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THE HEPATIC METABOLISM OF ETHIDIUM BROMIDE TO REACTIVE MUTAGENIC SPECIES: BIOCHEMICAL AND STRUCTURAL REQUIREMENTS

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Abstract—Ethidium bromide, which was known to undergo *N*-acetylation in rats, is shown to be metabolized by rat liver cytochrome P448 and a soluble protein to one or several reactive species which are detected in the *Salmonella* assay. This biotransformation depends on the presence of a free aromatic amino group at position -3, on the basis of a structure–mutagenic activity study of eleven phenanthridine derivatives. Using the *Salmonella* assay as a biological test for the production of reactive intermediates, we have attempted to purify this enzyme and demonstrated its lack of *N*-acetyl- or sulfotransferase activity. Ethidium bromide thus behaves as a mutagenic aromatic amine, the bioactivation of which differs from previously described mechanisms.

The trypanocidal drug ethidium bromide (3,8-diamino-5-ethyl-6-phenyl-phenanthridinium bromide) is known as a powerful mutagen in yeast, yielding a high proportion of cytoplasmic respiratory-deficient 'petite' mutants [1]. It is also able to cure bacteria from plasmids [2], but no bacterial mutagenic effects were recorded until McCann *et al.* [3] showed that frameshift mutations in several *Salmonella typhimurium* strains occurred in the presence of ethidium when the drug was activated by a 9000 *g* supernatant from Aroclor 1254® induced rat liver homogenate. Other workers reported similar results with various phenanthridinium derivatives [4, 5]. These experiments demonstrated that ethidium bromide could interact with nucleic acids not only by a reversible intercalation reaction [6, 7], which was currently admitted as the basis of its specificity towards the supercoiled DNAs of mitochondria or plasmids, but also through an irreversible, enzyme-mediated binding. Proof of the covalent nature of this reaction was obtained by the decreased yield of mutants produced by an excision-repair proficient strain compared to the isogenic *uvrB* derivative [5]. Some indications that covalent ethidium-DNA adducts could occur without external activation in yeast were also reported [8].

While the metabolic activation pathways of mutagens and carcinogens such as polycyclic aromatic hydrocarbons and amines are presently well understood in several cases, no attempt has been made to identify the reactive metabolite(s) responsible for the covalent binding of ethidium to DNA, and the enzyme species which carry out this transformation. The only metabolic route previously known to be available for ethidium is *N*-acetylation [9], which *by itself* does not lead to an increase of drug reactivity.

In this paper, we first report the results of a structure–activity study of eleven phenanthridinium derivatives, in order to relate ethidium bromide to a class of known mutagens. Then using Ames' *Salmonella* assay as a test of enzymatic activity, we demonstrate the existence of a soluble enzyme from rat liver, which is required together with the microsomal fraction for the biotransformation of ethidium to reactive mutagenic intermediate(s). Finally, we report about the partial purification of this enzyme and show that its activity is distinct from other known conjugations, which have been involved in the generation of reactive metabolites.

MATERIALS AND METHODS

Chemicals. Ethidium bromide was bought from Sigma. The two *N,N,N',N'*-tetramethyl compounds (see Fig. 1) were gifts from Dr. J. Markovits, Paris and the 5,6-dihydro derivative from Kakko Kogyo Ltd., Tokyo. Compounds MB 3427, MB 3016 and RD 16101 were kind gifts from Dr. M. J. Waring, Cambridge, U.K. The other phenanthridinium salts were prepared in this Laboratory [10] according to known procedures. The purity of all compounds was checked by high pressure liquid chromatography. They were dissolved in reagent-grade dimethylsulfoxide and kept at 4° in the dark. Compound VI was dissolved in ethanol and used under subdued light.

The bacteriological media were bought from Difco, the inorganic components from Merck A.G. Germany and the biochemicals from Sigma Co. DEAE Sephacel and Sephadex G100 were from Pharmacia and phosphocellulose (Cellex P) from Biorad.

Rat liver fractions. All operations were performed at 4° with sterile glassware, instruments and solutions. Male Sprague–Dawley rats weighing 250–300 g were induced either by 500 mg/kg Aroclor 1254 in olive oil intraperitoneally (the animals being killed 5 days later) or by 80 mg/kg phenobarbital in saline by the same way (sacrifice 3 days later). The rats

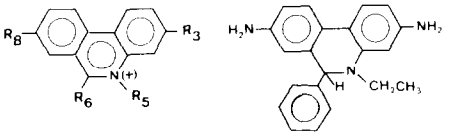

Fig. 1. Structure of phenanthridine derivatives. Left: Numbering of substituents. See Table 1 for denominations. Right: Structure of reduced ethidium.

Table 1. Indexing of the phenanthridine derivatives*

Substituents	Compound number I Ethidium	II	III MB3427	IV MB3016	V RD16101	VI reduced ethidium	VII	VIII	IX	X	XI
R_3	NH_2	$N(CH_3)_2$	—	—	$NHCOO-C_2H_5$	NH_2	NH_2	NH_2	CH_3CONH	CH_3CONH	$N(CH_3)_2$
R_5	C_2H_5	C_2H_5	CH_3	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	C_2H_5	—
R_6	C_6H_5	C_6H_5	C_6H_5	p-NH_2-C_6H_4	C_6H_5	(H) C_6H_5	C_6H_5	C_6H_5	C_6H_5	C_6H_5	C_6H_5
R_8	NH_2	$N(CH_3)_2$	—	NH_2	$NHCOO-C_2H_5$	NH_2	—	CH_3CONH	CH_3CONH	—	$N(CH_3)_2$

* The numbering of R substituents is made according to Fig. 1.

were anaesthesized with diethylether and 10–15 ml of sterile saline were perfused in the left ventricle after resection of the right one [11]. 20 ml saline were also injected in *vena porta* to wash the blood away. The livers were removed, minced with scissors and homogeneized in a Potter apparatus with 3 × weight of 0.2 M phosphate buffer (pH 7.2). The resulting homogenate was centrifuged for 30 min at 9000 *g* in a Beckman JA 20 rotor and the supernatant was used immediately or kept frozen at −80° (for up to 1 month). The protein concentration, determined by the method of Lowry [12] with bovine serum albumin as a standard, was about 30 mg/ml.

When necessary, the 9000 *g* supernatant was fractionated by centrifugation at 105,000 *g* during 1 hour into a supernatant and a microsomal pellet, which was resuspended in the same buffer, centrifuged for 1 more hour at the same speed and resuspended in a minimal volume of buffer. All soluble fractions from rat liver were sterilized by filtration on 0.45 μg detergent-free Millipore filters.

Spectral interactions studies with cytochrome P448. Cytochrome P448 was partly purified from the 100,000 *g* pellet of a 9000 *g* supernatant of Aroclor-induced rat liver homogenate. The microsomes were made 20% in sodium cholate and sonicated for 1 min. The resulting suspension was chromatographed on an affinity column of octamethylenediamine Sepharose 4B [13]. A carbon monoxide difference spectrum of the dithionite-reduced preparation indicated a content of 2.8 nmoles cytochrome P448 per mg protein, based on $\varepsilon = 91\ mM^{-1} \cdot cm^{-1}$ [14, 15]. Since ethidium and most other phenanthridinium derivatives absorb in the region of interest, the interaction studies were performed in double-compartment spectrophotometer cuvettes. The use of purified cytochrome P448 allowed to record difference spectra with sufficient spectral energy.

Enzyme purifications and assays. Rat or rabbit liver *N*-acetyltransferase (EC 2.3.1.5.) was assayed on isonicotinoylhydrazide as a substrate with CoA-S-Ac as a cofactor [16]. Phenolsulfotransferase (EC. 2.8.2) was assayed on phenol with a 3′-adenosinephosphate-5′-phosphosulfate generating system [17, 18]. *N*-Formyl-L-kynurenine formamidase (EC.3.5.1.9) was purified and assayed on *N*-acetylanthranilic acid as described by Santti [19].

In the chromatographic fractionation of the ethidium-activating enzyme, pooled or individual fractions were tested in duplicate at two or three amounts per plate, to check the linearity of the mutagenic effect vs. protein concentration.

Mutagenesis experiments. The determination of the mutagenic potency of the phenanthridinium

Table 2. Mutagenic properties of eleven phenanthridine derivatives*

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI
TA 98	400	2080	0	0	0	190	29	0	0	3	220
TA 1538	300	550	0	0	0	90	33	0	0	0.3	150
TA 1537	3	14	0	0	0	4	20	0	0	0	14
TA 100 and TA 1535	0	0	0	0	0	0	0	0	0	0	0

* *Salmonella* strains are listed in the leftmost column. The experiments were performed as described in Materials and Methods. The results are expressed in revertants per nmole of drug per plate. "0" indicates that at non toxic concentrations, the dose–response curve never exceeded twice the number of spontaneous revertants (about 15 for TA 1535, 150 for TA 100, 12 for TA 1537, 30 for TA 1538, 40 for TA 98). All determinations were done with Aroclor-induced rat liver homogenate (9000 *g* supernatant). No compound in this series was active without this preparation.

derivatives was carried out essentially as described by Ames [20]. The results (expressed as the number of revertants per nmole of chemical) were taken from the linear part of the dose–response curve whenever possible. Care was exercised to keep down the drug concentration on the petri plates to a level allowing the continuous growth of the background lawn of auxotrophic bacteria.

The five bacterial strains used in these studies were gifts of Dr. B. N. Ames, Berkeley CA. In each experiment, their phenotypic characters were checked according to published procedures [20]. Besides, routine positive controls were performed to control their ability of reversion to histidine prototrophy with methyl methanesulfonate (strain TA100), 4-nitroquinoline *N*-oxide (TA1535), and the frameshift mutagens 9-aminoacridine (TA1537) or 2-nitrofluorene (TA98 and TA1538). Aflatoxin B_1 was used to test the efficiency of the liver homogenates. All these standard mutagens gave yields of his^+ revertants comparable to those already published [20].

In reconstitution experiments, each petri plate received known amounts of microsomal (1 mg) and soluble (0.1 mg) proteins, unless otherwise specified. 1 ml of native or reconstituted fraction also contained 8 μmoles $MgCl_2$, 5 μmoles glucose-6-phosphate, 4 μmoles $NADP^+$ and 0.2 M phosphate buffer (pH 7.4). Fractions of the 100,000 *g* supernatant were also additioned with 0.13 unit glucose-6-phosphate dehydrogenase (EC 1.1.1.49). 0.5 ml of this mixture was poured on each plate.

RESULTS

Ethidium bromide as a mutagenic aromatic amine. Table 2 summarizes our findings in a study of eleven phenanthridine derivatives with Ames' *Salmonella* assay. Ethidium and three analogs had previously been studied with the same bacterial test by MacGregor and Johnson [4], but the limited number of substances prevented drawing conclusions about structure–activity relationships.

Table 2 shows that (a) blocking both aromatic amino groups by carbethoxylation (compound V) or acetylation (compound IX) results in complete loss of mutagenicity; (b) suppressing both of these groups (compound III) has the same effect; (c) on the other hand, tertiary amino groups have strong (compound XI) and extremely strong (compound II) mutagenic properties. The suppression of the 3-amino group in compound IV destroys the mutagenic activity and acetylation of the 3-monoamino derivative (compound X vs compound VII) results in a tenfold decrease in mutagenicity. The residual effect can possibly be explained by deacetylation by the liver homogenate.

These results led us to consider that ethidium bromide would follow the metabolic pathways already described for carcinogenic aromatic amines.

Table 3. Activation of ethidium bromide by components of differently pretreated liver homogenates*

	Number of his^+ revertants per plate		
	Aroclor pretreatment	Phenobarbital pretreatment	Artificial reconstitutions
Fraction of liver homogenate			
9000 *g* supernatant	1800	0	
microsomes	0	0	
cytosolic fraction	0	34	
reconstituted mixture	1766	52	
"Aroclor" microsomes + "phenobarbital" cytosol			1330
"Aroclor" microsomes + control cytosol			1800
"Phenobarbital" microsomes + Aroclor cytosol			140

* The *Salmonella* strain was TA 1538. The background of spontaneous revertants was 34. "0" indicates that the increase of the number of his^+ colonies was less than this figure. Other conditions as described in Materials and Methods.

Table 4. Inhibition by 7,8-benzoflavone of mutagenesis induced by ethidium bromide*

μg Ethidium per plate	μg 7,8-Benzoflavone per plate	His⁺ revertants per plate
	0	1530
1	20	111
	50	135
	0	3600
2	20	165
	50	113
	0	4400
5	20	240
	50	138

* The *Salmonella* strain was TA 98. The background number of revertants (80) has been substracted from these figures. 7,8-Benzoflavone was not mutagenic in this and larger dose ranges, with or without Aroclor-induced rat liver preparation.

N-Oxidation by mixed-function liver monooxygenases and conjugation by soluble enzymes were therefore investigated.

Specificity of the microsomal activation. Table 3 describes an experiment in which the 9000 *g* supernatant of differently pretreated animals was further fractionated by centrifugation at 105,000 *g*. As already mentioned [5], induction of liver monooxygenases by phenobarbital results in a nearly inactive metabolizing system. Furthermore, it is apparent that the activation of ethidium bromide requires both microsomal and soluble fractions from the 9000 *g* supernatant. Table 3 also shows that the lack of effect of phenobarbital-induced liver preparations can be ascribed to the nature of the microsomal fraction. The complementing soluble factor(s) may be drawn from animals pretreated with either phenobarbital, Aroclor or even solvent alone. The conclusion of these experiments is that the microsomal metabolism of ethidium bromide is carried out almost selectively by the forms of mixed-functions monooxygenases* which are induced by polycyclic aromatic hydrocarbons, namely cytochrome P448†. Moreover, at least one soluble factor is required for the transformation of ethidium to reactive mutagenic species.

Further evidence that cytochrome P448 is involved in this biotransformation comes from the experiment described in Table 4, showing the strong inhibitory effect of 7,8-benzoflavone on ethidium-induced mutagenesis.

* We have confirmed that no activation occurs in the absence of NADP(H) [5].

† Atlas *et al.* [21] have pointed out that ambiguities remain in the current nomenclature of the various forms of P450. We hereby use the denomination "P448" to differentiate our preparation of Aroclor-induced rat liver, which had a Soret peak at this wavelength, from cytochrome of uninduced or phenobarbital-induced rat liver. Specifically, we do not imply, at this stage of our work, a requirement for aromatic amide *N*-oxidation as associated with the blue-shifted electrophoresis band described in ref. [21]. The recent separation of cytochromes P_1-450 and P448 [22], will help to clarify this point, and work is being carried out in this direction in our Laboratory.

Spectral interaction studies on phenanthridines and cytochrome P448. The latter findings prompted us to study the interactions between phenanthridine derivatives and partly purified oxidized cytochrome P448 from Aroclor-induced rat liver microsomes. Figure 2 shows the difference spectra obtained when

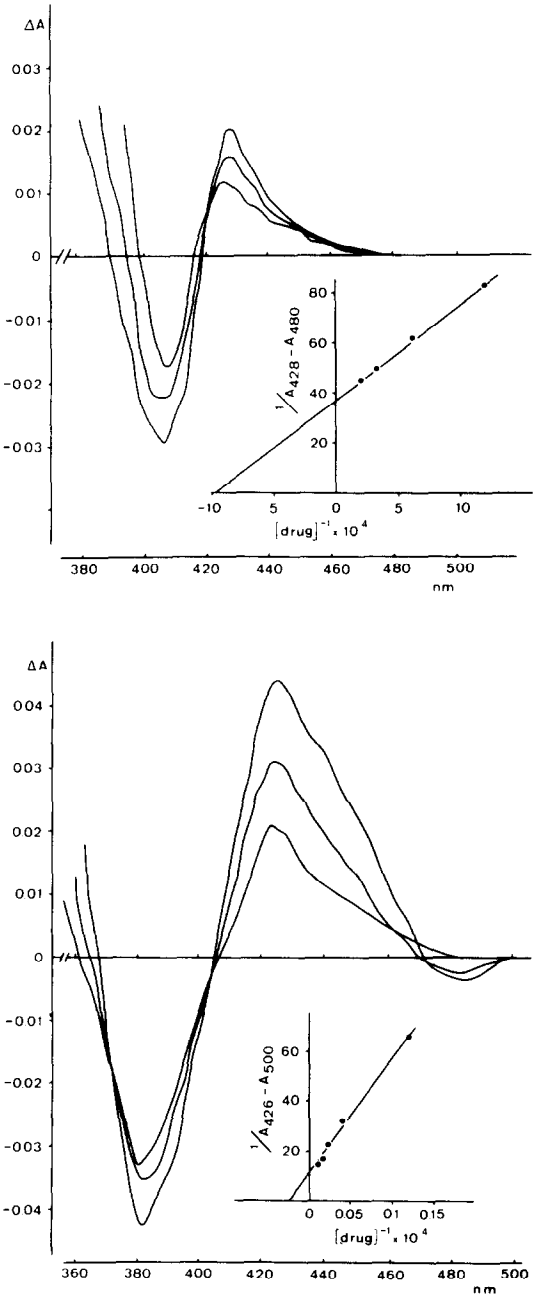

Fig. 2. Interaction of uncharged phenanthridines with cytochrome P448. (a) Reduced ethidium bromide (compound VI); (b) tetramethyl phenanthridine (compound XI). 1 ml cuvettes contained 4 nmoles cytochrome P448 in 50 mM Tris–HCl buffer (pH 7.5), 3 mM $MgCl_2$, 200 mM sucrose. Drug concentrations increased from 0.6 to 3.2 10^{-5} M (saturating ligand concentration). Insets: double reciprocal plots of Absorbance at 428 or 426 nm versus ligand concentration.

Table 5. Effect of heat or dialysis on the soluble factor*

		Addition to microsomes			
	9000 *g* sup.	—	native soluble fraction	dialyzed fraction	boiled fraction
Number of his$^+$ colonies	3120	52	2840	1980	103

* The bacterial strain was TA 98. 5 μg (10 nmoles) ethidium bromide were added per plate. The background number of spontaneous revertants (105) has been substracted. Microsomes were from Aroclor-induced rats.

compound VI (reduced ethidium) or XI (unquaternarized phenanthridine) were added to the sample cuvette. Lineweaver–Burk plots of these results yielded spectral dissociation constants equal respectively to 10^{-5} and $4 \cdot 10^{-5}$ M. The spectra are typical "type II" patterns [23], with maxima at 426 and 428 nm, respectively, indicating an interaction between nitrogen lone-pair electrons with the sixth coordination site of heme Fe^{3+}. It was not possible to attribute this effect to a particular nitrogen atom within each molecule.

On the other hand, addition of increasing amounts of ethidium bromide or compound II failed to produce any detectable spectral modification. Preincubation at 37° of the cytochrome suspension with ethidium or addition of NADPH to the mixture were also inneffective.

Proteic nature of the soluble factor. The soluble factor which is needed to complement cytochrome P448 in the activation reaction was assayed as described in Table 5. The 9000 *g* supernatant of Aroclor-induced rat liver homogenate was reconstituted with microsomes and 100,000 *g* supernatant which had been submitted to either extensive dialysis against 0.2 M phosphate buffer (pH 7.4), boiling for 10 min or no treatment. It can be deduced from Table 5 that this soluble factor is a heat sensitive macromolecule.

The proteic nature of this factor was demonstrated (Table 6) by incubating it with either micrococcal nuclease (EC 3.1.4.7), ribonuclease A (EC 3.1.4.22) or α-chymotrypsin (EC 3.1.21.1) before reconstituting the metabolizing mixture, and performing the mutagenesis assay on ethidium bromide. To avoid a possible destruction of the microsomal component of the metabolizing mixture, the protease inhibitor phenylmethylsulfonyl fluoride was added to the mixture of soluble fraction and chymotrypsin after 1 hr incubation at 37° with the latter. It is clear from Table 6 that hydrolysis of the nucleic acids has, if anything, a stimulatory effect upon the activation of ethidium bromide.

On the other hand, the mutagenic efficiency becomes completely lost after proteolytic digestion. Several control experiments are shown in Fig. 3, which demonstrate that phenylmethylsulfonyl fluoride is non-toxic to bacteria within the useful dose

Table 6. Effect of preincubation of rat liver 100,000 *g* supernatant extract with various hydrolytic enzymes* on mutagenesis by ethidium bromide*

Incubation of soluble factor	Reconstituted mixture
No enzyme	900
DNAse	1300
RNAse	1065
α-Chymotrypsin	0
Preinhibited chymotrypsin	555

* The experiments were performed on a 45–65% $(NH_4)_2SO_4$ cut of the 100,000 *g* supernatant. All enzymes were dissolved in 0.01 M Tris–HCl (pH 7.8), 0.1 M KCl, 2 mM $MgCl_2$. Amounts of enzymes per plate were: DNAse, 12 units; RNAse, 3 units; α-chymotrypsin, 2 units. Phenylmethylsulfonyl fluoride was added after incubation of the soluble fraction with the enzymes (150 μg/plate). Incubation lasted 1 hour at 37°. Inhibition of chymotrypsin (last row) was performed during 10 min at room temperature. In this case, no more phenylmethylsulfonyl fluoride was added before pouring the mixture on the plates. The strain was TA 98. Background (microsomes alone) has been subtracted. The combination of enzymes + liver extract, or enzymes + microsomes has no effect.

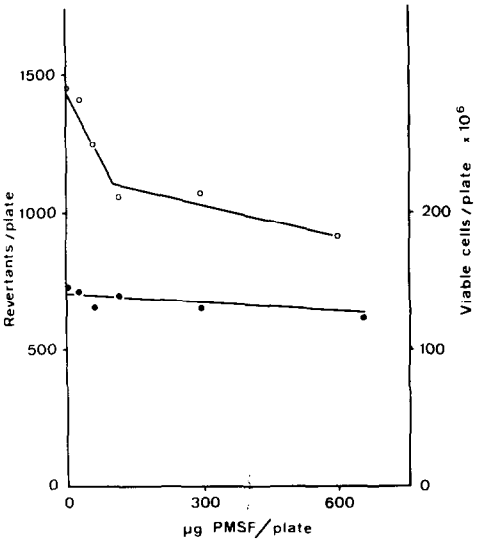

Fig. 3. Effect of phenylmethylsulfonyl fluoride on mutagenesis by ethidium bromide. Open circles: mutagenicity of 5 nmoles ethidium, in presence of 9000 *g* supernatant, and various amounts of inhibitor. Full circles: Number of viable bacteria per plate. Phenylmethylsulfonyl fluoride was dissolved (6 mg/ml) in ethanol. Dilutions of a bacterial culture were spread on minimal agar plates, supplemented with 0.1 ml 10^{-1} M histidine and 0.1 ml 10^{-3} M biotin, and additioned with 5 nmoles ethidium and 9000 *g* supernatant.

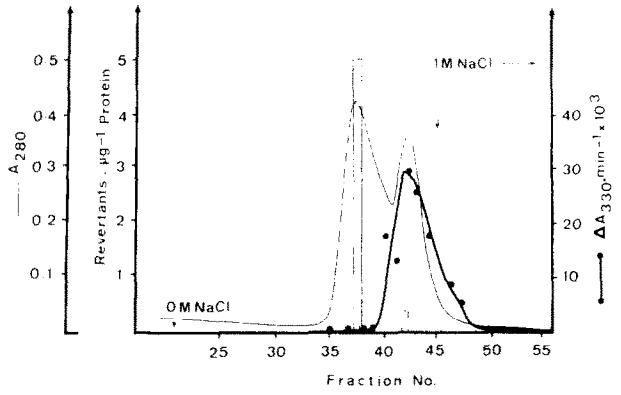

Fig. 4. Separation of formamidase and mutagenic activities on DEAE Sephacel. 6 mg of rat liver cytosolic proteins eluted from Sephadex G100 and possessing formylating activity were deposited on a 1.6 × 30 cm column of DEAE Sephacel equilibrated with 20 mM Na-K phosphate buffer pH 7.8. Arrows indicate the start and end of a linear 0–1 M NaCl gradient (no enzymatic or mutagenic activity was found in the wash-through). 40 drops (approx. 1.8 ml) fractions were collected. Bars indicate the number of TA98 revertants per plate, with 5 nmoles ethidium bromide and 2 mg microsomal proteins from Aroclor-induced rat liver. No activity was found besides the pooled fractions (35 to 39 and 40 to 44).

Table 7. Purification of the ethidium activating enzyme*

Step	Protein concn. (mg/ml)	Total amount of protein (mg)	Total activity†	Specific activity‡	Purification factor	Yield (%)
100,000 *g* supernatant	8.7	1650	2×10^6	1.26	1	100
DEAE	3.3	46	0.38×10^6	8.3	6.5	20
Phosphocellulose	0.87	2.6	0.14×10^6	53	42	7

* The DEAE Sephacel step was performed as described under Fig. 5. The second step consisted of mixing the pool of active fractions (after concentration over a PM10 Amicon membrane and dialysis against 20 mM phosphate buffer pH 6.7) with a slurry of 1 g phosphocellulose equilibrated with the same buffer. The mixture was centrifuged at 12,000 rpm for 20 min in a Beckman JA20 rotor, the supernatant decanted, and an equal volume of buffer added. The centrifugation was repeated and the supernatants were combined and assayed.

† Activity is computed as the number of TA98 revertants per plate that 1 nmole of ethidium bromide would yield in presence of the corresponding amount of soluble protein and 2 mg microsomal proteins from Aroclor-induced rat liver.

‡ Specific activity is similarly expressed as the number of revertants per µg soluble enzyme and nmole ethidium under the same conditions.

range, and that it exerts a limited (30%) but definite inhibitory effect on the metabolic activation of ethidium. This result explains why preincubation of chymotrypsin with this chemical before its addition to the incubation mixture does not completely protect the efficiency of the soluble factor, hence the somewhat decreased yield of revertants compared to control, as shown in Table 6. At last, similar findings were obtained with trypsin and proteinase K (data not shown). Phenylmethylsulfonyl fluoride was not mutagenic up to at least 150 µg per plate on strain TA98.

It is thus demonstrated that the soluble factor which complements the microsomal fraction in the activation of ethidium bromide is a protein.

Purification of the soluble enzyme. In view of the importance of the basic amino group in the mutagenicity of our phenanthridines, we looked for the possible role in the activation process of several activities which were known to yield conjugates or reactives metabolites from aromatic amines.

The *N*-formylating activity described by Santti [19] has been [24] dismissed as a source of reactive metabolites from *N*-hydroxy-acetylaminofluorene, but was nevertheless investigated. *N*-Acetylation* and *N,O*-sulfatation [25, 26] are known to generate nitrenium cations from derivatives of carcinogenic aromatic amines. We set up therefore to fractionate the 100,000 *g* supernatant, in order to see whether the mutagenic and conjugating activities would copurify or not.

We purified *N*-formyl-L-kynurenine formamidase and found that the first chromatographic step yielded coincident peaks of mutagenic and formylating activity (Sephadex G100). However, during the sec-

* The involvement of *N*-acetylation cannot be ruled out *a priori* in spite of the lack of mutagenicity of 8-acetyl ethidium, because it could exert itself at position 3- of ethidium or on an undetected metabolic intermediate.

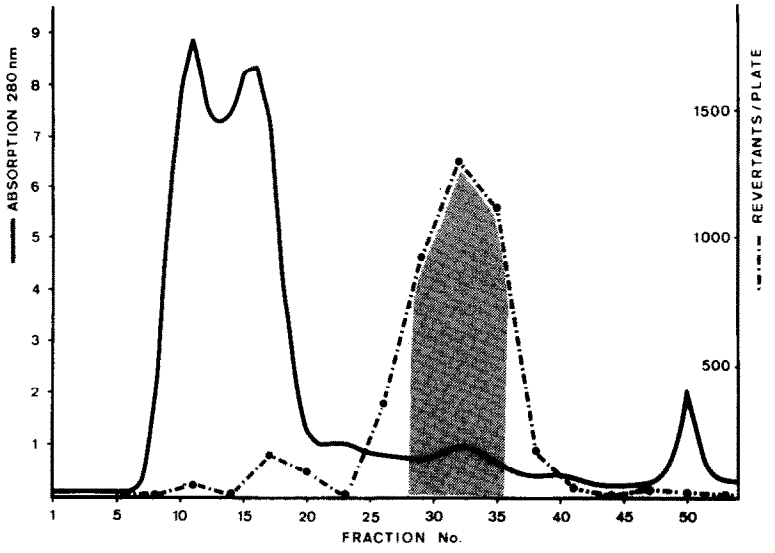

Fig. 5. Purification of the ethidium activating enzyme. All operations were performed at 4°. The cytosolic fraction was prepared from non-induced rat livers (5) as described in Materials and Methods, and was dialyzed against 25 mM phosphate buffer, pH 7.9, 1 mM dithiothreitol. It was deposited on top of a 2.5 × 90 cm column of DEAE Sephacel equilibrated with the same buffer. 400 drops (approx. 14 ml) fractions were collected. Activity was assayed on strain TA98, with 5 nmoles ethidium and 2 mg microsomal proteins from Aroclor-induced rat liver. Values are computed for 50 µl of each fraction per plate. Fractions 28 to 36 were pooled. Their activity and further purification is described in and under Table 7.

ond step (DEAE Sephacel) these two activities clearly separated (Fig. 4).

Then, we devised a two steps purification of the rat liver 100,000 *g* supernatant, using as a test of enzymatic activity the ability of the chromatographic fractions to complement microsomes in the biotransformation of ethidium to mutagen(s). Table 7 sums up this procedure and Fig. 5 shows a typical elution pattern for step 1 (step 2 was carried out batchwise).

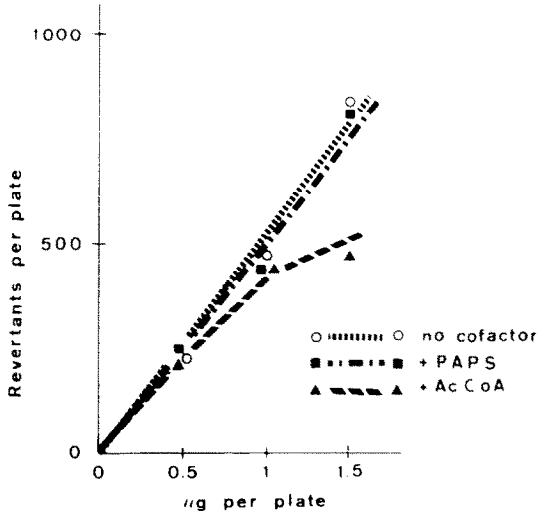

Fig. 6. Mutagenicity of ethidium bromide in presence of acetylation and sulfatation cofactors. A mixture of 0.1 ml bacterial culture (TA98), 0.1 ml 0.2 M phosphate buffer pH 7.4, 50 µl reconstituted metabolizing mixture (see text for pretreatment of the cytosolic part) was incubated with either 20 µM 3',5'-ADP + 10 mM p-nitrophenylsulfate, 0.6 mM AcCoA or no cofactor, for 20 min at 37° before pouring in 2 ml soft agar. Points are the mean of a duplicate determination. The background (32 revertants per plate) has been substracted.

During no phase of this purification could any acetylating or sulfating activity be detected in the active (mutagenic) pools of fractions. They could be measured, however, after concentrating by 45–65 per cent ammonium sulfate precipitation the eluate from high salt (1 M NaCl) washing of the DEAE Sephacel column and were found to represent respectively 0.21 and 672 units (total activity in eluate). This fraction was devoid of complementing activity in the mutagenesis test.

The purified enzyme quickly loses its activity. A third step of purification (on QAE Sephadex or hydroxyapatite) typically yielded specific activities of only three or four times the starting one. This is reflected in the poor factor of purification obtained in the first step, compared to what one would expect from the elution profile depicted in Fig. 5. Whether this results from denaturation or from loss of another component remains to be investigated.

Influence of cofactors on the mutagenicity of ethidium. Further evidence that acetylation and sulfatation are not involved in the activation of ethidium comes from the experiment depicted in Fig. 6. The reconstituted metabolizing mixture, bacteria and varying amounts of ethidium were mixed and incubated for 20 min at 37° with shaking (longer incubation times caused a decrease in the number of revertants in control as well as other experiments), in presence of a PAPS generating system, acetylcoenzyme A or no cofactor. According to [30], the soluble part of the metabolizing mixture had been pretreated with Sephadex G25 in order to remove

endogenous cofactors. Figure 6 shows that the addition of the necessary cofactors for acetylation or sulfatation has no effect on the yield of revertants, apart from a decrease at higher doses of ethidium, in presence of AcCoA. This could be attributed to formation of the non-mutagenic 8-acetylethidium.

DISCUSSION

The results of the structure–mutagenicity study which are summarized in Table 2 clearly point to the central role of the amino group borne by carbon-3, as demonstrated by the effect of its selective removal or blocking. Of particular interest is the fact that the 8-acetylated derivative of ethidium bromide, which is a metabolite found in the bile of rats injected with ethidium [9, 10] has lost the mutagenic properties of the parent molecule. This finding could be related to the ability of the substituent at position 8- to sustain shifts of electric charge or resonance structures. Work on the analog prothidium [4] (an 8-substituted secondary amine derivative) and our results with the tetramethyl compounds show that this position can be substituted by either primary, secondary or tertiary amino groups while keeping strong mutagenic properties. At last, reduction of the middle ring of the phenanthridine nucleus decreases only by half the mutagenic effects compared to ethidium. This reaction gives rise to a gauche carbon skeleton, which is unable to intercalate into DNA [27].

The apparent lack of affinity of ethidium bromide and its tetramethyl derivative for cytochrome P448 is consistent with the weak activation that can be found with microsomes alone, as shown in Table 3. It can be given at least two non-exclusive explanations: (a) only the uncharged derivatives are hydrophobic enough to interact with cytochrome P448; (b) the interaction with heme Fe^{3+} is mediated through the N_5 atom, and is therefore possible only when positive charge resulting from quaternarization is absent, either by reduction (compound VI) or by lack of substituent (compound XI).

As several other mutagenic or carcinogenic aromatic amines and amides, such as 2-naphtylamine or 2-acetamidofluorene, ethidium bromide needs at least two enzymatic components to undergo activation to reactive mutagenic species. Transacetylation [24, 28], deacetylation [29, 30] and *N,O*-sulfate ester formation [26] are recognized as soluble activities complementing the microsomal monooxygenase-mediated transformation of these substrates to reactive electrophilic species. We have shown that the activation of ethidium bromide is strictly P448 dependent, but that another factor, from the post-microsomal supernatant is also required for the reaction. The identification of this enzyme activity is hindered by the fact that much less is known about the activation of aromatic primary amines [31] than about amides such as 2-acetylaminofluorene and its *N*-hydroxy derivative.

Our experiments make it improbable that this factor should pertain to the acetyltransferase (or transacetylase [32]) and sulfotransferase family. It is however acknowledged that hidden activities (i.e., not detectable in the assays that we used) might be operative. This problem will find an answer only by the isolation of the metabolic intermediate, or at least of its products of reaction with nucleophiles, which is currently being carried out in our Laboratory.

Another question arises concerning the timing of the action of this protein: one could conceive that it acts as a conjugation enzyme on the product of microsomal metabolism (this would fail to explain the lack of affinity *in vitro* of ethidium towards cytochrome P448) or that it modifies the structure of the molecule so as to allow binding to the cytochrome. The latter hypothesis is attractive, since we have shown that uncharged phenanthridines do exhibit interaction spectra with cytochrome P448. In this case, the role of the soluble enzyme would be to remove the positive charge of quaternarized compounds. Preliminary experiments, however, have failed to detect any chemical modification of ethidium by the post-microsomal supernatant, with the exception of the CoA-S-Ac dependent 8-acetylation [10]. A third hypothesis would be that another microsomal enzyme, distinct from cytochrome P448, could also be involved in the pathway of activation.

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