MECHANISM FOR THE AUTOXIDATION OF HEMOGLOBIN BY PHENOLS, NITRITE AND "OXIDANT" DRUGS. PEROXIDE FORMATION BY ONE ELECTRON DONATION TO BOUND DIOXYGEN.

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Summary. The reaction of HbO_2 with phenols to produce metHb shows inverse rate dependence upon $[H^+]$, direct dependence upon $[HbO_2]$ and [phenol], and a rate that correlates with the electron donor characteristics of the reagents. Thus, the availability of an electron from an external agent permits facile reduction of O_2 to O_2^- and the reaction of HbO_2 with phenols gives rise to metHb and peroxide as reaction products. In contrast, with nucleophiles such as azide O_2 is displaced as superoxide. Since reduction of bound O_2 is seen to occur only by reductive displacement or by reaction with a single electron donor, Hb apparently owes its normal resistance to autoxidation to the isolation of the binding site from electron donors and nucleophiles and not to an unique kind of iron-O_2 bonding. Such reasoning explains the effects of structural abnormality that render M-type Hbs susceptible to oxidation. Also the oxidation of HbO_2 upon exposure to "oxidant drugs" is explicable in terms of the drugs acting as one electron reducing agents towards bound dioxygen.

Introduction: The ability of hemoglobin (Hb) to bind oxygen reversibly without the iron(II) complex undergoing oxidation to iron(III) is critical to the protein's physiological function in that the iron(III) form, metHb, is not able to bind O_2. However, a small amount metHb is formed in the normal erythrocyte and is reduced back to iron(II) enzymatically (1). Unusually high amounts of metHb of clinical importance can result from the presence of an abnormal amino acid substitution (M-type Hb) (2) or exposure to certain drugs or toxic agents (e.g. aryl amines, phenols, hydrazines, nitrites, copper) (3). Recently we described two key ways (4,5) that HbO_2 can undergo autoxidation [(iron(II) oxidation to iron(III))] with concomitant O_2 reduction. The first route which utilizes a one electron reduction of O_2 to O_2^- (superoxide) occurs through a slow proton - assisted reductive displacement of O_2^- (4) with displacement by chloride a normal occurrence in the erythrocyte (6). The second route involves a one electron transfer from an external donor to bound O_2. Then the combination of an electron from the external donor and an electron

from iron(II) of the protein allows the thermodynamically favored two electron reduction of bound dioxygen to peroxide to occur and opens up the possibility of a very rapid reduction reaction. Here we present more detailed evidence in support of the second route for oxygen reduction - a route which applies both to abnormal Hbs and to effects of drugs and toxic agents.*

Experimental: Hemoglobin was prepared and stored as described previously (4). The pH was maintained with 0.1 M buffers. Those employed were pH 6.4 maleate, pH 7.4 phosphate, pH 8.3 phosphate, pH 9.6 borate, pH 10.3 borate. Laboratory grade phenols were recrystallized from water.

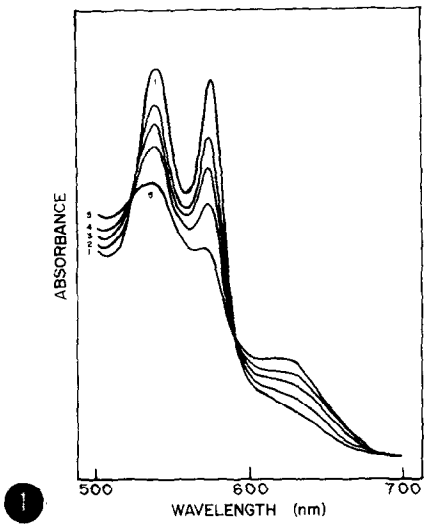
The hemoglobin solutions were prepared by diluting the stock solution to the appropriate concentration (0.04-0.10 mM) with buffer. The solution was brought to the required temperature (usually 30°) in a thermostatted bath, redox reagent added and the reaction followed at constant temperature on a Cary 17 spectrophotometer.

Results and Discussion. Phenols react with HbO_2 to give metHb and the progress of the reaction can be followed spectrophotometrically as shown in Figure 1 for the reaction with phenol. The excellent isosbestic points and the final (t_∞) spectrum serve to identify the unique reaction product as metHb. Despite the apparently "clean" chemistry the reaction was found to be kinetically complex and most of the data shown in Table I was obtained by the differential method using initial slopes. Such data is of inherently lower quality than that obtained by integral methods but has the advantage of minimizing the influence of changes that occur subsequent to the initial event. Rate constants obtained in this way give rise to the rate law

$$R = k[HbO_2]\,[Ph]/[H^+] \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots 1$$

where $[HbO_2]$ and $[Ph]$ represent the concentrations of oxyHb and the phenolic compound used as a reactant.

*Certain metals ions (e.g. ferricyanide, cupric) also promote metHb formation. However, present evidence suggests these agents oxidize deoxy and not oxyHb. In the case of copper, O_2 is envisioned as participating in the regeneration of Cu(II) from Cu(I) (7).



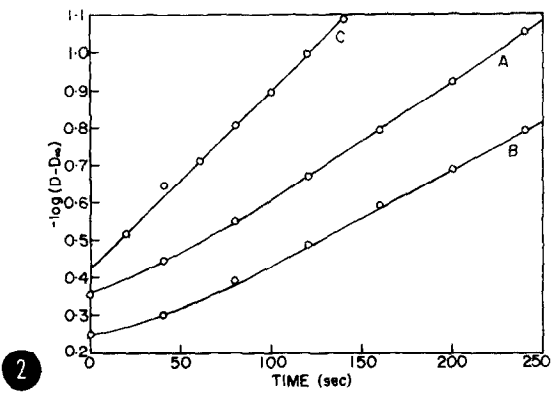


Figure 1. The reaction between HbO_2 and phenol at pH = 8.3 and 30° followed by visible spectra at various times from initiation 1. 0.2 hr; 2. 2.0 hr; 3. 14.8 hr; 4. 50.2 hr; 5. 95.0 hr.

Figure 2. Pseudo first order rate plots for the reaction between HbO_2 and hydroquinone at pH - 8.3 and 30°. A. hydroquinone = 4.8 mM; B. hydroquinone = 4.8 mM hydrogen peroxide - 10 mM; C. hydroquinone - 4.8 mM sodium azide = 10 mM.

These reactions between the phenols and HbO_2 are different in several ways from the reactions between nucleophiles (such as azide) and HbO_2 reported upon previously (4). The inverse hydrogen ion dependence suggests that the active phenol species is the phenolate ion and that a protonated protein intermediate is not involved. This is consistent with the observation that phenols undergo air oxidation more readily as the pH is increased. The rates of nucleophile induced displacements of superoxide from HbO_2 correlate well with the base strengths of the nucleophiles while the rates of the phenol induced reactions do not. Rather the correlation is with the order of the phenols as electron donors (p-nitrophenol < salicylic acid < phenol < resorcinol << hydroquinone). Particularly striking in this regard is the comparison between resorcinol (pK = 9.44, k_2 = 0.00042 M^{-1} min^{-1}) and hydroquinone (pK = 9.96, k_2 = 3.6 M^{-1} min^{-1}).

The very rapid reaction between hydroquinone and HbO_2 is seen (Figure 2) to deviate markedly from simple first order kinetics, but no other simple rate

Table I

Kinetic Parameters for the Reaction of

Oxyhemoglobin with Phenols at 30°

Phenol	pH	pK_a	$k^1_{app} \times 10^3$ (min^{-1})	Concn. (M)	k_2 ($M^{-1}min^{-1}$)
Hydroquinone	6.4	9.96	8.6	.033	.26
			5.6	.017	.33
	7.4		12	.0033	3.6
	8.3		110	.0032	34.4
			154	.0048	32.1
			187	.0063	29.7
			217	.0077	28.2
HQ + azide	7.4		158	.033	4.78
	8.3		420	.0088	47.7
			414	.0088	47.1
			456	.0088	51.9
			297	.0067	44.4
			156	.0031	50.4
	9.3		1320	.0033	400
Phenol	8.3	9.95	0.12	.042	.0029
	8.9		0.14	.040	.0036
	9.3		0.20	.040	.0050
	9.6		1.25	.103	.0121
	10.3		2.78	.111	.0250
Hydroquinone	7.4	9.96	116	.033	3.5
Resorcinol	7.4	9.44	.043	.103	0.00042
Phenol	7.4	9.95	.037	.103	0.00036
Salicylic Acid[a]	7.4	12.95	.0046	.103	0.000045
p-nitrophenol[a]	7.4	7.14	.010	.103	0.00010

[a]When the rate constants are corrected to equal phenolate ion concentrations salicylic acid is seen to react faster than p-nitrophenol.

law reproduced the data. The deviation from linearity in the first order rate plot was enhanced by the prior addition of hydrogen peroxide and removed by the addition of azide to the reaction mixture (Figure 2). In the presence of azide the reaction was found to follow pseudo first order kinetics through at least three half lives, and to exhibit a somewhat higher rate constant. In the absence of hydroquinone metHb reacts slowly with hydrogen peroxide. This reaction was quenched by the addition of azide to the reacting mixture or prevented entirely by the prior addition of azide to metHb. Additionally the mix-

ture remaining upon completion of the hydroquinone - HbO_2 reaction showed the characteristic peroxide oxidation of iodide to iodine.

These observations suggest that hydroquinone in its reaction with HbO_2 gives rise to the products metHb (observed spectroscopically), H_2O_2 (which gave the iodine reaction) and, presumably, semiquinone radical (detected by EPR) (reactions 1 and 2). The deviation from pseudo first order kinetics in the absence of azide is probably due to back reaction with the peroxide produced in the reaction. The back reaction was shown to be quenched by the addition of azide to the reaction mixture. This strong ligand binds to the iron(III) (reaction 3), blocks the back reaction and causes the apparent rate to increase.

$$1.\ HbO_2 + ArO^- \rightarrow HbO_2 + ArO\cdot$$

$$2.\ HbO_2^- + 2\ H_2O \rightarrow HbOH + H_2O_2 + OH^-$$

$$3.\ HbOH + N_3^- \rightarrow HbN_3 + OH^-$$

Thus, phenols appear to act as electron donors in their reactions with HbO_2 with the Hb oxidized by the action of reducing agent. Thus, the electron from the external reducing agent and an electron from iron must both be taken up by the bound dioxygen giving rise to peroxide and metHb as reaction products. Hence, O_2 becomes activated upon binding to the hemoglobin. Other single electron donors such as arylamines, dithionite (through the radical ion SO_2^- (8)) and nitrite reasonably act in a similar way (9). The nitrite reaction has been studied in connection with the present work and although the kinetics are complex the results are fully consistent with an initial step that involves a single electron transfer from nitrite to the bound dioxygen of HbO_2. After the completion of this work somewhat analogous observations for the reaction of hydroquinone with an oxygenated cobalt compound were reported (10).

There are then two, but only two, known ways in which dioxygen bound to hemoglobin (or myoglobin) can undergo reduction. One, the reductive displacement of superoxide by nucleophiles, is slow because of the high activation energy associated with the induced formation of the thermodynamically

unstable superoxide ion (4). Nevertheless, this represents the only reductive pathway open to dioxygen bound to hemoglobin under normal physiological conditions and, with chloride as the displacing nucleophile, can account for the normal (3-5%/day) production of metHb in erythrocytes (6). In the second process, the two electron transfer which gives rise to peroxide as the reduction product of dioxygen can take place only in the presence of an external one electron donor. However, since peroxide is a thermodynamically favored reduction product of dioxygen this reaction can be very rapid with a facile electron donor (e.g. hydroquinone). The two electron transfer involving one electron from the iron atom to which dioxygen is bound and one electron from another donor is probably relevant to oxygen utilizing (or producing) proteins such as cytochrome c oxidase. In such systems it seems reasonable that for rapid reduction the first step must involve a two electron transfer and as a consequence the dioxygen must be bound in such a configuration that it can receive virtually simultaneously two electrons from two different one electron donors.

The two electron reduction of dioxygen bound to hemoglobin through the intervention of an external one electron donor explains, in principle, a number of clinically significant oxidative problems involving hemoglobin. These range from the problems of genetic origin represented by the M-type Hbs (2) to those of drug and toxic agents induced oxidation of HbO_2. Thus the M-type Hbs in which tyrosine replaces a normal distal histidine contain a phenol residue that could readily act as the external electron donor and allow the two electron reduction of bound dioxygen to proceed readily (5). As shown here, phenols react with HbO_2 at neutral pH to yield peroxide rather than superoxide. The chemically induced autoxidation of hemoglobin has been examined extensively (11). By 1949, Lemberg and Legge (9) had compiled an extensive list of reagents including nitrite ion, aromatic amino and nitro compounds and organic nitrites. More recently much examination has been made of the effects of what Hopkins and Tudhope (12) refer to as "oxidant" drugs, which includes

such drugs as the antimalarials of the 8-aminoquinoline group, aspirin, sulfonamides and phenylhydrazines. These compounds are all active one electron donors and can readily act to promote the two electron reduction of dioxygen bound to hemoglobin in exactly the way described above for the phenols. Furthermore, Cohen and Hochstein (13) have established that peroxide is present in the erythrocyte following exposure to "oxidant" drugs. The peroxide so produced has been held responsible by Tudhope and Leece (14) for the Heinz body formation, cell fragility and hemolysis that often accompany the chemically induced metHb formation.

The rapid two electron pathway for the reduction of heme bound oxygen can be expected to have very wide applicability in biochemical processes as key initial steps in the reduction of dioxygen by oxygen utilizing proteins.

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