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THE NATURE OF THE "455 nm ABSORBING COMPLEX" FORMED DURING THE CYTOCHROME P450 DEPENDENT OXIDATIVE METABOLISM OF AMPHETAMINE

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Introduction

During their microsomal oxidative metabolism, a few compounds lead to intermediates able to react with reduced cytochrome P450 forming tight complexes with characteristic absorbances in the Soret region. They include methylene dioxyphenyl compounds (1), amphetamines (2,3), SKF 525A, SKF 26754 A(4), Lilly 18947 (5) and fluorene (6). Most of the corresponding stable complexes exhibit a characteristic Soret band around 455 nm and cause an inactivation of cytochrome P450 towards further mono-oxygenation reactions. The metabolite of amphetamine bound to reduced cytochrome P450, in the 455nm absorbing complex, is still not known. However Franklin (7) has recently shown that N-hydroxyamphetamine gives the 455 nm complex more rapidly and more extensively than amphetamine itself or its analogs, but only after oxidative metabolism.

Assuming that the active metabolite of amphetamine was derived from N-oxidation, the aim of this study was to identify it among the possible intermediates arising with the successive oxidation steps from amphetamine 1 to the nitro derivative 5 (Fig.1).

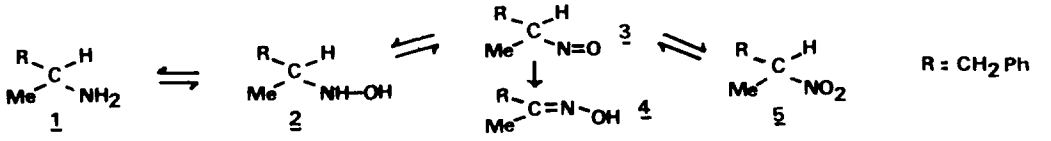

Fig.1 : Possible redox intermediates between amphetamine 1 and its corresponding nitrocompound 5

Our results are in favour of the nitroso compound 3, formed either by an oxidative route from 1 and 2 or by a reductive route from the nitro derivative 5, being the metabolite acting as a strong ligand of the Fe(II) of cytochrome P450 and thus responsible for the 455 nm absorbing spectrum.

Materials and methods : Hepatic microsomes were prepared from male Sprague-Dawley rats as described previously (8). The animals were pretreated with phenobarbital (three daily intra peritoneal injections of 80 mg/kg) and starved overnight before sacrifice. Spectrophotometric measurements were performed on an Unicam SP 800 spectrophotometer with microsomes at a concentration of 2-3 mg protein/ml in 0,1 M tris-HCl buffer, pH = 7,4. Protein concentrations were determined by the Biuret method (9). NADPH was purchased from Boehringer, nitro

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derivatives from Aldrich Chemicals ; d-amphetamine was a gift from Smith, Kline and French Laboratories. Horse myoglobin was purchased from Sigma Chemical Co and human hemoglobin was kindly provided by the "Laboratoire de Pathologie Moléculaire", group of Dr. Labie, CHU-Cochin, Paris.

Results

The oxime 4 having the next higher oxidation level after N-hydroxy amphetamine 2 and being the stable tautomer of the nitroso derivative 3, gives no difference spectra with rat liver microsomes either directly in a reducing medium (excess $Na_2S_2O_4$) or after pretreatment by NADPH and O_2 followed by dithionite reduction. On the contrary, the nitro compound 5, prepared by oxidation of 1 by CF_3CO_3H (10), interacts with rat liver microsomes, in the presence of excess sodium dithionite, leading to the formation of an intense difference spectrum with a peak at 455 nm. This result is in agreement with the first observation of Ullrich and al. (11) concerning the interaction of nitro-2 propane with dithionite-reduced microsomal cytochrome P450. Moreover, the difference spectrum produced by the nitro compound 5 is very similar to that obtained after oxidative metabolism of amphetamine (Fig.2).

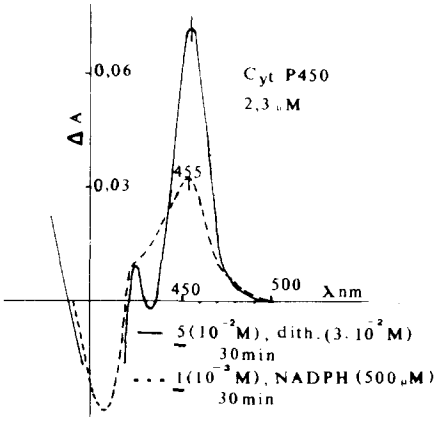

Fig.2: Difference spectra of rat liver microsomes produced by 5 and dithionite (—), and by oxidative metabolism of 1 (---)

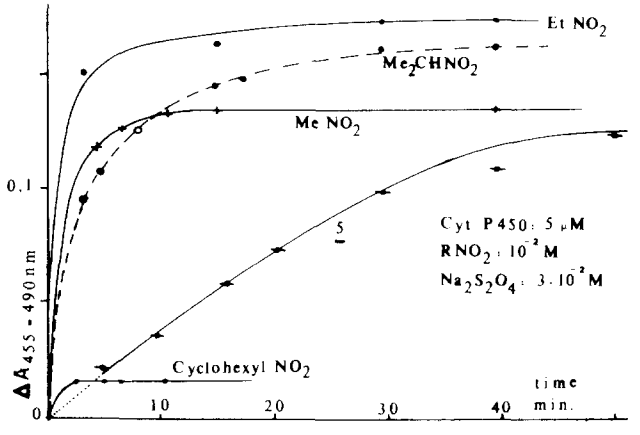

Fig. 3: Kinetics of appearance of the 455 nm peak from rat liver microsomes + nitrocompounds + dithionite.

The maximum absorbance of the 455 nm peak is only reached after 30 min. (Fig.3) and the Ks determined by the double reciprocal plot of spectral change versus 5 concentration. ($1/\Delta A_{455-490\ nm} = f(1/c)$) is 10^{-4} M. Several other aliphatic nitro compounds, RNO_2, with R = methyl, ethyl, n-pentyl, n-hexyl, cyclohexyl, cyclopentyl, benzyl, behave similarly. The corresponding spectra exhibit different maximum absorbances (Δ Am(455-490 nm) at saturation by the substrate and different kinetics of appearance of the 455 nm peak (Fig.3). However , C-tertiary nitro compounds, like 2-methyl-2-nitropropane and 2-methyl-2-nitro-3 phenyl propane do not induce the 455 nm spectrum formation under the same conditions.

Aliphatic nitro compounds also exhibit a strong interaction with other reduced hemo proteins, hemoglobin and myoglobin, but only in the presence of an excess of sodium dithionite (12). For instance, Fig.4 shows that deoxy HbA, under anaerobic conditions, does not give any spectral change in the presence of 10 mM nitromethane after 3 h. However, after addition of excess sodium dithionite a new spectrum appears with γ , β and α bands respec - tively at 420, 542 and 562 nm (Fig.4).

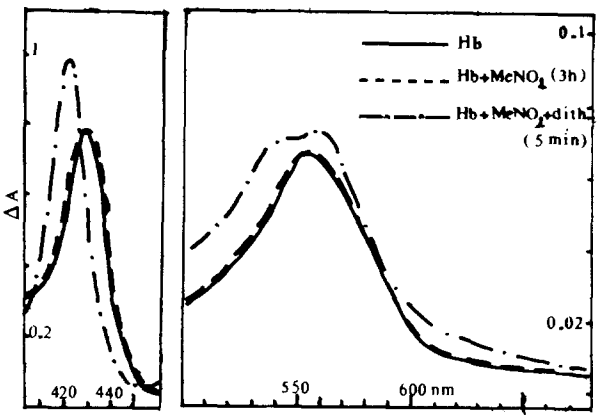

Fig.4: The effect of $MeNO_2$ on the absolute spectrum of HbA without and with sodium dithionite – Conditions : see (a).

Table 1: Spectral characteristics of Mb or HbA with $MeNO_2$ and dithionite, and of the HbA-C_6H_5NO complex (14).

	β	α	γ
Horse Mb (a)	546 nm	575	425
+ dithionite			
+ $MeNO_2$	(ε = 15)	(shoulder)	(163)
HbA (a)	542 (sh)	562	421
+ dithionite			
+ $MeNO_2$	(13,2)	(13,6)	(168)
HbA + C_6H_5NO	542 (sh)	562	422
(ref. 14)	(13,1)	(14)	(154)

(a) -Hemoprotein ($5.\ 10^{-5}$ MFe) in buffer PH = 7,4 t = 20°C; $MeNO_2$ (10^{-2}M) $S_2O_4Na_2$ (3.10^{-2}M) - ε in [$cm^{-1}.M^{-1}$]

The need of a reducing agent for the formation of the new HbA complex indicates that it is not derived from the direct interaction of deoxy HbA with the nitro compound but from its interaction with a metabolite formed by a reduction of the nitro compound with dithionite, either directly* or via the HbA heme. Concerning the nature of this metabolite, Table 1 lists the spectral characteristics of its HbA complex comparatively with those of the previously reported nitrosobenzene-HbA complex (14). These characteristics and the shape of the two spectra are strikingly similar.

Discussion

The spectral change induced by nitro-2 propane with rat liver microsomal cyt. P450 in the presence of excess sodium dithionite, reported by Ullrich and al. (11), is an example of a general phenomenon with aliphatic nitro compounds. Furthermore, the nitro compound 5 produces, under these conditions, the same spectral change and presumably the same cyt. P450 complex, as the one obtained from amphetamine 1 or N-hydroxy amphetamine 2 under oxidative conditions. Therefore, in the 455 nm absorbing complex, the entity bound to reduced cyt. P450 may be : a)- the nitro compound itself or a species having the same oxidation level, like the previously proposed anion (11).

b)- a reduced metabolite of the nitro compound.

The slow kinetics of appearance of the 455 nm spectrum with rat liver microsomes (Fig.3) and the lack of spectral interaction of HbA or horse Mb with nitro compounds in the absence of dithionite are strongly in favour of proposition b).

As it appears that the 455 nm absorbing complex is formed after reduction of 5 and as Franklin (7) showed that it is formed after oxidation of 2, the metabolite bound to reduced cyt. P450 in this complex must have the oxidation level of the nitroso compound 3 or its tautomer 4. As the oxime 4 fails to produce any spectral change with microsomal cyt. P450, we propose that the 455 nm absorbing complex corresponds to the binding of the nitroso compound 3 on the sixth coordination site of iron (II) in cyt P450 :

$$1 \text{ (or } 2) \xrightarrow[O_2]{\text{NADPH}} \left[R\text{-}\underset{\underset{O}{\|}}{N} \rightarrow \text{Fe(II) cyt. P450} \right] \xleftarrow{Na_2S_2O_4} 5$$

* Chemical reduction of nitro compounds by dithionite is known (13).

The same type of complex can explain the spectra obtained with Hb or Mb and aliphatic nitro compounds, with excess dithionite. Our hypothesis is suppported by the close similarity between the corresponding spectra and that of the known Hb-nitrosobenzene complex (14). Some complexes of nitroso compounds with transition metal ions are known, particularly with iron (II) derivatives (15), and we are currently investigating the nature of the metal - RNO bond, the strength of which could explain the great stability of the 455 nm absorbing complexes and, therefore, their inactivation of cyt. P450.

Furthermore, the biological occurence of nitroso compounds formed either by reductive metabolism of the corresponding nitro compounds or by oxidative metabolism of the amino derivatives that is to say the biological existence of the redox chain of Fig.1, was previously established : aromatic nitroso compounds derived from aniline or nitrobenzene (16, 17) or even very recently, aliphatic nitroso derivatives derived from amphetamines (18) have been isolated as metabolic intermediates in animals.

The correspondence between the failure to obtain the 455 nm complex either from Mephentermine (N-methyl-1,1-dimethyl-2-phenyl ethylamine) after oxidative metabolism, as noticed by Franklin (19) or from the corresponding nitro derivative 2-methyl-2-nitro-3-phenyl propane after reductive metabolism (vide supra), can be explained by the steric hindrance at the carbon α to nitrogen in these two derivatives, preventing the nitroso metabolite from binding to cyt. P450 Fe(II). Actually C-tertiary nitro compounds, like 2-methyl-2-nitro propane or 2-methyl-2-nitro-3-phenyl propane in the presence of excess dithionite, and 2-methyl-2-nitroso propane itself do not produce significant spectral changes with HbA or horse Mb. These preliminary results should lead to a correlation between the structure of some amino or nitro compounds and their ability to produce a 455 nm absorbing cyt.P450 complex after metabolisation.

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