CHEMICAL STRUCTURE OF ETHIDIUM BROMIDE METABOLITES EXTRACTED FROM RAT BILE

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1. Introduction

Ethidium bromide is one of the most studied DNA intercalating agents [1–4] and behaves as a trypanocidal agent [5]. Likewise it exhibits mutagenic properties [6] and some antitumor activity against the L1210 leukemia of mice [7]. These biological effects could be related to the biological transformation of the amino groups into electrophilic species as nitrenium cation of nitroxyl radical known to bind covalently to DNA [8,9]. Such an activation process was described for several strong antitumor drugs like endoxan [10] or ellipticine [11,12]. After photoactivation ethidium azide shows the same biological effects as those produced by ethidium itself [13]. This result seems to imply the covalent binding of ethidium to DNA which requires the previous biological activation of the drug.

To test this assumption and to design more active derivatives, a study of the ethidium metabolism was performed [14]. Two metabolites were extracted from rat bile and purified by high-performance liquid chromatography (HPLC) [14]. Here, the unambiguous determination of their chemical structure was achieved by mass and ^{1}H NMR spectroscopies. A possible pathway for biochemical transformation of ethidium is proposed.

2. Experimental

2.1. *Chemical*

Ethidium bromide was purchased from Sigma Chemical Co.

8-amino 5-ethyl 6-phenylphenanthridinium chloride I and 8-acetamido 5-ethyl 6-phenylphenanthridinium acetate II were synthetized as in [15] and crystallized from ethanol. Their purity was checked by thin-layer chromatography on silica gel plates with a 4:1:1 mixture of butanol:acetic acid:water as eluent.

2.2. *Purification of ethidium metabolites*

Biliary samples from rats treated successively with Aroclor 1254 [16] and ethidium as in [14] were collected during 7 h, incubated at 37°C for 20 h with an equal volume of β-glucuronidase (*Escherichia coli* type 1, Sigma Chemical Co.) in 0.1 M acetate buffer (pH 5) (2000 units/ml). The samples were then extracted with *n*-butanol (1/1, v/v) and centrifuged at 6000 × *g* for 20 min. The butanolic phase was lyophilized and the residue dissolved in methanol. The metabolites were then separated by HPLC on a Waters reverse phase μ-Bondapack-CN column (4.5 mm × 30 cm) using as eluent a degassed mixture of *n*-butanol:water:methanol, 90:8:2 (by vol.) containing ammonium acetate (5×10^{-3} M); flow rate 1.2 ml/min. The retention times are, respectively, 9, 12 and 14 min for B′, A and ethidium bromide. HPLC was performed on a Waters Associates instrument equipped with a 6000 A pump, a U6K injector and a 440 UV detector. Detection was made at $\lambda = 254$ nm and recorded with a Houston Instrument 5210 omniscribe chart recorder. Before NMR and mass spectrometry experiments, the samples from HPLC were chromatographed on Sephadex LH20 using ethanol:sodium acetate 0.1 M, 80:20 as eluent. The pure fractions were collected, then lyophilised.

2.3. *Mass spectrometry*

Mass spectra were obtained with a Varian MAT 311 A spectrometer by field desorption using an anode heating current of 15–25 mA.

2.4. ^1H *NMR spectroscopy*

^1H NMR spectra were recorded at 270 MHz in DMSO-d_6 on a Bruker WH 270 MHz operating at 20°C in the Fourier transform mode.

All chemical shifts are expressed in ppm with respect to the hexamethyldisiloxane (HMDS) signal used as an internal reference and are reliable to ± 0.01 ppm.

3. Results and discussion

Using HPLC techniques two ethidium metabolites, A and B were separated from the bile of rats treated with ethidium bromide after liver monooxygenase activation by Aroclor 1254 [14]. The minor metabolite B is a glucuroconjugated derivative of ethidium as shown by its transformation into a new compound B′ under the action of β-glucuronidase [14].

3.1. *Mass spectroscopy*

Fig.1 shows the mass spectra of ethidium bromide, metabolite A and deconjugated derivative B′ obtained by field desorption. In the mild conditions used, only the peaks corresponding to the quaternary ethidium ion and to the tertiary base after loss of the quaternary ethyl group are observed. However, based on the peak due to the ethidium ion at $m/z = 314$, the positions of the highest mass peaks in the spectra of A and B′ correspond to the introduction of an acetyl group in A and both an acetyl-group and a hydroxyl-group in B′. The structure of A was confirmed by the similarity of its mass spectrum and HPLC retention volume with those of an authentic sample of 8-monoacetamido-ethidium obtained by an unambiguous synthesis [15].

3.2. *NMR spectroscopy*

Apart from some reports on ethidium [17] and its dimeric analogs [18,19] no systematic NMR studies were done on phenanthridinium derivatives. Therefore, the structural determination of ethidium metabolites required a preliminary analysis of the NMR spectra of the 8-amino 5-ethyl 6-phenylphenanthridinium chloride I and its 8-acetyl derivative II, which were obtained by an unambiguous synthesis.

The NMR spectrum of I (fig.2b) shows an AMX part corresponding to the 8-amino substituted ring, in which the more shielded proton (6.38 ppm) was attributed to H_7 as for ethidium [17]. On the basis of this assignment, the H_9 and H_{10} protons were attributed from double resonance experiments whereas the remaining resonances were assigned starting from the H_1 signal, shifted downfield by the well-known peri effect.

The introduction of an acetyl group on the 8-amino substituent of I leads to the expected deshielding

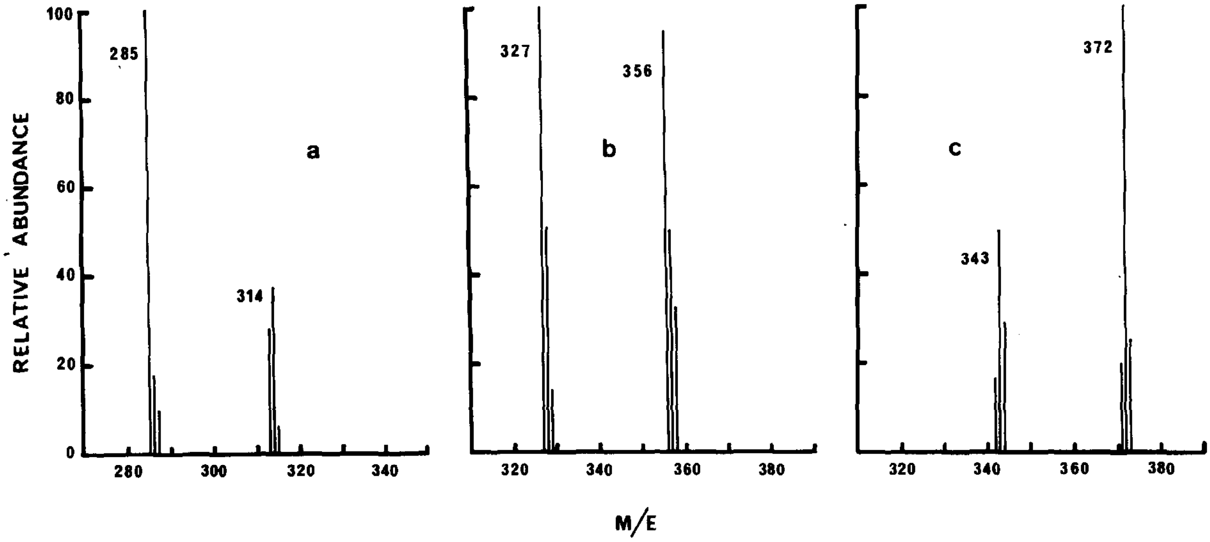

Fig.1. Mass spectra of (a) ethidium bromide, (b) metabolite A and (c) deconjugated derivative B′ performed by field desorption technique. Anode heating current is respectively 15, 25 and 25 mA.

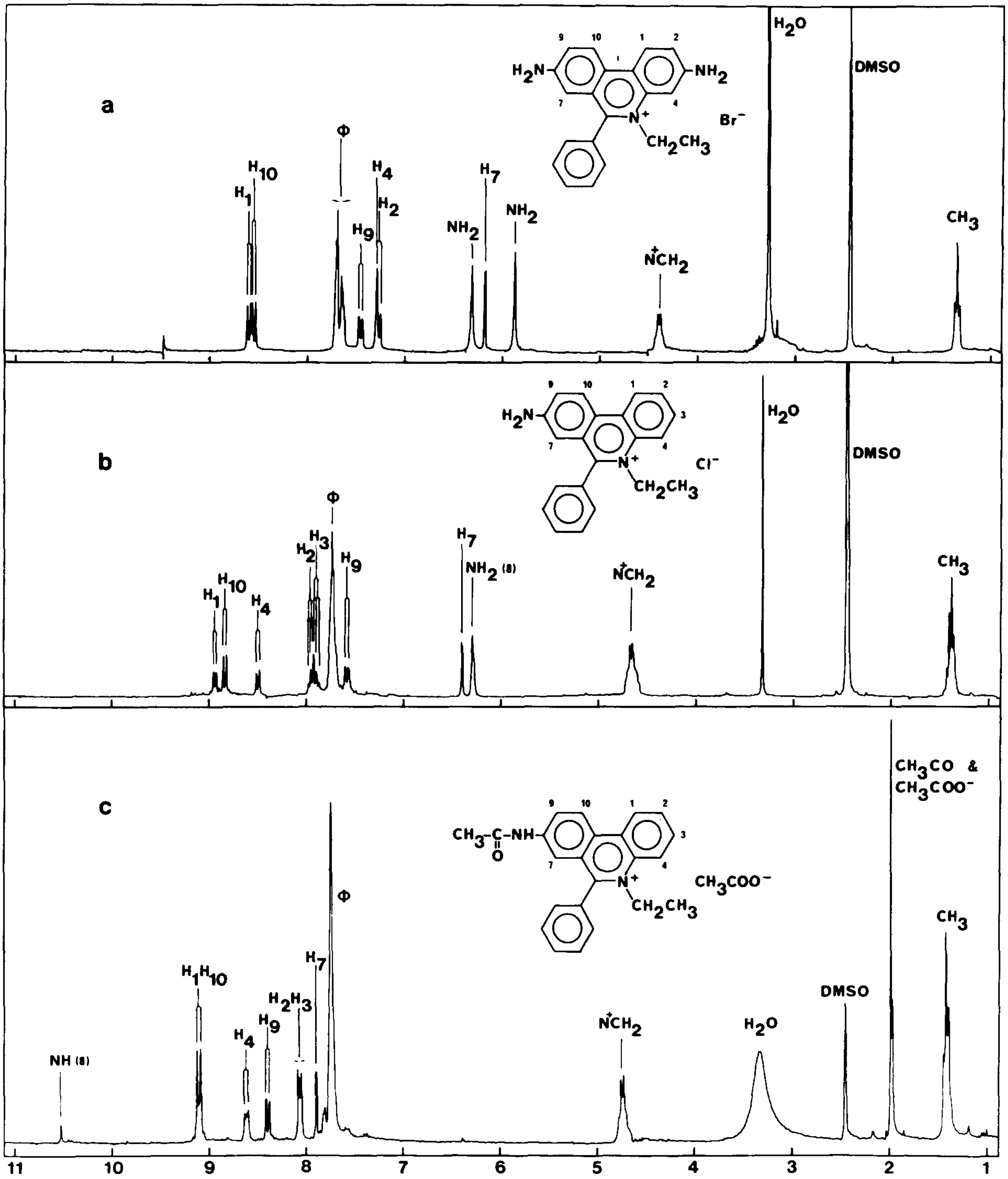

Fig.2. 270 MHz ^1H NMR spectra of (a) ethidium bromide, (b) 8-amino 5-ethyl 6-phenylphenanthridinium chloride I and (c) 8-acetamido 5-ethyl 6-phenylphenanthridinium acetate II in DMSO-d_6; $T = 20°C$.

Table 1

Chemical shifts of 8-amino 5-ethyl 6-phenylphenanthridinium chloride I and chemical shifts differences between 8-amino 5-ethyl 6-phenylphenanthridinium chloride I and 8-acetamido 5-ethyl 6-phenylphenanthridinium acetate II ($\Delta\delta_{II-I}$) or ethidium bromide ($\Delta\delta_{Et-I}$), respectively

Protons	1	2	3	4	7	9	10
δ_I	8.91	7.93	7.86	8.46	6.38	7.58	8.82
$\Delta\delta_{II-I}$	+0.15	+0.11	+0.18	+0.14	+1.50	+0.80	+0.27
$\Delta\delta_{Et-I}$	−0.33	−0.64	–	−1.14	−0.20	−0.10	−0.30

These increments, expressed in ppm, were obtained from the NMR spectra of I, II and ethidium bromide performed in DMSO-d_6. Positive values correspond to a deshielding effect

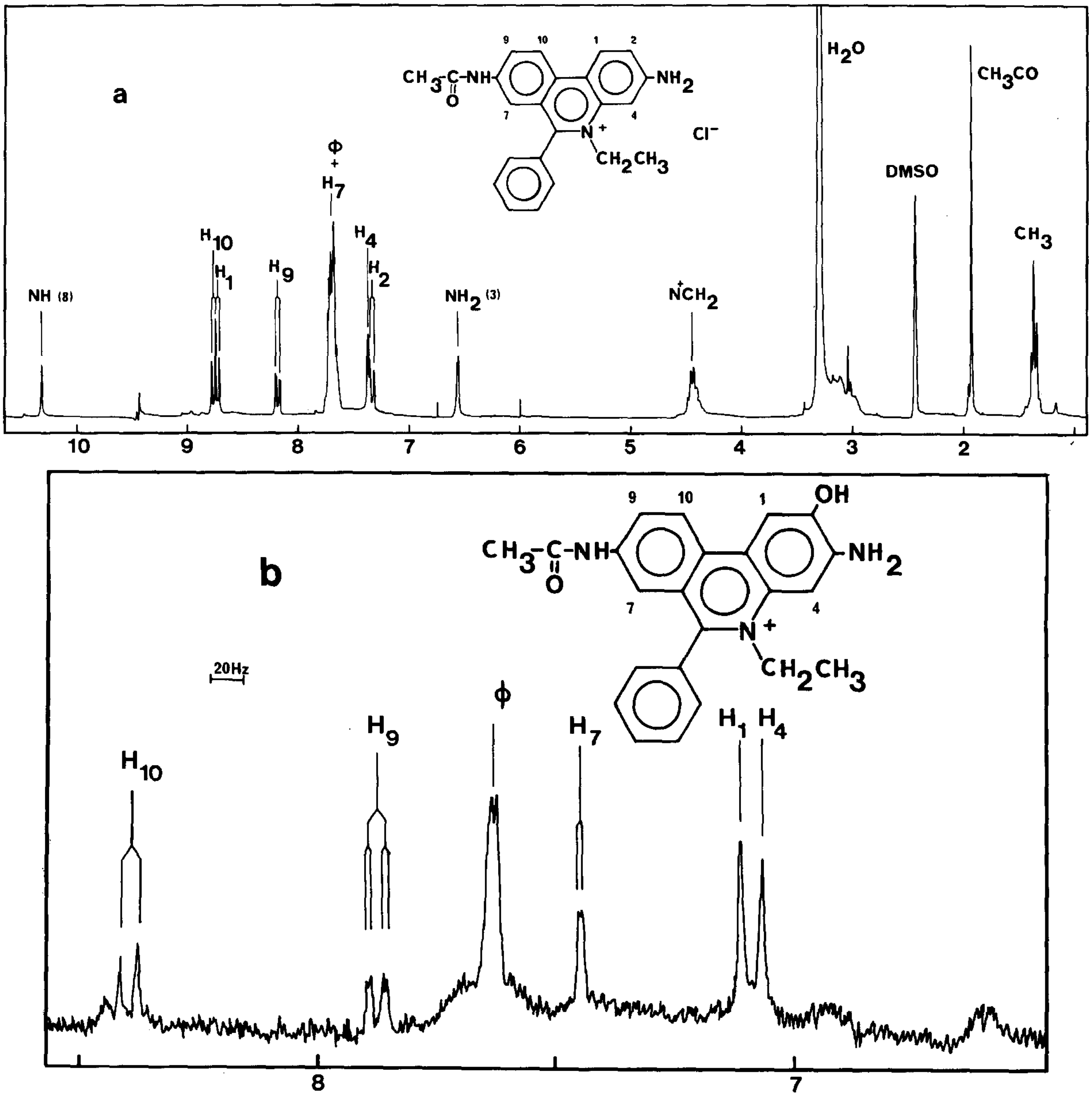

Fig.3. 270 MHz ^1H NMR spectra of (a) metabolite A and (b) deconjugated derivative B' in DMSO-d_6; T = 20°C.

Table 2

Comparison between computed (δ_{calc}) and observed (δ_{obs}) chemical shifts (in ppm) for the aromatic protons of metabolite A

Protons	1	2	4	7	9	10
δ_{calc}	8.73	7.40	7.46	7.68	8.28	8.79
δ_{obs}	8.73	7.33	7.37	7.70	8.10	8.77

The δ_{calc} were computed using experimental chemical shifts of I and the increments $\Delta\delta_{II-I}$ and $\Delta\delta_{Et-I}$ of table 1. The observed chemical shifts δ_{obs} were measured from the NMR spectrum of A performed in DMSO-d_6 at 20°C

effects (fig.2c) occurring mainly on the protons of the acetamido substituted ring as evidenced by the chemical shift differences $\Delta\delta_{II-I}$ (table 1). The additional peak at 1.96 ppm corresponds to the acetyl group (fig.2c). As compared to I, the NMR spectrum of ethidium (fig.2a) reflects the expected shielding effect due to the introduction of a 3-amino group as shown by the chemical shift differences $\Delta\delta_{ET-I}$ (table 1).

The NMR spectrum of the metabolite A (fig.3a) exhibits two ABX systems in the aromatic region and a methyl signal at 1.96 ppm. The signals corresponding, respectively, to the acetamido and the amino rings and therefore the position of the acetyl group were finally established from the very close agreement (table 2) between the experimental chemical shifts and those computed using the $\Delta\delta_{II-I}$ and $\Delta\delta_{ET-I}$ increments of table 1. Moreover, the methyl signal of the acetyl group appears at the same position (1.96 ppm) in the spectra of A and II. Therefore the methyl signal at 2.15 ppm in the spectrum of the 3,8-diacetylethidium (not shown) can be unambiguously assigned to the 3-acetyl group.

The very low quantity of the deconjugated minor metabolite B′ obtained from preparative HPLC of rat bile extracts treated by β-glucuronidase has required several lyophilisations in D_2O in order to remove contaminents from HPLC eluent (H_2O, *n*-butanol, ammonium acetate, methanol). Therefore all exchangeable protons of this deconjugated metabolite do not appear in its spectrum. The ^1H NMR spectrum of B′ exhibits a singlet at 1.94 ppm (3H) partially overlapped by the contaminant counter-ion acetate resonance (1.88 ppm). This signal can easily be assigned to a 8-acetamido group by comparison with those of compounds II and A. The two coupled signals at 4.31 ppm (quadruplet 2H) and 1.31 ppm (triplet 3H) confirm that this phenanthridinium derivative is still quaternized by an ethyl group.

The aromatic part (fig.3b) shows two independent AMX and A′X′ systems. Double resonance experiments demonstrate that the signals at 7.44 ppm (doublet J_{AX} = 2 Hz), 7.87 ppm (doublet of doublet J_{AM} = 9 Hz, J_{AX} = 2 Hz) and 8.33 ppm (doublet J_{AM} = 9 Hz) are coupled and therefore located on the same ring. The two remaining resonances at 7.06 ppm

Table 3

Chemical shifts of the aromatic protons of 8-acetylethidium (= metabolite A) and of the deconjugated derivative B′ following two possible structural assumptions (9- or 2-hydroxy derivatives)

Protons	1	2	4	7	9	10
8-acetyl ethidium = A	8.73	7.33	7.37	7.70	8.10	8.77
B′ = first assumption = 8-acetyl 9-hydroxy ethidium	8.33	7.87	7.44	7.06	–	7.11
B′ = second assumption = 8-acetyl 2-hydroxy ethidium	7.11	–	7.06	7.44	7.87	8.33

These experimental values were measured from the NMR spectra of A and B′ performed in DMSO-d_6

Table 4
Coupling constants of ethidium bromide, its metabolites A and B′ and the phenanthridinium derivatives I and II

	J_{1-2}	J_{1-3}	J_{2-3}	J_{2-4}	J_{3-4}	J_{7-9}	J_{9-10}
Ethidium bromide	9	–	–	2^{a}	–	2.5	9
8-amino 5-ethyl 6-phenyl phenanthridinium chloride I	8	2	8^{a}	2^{a}	8.5	2	9
8-acetamido 5-ethyl 6-phenyl phenanthridinium acetate II	8	2^{a}	8^{a}	2^{a}	8.5	2	9
8-acetyl ethidium = metabolite A	9	–	–	2^{a}	–	2	9
8-acetyl 2-hydroxy ethidium = metabolite B′	–	–	–	–	–	2	9

All J-values were directly measured on the spectra. When direct first order analysis of the spectra does not apply, [a] indicates that the given values are reliable to ± 1 Hz only

and 7.11 ppm exhibit neither ortho nor meta coupling since they are higher and sharper than the signals at 7.44 ppm and 7.87 ppm. Therefore these protons should be in *para* position one in respect to the other. The coupling constants of all the studied compounds are reported in table 4.

Two structures could agree with the spectrum of B′ (fig.3b), either 8-acetyl 9-hydroxy ethidium or 8-acetyl 2-hydroxy ethidium.

In the first case, the OH-group being on the 9 position, the AMX system should be assigned to H_1, H_2 and H_4 and the A′X′ part to H_7 and H_{10}. In that case, protons H_2 and H_4 would undergo a deshielding effect (see table 3) which cannot be explained by the introduction of the OH on the 9 position, since this group is a well-known upfield shift inducer. Therefore such a structure must be ruled out.

In the second structure the OH-group was located on the 2 position and the AMX system can be assigned to protons H_7, H_9 and H_{10} whereas the A′X′ part corresponds to H_1 and H_4. In this case, as expected, all the aromatic resonances are upfield shifted (table 3). Therefore the 8-acetyl 2-hydroxy ethidium structure could be attributed to this deconjugated metabolite B′. The larger than expected shielding effect on proton H_1 (1.62 ppm) using the shielding induced in benzene by an *ortho* OH group (0.56 ppm) [20] could be explained by three factors:

(i) B′ could be a phenolate ion and it is well-known that such ionization causes enhanced upfield shifts [21];

(ii) Large discrepancies between calculated and observed chemical shifts were found when two substituents are *ortho* to one another [21];

(iii) The substituent effects are more important in polycyclic systems than in benzene. Likewise the unexpected large shielding of H_{10} (0.44 ppm) could be related to a specific electronic distribution in the tricyclic quaternary compound.

4. Conclusion

In this study, the structure of the main metabolite of ethidium extracted from rat bile was unambiguously established as 8-acetyl ethidium (= 8-acetamido 3-amino 5-ethyl 6-phenyl phenanthridinium). The identity between the compound isolated from this biological medium and an analytical sample synthetized as in [15] indicates that in both conditions the acetylation occurs on the more basic amino group in accordance with [22]. The minor metabolite is the glucuroconjugated derivative of 8-acetyl 2-hydroxy ethidium (= 8-acetamido 3-amino 5-ethyl 2-hydroxy 6-phenylphenanthridinium).

Therefore, this study allows confirmation of the biological transformation process of ethidium bromide in liver cells proposed in [14]. This metabolic pathway involves a cytosolic *N*-acetyltransferase leading to 8-acetyl ethidium which could be hydroxylated through the action of microsomal monooxygenases (*P*-448 or P_1-450 cytochromes), followed by glucuro-

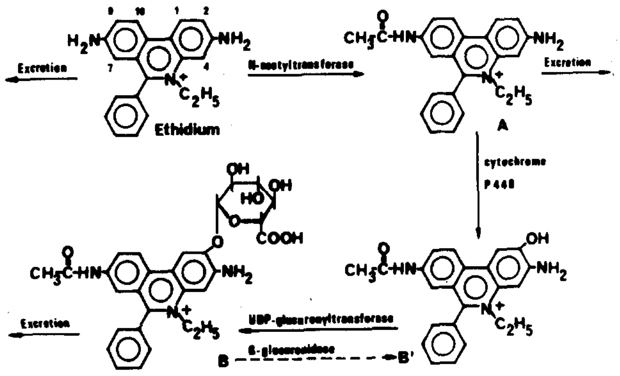

Fig.4. Simplified scheme of the ethidium metabolism in rat liver.

conjugation of the hydroxy derivative by UDP-glucuronyl transferase [14] (fig.4). These results agree well with [23] on the biological transformation of phenanthridinium drugs.

Compound B′ could arise from the transposition of an *N*-hydroxy amino-3 ethidium derivative resulting from the oxydation of the 3-amino group of compound A by liver enzymes. Formation of a highly mutagenic *N*-hydroxyamino derivative occurs in the case of 2-aminofluorene [24]. Whatever the biological mechanism involved in the formation of compound B′, its quinone–imine structure could allow its covalent binding to DNA in vivo. Synthesis of large quantities of B′ is now in progress to check this hypothesis.

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