of cytochrome P450 (ferricytochrome P450) which is added to a complete cytochrome P450 system which catalyzes aniline hydroxylation (14), and we sought to compare the systems directly (see Table II of the adjacent article (1)). However, Fig. 3S confirms that hemoglobin, added initially as oxyferrohemoglobin, will also give rise to a linear rate of p-aminophenol formation (Note: the [aniline] was 40 mM for Fig. 2S, 20 mM for Fig. 3S) (see Discussion of the preceding articles (1,2)).

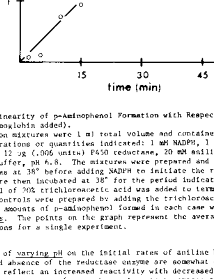

Fig. 3S - Linearity of p-Aminophenol Formation with Respect to Time (Oxyferrohemoglobin added).
Reaction mixtures were 1 ml total volume and contained the following at the concentrations or quantities indicated: 1 mM NADPH, 1 μM human oxyferrohemoglobin, 12 μg (.006 units) P450 reductase, 20 mM aniline and 20 mM K phosphate buffer, pH 6.8. The mixtures were prepared and preincubated for 2 - 3 minutes at 38° before adding NADPH to initiate the reaction. The mixtures were then incubated at 38° for the period indicated. At the appropriate time, 0.3 ml of 20% trichloroacetic acid was added to terminate each reaction. Zero time controls were prepared by adding the trichloroacetic acid before the NADPH. The amounts of p-aminophenol formed in each case was assayed as described under Assays. The points on the graph represent the average of duplicate determinations for a single experiment.

The effects of varying pH on the initial rates of aniline hydroxylation in the presence and absence of the reductase enzyme are somewhat different (Fig. 4S). Both curves reflect an increased reactivity with decreased pH. These results suggest that proton transfer may be an important component of the overall reaction scheme (see Discussion, adjacent article (1)). All subsequent reaction mixtures were buffered at pH 6.8, since this value represented the point at which the effect of the reductase was maximal. It is noted that the activity at pH 7.4 is still approximately 75% of that at pH 6.8.

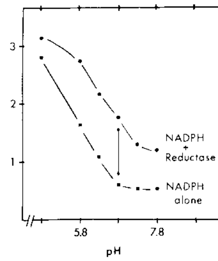

Fig. 4S - Dependence of the Initial Rate of Aniline Hydroxylation on pH.
Two series of reaction mixtures were set up as described under Fig. 2S, except for the following: For one series the reductase enzyme was omitted; and for both series the pH of the buffer was varied as shown, and the incubation period was 15 min. At the low end of the pH range, Na acetate buffer was tested as well as K phosphate. At the high end, Tris HCl was tested in addition to the K phosphate. Changes in either buffer composition or concentration (up to 0.1 M tested) caused little (if any) deviation from the results shown. Each point on the figure represents the average of at least two determinations.

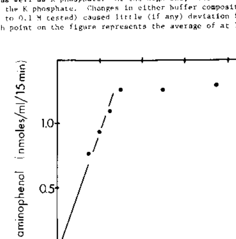

Fig. 5S - Dependence of Rate of Aniline Hydroxylation on Reductase Concentration.
Two series of reaction mixtures were set up as described under Fig. 2S, except for the following: for one series the reductase enzyme was omitted; for the other, the reductase concentration was varied as shown. The incubation period after addition of NADPH was 15 min. in all cases. The data plotted represent the difference between the amount of p-aminophenol formed in the presence of reductase minus p-aminophenol formed in the absence of reductase. Each point on the graph represents the average of at least two determinations.

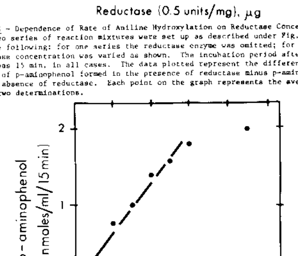

Fig. 6S - Dependence of Rate of Aniline Hydroxylation on Hemoglobin Concentration.
Reaction mixtures were set up as described under Fig. 2S, except that the concentration of hemoglobin was varied as shown, and the incubation period was 15 min. Each point on the graph represents the average of at least two determinations.

Fig. 5S illustrates that there is a linear dependence of the reaction velocity on reductase concentration up to ~ 10 μg/ml (0.005 units). Beyond this concentration there is no further effect; consequently, ~ 10 μg reductase was routinely employed to provide maximal response. A single preparation of reductase was used for all the experiments reported in these articles, but in separate experiments we confirmed that other preparations using either the procedure described above or the one described more recently (15) also support hemoglobin-catalyzed hydroxylation of aniline. Fig. 6S shows that the rate of aniline hydroxylation is linearly dependent on ferrihemoglobin concentration. The same linear concentration range is observed (i.e. 0 - ~ 1.2 nmoles Hb/ml) whether or not the reductase enzyme is present; in both cases, the line through the individual data points extrapolates to the origin (see also Table I, adjacent article (1)). A similar linear range of hemoenzyme concentration is generally observed for P450-catalyzed reactions.

Optimal conditions for catalysis of p-aminophenol formation by bovine hemoglobin and sperm whale myoglobin were determined in an analogous fashion. (Comparison of the various systems is given in Table II, adjacent article (1)).

DISCUSSION

Mechanism of the NADPH-mediated Reaction: The donation of electrons at steps 2 and 5 of Fig. 4 of the adjacent article (1) is indicated by the general symbol e^- rather than by a specific reducing agent(s), because the differential susceptibility to inhibitors suggested that the NADPH-mediated reaction and the reductase-mediated reaction might be different in this regard (Figures 2A,B; Table III (1)). The finding that NADPH works at all in the absence of the reductase enzyme indicates that it can directly reduce methemoglobin . It is known that NADPH reduces ferricyanide (16), and it has been reported that it reduces methemoglobin (17). We have confirmed the latter observation for our conditions and preparations of hemoglobin, and have shown that reduction of methemoglobin is enhanced by the reductase enzyme in the quantities used for the catalytic system. Although the question of $1e^-$ or $2e^-$ transfer by NADPH has not yet been resolved for its participation in dehydrogenase reactions (16), it is generally believed that direct hydride ($2e^-$) transfer is involved. It would seem likely, however, that reduction of 1e acceptors such as methemoglobin or metmyoglobin should occur via $1e^-$ transfer from NADPH. This necessarily means, then, that unstable free radicals must be formed in these systems, e.g. $NADPH^{\cdot}$, $O_2^{\cdot-}$, $\cdot OH$, etc. (It is known that $O_2^{\cdot-}$ spontaneously disproportionates to give HOOH and $O_2^{\cdot-}$ (18) and that the reactions of HOOH with $O_2^{\cdot-}$ or with ferrous iron (Hb^{2+}) may yield $\cdot OH$ (19,20)). Since it is unlikely that NADPH donates electrons directly to O_2, it follows that for the NADPH-mediated reaction the electron at step 2 (Figure 4 (1)) must be donated by one of these radicals, probably $O_2^{\cdot-}$ which is the most stable. That conclusion is consistent with the inhibition data; i.e. the NADPH-mediated reaction is very sensitive to superoxide dismutase (Figure 2B (1)), but much less sensitive to scavengers of other radicals (Table III (1)). The catalase effect (Figure 2A (1)) is best explained by postulating that a relatively stable complex containing bound peroxide (IIIb, Fig. 4 (1)) is formed during the course of the reaction. The action of catalase on any H_2O_2 dissociated from that complex would drive the equilibrium toward dissociation and lead to complete inhibition. This explanation may be applied to both the NADPH- and the reductase-mediated reactions. An alternate interpretation of the data for the NADPH-mediated system must also be considered, however. It has been suggested for various model hydroxylation systems that the ultimate hydroxylating agent may be $\cdot OH$ (20), and that this species may be formed by the Haber-Weiss reaction (19): $H_2O_2 + O_2^{\cdot-} \xrightarrow{H^+} \cdot OH + H_2O + O_2$. Thus, the reaction would be inhibited by catalase, superoxide dismutase and radical scavengers (ethanol, mannitol). The criterion for substantial inhibition by the alcoholic radical scavengers was not fulfilled for either the NADPH-mediated reaction or the reductase-mediated reaction. The ~ 40% inhibition of the former reaction at 1 M ethanol may suggest partial involvement of $\cdot OH$ in the reaction, or it may reflect a less specific effect of the organic solvent.
On the Nature of the Interaction of Aniline with Oxyhemoglobin: It was concluded that the most likely mechanism of interaction of aniline with oxyhemoglobin involves binding of aniline at a site other than the heme iron atom. That possibility was discussed in the adjacent article (2); the other possibilities which seem less likely are discussed here: (a) aniline might interact directly with the "ferric" heme iron atom of $[Hb^{2+}-O_2 \leftrightarrow Hb^{3+}-O_2^{\cdot-}]$, displacing $O_2^{\cdot-}$. On the basis of various types of spectral evidence (21,22), it has been proposed that the electronic configuration of oxyferrohemoglobin might be best represented as a charge transfer complex in which the iron atom possesses ferric character, i.e. $Hb^{3+}-O_2^{\cdot-}$. Wallace and Caughey have carried this concept further and have suggested that azide and even chloride may directly displace (nucleophilic attack) superoxide anion from HbO_2 and thus form diazide-liganded Hb^{3+} directly (23). Misra and Fridovich have suggested that shark oxyhemoglobin may spontaneously breakdown to $Hb^{3+} + O_2^{\cdot-}$ (24). It is generally agreed that regardless of its polarization the $Hb-O_2$ bond in mammalian hemoglobin is covalent; that O_2 binds reversibly as O_2, and that autoxidation in the absence of an external electron donor is initiated by oxidation of the heme ferrous iron atoms from which O_2 has dissociated (3,25-27). It seems unlikely that the acceleration of autoxidation by aniline (Figure 2) could be attributed to direct displacement of $O_2^{\cdot-}$. Aniline is a poorer nucleophile than azide and binds to Hb^{3+} much more weakly (3,28). Thus, it is also not feasible that the aniline-accelerated formation of Hb^{3+} from HbO_2 could be explained by aniline's displacing an equilibrium mixture of HbO_2 and $Hb^{3+} + O_2^{\cdot-}$ toward Hb^{3+}-AN. For that to be reasonable, the interaction constant derived from Figure 2B (2) would necessarily have to be ≥ 105 mM, i.e. the apparent dissociation constant for $AN-Hb^{3+}$ in the absence of $O_2^{\cdot-}$ (28).

(b) Wallace and Caughey (23) proposed that at alkaline pH, phenol may directly donate an electron to bound oxygen, thereby accelerating donation of a second electron by the iron atom to generate Hb^{3+} and peroxide. Although aniline is structurally related to phenol, it seems unlikely that the ionization potential of aniline at slightly acid pH would be compatible with direct electron transfer to O_2.

(c) Aniline might interact directly with the ferrous heme iron atom, either displacing O_2 directly or drawing the equilibrium toward $AN-Hb^{2+}$; hybrid, mixed ligand forms of ferrohemoglobin might occur where each type of ligand (O_2 and aniline) were bound to one or more separate heme iron atoms of the tetramer, e.g. $(O_2)_{1-3}Hb^{2+}-(AN)_{1-3}$. This possibility is unlikely because no consistent spectral changes were observed when aniline was mixed with Hb^{2+} (2). Since the spectrum of deoxyhemoglobin is unique in the visible region with a single broad band at 555 nm (Fig. 1S), while spectra of liganded ferrohemoglobin generally have α and β bands (3), some concentration-dependent spectral change would be expected if a stable $AN-Hb^{2+}$ complex were formed. (The occurrence of a transient spectral change when $Na_2S_2O_4$ was added to $CN-Hb^{2+}$ was interpreted to indicate transient $CN-Hb^{2+}$ ligation (3)). In addition, ligation of the ferrous iron atom would be expected to protect it from autoxidation rather than to accelerate the process. Techniques other than spectroscopy (e.g. equilibrium dialysis) could be employed to directly test for aniline-Hb^{2+} interaction, however, before this possibility is completely dismissed.

REFERENCES

1. Mieyal, J.J., Ackerman, R.S., Blumer, J.L., and Freeman, L.S. (1976), J. Biol. Chem. 251, adjacent article.
2. Mieyal, J.J., and Blumer, J.L. (1976), J. Biol. Chem. 251, adjacent article.
3. Antonini, E., and Brunori, M. (1971), Hemoglobin and Myoglobin in Their Reactions with Ligands, North Holland Publishing Co., Amsterdam, 435 pps.
4. Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M., and Conney, A.H. (1972) J. Biol. Chem. 247, 1727-1734.
5. Strobel, H.W., Lu, A.Y.H., Heidema, J., and Coon, M.J. (1970), J. Biol. Chem. 245, 4851-4854.
6. Nash, T. (1953), Biochem. J. 55, 416-421.
7. Schenkman, J.B., Remmer, H., and Estabrook, R.W. (1967), Mol. Pharmacol. 3, 113-123.
8. Autor, A.P., Kaschnitz, R.M., Heidema, J.K., and Coon, M.J. (1973), Mol. Pharmacol. 9, 93-104.
9. Lowry, O.H., Roberts, N.R., and Kapphahn, J.I. (1957), J. Biol. Chem. 224, 1047-1064.
10. Omura, T., and Sato, R. (1964), J. Biol. Chem. 239, 2370-2378.
11. Van Kampen, E.J., and Zijlstra, W.G. (1961), Clin. Chim. Acta 6, 538-544.
12. Brodie, B.B., and Axelrod, J. (1948), J. Pharm. Exp. Therap. 94, 22-38.
13. Bray, H.G., and Thorpe, W.V. (1954), in Methods of Biochemical Analysis (D. Glick, ed.), Vol. 1, 27-52, Interscience Publishers, New York.
14. Lu, A.Y.H., Jacobson, M., Levin, W., West, S.B., and Kuntzman, R. (1972), Archiv. Biochem. Biophys. 153, 294-297.
15. Vermilion, J.L., and Coon, M.J. (1974), Biochem. Biophys. Res. Comm. 60, 1315-1322.
16. Collowick, S.P., van Lys, J. and Park, J.H. (1966) in Compreh. Biochem. (M. Florkin and E.H. Stotz, eds.), Vol. 14, 1-98.
17. Brown, W.D., and Snyder, H.E. (1969), J. Biol. Chem. 244, 6702-6706.
18. Fridovich, I. (1972), Accts. Chem. Res. 5, 321-326.
19. Haber, F., and Weiss, J. (1934), Proc. Roy. Soc., Ser. A, 147, 332-351.
20. Walling, C. (1975), Accts. Chem. Res. 8, 125-131.
21. Wittenberg, J.B., Wittenberg, B.A., Peisach, J., and Blumberg, W.E. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 1846-1853.
22. Hoffman, B.M., and Petering, D.H. (1970), Proc. Natl. Acad. Sci. U.S.A. 67, 637-643.
23. Wallace, W.J. and Caughey, W.S. (1975), Biochem. Biophys. Res. Comm. 62, 561-567.
24. Misra, H.P. and Fridovich, I. (1972), J. Biol. Chem. 247, 6960-6962.
25. Kiese, M. (1974), Methemoglobinemia: A Comprehensive Treatise, CRC Press, Inc., Cleveland, 260 pages.
26. Ingraham, L.L. (1966) in Comprehensive Biochem. (M. Florkin and E.H. Stotz, eds.), Vol. 14, 424-446.
27. Mansouri, A., and Winterhalter, K.H. (1974), Biochem. 13, 3311-3314.
28. Mieyal, J.J., and Freeman, L.S. (1976), Archiv. Biochem. Biophys., in press.