Biochemical Pharmacology, Vol. 36, No. 18, pp. 2963–2972, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00
Pergamon Journals Ltd.

N-HYDROXY-*N*-ARYLACETAMIDES—IV*

DIFFERENCES IN THE MECHANISM OF HAEMOGLOBIN OXIDATION *IN VITRO* BETWEEN *N*-HYDROXY-*N*-ARYLACETAMIDES AND ARYLHYDROXYLAMINES

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(*Received* 4 *September* 1986; *accepted* 23 *March* 1987)

Abstract—In solutions of purified human haemoglobin *N*-hydroxy-4-chloroacetanilide (*N*-hydroxy-4ClAA) was one of the most active compounds and *N*-hydroxy-acetanilide (*N*-hydroxy-AA) was the least active compound among the six *N*-hydroxy-*N*-arylacetamides tested for ferrihaemoglobin ($HbFe^{3+}$)-forming activity. Co-oxidation of haemoglobin by *N*-hydroxy-4-chloroacetanilide was compared with that of *N*-hydroxy-4-chloroaniline(*N*-hydroxy-4ClA) and found to differ in the kinetics of $HbFe^{2+}$-oxidation, in the catalytic activity of the two compounds, in the activation energy, and in the product pattern, indicating that the mechanism by which *N*-hydroxy-*N*-arylacetamides oxidize oxyhaemoglobin *in vitro* is different from that of arylhydroxylamines. Attempts have failed to detect by EPR spectroscopy acetyl 4-chlorophenyl nitroxide radical, the postulated catalytically-active oxidation product of *N*-hydroxy-4-chloroacetanilide.

Oxyhaemoglobin from different species in isolated form was found to display various enzyme activities. Human haemoglobin A and haemoglobin S for example, showed esterase activity, i.e. catalysed the hydrolysis of 4-nitrophenylacetate [2]. Human haemoglobin was also found to display peroxygenase activity, i.e. to catalyse C-oxygenation in a manner typical of the monooxygenase reaction observed with hepatic microsomal cytochrome P-450. In the presence of NADPH-cytochrome P-450 reductase, NADPH, and oxygen, haemoglobin catalysed *p*-hydroxylation of aniline and *N*-demethylation of benzphetamine [3]. Dog and bovine haemoglobin were found to display peroxidase activity, i.e. to catalyse electron transfer from phenylhydroxylamine to oxygen, whether bound to haemoglobin or molecular oxygen [4], likewise shark haemoglobin and human haemoglobin catalysed electron transfer from epinephrine [5] and 4-dimethylaminophenol [6], respectively, to oxygen bound to haemoglobin. In a coupled reaction, phenylhydroxylamine reacted with oxyhaemoglobin in the presence of molecular oxygen to yield ferrihaemoglobin and nitrosobenzene, epinephrine yielded ferrihaemoglobin and adrenochrome, and 4-dimethylaminophenol gave the corresponding phenoxy radical. This phenoxy radical catalysed ferrihaemoglobin formation by 50–100 electron transfers per molecule 4-dimethylaminophenol.

Autoxidation of oxyhaemoglobin [$HbFe(II)^{\delta(+)}$–$O_2{}^{\delta(-)}$] can be accelerated by electron-donating organic molecules, oxygen being released as superoxide ($O_2{}^{\cdot}$), [5, 7–10]. Superoxide is a source for singlet oxygen ($^1O_2$) [11, 12] and rapidly dismutates to yield O_2 and H_2O_2 [13]; H_2O_2 in the presence of electron-donating molecules, such as superoxide, yields OH-radicals [14]. Thus, several reactive and electrophilic oxygen species are produced during haemoglobin autoxidation which may effect oxidation of electron-donating organic molecules.

Here we wish to report on the results of our experiments showing the differences in the mechanism of haemoglobin oxidation between *N*-hydroxy-*N*-arylacetamides and arylhydroxylamines.

Short communications on this subject have appeared [15–17].

MATERIALS AND METHODS

N-Hydroxy-N-arylacetamides. Preparation and properties of *N*-hydroxyacetanilide (*N*-hydroxy-AA), *N*-hydroxy-4-chloroacetanilide (*N*-hydroxy-4ClAA), *N*-hydroxy-3,4-dichloroacetanilide (*N*-hydroxy-3,4Cl_2AA), *N*-hydroxyphenacetin (*N*-hydroxy-4EAA), *N*-hydroxy-4-acetylaminobiphenyl (*N*-hydroxy-4AAB), and *N*-hydroxy-2-acetylaminofluorene(*N*-hydroxy-2AAF) were described in Part II of this series [18].

[Ring-^{14}C]-*N*-Hydroxy-4-chloroacetanilide was prepared from [ring-^{14}C]-*N*-hydroxy-4-chloroaniline and acetic anhydride in ether containing acetic acid. The preparation of [ring-^{14}C]-*N*-hydroxy-4-chloroaniline was carried out as described recently [19]. [Ring-^{14}C]-*N*-hydroxy-4-chloroacetanilide had a specific activity of 1700 dpm/nmol.

Preparation and properties of *N*-hydroxy-4-chloroaniline(*N*-hydroxy-4ClA),4-chloronitrosobenzene-(4-ClNOB), 4-chloroaniline(4-ClA), and 4-chloroacetanilide(4-ClAA) were also described in [18].

* Communication III in this series: [1].

Sodium dithionite, sodium nitrite, ammonium sulphamate, potassium ferricyanide, potassium cyanide, potassium thiocyanate, *N*-1-naphthylethylenediamine dihydrochloride, and 2-butanone were of analytical grade and purchased from E. Merck AG, D-6100 (Darmstadt). 4-Chloronitrobenzene was purchased from Fluka AG, CH-9740 Buchs SG, superoxide dismutase and 4-chloromercuribenzoate from Sigma Chemie GmbH, D-8028 Taufkirchen, and catalase from Boehringer-Mannheim GmbH, D-6800 Mannheim.

Sephadex® G 100 fine and Sephadex® G 25 were purchased from Deutsche Pharmacia GmbH, D-7800 Freiburg, and DE 52-cellulose (Whatman) from Vetter KG, D-6900 Wiesloch.

Solvents. Diethylether DAB 7 was distilled before use; methanol, benzene, chloroform, and acetic acid were of analytical grade and purchased from E. Merck, Darmstadt.

Purified human haemoglobin was prepared from outdated human blood. Fifty ml of the concentrated human erythrocytes were washed 5 times with an equal volume of 0.2 M phosphate buffer pH 7.4. After final centrifugation, the packed cells were haemolysed by adding 100–200 ml distilled water, some saponin, by sonication, and the haemolysate centrifuged at 18,800 *g* for 20 min. Seventy ml of the supernatant was diluted to a concentration of 10 g Hb per 100 ml and chromatographed on a column of Sephadex® G 100 fine (7 cm i.d. × 80 cm, ascending) using 0.01 M Tris–HCl buffer pH 8.3 as solvent. The fractions containing Hb were combined and chromatographed on a column of DE 52-cellulose (4 cm i.d. × 15 cm, descending) by using a linear chloride gradient from 750 ml 0.01 M Tris–HCl buffer pH 8.3 to 0.1 M Tris–HCl buffer pH 7.0. For concentration, the combined Hb fractions were applied to a column of DE 52-cellulose (3 cm i.d. × 8 cm) and left at +4° for some hours. Haemoglobin was eluted from the column with the solvent 0.5 M NaCl in 0.1 M Tris–HCl buffer pH 7.0, yield: 30–25 ml 10–14% purified Hb. This haemoglobin preparation was dialyzed for 12 hr at +4° against 0.2 M phosphate buffer pH 7.4 before use and was found to contain 1–3% ferrihaemoglobin. The preparation was essentially free from SOD and catalase [20].

Carbonylhaemoglobin. Carbon monoxide was passed through a solution of purified haemoglobin for 60 min at room temperature. After addition of a small amount of sodium dithionite, the solution was applied to a column of Sephadex® G 25. Carbonylhaemoglobin was eluted by using CO-saturated 0.2 M phosphate buffer, pH 7.4.

Ferrihaemoglobin was prepared by addition of twice the stoichiometric amount of potassium ferricyanide to a solution of purified haemoglobin. Purified ferrihaemoglobin was obtained by chromatography of the solution on a column of Sephadex® G 25 with 0.2 M phosphate buffer pH 7.4.

Haemoglobin with blocked SH-groups was prepared by allowing 4-chloromercuribenzoate (twice the stoichiometric amount of $HbFe^{2+}$) to react with purified haemoglobin in 0.2 M phosphate buffer, pH 7.4, for 30 min at room temperature. The reaction mixture was dialyzed for 12 hr against 0.2 M phosphate buffer, pH 7.4, at +4°; determination of the SH-groups according to Boyer [21] showed that the two reactive SH-groups had been blocked.

Globin was prepared from purified haemoglobin according to Teale [22]. A solution of haemoglobin was dialyzed against water for 12 hr, mixed at 0° with 1 M HCl to give a pH 1.9 and extracted twice with an equal volume of ice-cold 2-butanone. The aqueous phase containing globin was dialyzed against water and lyophilized to yield solid globin.

METHODS

U.v. absorption spectra and absorbancies were measured with a Varian Cary 118 spectrophotometer. Kinetics of $HbFe^{2+}$ oxidation were measured either with an Aminco-Morrow stopped-flow accessory of an Aminco DW 2a spectrometer or with a Zeiss PMQ 3 spectrophotometer.

$HbFe^{3+}$ *determination.* $HbFe^{3+}$ concentrations in solutions of haemoglobin were determined at 550 nm by measuring the increase in absorbance caused by addition of potassium cyanide to the sample cuvette according to Evelyn and Malloy [23] with the modification of Kiese [24]. For continuous monitoring at 630 nm either a split-beam spectrophotometer or a dual-wavelength spectrophotometer and a reference wavelength of 680 nm was used.

Determination of N-hydroxy-4-chloroaniline and 4-chloronitrosobenzene in blood, erythrocyte suspensions, and solutions of haemoglobin. N-Hydroxy-4-chloroaniline or 4-chloronitrosobenzene in blood, erythrocyte suspensions, or solutions of haemoglobin was determined as 4-chloronitrosobenzene according to Herr and Kiese [25], modified by Lenk and Sterzl [18]. Aliquots of 1 ml blood or suspension were haemolysed with 8 ml water, oxidized by 0.1 ml 10% potassium ferricyanide, and the mixture shaken with 6 ml CCl_4 for 10 min. After centrifugation, the aqueous layer was discarded and the CCl_4 phase washed twice with 10 ml 0.025 M H_2SO_4 to remove 4-chloroaniline, and once with water. To the CCl_4 phase, 2 ml acetic acid and 0.1 ml aqueous 20% sodium nitrite were added. After 15 min, 0.5–1.0 ml aqueous 50% ammonium sulphamate was added, and after a further 10 min, 0.1 ml aqueous 0.5% *N*-1-naphthylethylenediamine dihydrochloride. After 2 hr in the dark, the absorbance at 555 nm of the violet azo dye was read. A blank was prepared from blood, erythrocyte suspension, and haemoglobin solution without any additive. Calibration curves were obtained with increasing concentrations of *N*-hydroxy-4-chloroaniline.

Determination of 4-chloroaniline in blood and erythrocyte suspensions. Aliquots of 1 ml blood or erythrocyte suspension were haemolysed, oxidized, and shaken with 6 ml CCl_4 as described above. However, the CCl_4 phase was not washed with 0.025 M H_2SO_4, so that both, 4-chloroaniline *and* 4-chloronitrosobenzene were converted to the violet azo dye by the subsequent procedure. Absorbancies read at 555 nm were corrected for 4-chloronitrosobenzene and the residual absorbancies expressed as 4-chloroaniline by using a calibration curve obtained with increasing concentrations of 4-chloroaniline.

Extraction procedure. To determine the amounts of *N*-hydroxy-4-chloroacetanilide, 4-chloronitroso-

benzene, 4-chloronitrobenzene, 4-chloroaniline, and 4-chloroacetanilide, aliquots of haemoglobin solutions incubated at 37° with *N*-hydroxy-4-chloroacetanilide were extracted twice with 25 ml ether. The combined ether extracts were shaken twice with 15 ml 2 M NaOH to remove residual *N*-hydroxy-4-chloroacetanilide from the neutral and basic metabolites in the ether extracts (ether 1). The two alkaline phases were combined, acidified with 35 ml 2 M HCl, and extracted twice with 50 ml ether. The combined ether extracts were dried over Na_2SO_4 and evaporated *in vacuo* to yield the recovered *N*-hydroxy-4-chloroacetanilide (ether 2). To determine the residual amount of 4-chloronitrosobenzene bound to haemoglobin-Fe^{2+}, the solution was shaken for 15 min with 20 ml ether and 1 ml 10% potassium ferricyanide to yield ether 3 extract. The components of ether 1 and ether 3 were identified and determined by HPLC, and *N*-hydroxy-4-chloroacetanilide in ether 2 by u.v. spectroscopy.

Reaction mixtures of haemoglobin and either *N*-hydroxy-4-chloroaniline or 4-chloronitrosobenzene were extracted with ether to yield ether 1 extract; since no *N*-hydroxy-4-chloroacetanilide was present, no ether 2 extract was prepared. Ether 1 and ether 2 extracts were analysed by HPLC.

Assay for N-*hydroxy-4-chloroacetanilide, 4-chloronitrosobenzene, 4-chloronitrobenzene, 4-chloroaniline, and 4-chloroacetanilide.* Ether 1 and ether 3 extracts containing the neutral and basic metabolites of *N*-hydroxy-4-chloroacetanilide, *N*-hydroxy-4-chloroaniline, and 4-chloronitrosobenzene were analysed by HPLC with an isocratic basic system from Waters Ass. Inc. (Milford, MA), using a μ-Bondapak C_{18}-column and methanol–water = 70:30 (v/v) as solvent. The resolved metabolites were detected by their u.v. absorbance at 254 and 280 nm and quantified by comparing the area under the peaks with those of known amounts of authentic compounds. The following retention times (R_t) were observed at a flow rate of 2 ml/min and a pressure of 2500 psi: 4-chloronitrosobenzene (4.2 min), 4-chloronitrobenzene (3.7 min), 4-chloroaniline (2.2 min), and 4-chloroacetanilide (2.7 min).

Since *N*-hydroxy-4-chloroacetanilide disturbed HPLC of the neutral and basic metabolites by its tailing effect, we have removed its residual amounts from ether extracts by 2 M NaOH and re-extracted it into ether after acidification (ether 2). The amount of recovered *N*-hydroxy-4-chloroacetanilide was determined by u.v. spectroscopy by reading the absorbance of its (i) neutral methanol solution at $\lambda = 257$ nm using $\epsilon_{257} = 13980$ and (ii) alkaline methanol solution at $\lambda = 298$ nm using $\epsilon_{298} = 8490$.

EPR measurement. EPR measurements to detect acetyl 4-chlorophenyl nitroxide radical in solutions of purified haemoglobin containing *N*-hydroxy-4-chloroacetanilide were performed at room temperature with a Varian E 109 spectrometer as well as with a Bruker ER 200 D-SRC spectrometer.

RESULTS

Kinetics of haemoglobin oxidation by N-*hydroxy-4-chloroacetanilide*

To determine the order of the reaction in respect of the two reactants, haemoglobin-Fe^{2+} and *N*-hydroxy-4ClAA, and of the total reaction, initial velocities of $HbFe^{2+}$ oxidation were measured by keeping the concentration of one reactant constant and varying the concentration of the other or *vice versa*. On plotting $\log\frac{(dHbFe^{3+})}{dt}$ versus log ($HbFe^{2+}$) or log(*N*-hydroxy-4ClAA) from Table 1, straight lines were obtained with the slope of 1 (plots not shown), i.e. the reaction is first order in respect of ($HbFe^{2+}$) and (*N*-hydroxy-4ClAA), and the total reaction is second order. An expression for the rate of $HbFe^{3+}$ formation therefore is equation (1).

$$\frac{+\,d(HbFe^{3+})}{dt} = k \times (HbFe^{2+}) \times (N\text{-hydroxy-4ClAA}) \quad (1)$$

Table 1. Rate coefficient of haemoglobin oxidation by *N*-hydroxy-4-chloroacetanilide determined from initial velocities

N-hydroxy-4ClAA mM	$HbFe^{2+}$ mM	$HbFe^{3+}$* nM × sec^{-1}	k l × mol^{-1} × sec^{-1}
0.131	0.105	46.9	3.41
0.131	0.211	79.2	2.86
0.131	0.729	374.9	3.93
0.261	0.105	103.6	3.78
0.522	0.105	185	3.37
0.020	0.506	46	4.54
0.050	0.506	125.5	4.96
0.100	0.506	245.9	4.85
0.020	5.06	433.3	4.28
0.050	5.06	1531.2	6.05
0.100	5.06	2538.5	5.02
			M = 4.28 ± 0.28 (SEM)

* Formed at 37° and determined with a Zeiss PMQIII spectrometer at 630 nm; data in column 3 are corrected for haemoglobin autoxidation.

Table 2. Dependence of the catalytic activity on the molar ratio of *N*-hydroxy-4-chloroacetanilide to haemoglobin-Fe^{2+}

N-hydroxy-4ClAA mM	$HbFe^{2+}$ mM	$HbFe^{2+}$ produced after 2 hr* %	mM	equiv.
2.73	2.73	100†	2.73†	1
0.11	2.73	68.7	1.875	17
0.055	2.73	53.5	1.46	26
0.011	2.73	19.4	0.53	48
0.108	2.7	82	2.214	20
0.055	2.7	71	1.911	35
0.011	2.7	33	0.891	81
0.1	5.06	67	3.39	34
0.05	5.06	46.5	2.353	47
0.02	5.06	28.5	1.442	72

* Formed at 37° and determined at 550 nm with the cyanide method; data in column 3 are corrected for haemoglobin autoxidation.

† After 60 min incubation.

Rate coefficients for the second order oxidation were determined from the slope of the tangents on the linear segments of the recorded kinetics. Depending on the ratio of (*N*-hydroxy-4ClAA) to ($HbFe^{2+}$), linear segments were observed up to 7 min, during which time < 10% of total $HbFe^{2+}$ was oxidized. Rate coefficients, shown in Table 1, suggest that the rate of the reaction increased with increasing ratio of the two reactant concentrations.

When we determined the temperature-dependency of the reaction by varying the temperature of the reaction between 10 and 37°, we obtained an activation energy E_a of 12.7 kcal × mol^{-1} from the linear Arrhenius plots.

Catalytic activity of N-*hydroxy-4-chloroacetanilide*

When the ratio of *N*-hydroxy-4ClAA concentration to $HbFe^{2+}$ concentration was 1:25, each *N*-hydroxy-4ClAA oxidized 16 equivalents of $HbFe^{2+}$ in 2 hr. When the molar ratio was further increased to 1:245, each *N*-hydroxy-4ClAA oxidized 81 equivalents of $HbFe^{2+}$ in 2 hr, i.e. on increasing the molar ratio from 1:25 to 1:245, the catalytic activity of *N*-hydroxy-4ClAA increased about 4-fold (Table 2).

Kinetics of haemoglobin oxidation by N-*hydroxy-4-chloroaniline*

We have also studied the kinetics of haemoglobin oxidation by *N*-hydroxy-4ClA. The order of the reaction was also determined by keeping the concentration of one reactant constant and varying the concentration of the other and *vice versa*. Plots of log $\frac{(dHbFe^{3+})}{dt}$ versus log ($HbFe^{2+}$) or log(*N*-hydroxy-4ClA) were also linear with the slope of 1, i.e. the reaction is also first order in respect of (*N*-hydroxy-4ClA) and ($HbFe^{2+}$) and second order in respect of the total reaction (plots not shown).

Rate coefficients of the second order oxidation were determined from the slope of the tangents on the linear segments of the kinetics, which were recorded with an Aminco-Morrow stopped-flow apparatus (Table 3). The rate constant of 7800 l × mol^{-1} × sec^{-1} for *N*-hydroxy-4ClA as compared to 4.28 l × mol^{-1} × sec^{-1} for *N*-hydroxy-4ClAA showed that the arylhydroxylamine reacted 1800 times faster with $HbFe^{2+}$ than did the *N*-hydroxy-*N*-arylacetamide. In addition, the temperature dependency of the reaction, determined between 10 and 37°, gave an activation energy of E_a = 3.5 kcal × mol^{-1} from the linear Arrhenius plot.

Catalytic activity of N-*hydroxy-4-chloroaniline*

To determine how many equivalents of $HbFe^{2+}$ can be oxidized by each *N*-hydroxy-4ClA, the concentration of $HbFe^{2+}$ was kept constant (2.7 mM) and the concentration of *N*-hydroxy-4ClA varied. On addition of an equimolar concentration of *N*-hydroxy-4ClA, less than 1 equivalent of $HbFe^{3+}$ was formed instantaneously (0.7), but on addition of 1.35, 0.67, and 0.106 mM *N*-hydroxy-4ClA, 1 equivalent of $HbFe^{3+}$ (Fig. 1). After the rapid initial reaction a second slow phase of haemoglobin oxidation followed, by which 1 further equivalent of $HbFe^{3+}$ was formed in 2 hr.

Table 3. Rate coefficient of haemoglobin oxidation by *N*-hydroxy-4-chloroaniline determined from initial velocities

N-hydroxy-4ClA μM	$HbFe^{2+}$ μM	$HbFe^{3+}$* μM × sec^{-1}	*k* l × mol^{-1} × sec^{-1}
54	107	44.2	7642
107	107	87.7	7656
215	107	191.6	8327
429	107	378.6	8248
107	54	49.9	8642
107	107	87.7	7656
107	215	150.7	6551
			M = 7844 ± 300 (SEM)

* Formed at 37° and determined with the Aminco–Morrow stopped-flow accessory of an Aminco DW 2 A spectrometer at 630 nm and a reference wavelength of 680 nm.

Metabolites of N-*hydroxy-4-chloroacetanilide,* N-*hydroxy-4-chloroaniline, and* 4-*chloronitrosobenzene produced by co-oxidation with haemoglobin*

Two attempts have failed to establish a similar relationship between $HbFe^{3+}$ concentration and the concentration of 4-ClNOB with purified human haemoglobin and *N*-hydroxy-4ClAA as with $HbFe^{3+}$ in rat erythrocytes *in vitro* [1]. Concentrations of 5.1 and 13.7 mM haemoglobin-Fe^{2+} incubated with 100 and 330 μM *N*-hydroxy-4ClAA, respectively, produced increasing $HbFe^{3+}$ concentrations, but no increase in 4-ClNOB concentration. Obviously, the 4-ClNOB concentrations were below the sensitivity range of the modified Herr and Kiese method [18], i.e. ≤ 7 μM. That this is the case, was shown by HPLC, see Table 4. However, the Herr and Kiese

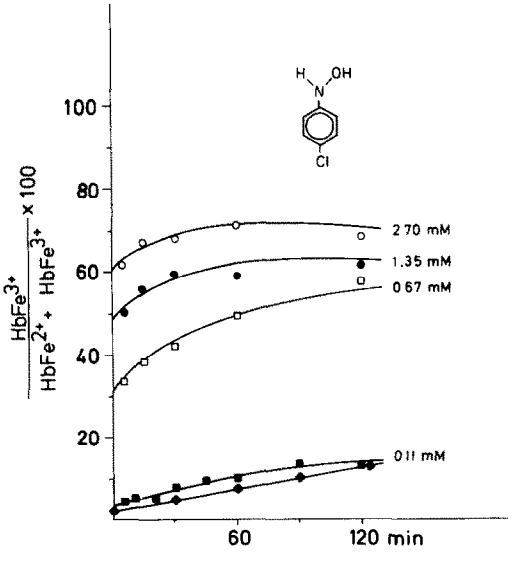

Fig. 1. Kinetics of ferrihaemoglobin formation *in vitro* by *N*-hydroxy-4-chloroaniline. A solution of purified human haemoglobin (2.24 mM $HbFe^{2+}$) in 0.2 M phosphate buffer pH 7.4 was incubated at 37° with various concentrations of *N*-hydroxy-4-chloroaniline indicated in the graph. Symbols represent data from single experiments with 2 ml reaction mixture each and are corrected for haemoglobin autoxidation (◆–◆–◆).

Table 4. Product pattern of *N*-hydroxy-4-chloroacetanilide, *N*-hydroxy-4-chloroaniline, and 4-chloronitrosobenzene produced by incubation at 37° with isolated human haemoglobin, a haemoglobin, whose SH-groups were blocked by *p*-chloromercuribenzoate, by carbonylhaemoglobin, ferrihaemoglobin and globin

Enzyme source:	—	—	3.13 mM $HbFe^{2+}$		3.11 mM PCMB-Hb		1 mM HbCO	2.6 mM $HbFe^{3+}$		0.42 mM Globin	3.3 mM $HbFe^{2+}$	3.3 mM $HbFe^{2+}$	
Substrate:	1.0 mM *N*-hydroxy-4-chloro-acetanilide		1.05 mM *N*-hydroxy-4-chloro-acetanilide		1.08 mM *N*-hydroxy-4-chloro-acetanilide		1.0 mM *N*-hydroxy-4-chloro-acetanilide	1.0 mM *N*-hydroxy-4-chloro-acetanilide		1.02 mM *N*-hydroxy-4-chloro-acetanilide	3.0 mM *N*-hydroxy-4-chloro-aniline	3.0 mM 4-chloro-nitroso-benzene	
Incubation time (min)	2	60	2	60	2	60	60	2	60	60	2	2	60
$HbFe^{3+}$ %	*	*	42	96	64	100	*	100	100	*	53	8.5	68.2
% Recovered	90	93.6	75.6	73.9	79.3	77.7	87.2	76.2	75	83	*	*	*
4-Chloroaniline (%)	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	0.5	0.1	0.5
4-Chloro-acetanilide (%)	0.1	0.1	1.8	5.0	0.8	0.3	0.1	0.7	1.5	0.1	†	†	†
4-Chloronitroso-benzene (%)	0.1	0.1	0.9	0.5	0.5	0.4	0.3	0.6	0.6	0.1	25.1	30.4	20.0
4-Chloronitro-benzene (%)	0.1	0.1	0.7	0.4	0.1	0.1	0.1	0.2	0.1	0.1	0.5	0.5	0.8
Means of N experiments	2		3		2		4	2		2	Single	Single	

* Not determined.

† Not detected.

‡ Trace amounts, not unambiguously identified.

Data are from N experiments with purified human haemoglobin incubated at 37° with *N*-hydroxy-4-chloroacetanilide, *N*-hydroxy-4-chloroaniline, and 4-chloronitrosobenzene for times indicated. Aliquots were analysed for $HbFe^{3+}$ and metabolites of *N*-hydroxy-4-chloroacetanilide, *N*-hydroxy-4-chloroaniline, and 4-chloronitrosobenzene as described under Methods.

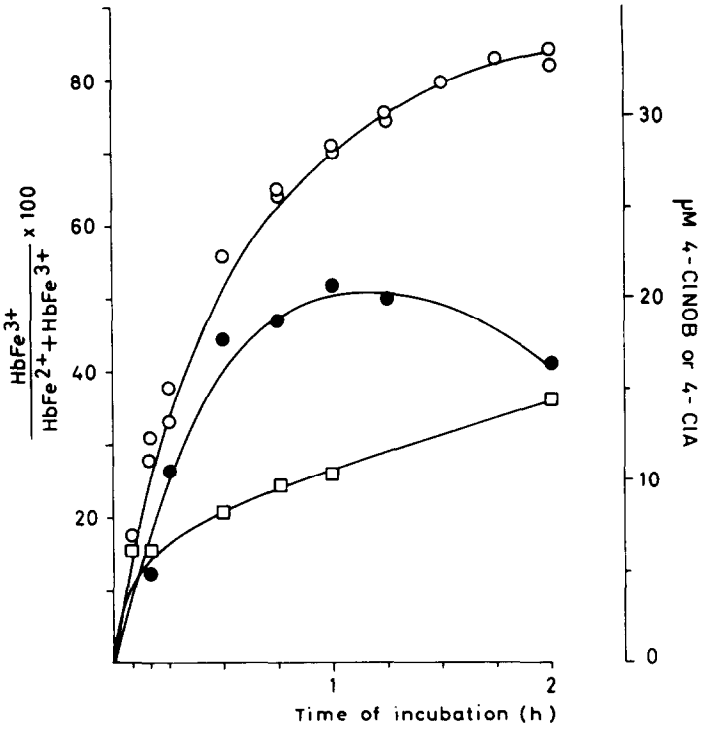

Fig. 2. Co-oxidation of haemoglobin-Fe^{2+} and *N*-hydroxy-4-chloroacetanilide in erythrocytes *in vitro*. Ferrihaemoglobin (○–○–○), 4-chloronitrosobenzene (●–●–●) and 4-chloroaniline (□–□–□) produced by washed human erythrocytes (8.5 mM $HbFe^{2+}$ after haemolysis; approx. 25 mM $HbFe^{2+}$ inside the red cell), suspended in 0.2 M Krebs–Ringer phosphate pH 7.4 without additives and incubated at 37° with 330 μM *N*-hydroxy-4-chloroacetanilide. Volume: 30 ml suspension; 0.1 ml was used for $HbFe^{3+}$ determination and 1.0 ml each for the determination of 4-chloronitrosobenzene and the sum of 4-chloronitrosobenzene and 4-chloroaniline.

method is superior over HPLC, because (i) major losses of the volatile 4-ClNOB and 4-ClA can be avoided and (ii) continuous monitoring of 4-ClNOB concentrations is less time-consuming. Its disadvantages, however, are (i) a certain lack of specificity and (ii) its limitation to those metabolites which can be diazotized.

However, on incubation of washed human erythrocytes (8.5 mM $HbFe^{2+}$ after haemolysis) with 330 μM *N*-hydroxy-4ClAA, a parallel increase of $HbFe^{3+}$, 4-ClNOB, and 4-ClA concentration was observed, indicating (i) accumulation of 4-ClNOB during haemoglobin oxidation and (ii) partial reduction of the newly-formed 4-ClNOB, which depended on external substrates of the glycolysis pathway, see Fig. 2. On incubation of purified haemoglobin (2.77–3.5 mM $HbFe^{2+}$) with 1.0 mM *N*-hydroxy-4ClAA, haemoglobin was completely oxidized after 1 hr (Table 4), and larger concentrations of 4-ClAA, 4-ClNOB, and 4-$ClNO_2B$ were determined as in the control experiment without haemoglobin. The concentration of 4-ClAA increased with increasing $HbFe^{3+}$ concentration, but the concentration of 4-ClNOB and 4-$ClNO_2B$ increased in the first two min and then declined. This is because 4-ClAA is neither volatile nor reactive and accumulated during haemoglobin oxidation. But 4-ClNOB and 4-$ClNO_2B$ are volatile and the former can react with the SH-groups of haemoglobin. To correct the data in Table 4 for losses of 4-ClNOB, we include the results of recovery experiments with *N*-hydroxy-4ClA and 4-ClNOB, because *N*-hydroxy-4ClA is instantaneously oxidized to 4-ClNOB and determined as such. The results of either experiment show that 2 min after the rapid initial reaction with haemoglobin, 75 and 70%, respectively, were lost.

When we analysed the reaction mixture of carbonylhaemoglobin or globin and *N*-hydroxy-4ClAA, we found metabolite concentrations similar as in the control experiment without haemoglobin, indicating that oxygen bound to the ferrohaem is an absolute requirement for the metabolization of *N*-hydroxy-4ClAA.

On addition of *N*-hydroxy-4ClAA to haemoglobin, whose SH-groups were blocked by 4-chloromercuribenzoate, we observed accelerated haemoglobin autoxidation and lower concentrations of the three metabolites. This is in agreement with the results of other groups [26–28].

When we incubated purified ferrihaemoglobin with *N*-hydroxy-4ClAA, we determined concentrations of the three metabolites which exceeded those of the control experiment, indicating that also ferrihaemoglobin is capable of oxidizing *N*-hydroxy-4ClAA, although to a lesser extent.

Search for ^{14}C-labeled haemoglobin. Purified haemoglobin (3.1 mM $HbFe^{2}$) was incubated with 3.17 mM ^{14}C-N-hydroxy-4ClAA at 37°. After 2 and 60 min aliquots were chromatographed on a Sephadex® G 75 with 2 mM phosphate buffer pH 7.4

as solvent. The eluate was collected in fractions of 5 ml, whose absorbance at 254 nm and radioactivity were determined.

Two peaks were found in the elution profile. Peak 1 contained the fraction with haemoglobin and peak 2 the fractions containing ^{14}C-*N*-hydroxy-4ClAA and metabolites. After 2 min incubation, 77% $HbFe^{3+}$ (mean of 2 experiments) and 1.5% of the added radioactivity, and after 60 min, a mean of 76% $HbFe^{3+}$ and 4.7% radioactivity, was determined in peak 1. Radioactivity in peak 2 was extracted into ether and analysed by HPLC to yield 80/77% *N*-hydroxy-4ClAA (recovered), 2.1/2.5% 4-ClAA, and 0.1/0.1% 4-ClNOB and 4-ClNO_2B, determined together. Twenty percent of the radioactive material in peak 1 was extracted into ether, but the amount of radioactive material was too low to be analysed by HPLC. These results indicated that binding of *N*-hydroxy-4ClAA or its metabolites to haemoglobin was negligible.

The effect of superoxide dismutase and catalase on N-hydroxy-4-chloroacetanilide-induced haemoglobin oxidation

To a solution of haemoglobin (0.11 mM $HbFe^{2+}$) was added 900–1000 U of superoxide dismutase and 0.11 mM *N*-hydroxy-4ClAA and the mixture incubated at 37°. Superoxide dismutase impaired *N*-hydroxy-4ClAA-induced haemoglobin oxidation by maximal 10% as compared with the experiment without SOD.

When haemoglobin (0.11 mM $HbFe^{2+}$) containing 15% $HbFe^{3+}$ was incubated with 900 U SOD per ml and 0.11 mM *N*-hydroxy-4ClAA, SOD impaired *N*-hydroxy-4ClAA-induced haemoglobin oxidation by 33%.

When oxyhaemoglobin was mixed with $HbFe^{3+}$ to yield a concentration of 50% $HbFe^{3+}$, and then incubated with 900 U SOD per ml and 0.1 mM *N*-hydroxy-4ClAA, the rate of haemoglobin oxidation was not significantly different from that in the absence of SOD. These results indicated that superoxide dismutase did not significantly affect haemoglobin oxidation by *N*-hydroxy-4ClAA.

The requirement of molecular oxygen for the N-hydroxy-4-chloroacetanilide- and N-hydroxy-4-chloroaniline-induced haemoglobin oxidation

After addition of an equimolar concentration of *N*-hydroxy-4ClAA to 0.11 mM haemoglobin-Fe^{2+} at room temperature, a slow increase in absorbance at 630 nm, a slow decrease at 576 and 540 nm, and a shift of the Soret band to 404 nm was observed as an expression of slow ferrihaemoglobin formation. When the experiment was performed with deoxyhaemoglobin under N_2, no spectroscopic changes of the deoxyhaemoglobin spectrum were observed during the first 15 min.

After addition of an equimolar concentration of *N*-hydroxy-4ClA to 0.11 mM haemoglobin-Fe^{2+} at room temperature, the absorption maxima of oxyhaemoglobin at 576, 540, and 413 nm disappeared and the Soret band shifted to 404 nm as an expression of ferrihaemoglobin formation. But after addition of

Table 5. Kinetic and catalytic properties of six *N*-hydroxy-*N*-arylacetamides in the oxidation of purified haemoglobin *in vitro*

	Kinetic properties	Catalytic properties: 5.04 mM haemoglobin-Fe^{2+}, 50 μM *N*-hydroxy-*N*-arylacetamide, $HbFe^{3+}$ produced in 3 hr			Catalytic properties: 5.04 mM haemoglobin-Fe^{2+}, 20 μM *N*-hydroxy-*N*-arylacetamide, $HbFe^{3+}$ produced in 3 hr		
	k $l \cdot mol^{-1} \cdot sec^{-1}$	%	μM	equiv. $HbFe^{2+}$ oxidized/ *N*-Hydroxy-*N*-arylacetamide	%	μM	equiv. $HbFe^{2+}$ oxidized/ *N*-hydroxy-*N*-arylacetamide
N-hydroxy-acetanilide	0.5 ± 0.02 (14)	13	655	13	6	302	15
N-hydroxy-4-chloro-acetanilide	3.4 ± 0.2 (36)	53	2671	53	31	1562	78
N-hydroxy-3,4-dichloroacetanilide	5.0 ± 0.2 (5)	44	2218	44	29	1462	73
N-hydroxyphenacetin	1.2 ± 0.03 (10)	29	1462	29	10	529	26
N-hydroxy-4-acetylamino-biphenyl	3.6 ± 0.2 (14)	30*	1754*	33*	15*	891*	42*
N-hydroxy-2-acetylamino-fluorene	2.6 ± 0.1 (10)	27	1361	27	12	630	31

Data in column 2 are means ± SE (number of observations in brackets) of the second order rate constants of haemoglobin oxidation at 37° by six *N*-hydroxy-*N*-arylacetamides. Concentrations applied: haemoglobin-Fe^{2+}: 0.11, 0.21, and 0.73 mM; *N*-hydroxy-*N*-arylacetamides: 0.11, 0.22, 0.33, 0.44, and 0.52 mM. Initial velocities of $HbFe^{3+}$-formation were monitored at 630 nm with a Zeiss PMQ III spectrophotometer at 37° with 3 ml reaction mixture.

Data in columns 3–8 are from single experiments with 50 or 20 μM *N*-hydroxy-*N*-arylacetamide and 5.04 mM haemoglobin-Fe^{2+} in 3 ml reaction mixture at 37° and are corrected for haemoglobin autoxidation. $HbFe^{3+}$ formation was determined for 3 hr at 550 nm by the cyanide method.

* In these experiments, haemoglobin-Fe^{2+}-concentration was 5.75 mM and the *N*-hydroxy-4-acetylaminobiphenyl-concentration 52.7 μM and 21.1 μM, respectively.

an equimolar concentration of *N*-hydroxy-4ClA to a solution of deoxyhaemoglobin (0.11 mM) in a Thunberg cuvette under N_2, no spectral changes of the absorption maxima at 553 and 428 nm and also no increase in absorbance at 630 nm were observed.

After addition of equimolar concentrations of 4-ClNOB to either oxy- or deoxyhaemoglobin (0.11 mM $HbFe^{2+}$) in a Thunberg cuvette under N_2, new absorption maxima at 561, 540, and 420 nm appeared as an expression of the ferrohaem adduct of 4-ClNOB. In addition, the absorbance at 630 nm increased, which was not an expression of $HbFe^{3+}$ formation, since it could be reversed by carbon monoxide.

These results demonstrated the absolute requirement of molecular oxygen as the third reactant in the *N*-hydroxy-4ClAA- as well as *N*-hydroxy-4ClA-induced haemoglobin oxidation.

Failure to detect acetyl 4-chlorophenyl nitroxide radical

After mixing 5 ml 1 mM haemoglobin-Fe^{2+} in 0.2 M phosphate buffer pH 7.4 with 10 µl of a methanol solution of *N*-hydroxy-4ClAA (final concn.: 1 mM), the reaction mixture was placed in a flat cell or a capillary and monitored for 30 min at the position where the expected signal was to appear, but it was not detectable. No other result was obtained when the experiment was repeated later with another spectrometer, but also at room temperature.

Substituent effects on the kinetics of haemoglobin oxidation

The kinetics of ferrihaemoglobin formation from four monocyclic *N*-hydroxy-*N*-arylacetamides, *N*-hydroxy-AA, *N*-hydroxy-4ClAA, *N*-hydroxy-3,4-Cl_2AA, and *N*-hydroxy-4EAA and the two polycyclic analogues *N*-hydroxy-4AAB and *N*-hydroxy-2AAF, have been compared with purified haemoglobin *in vitro*. The results (Table 5) show differences in the rate and efficacy of $HbFe^{3+}$ formation. *N*-Hydroxy-3,4Cl_2AA was the most active compound, followed by *N*-hydroxy-4ClAA and *N*-hydroxy-4AAB, and *N*-hydroxy-AA was the least active compound.

After 3 hr, each *N*-hydroxy-4ClAA oxidized 53, each *N*-hydroxy-3,4Cl_2AA 44, and each *N*-hydroxy-4AAB 33 equivalents of $HbFe^{2+}$, whereas *N*-hydroxy-AA oxidized only 13 equivalent of $HbFe^{2+}$. When the *N*-hydroxy-*N*-arylacetamide concentration was decreased by 60%, the catalytic activity of *N*-hydroxy-4ClAA increased by 47%, of *N*-hydroxy-3,4Cl_2AA by 66%, and of *N*-hydroxy-4AAB by 27%, indicating that not simply one substituent effect exists, but that inductive and conjugative effects of the aryl substituents govern the kinetic properties and catalytic activities of *N*-hydroxy-*N*-arylacetamides.

DISCUSSION

Differences in the mechanism of haemoglobin oxidation between N-*hydroxy*-N-*arylacetamides and arylhydroxylamines*

The results presented here show that the mechanism by which *N*-hydroxy-*N*-arylacetamides oxidize haemoglobin-Fe^{2+} *in vitro* is distinct from that of arylhydroxylamines, although either reaction is second order in respect of the total reaction and first order in respect of the two reactants. Yet, it has to be assumed that the rate-limiting step of the two reactions is different, because different products are formed by the co-oxidation of haemoglobin with either *N*-hydroxy-*N*-arylacetamides or arylhydroxylamines.

The reaction of *N*-hydroxy-4ClAA with oxyhaemoglobin is slow, k being $4.31 \times mol^{-1} \times sec^{-1}$ at 37° as compared with the instantaneous reaction of *N*-hydroxy-4ClA with oxyhaemoglobin, k being $7800\ l \times mol^{-1} \times sec^{-1}$. The activation energy $E_a = 12.7$ kcal $\times$ mol^{-1} for the reaction of *N*-hydroxy-4ClAA was much higher than that for *N*-hydroxy-4ClA (3.5 kcal $\times$ mol^{-1}). The activation energy of 12.7 kcal $\times$ mol^{-1} is closer to the value of 14 kcal $\times$

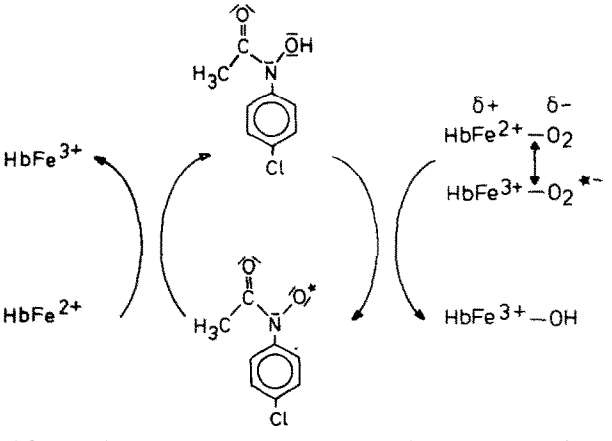

Fig. 3. Catalysis of haemoglobin oxidation by *N*-hydroxy-4-chloroacetanilide. The mechanism of haemoglobin oxidation catalysed by *N*-hydroxy-4-chloroacetanilide is depicted; we consider the formation of acetyl 4-chlorophenyl nitroxide the rate-limiting step in the second order oxidation; since the superoxide dismutase and catalase did not affect *N*-hydroxy-4-chloroacetanilide-induced haemoglobin oxidation, we assume that the dioxygen moiety of oxyhaemoglobin is sequentially reduced to water *in situ*, i.e. oxyhaemoglobin acts as superoxidase.

mol^{-1} determined from the half-wave potential of the chemical one-electron oxidation of *N*-hydroxy-4ClAA than the value of 22.1 kcal × mol^{-1} (≙ 960 ± 10 mV, Riedl [29]) for the irreversible two-electron oxidation, therefore one-electron oxidation is more likely to happen. It may be assumed that the *N*-hydroxy-4ClAA-induced oxidation of oxyhaemoglobin is a three-center reaction of the hydroxamic acid with haemoglobin-Fe^{2+} as the electron donors and the dioxygen moiety of oxyhaemoglobin as the electron acceptor, see Fig. 3. Electron transfer from haemoglobin-Fe^{2+} to the dioxygen moiety, which is normally rather slow, is accelerated by the hydroxamic acid which acts as a catalyst, and whose occasional self-reaction gives rise to only small concentrations of the three metabolites, 4-ClAA, 4-ClNOB, and 4-$ClNO_2B$. Forrester *et al.* [30], who found that chemical oxidation of *N*-hydroxy-AA and *N*-hydroxy-4AAB by PbO_2 in benzene yielded the corresponding *N*-arylacetamides, nitroso- and nitroarenes, and *N,O*-bis-acetylarylhydroxylamines, proposed a mechanism for the self-reaction of secondary aromatic nitroxides. However, the results of Riedl [29] on the chemical oxidation of *N*-hydroxy-4ClAA suggested that this scheme has to be modified, because the product pattern depended on the reactant concentration, solvent, and the oxidant.

Apparently the dioxygen moiety is sequentially reduced to water, since we found no evidence for the presence of superoxide or hydrogen peroxide in the reaction mixture.

N-Hydroxy-4ClAA oxidized up to 81 equivalent of $HbFe^{2+}$, indicating its catalytic activity. The finding that only 7% of the applied *N*-hydroxy-4ClAA was accounted for by the three metabolites, supports the idea that the preponderant amount of the catalytically-active molecule returned to the hydroxamic acid and only a few underwent self-reaction. We assume that it is acetyl 4-chlorophenyl nitroxide radical which is formed from *N*-hydroxy-4ClAA by one-electron oxidation. Such an idea is in agreement with the view of Michaelis [31], that oxidation of organic molecules proceeds in successive univalent steps and that the intermediate is a radical, which is a prerequisite for the reversibility of an oxidation-reduction system. Such a view is also in agreement with the laws of thermodynamics, since electrons can flow from haemoglobin-Fe^{2+} ($E_{1/2}$ = +125 mV, Antonini *et al.* [32]) to acetyl 4-chlorophenyl nitroxide ($E_{1/2}$ = +610 ± 10 mV ≙ 14.0 ± 0.2 kcal, Riedl [29]) and on to the dioxygen moiety of oxyhaemoglobin to yield either H_2O_2 ($E_{1/2}$ = +900 mV, James [33]) or H_2O ($E_{1/2}$ = +800 mV[$O_2 \rightarrow H_2O$] or +1350 mV[$H_2O_2 \rightarrow H_2O$], James [33]). Also in agreement with the laws of thermodynamics is the observation that *N*-hydroxy-4ClAA is not oxidized by molecular oxygen in buffer pH 7.4 [$O_2 + e \rightarrow O_2^{\cdot}$; $E_{1/2}$ = −400 mV], James [33]). Since our attempts have failed to directly demonstrate the presence of acetyl 4-chlorophenyl nitroxide radical by EPR spectroscopy in reaction mixtures of oxyhaemoglobin and *N*-hydroxy-4ClAA, we assume that its stationary concentration during the initial fast phase of the oxidation must be below the sensitivity of the method, i.e. $<10^{-5}$M.

In contrast, the *N*-hydroxy-4ClA-induced haemoglobin oxidation was different, in that each *N*-hydroxy-4ClA reacted only once with haemoglobin-Fe^{2+}, indicating its lack of catalytic activity in the absence of reducing equivalents. Furthermore, the second order oxidation of haemoglobin was 1800-fold faster, the activation energy of the reaction much lower, and two electrons were transferred from each *N*-hydroxy-4ClA to the oxygen moiety of oxyhaemoglobin, since 4-ClNOB is the oxidation product. The activation energy of 3.5 kcal × mol^{-1} is of the same order of magnitude as the electric energy of formation obtained from the half-wave potential of the couple *N*-hydroxy-4ClA/4-ClNOB, $E_{1/2}$ = +80 ± 15 mV ≙ 1.8 ± 0.35 kcal, Riedl [29]) and reflects the energetically-favoured transfer of *two* electrons.

Data on the kinetic properties of arylhydroxylamines are scanty. In their experiments with isolated human haemoglobin, Eyer *et al.* [20] determined the second order rate constant for the unsubstituted phenylhydroxylamine to 2350 l × mol^{-1} × sec^{-1}, the *p*-chloro-substituted arylhydroxylamine surpassed the unsubstituted analogue 3.3-fold in its activity.

Hustedt and Kiese [34] found that the rate of $HbFe^{3+}$ formation by *N*-hydroxy-AA in haemolysate of bovine erythrocytes was proportional to the concentration of $HbFe^{2+}$ and *N*-hydroxy-AA and assumed a second order reaction.

In contrast to the results of Elbaum and Nagel [2] that haemoglobin displayed esterase activity towards 4-nitrophenylacetate, we found no evidence for the enzymic *N*-deacetylation of *N*-hydroxy-4ClAA by haemoglobin, which would have made *N*-hydroxy-4ClA the actual ferrihaemoglobin-forming molecule.

Substituent effects in the oxidation of haemoglobin by N-*hydroxy*-N-*arylacetamides*

We determined the kinetic properties and catalytic activities of the four monocyclic *N*-hydroxy-*N*-arylacetamides, *N*-hydroxy-AA, *N*-hydroxy-4ClAA, *N*-hydroxy-3,4Cl_2AA, and *N*-hydroxy-4EAA and the two polycyclic analogues, *N*-hydroxy-4AAB and *N*-hydroxy-2AAF. The second order rate constants show (Table 5), that *N*-hydroxy-3,4Cl_2AA, *N*-hydroxy-4ClAA, and *N*-hydroxy-4AAB were among the most active compounds and *N*-hydroxy-AA was the least active compound tested.

The catalytic activity of the six *N*-hydroxy-*N*-arylacetamides was determined by monitoring $HbFe^{3+}$ concentrations for 3 hr with 50 and 20 μM *N*-hydroxy-*N*-arylacetamides and 5.04 mM haemoglobin-Fe^{2+}, i.e. a 100- and 250-fold excess of haemoglobin-Fe^{2+}, respectively. *N*-hydroxy-4ClAA showed the highest catalytic activity, followed by *N*-hydroxy-3,4Cl_2AA and *N*-hydroxy-4AAB, and *N*-hydroxy-AA was the least active compound (Table 5). On reducing the hydroxamic acid concentration by 60%, the catalytic activity of *N*-hydroxy-4ClAA increased by 47%, that of *N*-hydroxy-3,4Cl_2AA even by 64%, and that of *N*-hydroxy-4AAB by 27%. We assume that the catalytically-active oxidation products of *N*-hydroxy-4ClAA and *N*-hydroxy-34Cl_2AA, presumably the corresponding acetyl chlorophenyl nitroxides, are especially stabilized by the (−I)- and (+M)-effect of the Cl-atoms, by which the unpaired electron of the nitroxide–oxygen is delocalized to a larger

extent than by any other substituent. The catalytic activity of *N*-hydroxy-4AAB is probably much lower, because the much weaker (−I)- and (+M)-effect or the weak (−M)-effect of the 4-phenyl substituent governs its oxidation to the corresponding nitroxide and in turn its reduction to the corresponding hydroxamic acid.

Further reaction of the ferrohaem-nitrosoarene adduct

The second slow phase of haemoglobin oxidation which followed the rapid initial phase (Fig. 1) continued, until all $HbFe^{2+}$ was oxidized. The results with 4-ClNOB (Table 4) show, that the concentration of 4-ClA and 4-$ClNO_2$B increased with $HbFe^{3+}$ concentration. Probably part of the electrons which were transferred from $HbFe^{2+}$ to oxygen [35] have sequentially reduced 4-ClNOB to 4-ClA. But we cannot explain at present, whether 4-$ClNO_2$B was formed by autoxidation of 4-ClNOB or by disproportionation of the other product of this one-electron transfer which may be the nitrosoarene radical anion (Russel *et al.* [36]). 4-ClA then could be the other product of the disproportionation.

Hirota and Itano [37], who studied the influence of ring substituents on the binding of nitrosoarenes to haemoglobin-Fe^{2+}, also observed slow conversion of the ferrohaem–nitrosoarene complexes into unliganded ferrihaemoglobin, but did not determine the product pattern.

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