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DIFFERENCES IN THE REACTIONS OF ISOMERIC *ORTHO*- AND *PARA*-AMINOPHENOLS WITH HEMOGLOBIN*

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Abstract—The metabolites of phenacetin, 2-hydroxyphenetidine and 4-nitrosophenetol, rapidly produced ferrihemoglobin both *in vivo* (dogs) and *in vitro*. At low concns, 2-hydroxyphenetidine was superior to 4-nitrosophenetol in ferrihemoglobin formation. The kinetics of ferrihemoglobin formation by 2-hydroxyphenetidine in solutions of purified human hemoglobin was biphasic and exhibited an unusual dose response. Similar to *p*-aminophenols, 2-hydroxyphenetidine was oxidized by oxyhemoglobin, and the oxidation product(s) were reduced by ferrohemoglobin with the formation of ferrihemoglobin. In addition, these oxidation products condensed to 2-amino-7-ethoxy-3*H*-phenoxazine-3-one (u.v., i.r., ^{1}H-NMR and mass spectroscopy). This metabolite produced ferrihemoglobin by itself and was responsible for the slow phase of ferrihemoglobin formation observed with 2-hydroxyphenetidine. This condensation reaction, which was also observed with 2-aminophenol, prevented thioether formation of the transient *o*-quinonimines with the cysteine residues of hemoglobin and reduced glutathione as observed with 4-aminophenol and 4-dimethylaminophenol. Phenoxazone formation, which depends on the square of the *o*-quinonimine concn, was negligible at micromolar concns. At similar concns addition reactions to thiols prevailed also with 2-hydroxyphenetidine and 2-aminophenol. Other electrophilic reactions, e.g. with primary amino groups of amino acids, were insignificant. These dose-dependent differences in the reactions of isomeric aminophenols may explain the low nephrotoxicity of those *o*-aminophenols capable of forming phenoxazones when given in a single dose. This self-detoxication of some *o*-quinonimines, however, should not function during long-term exposure to repetitive low doses of such *o*-aminophenols.

Studies on the *p*-phenetidine metabolism *in vivo* [2–4] and in the isolated perfused rat liver‡ revealed an overproportional increase in 2-hydroxyphenetidine (2-OHPt)§ formation at increasing doses of *p*-phenetidine. As an autoxidizable *o*-aminophenol, 2-OHPt was supposed to be potentially nephrotoxic. The studies of Calder *et al.* [5], however, indicate that 2-OHPt as well as 2-AP is essentially not nephrotoxic in contrast to 4-AP. This difference in nephrotoxicity is poorly understood, because all these aminophenols quickly form ferrihemoglobin, both *in vitro* and *in vivo*. Consequently, *o*-aminophenols should also be able to form an electrophilic intermediate. Concerning 2-OHPt, reports on its ferrihemoglobin forming activity are conflicting [6–9]. Therefore, we decided to reexamine the kinetics of ferrihemoglobin formation by 2-OHPt.

Our initial experiments have shown that 2-OHPt is a potent ferrihemoglobin-forming agent in both dog and human blood as well as in solutions of purified human hemoglobin. Surprisingly, the kinetics of ferrihemoglobin formation by 2-OHPt were biphasic: after a rapid initial ferrihemoglobin formation the second phase showed virtually no endpoint. Additionally, in contrast to *p*-aminophenols, 2-OHPt was apparently not bound to hemoglobin and did not react with reduced glutathione. Since this pattern was also shared by 2-AP, we suspected that *o*-aminophenols might react in a basically different manner to the *para* isomers. To prove this hypothesis, we compared isomeric aminophenols in their reactions with hemoglobin. In this well-defined model system, activation reactions by the heme-activated oxygen and toxic actions, i.e. ferrihemoglobin formation and covalent binding, can be studied simultaneously.

MATERIALS AND METHODS

Female beagles, 9–15 months old, body wt 9.5–11 kg, were fed a standard diet and received water *ad lib*.

Red cells were obtained from freshly drawn and heparinized blood and washed 5 times with a five-fold vol. of 0.2 M phosphate buffer, pH 7.4.

Hemolysates and purified human hemoglobin were prepared as reported [10].

Hemoglobin with blocked SH groups was prepared by reacting purified human hemoglobin with a 10-

* A preliminary report has been presented at the 23rd Spring Meeting of the Deutsche Pharmakologische Gesellschaft at Mainz (1982) [1].

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§ Abbreviations: 2-AP, 2-aminophenol; 4-AP, 4-aminophenol; *p*CMB, *p*-chloromercuribenzoic acid; 2-DMAP, 2-dimethylaminophenol; 4-DMAP, 4-dimethylaminophenol; 4-NOPt, 4-nitrosophenetol; 2-OHPt, 2-hydroxyphenetidine.

fold excess of *p*CMB at room temp, pH 7.4, for 30 min. The hemoglobin solution was then dialyzed against phosphate buffer. Complete blockage was checked by the method of Boyer [11].

4-NOPt was synthesized by persulphate oxidation of 4-phenetidine similar to the method reported by Hirota and Itano [12]. A mixture of finely powdered potassium persulphate (12.5 g) and conc. sulphuric acid (12.5 ml) was stirred at room temp for 1 hr and then poured into ice water (1 l.). This solution was adjusted with solid potassium carbonate to pH 8.4 and then with glacial acetic acid to pH 6.4. On addition of 4-phenetidine hydrochloride (50 mmoles), the solution quickly turned green and was vigorously stirred at 0° for 5 min. The resulting 4-NOPt was extracted twice with 500 ml ether and the unreacted 4-phenetidine reextracted with 0.1 N sulphuric acid. The ether was dried over anhydrous sodium sulphate and evaporated to about 10 ml. On cooling to −20°, 4-NOPt crystallized. For further purification, the material was steam distilled (yield 34 mmoles). This 4-NOPt preparation still contained 13% of 4-nitrophenetol (which might co-crystallize with 4-NOPt [13, 14]) and therefore the steam distillation procedure was repeated twice. The final product (yield 18 mmoles) contained less than 1% of 4-nitrophenetol and melted at 33° as published [15].* The molar absorbance of an ethereal solution was 18,100 at 336 nm.

2-OHPt was obtained from 2-hydroxyphenacetin (EGA-Chemie, Steinheim, F.R.G.) by acid hydrolysis: 2.56 mmoles 2-hydroxyphenacetin was suspended in 50 ml 1 N hydrochloric acid and hydrolyzed at 100° for 2 hr. Unreacted material was removed with ether. After neutralization, 2-OHPt was extracted with ether and, after drying the ether over anhydrous sodium sulphate, precipitated as the hydrochloride (74% yield). The base melted at 152° (147–149° [18]), was 98% pure [TLC on silica gel plates with chloroform:methanol (92.5:7.5), R_f = 0.47; HPLC on μ-Bondapak C_{18}, methanol:2 mM sodium phosphate, pH 7.4 (68:32), R_{vol} = 4.2 ml], and contained less than 2% of 2-amino-7-ethoxy-3*H*-phenoxazine-3-one (u.v., TLC and HPLC).

[^{14}C, U-ring]2-OHPt), was biosynthesized. One mmole phenacetin (NEN, Boston, MA) (0.28 mCi/mmole) was completely hydrolyzed in 5 ml 1 N hydrochloric acid at 100° within 2 hr. After neutralization 0.97 mmoles [^{14}C]phenetidine was extracted with ether. For ring hydroxylation, 0.35 mmoles [^{14}C]phenetidine, dissolved in 120 ml Krebs–Henseleit buffer, was perfused, recirculating through an isolated rat liver (7.3 g, 4 ml/g/min [19]) for 5 hr. After extraction with ether, 40% of the radioactivity was found in the aqueous phase which was treated with 1 N hydrochloric acid at 100° for 2 hr to hydrolyze the ethereal sulphate of 2-OHPt which was formed by the liver. After neutralization, [^{14}C]2-OHPt was extracted with ethyl acetate and acetylated with acetic anhydride. The [^{14}C]2-hydroxyphenacetin formed was extracted with 0.1 N sodium hydroxide. For further purification, the compound was chromatographed on silica gel plates with chloroform: methanol (95:5) (R_f = 0.48). The compound was radiochemically pure (TLC) (HPLC on μ-Bondapak C_{18}, methanol:water (3:7), R_{vol} = 11.8 ml) and the yield was 8%. Prior addition to hemoglobin [^{14}C]2-hydroxyphenacetin was hydrolyzed as described.

2-Amino-7-ethoxy-3*H*-phenoxazine-3-one, the condensation product from 2-OHPt: 1.95 mmoles 2-OHPt was autoxidized in 500 ml sodium borate (0.2 M, pH 8.5) at 37° overnight. The reaction mixture was extracted exhaustively with dichloromethane. The phenoxazone was crystallized by evaporation of the solvent. After two recrystallizations from hot acetone, 0.54 mmoles (55% of theory) was obtained. The compound melted at 256° (257° [20]) and was pure [TLC on silica gel plates with chloroform:methanol (95:5), R_f = 0.70; HPLC on μ-Bondapak C_{18}, methanol:2 mM sodium phosphate, pH 7.4 (68:32), R_{vol} = 9.4 ml]. The mol. wt for 2-amino-7-ethoxy-3*H*-phenoxazine-3-one of 256 was confirmed by mass spectroscopy and showed fragment ions of *m/z* 229 $[M - HCN]^+$, 228 $[M - CO]^+$, and 227 $[M - C_2H_5]^+$. The structure was further confirmed by ^{1}H-NMR in deuterated trifluoroacetic acid: 7.65 (d, 1, J = 9 Hz), 7.25 (m, 2), 6.93 (s, 1), 6.77 (s, 1), 4.29 (q, 2, J = 7 Hz, $-CH_2-$), 1.55 ppm (t, 3, J = 7 Hz, $-CH_3$). Fig. 1 shows the i.r. spectrum in KBr (upper panel) and the electronic spectrum in methanol (lower panel). The compound was sparingly soluble in most solvents. For addition to hemoglobin solutions (1/100 v/v), the compound was dissolved in dioxane and stored as a 20 mM stock solution.

2- and 4-AP were purchased from Merck (Darmstadt, F.R.G.).

[U-^{14}C]2-AP was prepared from [U-^{14}C]aniline hydrogen sulphate [sp. act. 102 mCi/mmole; 98% purity (Amersham Buchler, Braunschweig, F.R.G.)] according to Boyland *et al.* [21]: 2.45 μmoles [^{14}C]aniline hydrogen sulphate was oxidized by potassium persulphate in potassium hydroxide solution. The resulting 2-AP sulphate was purified by preparative HPLC on μ-Bondapak C_{18} using the paired-ion chromatography technique [methanol: tetrabutylammonium phosphate, 10 mM (40:60 v/v) R_{vol} = 6.4 ml]. Finally, the 2-AP sulphate ester was chromatographed on Sephadex LH 20 (1 × 200 cm) with water to remove the tetrabutylammonium phosphate (yield 9%). This product was mixed with inactive 2-AP and hydrolyzed in conc. hydrochloric acid at 100° for 30 min. The resulting 2-AP preparation (5 μCi/μmole) contained about 7% of 2-AP sulphate ester and traces of 2-amino-3*H*-phenoxazine-3-one. The sulphate ester was inert during incubation with hemoglobin and glutathione.

2-Amino-3*H*-phenoxazine-3-one, the condensation product from 2-AP, was prepared in the same way as 2-amino-7-ethoxy-3*H*-phenoxazine-3-one. After autoxidation and extraction with dichloro-

* Uehleke [16] who used peracetic acid for 4-phenetidine oxidation reported a 2–5% yield of pure 4-NOPt. The synthesis of 4-NOPt by oxidation of *N*-hydroxy-4-phenetidine which was prepared by zinc dust reduction of 4-nitrophenetol according to Rising [15] apparently gave low and poorly reproducible yields of 5–20% [16] and 35% [17] respectively, with some 20% of 4-nitrophenetol as revealed by HPLC. We therefore recommend the method of Hirota and Itano [12] which in our case gave reproducibly some 35% of pure 4-NOPt.

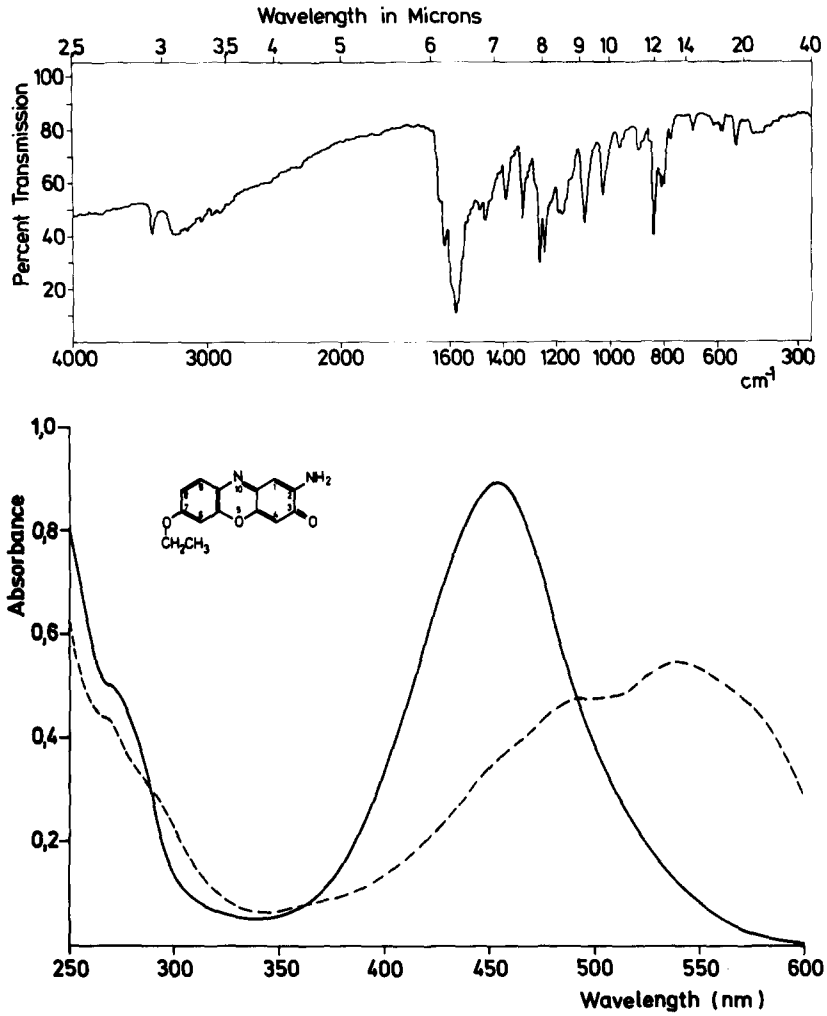

Fig. 1. 2-Amino-7-ethoxy-3*H*-phenoxazine-3-one, the condensation product of 2-hydroxyphenetidine. Upper panel: i.r. spectrum in KBr. Lower panel: electronic spectrum in methanol (0.04 mM) and in 0.2 N methanolic HCl (broken line).

methane, this phenoxazone was obtained with a 21% yield after two recrystallisations. The compound melted at 257° (225–257° [22]) and was pure [TLC on silica gel plates with chloroform:methanol (95:5), $R_f = 0.64$; HPLC on μ-Bondapak C_{18}, methanol: water (8:2), $R_{vol} = 4.5$ ml]. The structure of 2-amino-3*H*-phenoxazine-3-one was confirmed by u.v., vis, ^{1}H-NMR and mass spectroscopy; the obtained data agreed with those published [23–26]. Due to the very poor solubility, the compound was dissolved in dioxane prior to addition to hemoglobin solutions (1/100 v/v).

2-DMAP was a generous gift from Prof. Dr G. Renner, Institut für Pharmakologie und Toxikologie der Universität München (München, F.R.G.). The compound was synthesized according to Huisgen *et al.* [27, 28] and was pure [TLC on silica gel plates with chloroform:*n*-hexane:ethyl acetate (45:45:10), $R_f = 0.75$].

[U-^{14}C]2-DMAP was prepared by NEN; the compound (as hydrochloride) was mixed with inactive 2-DMAP to give a final sp. act. of 0.03 μCi/μmole (radiochemical purity 98%).

4-DMAP hydrochloride and [U-^{14}C]4-DMAP (sp. act. 9 μCi/μmole, 99% purity) were prepared by Farbwerke Hoechst.

All other reagents were commercially available products.

Ferrihemoglobin and total hemoglobin were measured by the method reported by Kiese [29].

Reduced glutathione (GSH) was determined as *S*-lactyl-glutathione after transformation by glyoxalase I in the presence of methylglyoxal according to Racker [30].

Glutathione disulfide (GSSG) was determined enzymatically by the oxidation of NADPH in the presence of glutathione reductase according to Rall and Lehninger [31].

Lysine and histidine were estimated by a Multichrom M amino acid analyzer (Beckman, München, F.R.G.).

HPLC was performed with a chromatograph ALC/GPC 244 (Waters, Milford, MA) on μ-Bondapak C_{18} or μ-Porasil (4 mm i.d. × 30 cm) at 2 ml/min flow rate and detection at 254 nm.

[^{14}C]2-AP was determined by isotope dilution

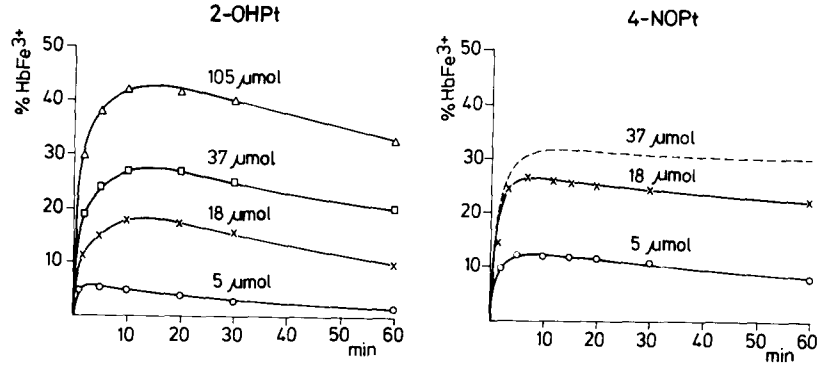

Fig. 2. Ferrihemoglobin formation in dogs after intravenous injection of 2-hydroxyphenetidine (2-OHPt) and 4-nitrosophenetol (4-NOPt) (µmoles/kg). The broken line represents results from Baader *et al.* [44].

analysis. 0.5 ml of a sample was mixed with 0.1 mg of inactive 2-AP and extracted with 2.5 ml ether. After evaporation the reduced vol. was spotted on silica gel plates and chromatographed with chloroform:methanol (95:5). The 2-AP-containing bands were extracted with ether. By reading the u.v. absorbance at 290 nm (molar absorbance = 3240) and determining the radioactivity the concn of the radioactive 2-AP in the sample was calculated.

[^{14}C]2-Amino-3*H*-phenoxazine-3-one was similarly determined using a molar absorbance coefficient of 24,500 at 426 nm.

Covalent binding of radioactive metabolites to hemoglobin was determined in precipitates after several washings with trichloroacetic acid and methanol. Precipitates were dissolved in Soluene 100 as described later.

Radioactivity in colourless solutions was measured in Bray's solution with a Packard Tri-Carb 2660 scintillation spectrometer using an external standard. Radioactivity in red cells and hemoglobin containing solutions was determined in Insta-Gel (Packard) after decolorization in Soluene 100 (Packard) and H_2O_2. All results have been corrected for recovery and background radiation.

M.P. determinations were carried out on a Leitz heating microscope 350. All m.ps have been corrected.

Means are given ± S.E.M. within 95% confidence interval.

RESULTS

Reactions of 2-OHPt and 4-NOPt with hemoglobin

After intravenous injection to beagles, 2-OHPt rapidly formed ferrihemoglobin. Maximal concns of ferrihemoglobin were attained in less than 15 min. 4-NOPt was even more efficacious and produced maximal ferrihemoglobin concns within 5 min, as shown in Fig. 2. *In vitro*, however, ferrihemoglobin formation in washed red cells of dogs showed a different dose response. As illustrated in Fig. 3, at low concns (0.2 mM) 2-OHPt formed ferrihemoglobin more rapidly than 4-NOPt. At 1 mM concn, the yield of ferrihemoglobin was only doubled by 2-OHPt, whereas 4-NOPt produced a five-fold amount. Additionally, the kinetics of ferrihemoglobin formation by 2-OHPt was apparently biphasic.

To rule out other influences, ferrihemoglobin formation was studied further with purified human hemoglobin. As depicted in Fig. 4, ferrihemoglobin formation still proceeded biphasically and was increased only slightly by increasing the concn of 2-OHPt. Ether extracts of these reaction mixtures revealed a rapid disappearance of 2-OHPt ($t_{\frac{1}{2}}$ = 1 min). Instead, a yellow compound was formed which turned purple during reextraction with hydrochloric acid. The isolated metabolite was shown to be 2-amino-7-ethoxy-3*H*-phenoxazine-3-one by u.v., vis, i.r., ^{1}H-NMR and mass spectroscopy.

This metabolite produced several equivalents of ferrihemoglobin by itself (Fig. 5) and thus seemed responsible for the slower second phase of ferrihemoglobin formation observed after addition of 2-OHPt. Only part of the 2-OHPt which had reacted with hemoglobin was recovered as 2-amino-7-ethoxy-3*H*-phenoxazine-3-one. Since phenoxazone formation is regarded as a condensation of two *o*-quinonimine molecules [22, 24], part of the oxidized 2-OHPt might have also reacted with nucleophiles of hemoglobin.

When ^{14}C-labeled 2-OHPt (0.2 mM) had reacted with 3 mM purified human hemoglobin for 30 min, 1/3 of the radioactive material was covalently bound to hemoglobin; 2/3 was extracted together with hemin by acid methylethylketone in the procedure

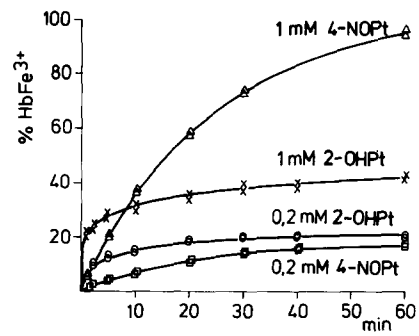

Fig. 3. Ferrihemoglobin formation in red cells of dogs by 2-hydroxyphenetidine (2-OHPt) and 4-nitrosophenetol (4-NOPt). 2-OHPt and 4-NOPt (0.2 and 1 mM, respectively) were incubated with washed red cells and suspended in 0.2 M phosphate, pH 7.4 (12 g Hb/100 ml), containing 10 mM glucose at 37° under air (two experiments).

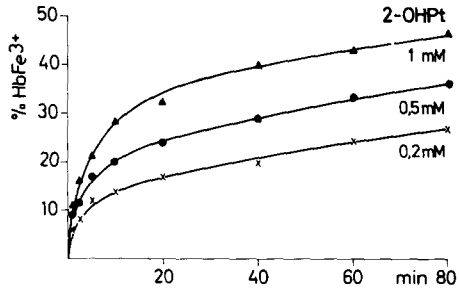

Fig. 4. Ferrihemoglobin formation by various 2-hydroxyphenetidine concns incubated with purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air (means of three experiments; corrected for autoxidation of hemoglobin).

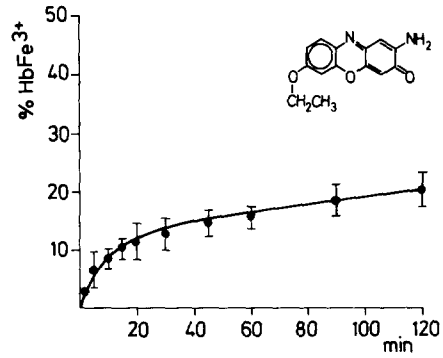

Fig. 5. Ferrihemoglobin formation by 2-amino-7-ethoxy-3*H*-phenoxazine-3-one, the condensation product of 2-hydroxyphenetidine. 2-Amino-7-ethoxy-3*H*-phenoxazine-3-one (0.2 mM) was incubated with purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air (corrected for autoxidation of hemoglobin; means of five experiments).

of Teale [32]. After chromatography of the aqueous phase containing the globin moiety on Sephadex G-75 fine (2.5 × 40 cm), all the radioactive material was eluted together with the protein and was precipitated by trichloroacetic acid. 2-OHPt presumably had reacted with the reactive cysteine residues of hemoglobin. When 2-OHPt (1 mM) was incubated with purified human hemoglobin (3 mM) for 1 hr, 0.28 mM of the reactive SH groups were blocked. GSH competed for the same binding site and was correspondingly diminished. Lysine and histidine, on the other hand, apparently did not react (cf. Table 1). These results indicated a diminished reactivity of oxidized 2-OHPt towards thiols as compared to another aminophenol, 4-DMAP, which has been extensively studied in our laboratory [33–36]. In order to decide whether there are basic differences in the reactions of *o*-and *p*-aminophenols, reactions of isomeric *o*- and *p*-aminophenols with hemoglobin were systematically studied.

Reactions of 2- and 4-AP with purified human hemoglobin

2-AP produced ferrihemoglobin more rapidly than 4-AP. As shown in Fig. 6, the initial velocity of ferrihemoglobin formation by 2-AP was 3 times higher than by 4-AP. This difference was even more pronounced in suspensions of washed human red cells, where 2-AP increased the ferrihemoglobin content 15 times faster than 4-AP. Interestingly, ferrihemoglobin formation by 2-AP was faster in red cells as compared to the hemoglobin solution whereas 4-AP was more efficacious in the hemoglobin solution. To study the fate of both aminophenols, they were incubated with various concns of hemoglobin and extracted with ether. In extracts from incubations with 2-AP, a yellow compound was detected which resembled the phenoxazone isolated from 2-OHPt. It was proved to be 2-amino-3*H*-phenoxazine-3-one by u.v., vis, i.r., ^{1}H-NMR and mass spectroscopy. In contrast, ether extracts from incubations with 4-AP were virtually colourless and contained only decreasing amounts of 4-AP. As shown in Fig. 7, oxyhemoglobin markedly accelerated the disappearance of both aminophenols and enhanced the corresponding phenoxazone formation from 2-AP. Obviously, 2-amino-3*H*-phenoxazine-3-one accounted for the missing 2-AP. Similar to 2-amino-7-ethoxy-3*H*-phenoxazine-3-one, 2-amino-3*H*-phenoxazine-3-one slowly formed many equivalents of ferrihemoglobin. The missing 4-AP probably was covalently bound to hemoglobin since the reactive SH groups of hemoglobin were diminished after reaction with 4-AP. Table 2 shows that 2 mM 4-AP blocked 0.91 mM of the reactive SH groups whereas 2-AP blocked only 0.1 mM. Instead, 1.66 mM 2-amino-3*H*-phenoxazine-3-one equivalents were formed. Glutathione prevented the

Table 1. Reactions of 2-hydroxyphenetidine with reduced glutathione (GSH), lysine (LYS) or histidine (HIS) in solutions of purified human hemoglobin

Additive			Found after 60 min		Found after 120 min		
GSH (mM)	LYS (mM)	HIS (mM)	GSH (mM)	LYS (mM)	GSH (mM)	LYS (mM)	HIS (mM)
4.75	5	5	3.45	4.87	3.21	4.68	4.67
4.75	1		3.60	n.d.	3.09	0.96	
0.95	5		0.31	4.98	0.45	4.87	
0.95	1		0.39	n.d.	0.44	0.97	

2-Hydroxyphenetidine (2 mM) was incubated with GSH, LYS or HIS in solutions of purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air for 60 and 120 min, respectively. Determined glutathione represents GSH + 2GSSG; n.d. = not determined.

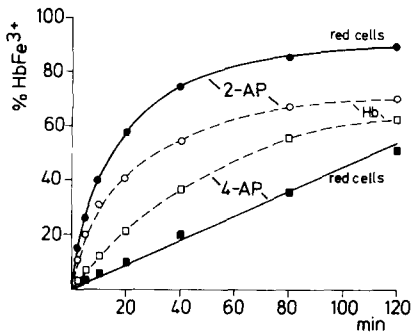

Fig. 6. Ferrihemoglobin formation by 2- and 4-aminophenol (2- and 4-AP). Purified human hemoglobin (3 mM Fe, broken lines) or washed human red cells (5 g Hb/100 ml) were incubated with 2- and 4-AP (0.5 mM) in 0.2 M phosphate, pH 7.4, at 37° under air (corrected for autoxidation of hemoglobin).

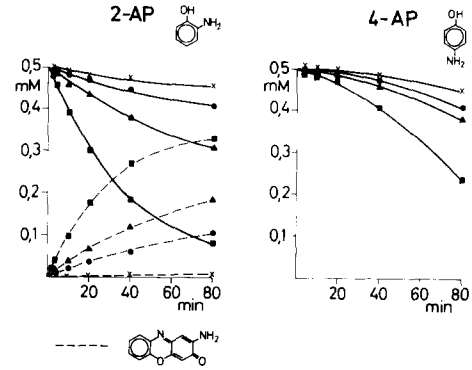

Fig. 7. Influence of hemoglobin concn on the decrease in 2-aminophenol (2-AP) and increase in 2-amino-3*H*-phenoxazine-3-one formation, and on the decrease in 4-aminophenol (4-AP), respectively. 2- or 4-AP (0.5 mM) were incubated in 0.2 M phosphate, pH 7.4, (×) and various concns of purified human hemoglobin at 37° under air: 0.06 (●), 0.6 (▲), 3 mM (■) Fe. (Broken lines give equivalent concns of 2-amino-3*H*-phenoxazine-3-one formed.)

covalent binding of 4-AP to hemoglobin and was diminished by 2.7 mM. Some reaction had probably also occurred with 2-AP because glutathione was diminished by 0.9 mM. Lysine and histidine had virtually no effect.

At this stage, we concluded that oxidized *o*-aminophenols preferred the phenoxazone condensation reaction instead of electrophilic addition. Since phenoxazone formation needs two molecules of an oxidized *o*-aminophenol, this condensation should be diminished at low *o*-aminophenol concns in favour of addition reactions. Therefore the following experiments were conducted with radioactively labeled 2-AP at micromolar concns.

As shown in Fig. 8, 2-AP at micromolar concns bound preferably to hemoglobin whereas phenoxazone formation prevailed at concns above 0.1 mM. In this experiment, 2-AP had reacted with purified human hemoglobin for 24 hr to transform 2-AP completely. To demonstrate the covalent character of that binding, a sample of hemoglobin incubated with 5 μM ^{14}C-labeled 2-AP was chromatographed on Sephadex G-75 fine. As expected from Fig. 8, 88% of the radioactive material co-chromatographed with hemoglobin. This radioactive material was precipitated by trichloroacetic acid and was insoluble in methanol. GSH (2 mM) accelerated the disappearance of 2-OHPt (5 μM) and effectively diminished covalent binding to hemoglobin, as illustrated in Fig. 9. When the hemoglobin was pretreated with *p*CMB to block the reactive SH groups, covalent binding of [^{14}C]2-AP (5 μM) to hemoglobin was reduced to 1/5 of the amount observed with the native hemoglobin.

These experiments revealed that isomeric aminophenols react very similarly, except that *o*-aminophenols can additionally form phenoxazones. To confirm this hypothesis, we extended our studies to reactions of 4- and 2-DMAP, an *o*-aminophenol which cannot form a phenoxazone because it lacks a primary amino group.

Reactions of 2- and 4-DMAP with hemoglobin

Ferrihemoglobin formation in purified human hemoglobin by 4-DMAP was about two orders of magnitude faster than by 2-DMAP. Apart from these kinetic differences, both dimethylaminophenols reacted very similarly. As illustrated in Fig. 10, the covalently bound proportion of both dimethylaminophenols fully accounted for the missing dimethylaminophenol.

Table 2. Reactions of 2- and 4-aminophenol (2- and 4-AP) with reduced glutathione (GSH), lysine (LYS) and histidine (HIS) in solutions of purified human hemoglobin

	Control Found	+ GSH Found		+ LYS Found		+ HIS Found	
	HbSH (mM)	GSH (mM)	HbSH (mM)	LYS (mM)	HbSH (mM)	HIS (mM)	HbSH (mM)
Hemoglobin	1.53	4.4	1.5	3.4	1.5	4.7	1.5
Hemoglobin + 2-AP	1.42	3.51	1.5	3.3	1.41	4.7	1.4
Hemoglobin + 4-AP	0.63	1.72	1.46	3.2	0.59	4.68	0.56

2-AP and 4-AP (2 mM) were incubated with GSH, LYS and HIS in solutions of purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air for 60 min. Determined glutathione represents GSH + 2GSSG; reactive SH groups of hemoglobin (HbSH) were determined according to Boyer [11].

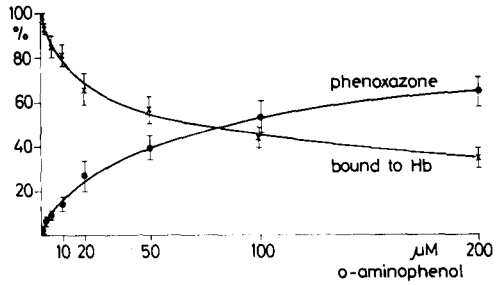

Fig. 8. Dependence of covalent binding and phenoxazone formation on 2-aminophenol concn. [^{14}C]2-Aminophenol was incubated with purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air for 24 hr.

GSH in excess completely prevented covalent binding of 4-DMAP to hemoglobin and markedly reduced this proportion in the case of 2-DMAP. Additionally, the degradation of both dimethylaminophenols was accelerated in the presence of reduced glutathione. When 4-DMAP (0.6 mM) reacted with purified human hemoglobin (3 mM) for 20 hr, 0.6 mM of the reactive SH groups of hemoglobin was blocked. Under similar conditions, 2-DMAP blocked 0.35 mM SH groups, although more than 0.5 mM 2-DMAP disappeared.

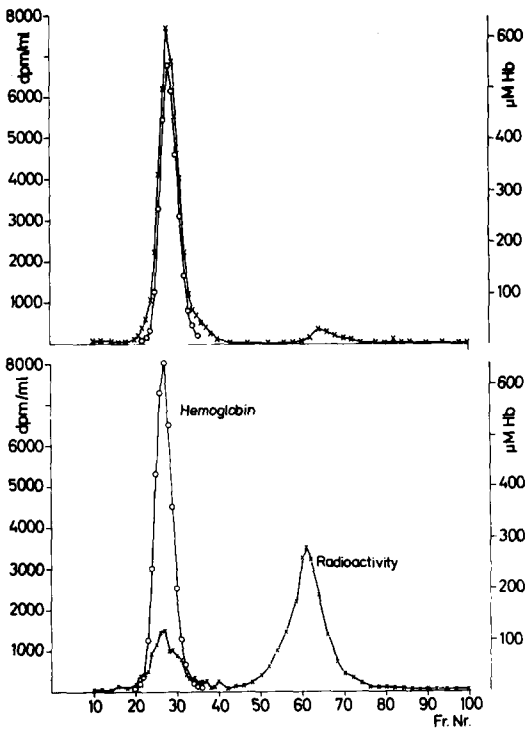

Fig. 9. Elution profile of purified human hemoglobin and radioactivity after reaction with [^{14}C]2-aminophenol, and the effect of GSH. [^{14}C]2-Aminophenol (5 µM), incubated with hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air for 20 hr, was chromatographed on Sephadex G-75 fine (2.5 × 40 cm) with 2 mM phosphate, pH 7.4. Upper panel: without GSH. Lower panel: incubated with 2 mM GSH.

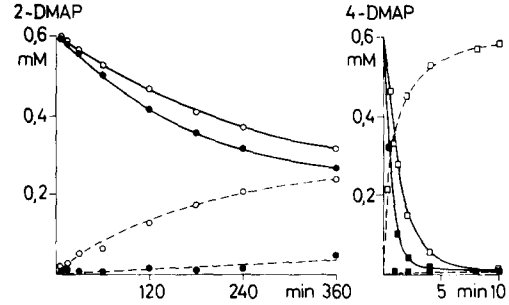

Fig. 10. Influence of GSH on the decrease of 2- and 4-dimethylaminophenol (2- and 4-DMAP) and on covalent binding to hemoglobin. [^{14}C]2-DMAP and [^{14}C]4-DMAP, 0.6 mM each, were incubated with purified human hemoglobin (3 mM Fe in 0.2 M phosphate, pH 7.4) at 37° under air in the absence (open symbols) and presence of 2 mM GSH (closed symbols). Broken lines show the radioactive material covalently bound to hemoglobin in the absence (open symbols) and presence of GSH (closed symbols).

DISCUSSION

From these experiments it is obvious that *o*- and *p*-aminophenols react very similarly with hemoglobin. 2- and 4-AP are oxidized by the activated oxygen [37] in oxyhemoglobin (cf. Fig. 7). Their oxidation products are reduced by ferrohemoglobin, with the formation of ferrihemoglobin. Hence, aminophenols catalytically transfer electrons from ferrohemoglobin to oxygen [33, 38]. This catalyzed ferrihemoglobin formation is terminated by side reactions with consumption of the catalyst. Here, *o*- and *p*-aminophenols react differently.

p-Aminophenols are covalently bound to hemoglobin, mainly to the reactive SH groups, or form thioethers with GSH in the red cell [33, 34]. In contrast, *o*-aminophenols with a primary amino group condense to phenoxazones after their oxidation [39]. The isolated phenoxazones from 2-AP and 2-OHPt are still weak ferrihemoglobin-forming agents by themselves, but are virtually not covalently bound to hemoglobin. Presumably, phenoxazone formation is responsible for the biphasic kinetics of ferrihemoglobin formation in the presence of 2-OHPt. Although all the 2-OHPt had been consumed within 10 min, ferrihemoglobin formation still proceeded and can be attributed to the 2-amino-7-ethoxy-3*H*-phenoxazine-3-one formed in this reaction (cf. Figs. 4 and 5).

The condensation reaction leading to phenoxazones raises a special kinetic problem. Whereas the electrophilic addition of the *o*-quinonimines to the abundant hemoglobin and glutathione follows pseudo-first-order kinetics, the condensation to phenoxazones is clearly a second-order reaction. This implies that phenoxazone formation depends on the square of *o*-quinonimine and hence *o*-aminophenol concn. For example, at 2 mM 2-AP more than 90% of the 2-amino-3*H*-phenoxazine-3-one was formed in the reaction with hemoglobin, whereas at 2 µM 2-AP this proportion was reduced to less than 7% and covalent binding prevailed (cf. Fig. 8). At this low 2-AP concn, the intermediate *o*-quinonimine reacted essentially as the *para* isomer and formed

thioethers with cysteine residues of hemoglobin or GSH. Other electrophilic reactions, e.g. with primary amino groups of amino acids, are apparently negligible. Neither histidine nor lysine competed with the SH groups of hemoglobin (cf. Tables 1 and 2). Correspondingly, blocking of the SH groups of hemoglobin prior to 2-AP addition substantially diminished covalent binding. These results are in contrast to the findings of King *et al.* [40] but agree with those of Hadler and Erwin [41].

The similar behaviour of isomeric aminophenols in hemoglobin solutions observed with 2- and 4-AP only at low concns was confirmed with 2- and 4-DMAP even at high concns. This is because 2-DMAP lacks the primary amino group essential for phenoxazone condensation. Surprisingly, 2-DMAP reacted much slower with hemoglobin than did 4-DMAP. This difference in the isomerism, however, is not shared by all *o*-aminophenols since 2-AP was superior to 4-AP in ferrihemoglobin formation (cf. Fig. 6). Rather, steric hindrance seems to be responsible for the weak reactivity of 2-DMAP in human hemoglobin solutions, because the reactivity with dog hemoglobin was considerably higher and approached that of 4-DMAP.

The puzzling influence of red cells on the reaction rates of 2- and 4-AP with hemoglobin as illustrated in Fig. 6 deserves comment. By the nonhomogeneous distribution of hemoglobin in suspensions of red cells, the concn of hemoglobin at the site of reaction is about 7 times higher than in homogeneous solutions with the same hemoglobin content. Since the velocity of oxidation of 2-AP strongly depends on hemoglobin concn (cf. Fig. 7), ferrihemoglobin formation is accelerated in the concentrated hemoglobin solution within red cells. This influence is less significant in the case of 4-AP. Moreover, oxidized 4-AP is trapped by the cellular GSH. Hence, diminished ferrihemoglobin formation results as was also reported for 4-DMAP [34].

4-AP, a long known metabolite of phenacetin [42, 43], was earlier ruled out as the candidate responsible for the occasionally observed methemoglobinemia after phenacetin application [44]. Kiese instead considered *N*-oxygenated products of phenetidine as the causative metabolites. In fact, 4-NOPt produced roughly 6 times more ferrihemoglobin in dogs than 4-AP. This potent ferrihemoglobin forming activity of 4-NOPt was confirmed by our results. Moreover, 2-OPHt, though only half as active, should also be considered. Whereas Kiese and Weger [6] reported only faint ferrihemoglobin-forming activity for 2-OHPt, Shahidi *et al.* [7–9] presented evidence for the involvement of 2-OHPt in methemoglobinemia after phenacetin ingestion.

The importance of 2-OHPt was also supported by Dubach and Raaflaub [3], who found an overproportional production of 2-OHPt with increasing doses of phenacetin in humans. Consequently, Calder *et al.* [5] also included 2-OHPt in their study on potentially nephrotoxic metabolites of phenacetin. Surprisingly, neither 2-OHPt nor 2-AP exhibited any significant nephrotoxicity, in contrast to 4-AP. This is a further example of the lack of correlation between ferrihemoglobin formation and organ toxicity as already deduced by Neish [45]. According to our experiments, the results of Calder *et al.* [5] seem plausible, since at such high *o*-aminophenol concns the ultimate toxic *o*-quinonimines detoxify themselves by phenoxazone formation.

The dependence of the different reaction pathways on 2-AP concn (cf. Fig. 8) presumably has a more general toxicological significance. One would expect that long-term exposure to low doses of those *o*-aminophenols, which are capable of forming a phenoxazone, leads to a greater extent of covalent binding than a single exposure to the same total load, where self-detoxication prevails. This phenomenon should be kept in mind in considering the safety of threshold doses during long-term exposure. For risk assessment, toxicological data are usually extrapolated to infinitesimally low doses implying that toxicity and covalent binding are proportional to the dose [46] or even underproportional as suggested by Cornfield [47] and Gehring and Blau [48]. This "saturation hypothesis" implies some safety below a threshold dose. From our results with *o*-aminophenols, this ratio may even be overproportional when the dose is lowered. Hence, extrapolation to the inaccessible region at low doses seems questionable as long as reaction mechanisms underlying the toxicity are unknown.

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