Biochemical Pharmacology, Vol. 27, pp. 1229–1237.

0006-2952/78/0415-1229$02.00/0

INTERACTION OF ALIPHATIC *N*-HYDROXYLAMINES WITH MICROSOMAL CYTOCHROME P450: NATURE OF THE DIFFERENT DERIVED COMPLEXES AND INHIBITORY EFFECTS ON MONOXYGENASES ACTIVITIES

DANIEL MANSUY†, EVELYNE ROUER*, CHARLES BACOT, PIERRE GANS, JEAN CLAUDE CHOTTARD and JEAN PAUL LEROUX*

Laboratoire de chimie de l'Ecole Normale Supérieure, associé au C.N.R.S. n°32, et Université Paris V, 24 rue Lhomond, 75231 Paris Cedex 05, France and *Laboratoire de Biochimie Médicale, Faculté de Médecine Necker-Enfants Malades, Université Paris V, 156 rue de Vaugirard, 75730 Paris Cedex 15

(*Received* 11 *July* 1977; *accepted* 3 *October* 1977)

Abstract—Primary *N*-hydroxylamines, RR'R"CNHOH, produce difference spectra of liver microsomes from variously pretreated rats with peaks around 420 nm and troughs around 392 nm which are interpreted as the result of the hydroxylamines binding to cytochrome P450-Fe (III) through their oxygen atom. The hydroxylamines interact with dithionite–or NADPH–anaerobically reduced microsomes giving peaks around 423 nm. In the presence of NADPH, oxygen and microsomes, all the hydroxylamines of the type RR'CHNHOH lead to 455 nm difference spectra which should correspond to cytochrome P450-Fe (II)–RR'CHNO complexes. These hydroxylamines also act as strong inhibitors of aniline hydroxylase, *p*-nitroanisole-*O*-demethylase and 7-ethoxycoumarin-*O*-dealkylase activities of PB-pretreated rat liver microsomes. In most cases, they are better inhibitors than metyrapone or SKF 525A. These inhibitory effects of hydroxylamines are related to their ability to lead to 455 nm absorbing complexes; For instance, 1-hydroxylamino-2phenyl-ethane exhibits the best apparent Ks for the 455 nm absorbing complex formation and is also the best inhibitor of microsomal 7-ethoxycoumarin-*O*-dealkylase ($I_{50} = 0.5\ \mu M$).

Several amines, including drugs and carcinogens, are metabolized into *N*-hydroxylamines [1–4] and this has been shown to be an important step for expression of the toxicity of various amines [1, 5]. The hepatic microsomal oxidation of some aliphatic *N*-hydroxylamines has been shown to be NADPH- and oxygen-dependent [6]. Moreover, during their hepatic microsomal oxidations, *N*-hydroxy-amphetamine [7] and 1-hydroxylamino 2-phenyl ethane [8] have been shown to form stable 455 nm absorbing cytochrome P450 complexes.

The formation of the *N*-hydroxy-amphetamine derived complex has been obtained with purified cytochrome P450 or P448 and requires NADPH-cytochrome-*c* reductase and a phospholipid in addition to NADPH and oxygen [9]. It also happens during microsomal oxidation of amphetamines, which are precursors of *N*-hydroxyamphetamine, in respect to their *N*-oxidation state [10–13]. Some other aminodrugs and SKF 525 A and related compounds are also able to lead to 455 nm absorbing complexes after oxidative metabolism [14, 15]. Moreover, it has been reported that the formation of such complexes from benzphetamine, SKF 525 A, amphetamine and *N*-hydroxyamphetamine leads to an important inhibition of some microsomal monoxygenases [10, 16, 17].

We showed recently that the 455 nm absorbing complex obtained by oxidation of *N*-hydroxy-amphetamine can also be formed by reduction of "nitroamphetamine",1-nitro-2-phenylpropane [18], and that similar complexes can be formed by reduction of almost any primary and secondary nitroalkanes [19].

We concluded that the corresponding nitrosoalkane monomers are the actual ligands in these cytochrome P450-Fe (II) complexes [18–19]. This is in agreement with a recent report on the formation of a 455 nm absorbing complex during interaction of 1-nitroso-2 phenyl propane with NADPH- or dithionite- reduced microsomes [8]. Moreover, we have very recently isolated nitrosoalkanes-Fe (II) porphyrins complexes [46], exhibiting properties very similar to those of myoglobin-Fe (II)-nitrosoalkanes complexes [47].

As the formation of the cytochrome P450-Fe(II)-RR'CHNO complexes upon reduction of nitroalkanes $RR'CHNO_2$ is quite general [19], one could expect that such complexes could be formed from any amino, $RR'CHNH_2$, or hydroxylamino, RR'-CHNHOH, compound, provided that it can be oxidised to the corresponding nitrosoderivative by the microsomal enzymes. In order to very this hypothesis, we studied the interactions of various aliphatic primary *N*-hydroxylamines with rat liver

† To whom reprint requests should be addressed: Laboratoire de Chimie de l'Ecole Normale Supérieure, 24 rue Lhomond, 75231 Paris Cedex 05, France.

microsomal cytochrome P450 in the conditions of oxidative metabolism. We also studied the interactions of cytochrome P450, in the oxidised or reduced state, with the hydroxylamines alone. Each kind of interaction leads to a different type of cytochrome P450 complex. This paper also reports the inhibitory effects of the hydroxylamines on aniline hydroxylase, para-nitroanisole-*O*-demethylase and 7-ethoxy-coumarin-*O*-dealkylase and compares them to the effects of typical inhibitors of microsomal monoxygenases as metyrapone and SKF 525 A.

MATERIALS AND METHODS

Hepatic microsomes were prepared from male Sprague–Dawley rats as described previously [20]. Sodium phenobarbital was given i.p. in a daily dose of 80 mg/kg body wt for 3 days and 3-methylcholanthrene, dissolved in corn oil, i.p., in a dose of 20 mg/kg for 2 days. Protein and cytochrome P450 concentrations were determined according to Gornall *et al.* [21] and to Omura and Sato [22]. Spectrophotometric measurements were performed on an Aminco DW2 spectrophotometer with microsomes at a concentration of 1.5–4 mg protein/ml in 0.1 M phosphate buffer, pH = 7.4. Kinetics were recorded by use of the dual wavelength mode. *N*-methylhydroxylamine and 2-phenylethanol were purchased from Aldrich chemicals. 2-hydroxylamino-propane, m.p.: 87°, litt. [23] 87° and 2-hydroxylamino-2-methyl-propane, m.p. point: 65°, litt [24]: 64–65° were prepared by reduction by Zn-NH_4Cl [25] of 2-nitro-propane and 2-methyl-2-nitropropane. 1-hydroxylamino-2-phenyl ethane, m.p.: 64°, litt [26]: 63–64°, and 2-hydroxylamino-1-phenyl propane, m.p.: 89°, litt. [26]: 84°, were obtained respectively by reduction by $LiAlH_4$ of 1-nitro-2-phenyl-ethylene and 2-nitro-1-phenylpropane [24]. 1-benzoyloxyamino-2-phenyl ethane was prepared by reaction of 2-phenylethylamine with dibenzoyl peroxide [25]. NADPH cytochrome-*c* reductase was determined by the method of Masters *et al.* [28]. The activities of *p*-nitroanisole *O*-demethylase, aniline hydroxylase and 7-ethoxy-coumarin-*O*-dealkylase were assayed respectively by the methods described by Netter *et al.* [29], Kato *et al.* [30] and Ullrich *et al.* [31]. For the assays, the microsomes were suspended in a 50 mM Tris–HCl buffer pH 7.4 containing 150 mM KCl, 10 mM $MgCl_2$ and a NADPH-generating system consisting in 1.2 mM glucose-6-phosphate, 0.1 mM $NADP^+$ and glucose-6 phosphate dehydrogenase (from Boehringer) 0.4 μg/ml. The activities were all determined at 37°, the substrates concentrations being: *p*-nitro-anisole, 1.2 mM, aniline 1.25 mM and 7-ethoxy-coumarin 0.1 mM.

RESULTS

Interaction of aliphatic primary N-*hydroxylamines, RR'R"*CNHOH 1, *with oxidized microsomal cytochrome P450.* All the tested hydroxylamines 1 ($R = R' = R'' = H$; $R = R' = R'' = CH_3$; $R = R' = CH_3$, $R'' = H$; $R = R' = H$, $R'' = CH_2Ph$; $R = H$, $R' = CH_3$, $R'' = CH_2Ph$) produce a difference spectrum of rat liver microsomes with a peak around 420 nm and a trough around 390 nm (Fig. 1, Table 1) as previously described for *N*-hydroxyamphetamine [7]. The position of the peak and its intensity are greatly dependent upon the rats pretreatment. In each case where it was investigated, one observes a blueshift of the Soret peak when passing from 3-methylcholanthrene (3-MC)-pretreated-(421–427 nm) to control (419 nm) and to phenobarbital (PB)-pretreated (415–420 nm) rats. Moreover, the maximum intensities of the peaks are 4-fold greater after 3 MC induction compared to PB induction. The hydroxylamine having three alkyl substituents on the carbon α to nitrogen, 1 ($R = R' = R'' = CH_3$), produces no significant difference spectrum with control or PB-induced rat microsomes and a less intense spectrum than the other hydroxylamines with 3-MC-induced rat microsomes. The apparent spectral dissociation constants, K_s, for these "420 nm" peak formations vary very much with the structure of the hydroxylamine, 1-hydroxylamino-2-phenyl-ethane having the best affinity ($K_s = 6 \times 10^{-6}$ M), a 1000-fold better than that of methylhydroxylamine.

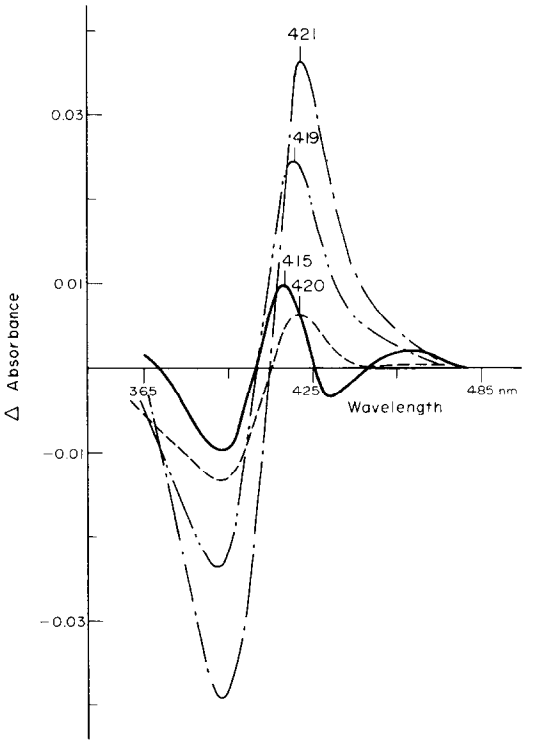


Fig. 1. Difference spectra of liver microsomes from differently pretreated rats produced by 1-hydroxylamino-2-phenyl ethane and 2-phenyl-ethanol. The two cuvettes contained 2 μM cytochrome P450. 1-hydroxylamino-2-phenyl ethane is added to the sample cuvette containing microsomes from control (— · —), PB-(——) and 3-MC (— — —) pretreated rats: respective hydroxylamine concentrations: 5, 0.16 and 0.5 mM; respective cytochrome P450 content/mg protein: 0.44, 2.1 and 1.4 nmoles. (---) addition of 1 mM 2-phenyl-ethanol to the sample cuvette containing microsomes from PB-pretreated rats (1.7 nmoles cyt. P450/mg protein).

As previously reported for *N*-hydroxyamphet-

Table 1. Spectral interactions of aliphatic primary *N*-hydroxylamines $R_1R_2R_3CNHOH$ with aerobic liver microsomes from differently pretreated rats. Apparent spectral dissociation constants, K_S, were obtained from Lineweaver–Burk plots: $1/\Delta A$ (λ max–λ min) = f($1/[R_1R_2R_3CNHNOH]$). All values represent means ± S.E. from 2–5 experiments

	R_1	H	H	CH_3	H	H
	R_2	H	CH_3	CH_3	H	CH_3
	R_3	H	CH_3	CH_3	CH_2Ph	CH_2Ph
	Induction					
λ Max.	none	N.D.	—†	N.D.‡	419	419
(nm)	PB	420	418	—	415	415
	3MC	N.D.	423	427	421	421
Δ Amax.*	none	N.D.	—	N.D.	25 ∓ 3	23 ∓ 3
(λ max–λ min)	PB	10 ∓ 2	11 ∓ 4	—	18 ∓ 2	11 ∓ 2
× cm^{-1}	3MC	N.D.	40 ∓ 3	20 ∓ 3	42 ∓ 4	42 ∓ 4
× P450 mM^{-1}						
Ks§	none	N.D.	—	—	{ 0.02 ∓ 0.01 { 0.3 ∓ 0.2	{ 1 ∓ 0.5 { 9 ∓ 3
($\times 10^4$ M)	PB	90 ∓ 30	170 ∓ 50	—	{ 0.06 ∓ 0.01 { 0.3 ∓ 0.2	{ 0.56 ∓ 0.1 { 3 ∓ 1
	3MC	N.D.	150 ∓ 20	3 ∓ 1	{ N.D. { 0.4 ∓ 0.1	{ 0.7 ∓ 0.2 { 3 ∓ 1
Presence of	none	N.D.	—	—	—	Shoulder
a peak	PB	—	—	—	+	+
around	3MC	N.D.	—	—	—	—
455 nm						

* Obtained with 5×10^{-2} M hydroxylamine. All the described difference spectra exhibit a trough around 390 nm.
† No formation of a significant difference spectrum
‡ N.D. = not determined
§ The Lineweaver–Burk plots for *N*-hydroxyamphetamine and 1-hydroxylamino-2-phenyl-ethane (10^{-7} to 10^{-3} M) are clearly biphasic, leading to the two extrapolated K_s values. Above 10^{-3} M, the curves deviate from linearity with a larger slope.

amine [7], the hydrophobic hydroxylamine, 1-hydroxyl-amino-2-phenyl-ethane, interacts with microsomes of PB-pretreated rats leading to a second peak around 455 nm and a second trough around 427 nm. It is noteworthy that this phenomenon occurs only during interaction of the hydrophobic hydroxylamines with microsomes of PB-pretreated rats.

The difference spectra of oxidized microsomes exhibiting a peak around 420 nm and a trough around 390 nm have been called "reverse type 1" or "modified type 2" spectra [32]. A proposed explanation of such spectra produced by alcohols or other compounds having an oxygen with an accessible lone-pair of electrons is the coordination of these compounds by their oxygen atom to the iron (III) of cytochrome P450 [33, 34]. Primary *N*-hydroxylamines could bind to heme-Fe(III) of cytochrome P450 either by their oxygen or nitrogen atom. In order to know if one of these modes of binding could explain the observed difference spectra we tested the spectral interactions of microsomes with various compounds related to 1-hydroxylamino-2-phenyl-ethane having different *N* or *O*-substituents (Table 2). 2-Phenyl-ethanol behaves similarly to 1-hydroxylamino-2-phenyl-ethane; its acetate, with a more hindered and deactived oxygen atom, produces only a type 1 difference spectrum at concentrations lower than 10^{-4} M and a weak 420 nm spectrum at higher concentrations (Table 2). Similarly, when the hydroxylamine oxygen atom is hindered and electronically deactivated as in its *O*-benzoylated-derivative, it does not lead to the formation of a 420 nm spectrum (Table 2). However, *t*-butylhydroxylamine with its hindered nitrogen atom (it is known that *t*-butylamine

Table 2. Spectral interactions of various *N* or *O*-substituted analogs of 1-hydroxylamino-2-phenyl ethane with oxidized or reduced microsomal cytochrome P450 from PB-pretreated rats

Compounds* $PhCH_2CH_2$-X	oxidized cyt. P450			reduced cyt. P450
×	λ max. (nm)	λ min	Δ A§ × cm^{-1} × P450 mM^{-1}	λ max. (nm)
NHOH	415	392	8.5	422
OH	420	392	7	—
$OCOCH_3$	385†	424	4	—
$NHOCOC_6H_5$	—‡	—	—	—

* 10^{-4} M; (†) a reverse type 1 spectrum (λ max = 420 nm, λ min = 392 nm) appears only at concentrations higher than 10^{-3} M
‡ No significant difference spectrum; § Δ A (λ max–λ min)

does not bind to cytochrome P450-Fe(III) [35]), is able to lead to the formation of a 420 nm spectrum, at least with microsomes of 3-MC pretreated rats (Table 1).

Interaction of aliphatic primary N-hydroxylamines, RR'R" CNHOH with reduced cytochrome P450, in anaerobic conditions. The addition of the hydroxylamines, 1 (R = R' = R" = H; R = R' = CH_3, R" = H; R = R' = H, R" = CH_2Ph and R = H, R' = CH_3, R" = CH_2Ph) at high concentrations ($> 10^{-3}$ M) to a liver microsomal suspension previously reduced by excess dithionite, produces a difference spectrum with a peak around 423 nm (Fig. 2). With lower concentrations (10^{-5} M) of the hydrophobic hydroxylamines 1 (R = R' = H, R" = CH_2Ph and R = H, R' = CH_3, R" = CH_2Ph), this spectrum appears with similar intensity, but decreases gradually with time. This phenomenon is probably related to a side reaction: the known chemical destruction of the hydroxylamines by dithionite [36]. The 423 nm difference spectra are equally produced by interaction of the hydroxylamines with NADPH reduced microsomes in anaerobic conditions (Fig. 2). The spectra are not affected by the addition of excess NADH to both cuvettes, indicating that it does not correspond to reduced minus oxidized cytochrome b5. This is further supported by the stability of the 423 nm spectra after addition of excess dithionite to both cuvettes. Upon addition of carbon monoxide or *n*-butyl-isocyanide to the 423 nm absorbing complexes, the 423 nm peak is rapidly replaced respectively by a 450 nm peak or by two peaks at 430 and 455 nm (Fig. 3). One should note that the disappearance of the 423 nm peak upon CO addition and its replacement by a 450 nm peak, the intensity of which corresponds to the total initial cytochrome P450, permit to exclude that the 423 nm peak derives from any type of cytochrome P450 denaturation.

Reaction of the hydroxylamines RR'R"CNHOH with aerobic microsomes in the presence of NADPH. Addition of NADPH to an aerobic suspension of microsomes containing any of the hydroxylamines 1 (R = R' = R" = H; R = R' = CH_3, R" = H; R = R' = H, R" = CH_2Ph; R = H, R' = CH_3, R" = CH_2Ph), causes an immediate transformation of the original 420 nm spectrum to a 455 nm spectrum (Fig. 4a). However, *t*-butylhydroxylamine fails to produce this spectrum in the same conditions. This last result agrees with the reported inability of 2-hydroxylamino-2-methyl-1-phenyl propane, to form a 455 nm absorbing complex [8]. The same 455 nm absorbing complex can be equally obtained when the 423 nm absorbing complex

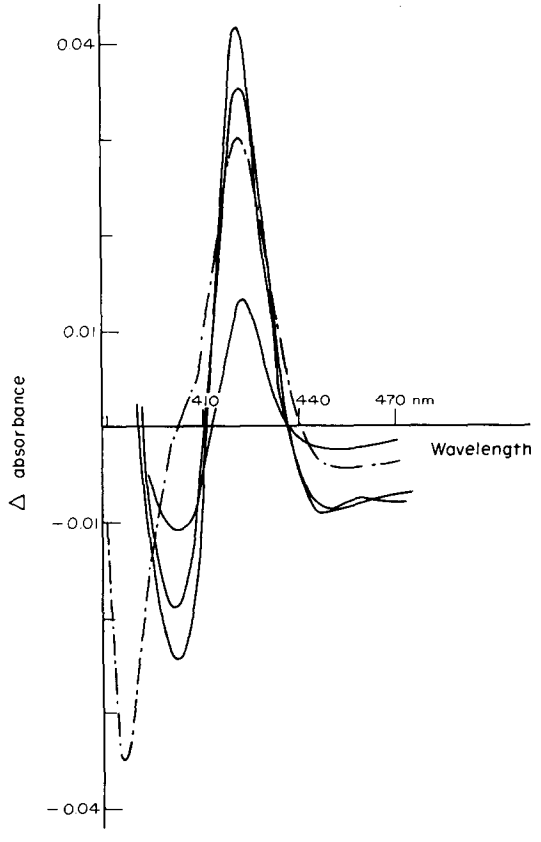


Fig. 2. Difference spectra of rat liver microsomes reduced by dithionite or by NADPH in anaerobic conditions, with hydroxylamines. (——): the two cuvettes contained 2 μM cytochrome P450 from liver microsomes of PB-pretreated rats (1.8 nmole cyt. P450/mg protein) and 10 mM sodium dithionite. Increasing amounts of 1-hydroxylamino-2-phenyl ethane (0.1, 0.5 and 1 mM) are added to the sample cuvette. (— · —): the two cuvettes containing 1.3 μM cytochrome P450 from liver microsomes of PB-pretreated rats (1.5 nmole cyt. P450/mg protein) and 5 mM NADPH were degassed under argon. 1 mM 2-hydroxylaminopropane is then added to the sample cuvette.

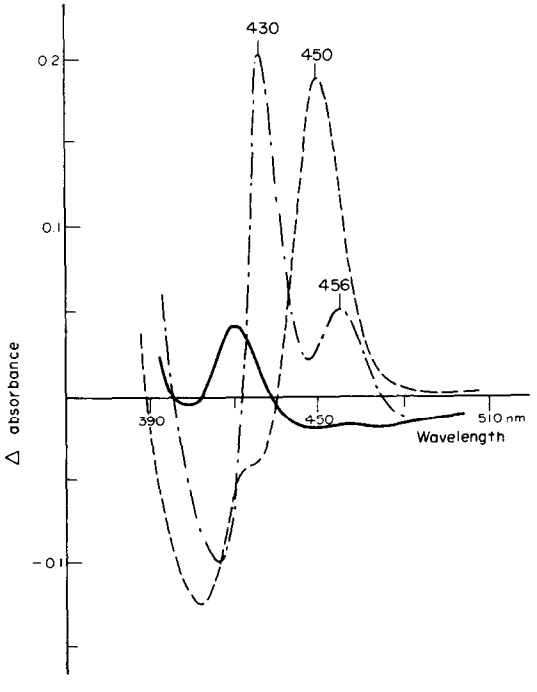


Fig. 3. Effect of carbon monoxide or *n*-butyl-isocyanide upon the "423 nm difference spectrum" produced by 1-hydroxylamino-2-phenyl ethane and reduced cytochrome P450. The two cuvettes contained 2.1 μM cytochrome P450 (1.5 nmoles/mg microsomal protein) and 10 mM dithionite. (——): addition of 1 mM 1-hydroxylamino-2-phenyl ethane to the sample cuvette. Further addition of 7 mM *n*-butyl-isocyanide (— · — ·) or 1 mM CO (– – –) to the same cuvette

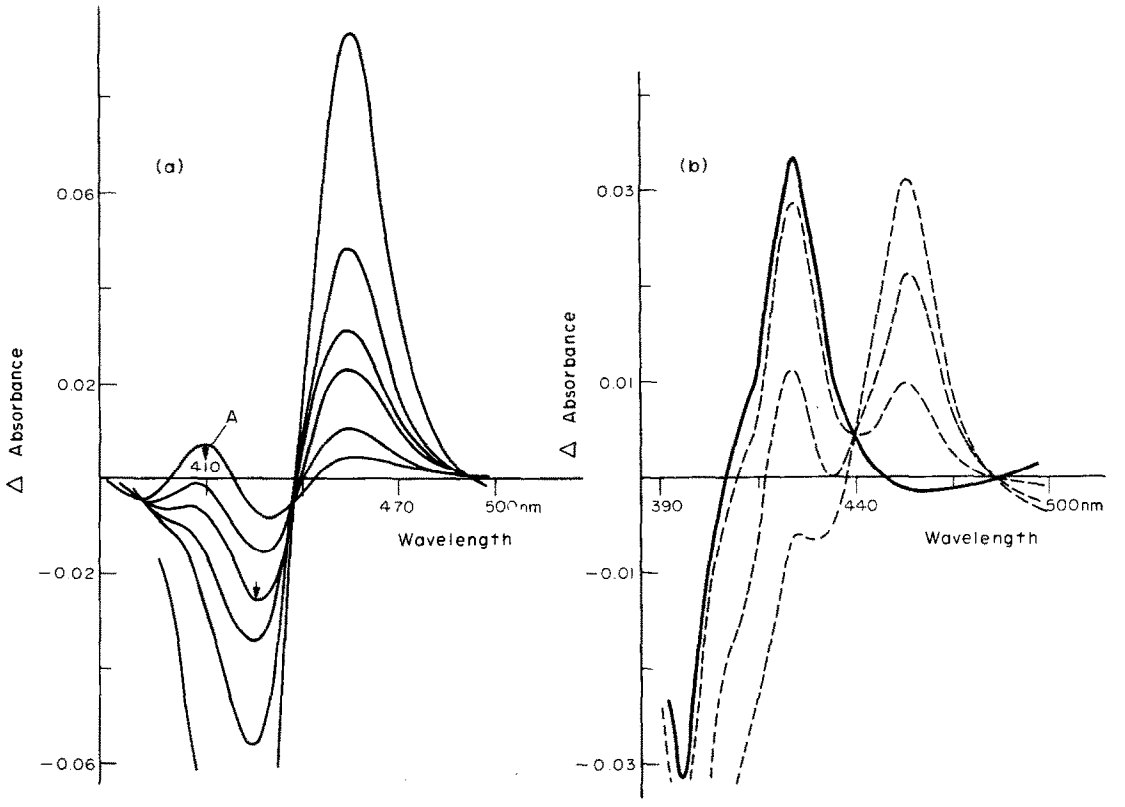


Fig. 4. Formation of 455 nm absorbing nitrosoalkane-cytochrome P450-Fe(II) complexes either by addition of NADPH to a microsomal cytochrome P450-Fe(III)-hydroxylamine complex in the presence of oxygen or by addition of oxygen to a microsomal cytochrome P450-Fe(II)-hydroxylamine complex previously obtained under anaerobic conditions.

Fig. 4a. The two cuvettes contained 1.8 μM cytochrome P450 (1.3 nmoles/mg microsomal protein). Curve A corresponds to the addition of 2 mM 1-hydroxylamino-2-phenyl-ethane to the sample cuvette. The others were registered after 0.5, 1, 2 and 3 min after 0.02 mM NADPH addition. The last one was then recorded after addition of 1 mM NADPH.

Fig. 4b. The two cuvettes containing 1 μM cytochrome P450 (1.5 nmoles/mg microsomal protein) and 5 mM NADPH were degassed under argon. (——): addition of 1 mM 2-hydroxylamino propane to the sample cuvette (---): further opening of both cuvettes to the air.

Table 3. Characteristics of the 455 nm absorbing complexes formed from various hydroxylamines and liver microsomes of PB-pretreated rats in the presence of 10^{-3} M NADPH. All values represent means ± S.E. from 2–5 experiments

R_1	H	H	CH_3	H	H
R_2	H	CH_3	CH_3	H	CH_3
R_3	H	CH_3	CH_3	CH_2Ph	CH_2Ph
λ max (nm)	455	457	—	455	455
ΔA max* (455–490 nm) × cm^{-1} × P450 mM^{-1}	6 ∓ 1	29 ∓ 3	—	44 ∓ 7	60 ∓ 3
"K_s"† (× 10^4 M)	5 ∓ 5	10 ∓ 5	—	0.06 ∓ 0.01	0.33 ∓ 0.05
Initial rate of complex formation (ΔA (455–490 nm) × cm^{-1} × P450 mM^{-1} × min^{-1})	3 ∓ 1	13 ∓ 2	—	55 ∓ 6	110 ∓ 15

* $R_1R_2R_3CNHOH$ concentrations: $R_1 = R_2 = R_3 = H$ and $R_1 = R_2 = CH_3$, $R_3 = H$, 5×10^{-2} M; $R_1 = R_2 = H$, $R_3 = CH_2Ph$ and $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_2Ph$, 10^{-3} M.

† Since metabolism is involved in the formation of the 455 nm complexes, one cannot determine an actual K_s. The reported "K_s" are the hydroxylamines concentrations leading to the half ΔA max of the 455 nm complex, the maximum hydroxylamines concentrations used being those indicated in the preceding footnote.

Table 4. Comparison of the inhibitory effects of aliphatic hydroxylamines and of metyrapone and SKF 525A on some monoxygenases activities of liver microsomes from PB-pretreated rats*

Inhibitor	% Inhibition of 7-ethoxycoumarin dealkylase† (number of experiments) means ± S.E.	% Inhibition of aniline hydroxylase‡ (number of experiments) means ± S.E.	% Inhibition of p-nitroanisole demethylase‡ (number of experiments) means ± S.E.
N-Methyl-hydroxyl-amine	49 ∓ 3 (5)	64 ∓ 6 (3)	35 ∓ 7 (5)
2-Hydroxylamino propane	63 ∓ 4 (8)	68 ∓ 8 (3)	34 ∓ 4 (5)
1-Hydroxylamino 2-phenyl ethane	87 ∓ 1 (7)	65 ∓ 5 (3)	51 ∓ 3 (5)
N-Hydroxy-amphetamine	81 ∓ 3 (4)		55 ∓ 1 (3)
Metyrapone	77 ∓ 1 (5)	27 ∓ 5 (4)	49 ∓ 6 (4)
SKF 525 A	51 ∓ 7 (3)	73 ∓ 5 (3)	29 ∓ 5 (4)

* Conditions as indicated in Methods. The microsomal suspension containing the NADPH-generating system and the inhibitor is first incubated during 5 min at 37°. Then, the reaction is started by addition of the substrate. In the absence of inhibitors, the 7-ethoxycoumarin-*O*-dealkylase, aniline hydroxylase and *p*-nitroanisole-*o*-demethylase activities were respectively 1.70 ∓ 0.06, 0.55 ∓ 0.03 and 16.5 ∓ 1.2 nmoles of product (umbelliferone, *p*-hydroxyaniline or *p*-nitrophenol) × min^{-1} × mg $protein^{-1}$.

† Concentrations of all inhibitors: 10^{-5} M, except *N*-methyl-hydroxylamine and 2-hydroxylamino-propane (10^{-4} M).

‡ Concentrations of all inhibitors : 10^{-3} M.

formed by interaction of the hydroxylamine with NADPH-reduced microsomes in anaerobic conditions, reacts with added oxygen (Fig. 4b). The characteristics of the 455 nm absorbing complexes formed from various hydroxylamines are compared in Table 3. It is noteworthy that the "K_s" corresponding to the 1-hydroxylamino-2-phenyl-ethane derived complex is as low as 6×10^{-6} M. These 445 nm absorbing complexes are all stable to dithionite [18] and remain unchanged after addition of strong cytochrome P450 ligands like metyrapone or CO. They are however destroyed by ferricyanide oxidation [7, 13, 19].

Inhibitory effects of the hydroxylamines, RR'-CHNHOH, on microsomal hydroxylation reactions. The action of four hydroxylamines RR'CHNHOH (R = R' = H; R = R' = Me; R = CH_2Ph, R' = H; R = CH_2Ph, R' = Me) on the *O*-dealkylation of 7-ethoxy-coumarin, the *O*-demethylation of para-nitroanisole and the aniline hydroxylation by liver microsomes of PB-pretreated rats are compared to the action of previously described monoxygenases inhibitors [38] (Table 4). The hydroxylamines (10^{-3} M) are more efficient than metyrapone to inhibit aniline hydroxylase activity of PB-pretreated rats microsomes, but comparable to SKF 525A which has been also reported to produce a 455 nm absorbing complex after its oxidative microsomal metabolism [15]. Para-nitroanisole-*O*-demethylase activity of PB-induced rat liver microsomes is inhibited to the same extent by the hydrophobic hydroxylamines and metyrapone (I_{50} around 10^{-3} M). All the hydroxylamines give important inhibitions of the 7-ethoxycoumarin-*O*-dealkylase activity of PB-induced rat liver microsomes, 1-hydroxyl-amino-2-phenyl-ethane and *N*-hydroxy-amphetamine leading to a greater extent of inhibition than metyrapone and SKF 525A.

As shown in Fig. 5, 1-hydroxylamino-2-phenyl-ethane inhibits the microsomal-*O*-deethylation of 7-ethoxycoumarin in a non-competitive manner. The I_{50} value, 0.4 μM, obtained for this hydroxyl-amine is much lower than those corresponding to 2-hydroxylamino-propane (40 μM) or to metyrapone (4 μM: this value agrees with that which can be inferred from the work of Ullrich *et al.* [39] and which lies around 5 μM). As the hydroxylamines are

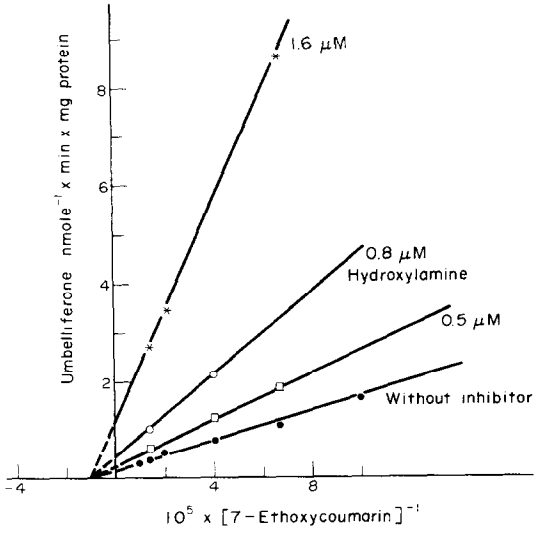


Fig. 5. Kinetics of the inhibition of 7-ethoxy-coumarin dealkylase activity of liver microsomes from PB-pretreated rats by 1-hydroxylamino-2-phenyl ethane. (Cf. Table 4.)

prone to give redox reactions [40], we looked for their possible interaction with NADPH-cytochrome-*c* reductase. In fact, the four hydroxylamines whose inhibitory effects have been tested (Table 4), exhibit no significant effect on the NADPH-cytochrome-*c* reductase activity of liver microsomes of PB-pretreated rats, in the conditions previously used for the inhibition experiments, even at high hydroxylamines concentration (10^{-3} M). The hydroxylamines inhibitory properties could be related to the formation of the corresponding cytochrome P450-Fe(II)-nitrosoalkane complexes, as indicated by the rough relationship between the effects of the four studied hydroxylamines on some microsomal monoxygenase activities and their ability to form 455 nm absorbing complexes (Tables 3 and 4). Accordingly, the inhibitory effect of 1-hydroxylamino-2-phenyl ethane or 2-hydroxylamino-propane on the microsomal *O*-dealkylation of 7-ethoxycoumarin significantly increases, when the microsomes are preincubated with the hydroxylamine and NADPH before 7-ethoxycoumarin addition (Fig. 6). This effect is only important during the first 5 min of incubation, a time which is necessary for the maximum formation of the 455 nm absorbing complex (Table 3). It is noteworthy that the influence of the preincubation is much greater for 2-hydroxylamino-propane than for 1-hydroxylamino-2-phenylethane. This is related to the greater rate and extent of formation of the 455 nm absorbing complex with the hydrophobic hydroxylamine (Table 3).

DISCUSSION

Various complexes of oxidized cytochrome P450 with ligands containing accessible oxygen atoms like alcohols, carbonyl compounds and ethers have been previously reported with Soret peak around 420 nm [33, 34, 39]. Very recently, Nebert *et al.* [34], from spectral studies of the interaction of *n*-butanol with microsomes, concluded that the endogenous sixth ligand of the low-spin cytochrome P450-Fe(III) *in vivo* could be a hydroxyl group or a group of similar ligand field strength from an adjacent amino acid residue. Thus, the reverse type I spectral change produced by an alcohol should correspond to its coordination through its oxygen atom to the sixth vacant position of the originally high-spin pentacoordinated cytochrome P450-Fe(III) complex. From the results of the spectral interactions of oxidized cytochrome P450 with compounds related to aliphatic hydroxylamines having various *N* or *O*-substituents (Tables 1 and 2), it seems likely that the hydroxylamines are bound through their oxygen atom to the iron (III) of cytochrome P450 (scheme 1). If the corresponding 420 nm difference spectrum is due to the transformation of originally high-spin cytochrome P450- Fe(III) to low-spin cytochrome P450-Fe(III), one should expect its intensity to be higher the greater the proportion of high-spin cytochrome P450-Fe(III) originally in the microsomal preparation. Accordingly we found (Table 1) an increase of the intensity of the 420 nm peak in the order: 3-methycholanthrene-treated rats > control rats > phenobarbital-treated rats. A similar result has been recently reported by Nebert *et al.* [34] for the spectral interactions of *n*-butanol with microsomes from differently pretreated animals.

We have seen that the 423 nm difference spectra obtained by interaction of the hydroxylamines with dithionite-reduced microsomes are neither due to cytochrome b5 nor to cytochrome P420. They should correspond to an interaction of the hydroxylamines with reduced cytochrome P450, presumably their binding to heme-Fe(II). However, one cannot exclude that they derive from the interaction of cytochrome P450 with a hydroxylamine metabolite formed in microsomes in the presence of NADPH in anaerobic conditions or dithionite. Contrary to 1-hydroxylamino-2-phenyl-ethane, its benzoate does not produce a 423 nm spectrum (Table 2). This suggests that the binding of the hydroxylamine occurs through its oxygen atom to cytochrome P450-Fe(II) in the 423 nm absorbing complex (scheme 1). It is noteworthy that such a coordination of oxygenated compounds to cytochrome P450-Fe(II), which is speculative at this time, is not usual. Accordingly, we did not observe any spectral change with an alcohol (Table 2). However, Kato *et al.* [41] have reported the formation of a reduced cytochrome P450 complex with tiaramide *N*-oxide, exhibiting a 424 nm spectrum similar to those obtained with the hydroxylamines.

Two conditions are required for the formation of a 455 nm absorbing nitrosoalkane-cytochrome P450 complex from an hydroxylamine: the iron of cytochrome P450 should be reduced and the hydroxylamine should be oxidised in the proximity of the heme (scheme 1). This proximity is required otherwise the unstable nitroso-compound formed, RR'CHNO, would irreversibly give its oxime tautomer [37] instead of coordination of Fe(II). These conditions are fulfilled when oxygen and NADPH

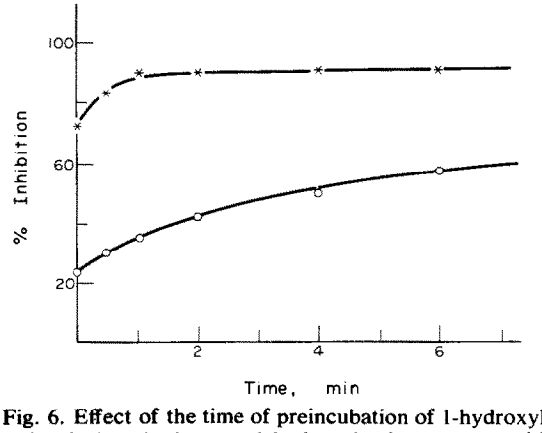
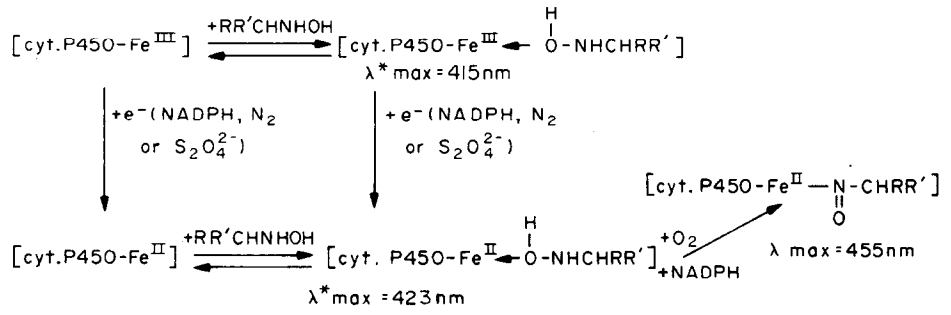


Fig. 6. Effect of the time of preincubation of 1-hydroxylamino-2-phenyl ethane or 2-hydroxylamino-propane with rat liver microsomes and NADPH on the inhibition of 7-ethoxy-coumarin dealkylase by these hydroxylamines. Liver microsomes from PB-pretreated rats suspended in Tris–Buffer pH 7.4, (0.5–1 mg protein/ml) are preincubated at 37° in the presence of 150 mM KCl, 10 mM $MgCl_2$, a NADPH-generating system (see Methods) and 10^{-5} M 1-hydroxylamino-2-phenyl ethane (—*—) or 10^{-4} M 2-hydroxylamino-propane (-°-°). The dealkylation activity is then measured after the addition of 2×10^{-4} M 7-ethoxy-coumarin. In the absence of inhibitors and without preincubation, the basal dealkylase activity was 2.12 ∓ 0.04 nmole umbelliferone/min/mg protein.

Scheme 1



* λ max of the difference spectrum of PB-induced rat liver microsomes.

permit the monoxygenases-dependent oxidation of the hydroxylamine in the hydrophobic pocket of cytochrome P450 itself. As expected, the higher the hydrophobicity and the affinity of an hydroxylamine for cytochrome P450, the faster and the more extensive its 455 nm complex formation (Tables 1 and 3). A very partial nitrosocomplex formation occurs by interaction of the hydrophobic hydroxylamines with aerobic microsomes from phenobarbital-pretreated rats in the absence of NADPH (Fig. 1). This explains the peculiar difference spectra obtained by interaction of the hydrophobic hydroxylamines with such microsomes, which are the sum of a reverse type 1 difference spectrum and a 455 nm spectrum (Fig. 1 [7]). The corresponding nitrosoalkane complex formed in these conditions derives from a direct chemical reaction between cytochrome P450-Fe(III) and the hydroxylamine. The formation of Fe(II)—nitrosoalkane complexes has been observed during chemical reactions of the same hydroxylamines with either porphyrins-Fe(III) or Fe(III)-methemoglobin and metmyoglobin [46]. However, in these cases, the nitrosocomplexes formation proceeded much faster and to a greater extent than with cytochrome P450. It is noteworthy that the nitrosoalkane-cytochrome P450 complexes formation in the absence of NADPH, was only observed after PB-induction. This could be related to the particular ability of microsomes from PB-pretreated rats to give 455 nm spectra after oxidative metabolism of amphetamines or *N*-hydroxyamphetamine [7].

In the 455 nm absorbing complexes, the nitrosoalkane ligands could be bound to Fe(II) either by nitrogen or oxygen. Nitrosobenzene has been recently shown to be coordinated by nitrogen to Fe(II) in phtalocyanin complexes [42]. Moreover, tertiary nitrosoalkanes [18, 8] like amines [35] or thiols [43] having a trialkylsubstituted carbon in α position of the hetero-atoms, are unable to bind to cytochrome P450-iron because of steric hindrance around nitrogen. This suggests that nitrosoalkanes are bound through their nitrogen atom to cytochrome P450-Fe(II) in the 455 nm absorbing complexes.

The important inhibitory effect displayed by the hydroxylamines RR'CHNHOH, on various microsomal monoxygenase activities seems to be related to their ability to form, in the conditions of metabolic hydroxylations, 455 nm absorbing complexes whose nitrosoalkane ligands are not displaced by oxygen. This is supported by the non-competitive type of inhibition observed with 1-hydroxylamino-2-phenyl ethane (Fig. 5) and by the increase of their inhibitory effects after preincubation of the hydroxylamines with microsomes, NADPH and oxygen, conditions required for the complete formation of the nitrosoalkane complexes.

According to our results, the formation of the 455 nm absorbing inhibitory complexes during microsomal oxidative metabolism of hydroxylamines, first observed by Franklin with *N*-hydroxyamphetamine [7] is a general phenomenon. Such complexes will certainly be observed during microsomal metabolic oxidation of several amines, generalizing previous results on amphetamines (10–19], SKF 525A [15] and other aminodrugs [14]. Provided that it can be first metabolically transformed into a hydroxylamine, RR'CHNHOH, any amine could lead to a 455 nm absorbing complex.

Thus, the failure of desmethylamphetamine, contrary to amphetamine [11], to give a 455 nm absorbing complex can probably be related to its lesser ability to be *N*-hydroxylated, as the corresponding hydroxylamine RR'CHNHOH ($R = CH_2Ph$, $R' = H$) leads rapidly to the nitrosoalkane complex [8]. On the contrary, phentermine is *N*-hydroxylated [44, 45] but does not lead to a 455 nm absorbing complex [8]. This is related to the inability of this nitrosometabolite to bind to cytochrome P450-Fe(II), as it is the case for all the tertiary nitroalkanes, too hindered around nitrogen [8, 18]. The general formation of nitrosoalkane-cytochrome P450 complexes and their important inhibitory properties on monoxygenases should be taken into account in pharmacology, especially in the case of drugs associations including an aminocompound susceptible to a metabolic oxidation to a primary or secondary nitrosoderivative.

Acknowledgements—We thank Miss M. Carlier for skillful technical assistance. This work was supported in part by C.N.R.S. grant ATP no. 2647 and by D.G.R.S.T. grant no. BRD 75-7-1614.

REFERENCES

1. M. Kiese, *Pharmac. Rev.* **18**, 1091 (1966).
2. H. Uehleke, *Xenobiotica* **1**, 327 (1971).
3. A. H. Beckett, *Xenobiotica* **1**, 365 (1971).
4. D. M. Ziegler, D. Jollow and D. E. Cook, in *Proc. Third Int. Symposium on Flavins and Flavoproteins* (Ed. H. Kamin), 507, University Park Press, Baltimore (1971).

5. J. A. Miller and E. C. Miller, *Proc. Exp. Tumor Res.* **11**, 273 (1969).
6. F. F. Kadlubar, E. M. Mc Kee and D. M. Ziegler, *Archs Biochem. Biophys.* **156**, 46 (1973).
7. M. R. Franklin, *Molec. Pharmac.* **10**, 975 (1975).
8. J. Jonsson and B. Lindeke, *Acta Pharm. Suecica* **13**, 313 (1976).
9. J. C. Kawalek, W. Levin, D. Ryan and A. Y. H. Lu, *Drug. Metab. Disp.* **4**, 190 (1976).
10. J. Werringloer and R. W. Estabrook, *Life Sciences* **13**, 1319 (1973).
11. M. R. Franklin, *Xenobiotica* **4**, 133 (1974).
12. R. C. James and M. R. Franklin, *Biochem. Pharmac.* **24**, 835 (1975).
13. J. Werringloer and R. W. Estabrook, *Archs Biochem. Biophys.* **167**, 270 (1976).
14. M. K. Buening and M. R. Franklin, *Drug. Metab. Disp.* **2**, 386 (1974).
15. J. B. Schenkman, B. J. Wilson and D. L. Cinti, *Biochem. Pharmac.* **21**, 2373 (1972).
16. M. K. Buening and M. R. Franklin, *Drug Metab. Disp.* **4**, 244 (1976).
17. M. R. Franklin, *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1027 (1976).
18. D. Mansuy, P. Beaune, J. C. Chottard, J. F. Bartoli and P. Gans, *Biochem. Pharmac.* **25**, 609 (1976).
19. D. Mansuy, P. Gans, J. C. Chottard and J. F. Bartoli, *Eur. J. Biochem.* **76**, 607 (1977).
20. U. Frommer, V. Ullrich and H. J. Staudinger, *Z. Physiol. Chem.* **351**, 903 (1970).
21. A. G. Gornall, C. J. Bardwill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
22. T. Omura and R. Sato, *J. biol. Chem.* **177**, 751 (1949).
23. A. I. Ryer and G. B. L. Smith, *J. Am. Chem. Soc.* **73**, 5675 (1951).
24. W. D. Emmons, *J. Am. Chem. Soc.* **79**, 5739 (1957).
25. Beckmann, *Ann.* **365**, 201 (1908).
26. R. T. Gilsdorf and F. F. Nord, *J. Am. Chem. Soc.* **74**, 1837 (1952).
27. G. Zinner, *Arch. Pharm.* **296**, 57 (1963).
28. B. S. S. Masters, J. Baron, W. E. Taylor, E. L. Isaacson and J. Lospalluto, *J. biol. Chem.* **246**, 4143 (1971).
29. K. J. Netter and G. Siedel, *J. Pharmac. exp. Ther.* **146**, 61 (1964).
30. Y. Imaï, A. Ito and R. Sato, *J. Biochem. Jap.* **60**, 417 (1966).
31. V. Ullrich and P. Weber, *Hoppe-Seyler's, Z. Physiol. Chem.* **353**, 1171 (1972).
32. J. B. Schenkman, D. L. Cinti, S. Orrenius, P. Moldeus and R. Kraschniz, *Biochemistry* **11**, 4243 (1972).
33. Y. Yoshida and H. Kumaoka, *J. Biochem.* **78**, 455 (1975).
34. D. W. Nebert, K. Kumaki, M. Sato and H. Kon, *Hoppe-Seyler's Z. Physiol. Chem.* **357**, (1976).
35. C. R. E. Jefcoate, J. L. Gaylor and R. L. Calabrese, *Biochemistry* **8**, 3455 (1969).
36. L. F. Audrieth and M. Sveda, *J. Org. Chem.* **9**, 89 (1944). J. Aloy and P. C. Rabaut, *Bull. Soc. Chim. Fr.* **33**, 654 (1905).
37. J. H. Boyer, in *The Chemistry of the Nitro and Nitroso Groups* (Ed. H. Feuer) Part 1 (1969).
38. G. J. Mannering, in *Fundamentals of Drug Metabolism and Drug Disposition* (Eds B. N. La Du, G. Mandel and E. L. Way), p. 206 Williams and Wilkins Co. (1971).
39. V. Ullrich, P. Weber and P. Wollenberg, *Biochem. biophys. Res. Comm.* **64**, 808 (1975).
40. N. V. Sidgwick, in *The Organic Chemistry of Nitrogen* Clarendon, Oxford, 304 (1966).
41. M. Sugiwa, K. Iwasaki and R. Kato, *Molec. Pharmac.* **12**, 322 (1976).
42. J. J. Watkins and A. L. Balch, *Inorg. Chem.* **14**, 2720 (1975).
43. W. Nastainczyk, H. H. Ruf and V. Ullrich, *Chem. biol. Interact.* **14**, 257 (1976).
44. A. H. Beckett and P. M. Belanger, *J. Pharm. Pharmac.* **26**, 205 (1974).
45. A. K. Cho, B. Lindeke and C. Y. Sum, *Drug Metab. Disp.* **2**, 1 (1974).
46. D. Mansuy, P. Battioni, J. C. Chottard and M. Lange, *J. Am. Chem. Soc.* **99**, 6441 (1977).
47. D. Mansuy, J. C. Chottard and G. Chottard, *Eur. J. Biochem.* **76**, 617 (1977).