Replacing the Mixed Function Oxidase *N*-Demethylation by Hydrogen Peroxide and either Hemoglobin or Ferrous Sulfate

Shmuel Zbaida[1,2], Dalia Gilhar[1], and Judith Silman-Greenspan[1]

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Abstract: Formaldehyde liberated from *N*-demethylation of aminopyrine, *N*-methylpiperazine and from the optically active (+)- and (−)-ephedrine was quantitated from the incubation mixtures of hepatic microsomes and from reactions of hydrogen peroxide with either hemoglobin or ferrous sulfate. Stereoselectivity was observed only in incubations with hepatic microsomes. *N*-Formyl derivatives were isolated from the reactions of *N*-ethylmorpholine, aminopyrine, *N*-methylpiperazine and *N,N'*-dimethylpiperazine with ferrous sulfate and hydrogen peroxide (Fenton reagent). Electron-withdrawing groups (NH, NCH_3, O) at the fourth position to the *N*-alkyl group facilitate *N*-dealkylation with Fenton reagent.

The microsomal mixed function oxidase system featuring cytochrome P-450 catalyzes a variety of different reactions such as *N*-, *O*- and *S*-dealkylation, aliphatic, aromatic and *N*-hydroxylation, epoxidation, sulfoxidation, dehalogenation, desulfurization and deamination (1–3). The catalytic cycle of cytochrome P-450 is believed to involve reduction of oxygen to peroxide followed by formation of the perferryl ion species **1** (4, 5).

$$\text{P-450}\cdot\text{Fe(III)} \xrightarrow[2e^-,\,2H^+]{O_2} \text{P}\cdot\text{450}\cdot\text{Fe(III)}\cdot H_2O_2$$

$$\longrightarrow \text{P-450}\cdot\text{Fe(III)}\begin{matrix}\\ |\\ O\end{matrix} + H_2O$$

1

This assumption is supported by the fact that a number of peroxidative agents can replace NADPH, the reductase and oxygen as co-reactants in most of the hydroxylation reactions of microsomal cytochrome P-450 (6, 7). Furthermore, it has been established that a mixture of Fe (II) and H_2O_2 (Fenton reagent) is a useful reagent for hydroxylation and epoxidation of hydrocarbons (4). To date, no reactions of *N*-dealkylation by either Fenton reagent or hemoglobin-hydrogen peroxide have been described.

A comparison betwen the *N*-dealkylation catalyzed by cytochrome P-450 to that performed by inorganic and hemoproteinic iron models with peroxides is essential for the evaluation of events occurring at the cytochrome P-450 catalytic site.

Materials and Methods

'Sabra' rats (male, 80 to 100 g each) were administered phenobarbital in their drinking water for 5 days. Microsomes were prepared by standard procedures (8, 9). The concentration of cytochrome P-450 was determined by the method of Omura and Sato (10) and protein was determined by the method of Lowry (11).

Incubations with Hepatic Microsomes

Incubations (in duplicate) were performed in 1.5 ml for 15 minutes at 37°C with 50 mM potassium phosphate buffer (pH = 7.4), 4 mg of phenobarbital-induced rat liver microsomes (2 nmoles P-450/mg protein), an NADPH generating system (7.2 mM *d*-glucose-6-phosphate, 0.66 mM $NADP^+$ and 0.06 units/ml glucose-6-phosphate dehydrogenase), 5 mM $MgCl_2$, 2 mM semicarbazide hydrochloride and 0.66 mM substrate as hydrochloride salt. Reaction controls contained all components except microsomes.

Incubations with Hemoglobin and Hydrogen Peroxide

Incubations (in duplicate) were performed in 1.5 ml for 15 minutes at 37°C with 50 mM potassium phosphate buffer (pH = 7.4), 0.15 mM hemoglobin (bovine), 0.33 mM hydrogen peroxide (30%), 2 mM semicarbazide hydrochloride and 0.66 mM substrate as hydrochloride salt. Reaction controls contained all components except hydrogen peroxide.

Reactions with Fenton Reagent

The *N*-alkylamine substrates (as hydrochloride salts) in water were treated with one equimolar amount of ferrous sulfate and two fold excess of hydrogen peroxide (30%) at room temperature (25°C) for 15 minutes. Work-up afforded the *N*-formyl products.

N-Methyl-*N'*-formylpiperazine **(4)** and *N,N'*-diformylpiperazine **(5)** were isolated from the reaction mixture of *N*-methylpiperazine **(3)**. The diformyl compound **5** was also isolated (18%) from the reaction of *N,N'*-dimethylpiperazine **(6)**. 1-Phenyl-2-formyl-3-methyl-4-aminoformyl-3-pyrazoline-5-one **(7)** was isolated from the reaction of aminopyrine **(2)**, and N-formylmorpholine **(9)** was isolated from the reaction of *N*-ethyl-morpholine **(8)** (Figure 1). The properties of the products are summarized in Table I.

Formaldehyde Quantitation

Formaldehyde liberated by *N*-demethylation was quantitated according to Nash (15) as modified by Cochin and Axelrod (16).

Results and Discussion

Formaldehyde (nmoles/min/nmole P-450) liberated by the *N*-demethylation of aminopyrine, **2** (1.4), *N*-methylpiperazine, **3** (1.1) and the optically active (+)-ephedrine (0.4) and (−)-ephedrine (0.25) was quantitated in the incubation mixtures with the hepatic microsomes. The stereoselectivity of cytochrome P-450 for ephedrine observed in these experiments was primarily associated with cytochrome P-450 substrate binding (17). However, in contrast to the incubations of ephedrine with rat liver microsomes, no stereoselectivity was observed using hemoglobin (0.15 mM) and hydrogen peroxide (0.33 mM). Fifteen μM formaldehyde was quantitated after 15 minutes at room temperature from both (+)- and (−)-ephedrine. This is probably a conse-

[1] Department of Pharmacy, School of Pharmacy, The Hebrew University, POB 12065, Jerusalem 91120, Israel
[2] Correspondence address

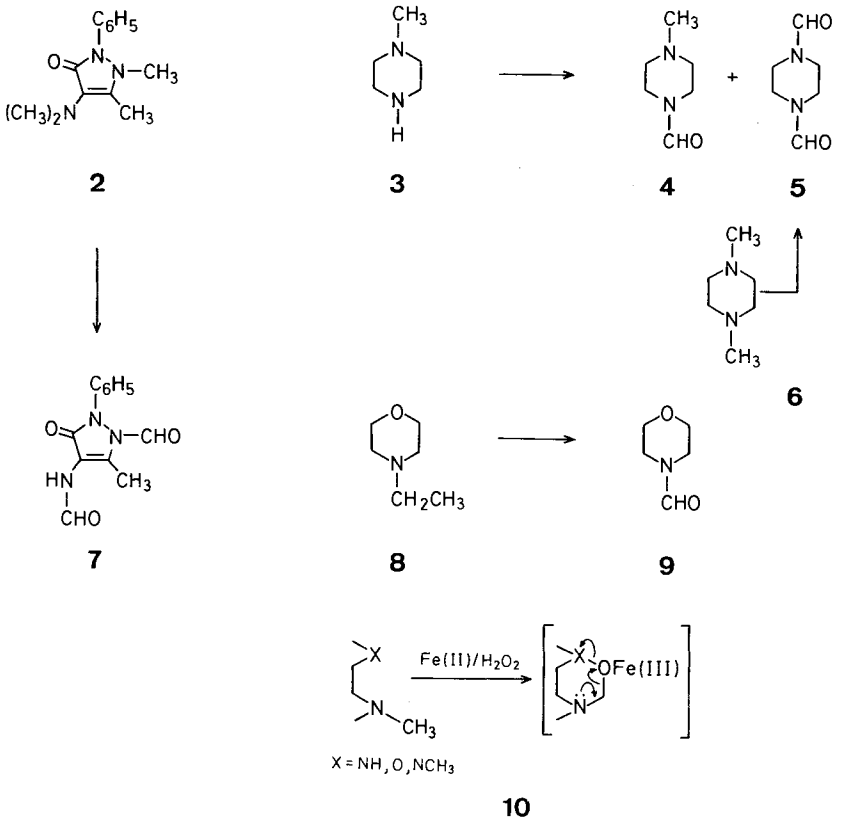

Fig. 1 Chemical structures of *N*-formyl products.

quence of globin lacking a substrate binding site. Formaldehyde was also quantitated from aminopyrine (**2**, 91 μM) and *N*-methylpiperazine (**3**, 25 μM) after 15 minutes from reactions performed as above with hemoglobin-hydrogen peroxide.

These results obtained with hepatic microsomes and hemoglobin-hydrogen peroxide support a mechanism of *N*-demethylation associated in both cases with ferryl or perferryl active species. Furthermore, a type II spectral change ensued by titrating the binding of amines to cytochrome P-450. Such spectral changes obtained as difference spectra indicate coordination of the amines to the heme moiety of cytochrome P-450 (18). A logical experimental approach to examine this hypothesis is to perform the *N*-demethylation by Fenton reagent. However, in contrast to the hepatic microsomes, no reaction is observed for *N,N*-dimethylaniline with the use of Fenton reagent. On the other hand we found that aliphatic amines containing N or O at the fourth position to the *N*-alkyl group were easily *N*-dealkylated by Fenton reagent with subsequent liberation of formaldehyde. Liberated formaldehyde was quantitated (15 minutes, 25°C) from a reaction mixture containing ferrous sulfate (0.66 mM), an equimolar amount of substrate and hydrogen peroxide (1.33 mM) from both aminopyrine (**2**, 150 μM) and *N*-methylpiperazine (**3**, 60 μM).

The formation of the *N*-formyl compounds **4** and **5** from *N*-methylpiperazine **(3)**, (Figure 1) in addition to formaldehyde, probably occurs via a recombination of formic acid (oxidation of formaldehyde) with *N*-dealkylated amines. However, we have no satisfactory explanation for the formation of *N*-formylmorpholine from *N*-ethylmorpholine. Since the existence of additional heteroatoms (N, O) at the fourth position to the N-alkyl group facilitate the *N*-dealkylation by Fenton reagent, we assume the formation of a perferryl six membered ring intermediate **10**. Similarly, oxidation of cyclohexanol by Fenton reagent in acetonitrile-water (1:9) leads predominantly to *cis*-1,4-cyclohexanediol (19).

It has been postulated (4, 5) that the normal reduction-oxygenative mechanism of the mixed function oxidase and the peroxidative pathway operate via a ferryl or perferryl intermediate. However, unlike the *N*-demethylation performed by Fenton reagent in which an additional electronegative residue is essential for the *N*-demethylation, there is no evidence to indicate that a similar mechanism involving a six-membered ring intermediate is operative in the *N*-demethylation reactions catalyzed by microsomes or by hemoglobin and hydrogen peroxide. Studies to clarify these points and their relevance to the cytochrome P-450 catalytic site are in progress.

Table I. The Properties of *N*-Formyl Products[a]

No.	Conversion %	mp/bp°C	νC=0 cm^{-1}	*m/e* M^+ (relative intensity)	NMR 300 MHz δ formyl ppm	δ others ppm
4	17	bp 102-4[12]	1650	128 (100%)	7.994 (1H s)	3.540, 3.354 (2Ht, J = 4.80 Hz) each, 2.322–2.295 (4H m) 2.286 (3H s)
5	18	mp 127.5[13]	1660	142 (20%)	8.113 (2H s)	3.556, 3.442 (4H s) each
7[b]	3	oil	1660	245 (37%)	7.438 (2H s)	7.272–7.115 (5H m) 1.552 (3H s)
9	6[c]	bp 236-7[14]	1660	115 (23%)	7.979 (1H s)	4.240, 3.58 (4H m) each

(a) Satisfactory elemental analysis (C, H, N) were obtained for all compounds.
(b) UV(EtOH) 220 (3.16), 270 (2.87); **7** was isolated in addition to other products of ring cleavage.
(c) In addition, 3-hydroxy-*N*-ethylmorpholine was isolated, 20% conversion, oil, νOH 3300 cm^{-1}, nmr ($CDCl_3$) δ4.910 ½H t (J=2.654 Hz) 4.93 ½H t(J=2.211 Hz), 4.692 1H bs (OH) 3.981–3.908 2H m, 3.625–3.555 2H m, 2.558 1H d(J=2.211Hz), 2.520 1H d(J=2.654 Hz), 2.368, 2.320 2H dq(J = 7.519 Hz), 1.009 3H t(J=7.519 Hz). Molecular weight 131, found m/e 131 (80%)

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Effect of Mangiferin, a Naturally Occurring Glucosylxanthone, on Reproductive Function of Rats

Sukumar Chattopadhyay[1], Utpala Chattopadhyay[1], Santyeswar P. Shukla[2], and Shibnath Ghosal[2,3]

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Abstract: Female albino rats, treated with mangiferin at daily doses of 5 mg/100 g ip for 3 days during mid-gestation, showed complete fetal resorption. Administration of a mangiferin-Cu^{2+} (1:1) complex, along with mangiferin, attenuated the effect of mangiferin and restored the completion of gestation in 60% of the rats. The mother rats that completed gestation successfully did not show any post-natal abnormality, and the litters born were also normal. Mangiferin treatment of non-gestating female rats caused little or no changes in their organ weights. However, changes in the protein and protein-DNA ratios of several organs were statistically significant. Mangiferin also modified the ascorbic acid retention capacity of adrenal glands of male rats *in vitro*. These findings are appraised in view of mangiferin as a potential antifertility agent.

[1] Department of Physiology, Institute of Medical Sciences, Banaras Hindu University
[2] Department of Pharmaceutics, Institute of Technology, Banaras Hindu University
[3] Correspondence address Prof. Ghosal, Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Banaras Hindu University, Varanasi-221005, India

Mangiferin (1,3,6,7-tetrahydroxyxanthone-C_2-β-D-glucoside) is widely distributed in higher plants. The glucosylxanthone was isolated in substantial amounts from *Canscora decussata* Schult (family Gentianaceae) (1), *Swertia chirata* Buch.-Ham. (Gentianaceae) (2), and *Mangifera indica* Linn. (Anacardiaceae) (3), following their reputation as valuable plant drugs in the Indian systems of medicine. The current study is part of a program to evaluate the potential of phytochemicals as antifertility agents (4, 5). It was prompted by the following observations. (i) Ingestion of tender leaves of *M. indica*, which contain 2% mangiferin (3), caused 'anti-spring flush' (decrease in yield of milk) in dairy cattle (5). Chemical agents which inhibit prolactin synthesis (cause of 'anti-spring flush') have been found to also interrupt pregnancy (6). (ii) Mangiferin was previously found to inhibit monoamine oxidase in the CNS of albino mice and rats (2, 7–9). Inhibition of pregnancy in mammals by naturally occurring CNS active agents is a well documented phenomenon (10). Thus, an antifertility effect of mangiferin was considered a reasonable possibility.

Materials and Methods

Mangiferin was isolated from tender leaves of *Mangifera indica* Linn. (cv Banarasi Langra) according to a published procedure (3).

Animals

Rats of the CF strain were bred and maintained, under controlled conditions (12 h light, 22 ± 3° C), with Hindusthan Lever food pellets and tap water ad libitum. Female rats (150–200 g) with regular estrus cycle were mated with male rats of proven fertility. Presence of sperms in vaginal smear of female rats, in estrus, was taken as day 1 of pregnancy. Pregnancy was confirmed by laparotomy on parallel groups of rats.

Gestation

Female rats showing presence of sperms in the vaginal smear were given mangiferin, suspended in saline, intraperitoneally (ip) at doses of 1.0, 2.5 and 5 mg/100 g body weight (b.w.) day^{-1}, for 3 days at mid-gestation, i. e. on day 12, 13, and 14. The rats were sacrificed along with those treated with the vehicle (saline) on day 20 of gestation, and the uteri were examined for the presence of normal fetus or signs of resorption. Distinct areas of hemorrhage and sign of tissue debris were taken as points of resorption. A total dose of 15 mg of