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THE DEMETHYLATION OF *m*-METHYL ORANGE AND METHYL ORANGE *IN VIVO* AND *IN VITRO*

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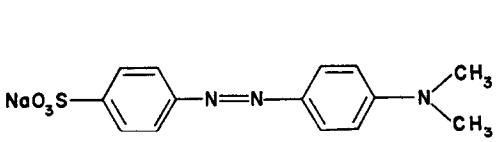
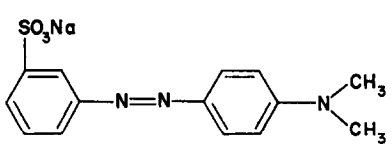
Abstract—Rats given *m*-methyl orange and methyl orange intraperitoneally and intravenously excrete the monomethylated and completely demethylated dyes in urine and in bile. Mouse liver microsomes also demethylate methyl orange. The results indicate that factors other than lipid solubility are important in the microsomal metabolism of drugs.

THE drug metabolizing enzymes of mammals are exceptionally non-specific and catalyse the oxidation of a wide range of substrates.[1] The only requirement noted so far for attack on a drug is a sufficient lipid solubility.[2] Recently we described an exception to this generalization when we found that rats hydroxylate 2-phenylazo-1-naphthol-5-sulphonic acid and the isomeric 4-sulphonic acid in the *para* position of the phenyl ring.[3] We have now extended this observation to *N*-dealkylation, an important reaction in amine metabolism. The present communication is concerned with the demethylation of methyl orange (I) and *m*-methyl orange (II) *in vivo* by rats and the *in vitro* demethylation of (I) by mouse liver microsomes.



I



II

EXPERIMENTAL

Methyl orange was a commercial sample purified by crystallization from water. *m*-methyl orange was prepared from diazotized metanilic acid and dimethylaniline.[4] 4′-sulpho-4-methylaminoazobenzene and 3′-sulpho-4-methylaminoazobenzene were prepared by coupling the respective diazotized aminobenzene sulphonic acids with methylaniline. The resulting crude diazoamino compounds were rearranged to the aminoazo compounds by heating in a mixture of methylaniline and its hydrochloride.[5] The reaction mixtures were made alkaline with Na_2CO_3 and excess amines extracted with ether. Evaporation of the aqueous phase gave the crude aminoazo compound as the sodium salt. These were characterized as the S-benzylisothiouronium salts. S-benzylisothiouronium-4-methylaminoazobenzene-4′-sulphonate had m.p. 187–9°. Found: C, 55·4; H, 5·2; N, 15·1%; $C_{21}H_{23}N_5O_3S_2$ requires C, 55·2; H, 5·1; N, 15·3%.

S-Benzylisothiouronium-4-methylaminoazobenzene-3'-sulphonate had m.p. 182–4°. Found: C, 55·0; H, 5·0; N, 15·0%; $C_{21}H_{23}N_5O_3S_2$ requires C, 55·2; H, 5·1; N, 15·3%.

4'sulpho-4-aminoazobenzene was prepared by heating 4-acetamidoazobenzene (1·2 g) with chlorosulphonic acid (4·5 ml) on the water bath for 1 hr. The mixture was basified with 20% aqueous NaOH and boiled for 10 min. After filtration, the crude sodium salt was obtained by evaporation and characterized as the *S-benzylisothiouronium* salt, m.p. 210–11°. Found: C, 54·2; H, 4·7; N, 15·8%. $C_{20}H_{21}N_5O_3S_2$ required C, 54·2; H, 4·9; N, 15·3%.

Tritiated methyl orange was prepared from diazotized sulphanilic acid generally labelled with tritium[6] (100 mg; $4·6 \times 10^5$ dis/min/mg) and dimethylaniline.[4] Recrystallization from water to constant count gave 230 mg (70%) of radioactive dye containing $2·44 \times 10^5$ dis/min/mg.

METHODS

Metabolic experiments. White male rats (300–400 g) were given dye (5 mg) in water by intraperitoneal injection. Urine was collected for 24 hr. Biliary metabolites were obtained from cannulated rats after an intravenous injection of dye (2 mg) in water.

Quantitative excretion of methyl orange. Quantitative data for methyl orange were obtained using the radioactive dye. For urinary studies rats were given the dye (2 mg) by intraperitoneal injection in water. Urine was collected after 24 and 48 hr. Biliary excretion was estimated after injecting the dye (2 mg) intravenously and collecting bile for 6 hr. The urine or bile was diluted to a suitable volume and assayed for total radioactivity. The individual radioactive compounds were separated by thin layer chromatography (see below) and eluted from the silica with methanol. The solvent was removed and the residue counted. All assays were carried out in triplicate.

Radioactive counting. Radioactive compounds were assayed by liquid scintillation counting. The solvent used was 10% ethanol in toluene containing 5 g of PPO and 50 mg POPOP per l. Two drops of 1M hyamine hydroxide in methanol was added to each vial. Quenching was estimated using tritiated toluene as an internal standard.

Mouse liver microsomes. Male albino mice were starved overnight, killed and the livers perfused with cold isotonic (1·15%) KCl. The livers were then removed, washed with cold aqueous KCl, surplus moisture removed, and weighed. A 33% (w/w) homogenate in KCl was prepared in a glass homogenizer fitted with a motor driven Teflon pestle. This was centrifuged at 9000 *g* for 20 min at 0°. The supernatant was decanted. Part was reserved and the remainder centrifuged at 100,000 *g* for 60 min at 0°. The supernatant was reserved, and the microsomal fraction was resuspended in KCl and centrifuged at 100,000 *g*. The supernatant KCl was removed and the microsomal pellet suspended in sufficient 0·1 M phosphate buffer, pH 7·4, so that 1 ml was equivalent to 1 g of liver.

Demethylation in vitro *of methyl orange.* Methyl orange (5 μM) was incubated with the following mixtures: (a) 9000 *g* supernatant (3 ml = 1 g liver), nicotinamide (100 μM), NADP (0·2 μM), glucose-6-phosphate (6 μM), MgCl (25 μM), made up to a final volume of 5 ml with 0·1 M phosphate buffer, pH 7·4.

(b) Microsomal suspension (= 1 g liver), 100,000 *g* supernatant (1 ml), co-factors as in (a) except that 30 μM of glucose-6-phosphate was added. The final volume was made to 5 ml with 0·1 M phosphate buffer.

(c) Microsomal suspension (= 1 g liver) with co-factors and buffer as in (b); glucose-6-phosphate-dehydrogenase (trace) was added in place of the 100,000 *g* supernatant.

Control mixtures for these experiments were identical to those outlined above except that (a) contained no 9000 *g* supernatant and (b) and (c) no microsomal suspension. The incubations were carried out under air in a shaking water bath at 37° for 30 min.

The products from these reactions were identified as set out below.

Chromatography. The metabolites from *m*-methyl orange were separated by paper chromatography in the following solvents: (A) *n*-butanol-acetic acid-water (4:1:5), (B) 1% aqueous NaCl. Methyl orange metabolites were separated on paper by the following solvents: (C) *n*-butanol-ethanol-conc. NH_3-water (4:4:1:2) and (D) 2% aqueous KCl. Complete separation of the methyl orange metabolites was carried out by thin layer chromatography (TLC) on silica gel with solvents E, ethyl acetate-ethanol (2:1) and F, water.

U.v. spectra were measured on a Unicam SP 700 recording spectrophotometer. The spectra of the metabolites were obtained after repeated chromatography. The purified compounds were eluted from paper or silica gel with distilled water and the pH adjusted to 7 before recording the spectra.

RESULTS AND DISCUSSION

Paper chromatography of the urine and bile of rats dosed with *m*-methyl orange (Fig. 2) showed three coloured spots in solvents (A) and (B) (Table 1). Metabolite (*a*)

TABLE 1. R_f VALUES AND ABSORPTION MAXIMA OF DYES AND METABOLITES

Compound	R_f Value (paper)				R_f Value (TLC)		λmax
	Solvent				Solvent		(mμ)
	A	B	C	D*	E	F	
m-Methyl orange	0·6	0·2					467
3′-Sulpho-4-methyl-aminoazobenzene	0·5	0·6					410
Metabolite a	0·6	0·2					470
Metabolite b	0·5	0·6					410
Metabolite c	0·45	0·75					390
Methyl orange			0·7	1	0·65	0·5	470
4′-Sulpho-4-methyl-aminoazobenzene			0·6	2	0·7	0·75	415
4′-Sulpho-4-amino-azobenzene			0·6	3	0·75	0·9	395
Metabolite a			0·7	1	0·65	0·5	470
Metabolite b			0·6	2	0·7	0·75	415
Metabolite c			0·6	3	0·8	0·9	390

* The figures here represent the order of increasing distance from the starting line since the chromatogram required 72 hr running to achieve separation.

was unchanged dye and metabolite (b) was 3′-sulpho-4-methylaminoazobenzene, confirmed by identical chromatographic behaviour and u.v. spectra. Compound (c) must be 3′-sulpho-4-aminoazobenzene, but we have not been able to obtain a pure specimen of this compound although spectroscopic data have been reported for it.[7]

Metabolite (c) has λ_{max} 390 mμ at neutral pH, in agreement with Thiel and Peter.[7] The spectrum was similar to that of 4-aminoazobenzene[8] and also with that of 4′-sulpho-4-aminoazobenzene reported here (Table 1).

Examination of the urine and bile of rats dosed with methyl orange (Fig. 1) gave similar results to those obtained with *m*-methyl orange. The demethylated products were best separated from methyl orange (Fig. 1) by thin layer chromatography on silica gel in solvents (E) and (F). Metabolite (b) was identical with 4′-sulpho-4-methylaminoazobenzene and metabolite (c) was identical with 4′-sulpho-4-aminoazobenzene in chromatographic behaviour and u.v. spectra (Table 1). Metabolite (a) was unchanged methyl orange.

The quantitative excretion of methyl orange and its metabolites was estimated in urine and bile using tritiated methyl orange labelled only in the *p*-sulphophenylazo ring. The results are set out in Table 2. After an intraperitoneal injection, rats excreted more than 50 per cent of the radioactivity in 24 hr and more than 70 per cent

TABLE 2. EXCRETION OF RADIOACTIVE METABOLITES IN URINE AND BILE AFTER DOSES OF TRITIATED METHYL ORANGE

	% Dose excreted*	% Excreted as methyl orange*	% Excreted as 4′-sulpho-4-methylamino-azobenzene*	% Excreted as 4′-sulpho-4-aminoazobenzene*
Urine (0·24 hr)†	55·7 (±0·8)	2·8 (±0·5)	7·8 (±0·4)	25·4 (±0·8)
Urine (24·48 hr)†	16·4 (±0·7)			
Bile (0·6 hr)†	69·0 (±1·8)	12·4 (±0·2)	30·8 (±0·9)	26·3 (±0·4)

* Figure in brackets is standard deviation.
† 4 animals used in each experiment.

after 48 hr. The amount of activity in each excreted dye was determined in urine collected for 24 hr. 4-Amino-4′-sulphoazobenzene was the major radioactive dye. Methyl orange was only a small percentage of the total material excreted. However, the radioactivity of the dyes does not account for all the activity excreted. The remainder is presumably due to sulphanilic acid formed by the *in vivo* reduction of methyl orange. Preliminary experiments with a single rat indicated the correctness of this assumption.

The biliary excretion of radioactivity was high (Table 2) , being about 70 per cent after 6 hr. All the radioactivity was accounted for as methyl orange or its demethylated metabolites. The latter made up the bulk of the activity and indicated the efficiency with which the rat was able to demethylate methyl orange. The high biliary excretion may indicate an enterohepatic circulation of these dyes.

The quantitative excretion of *m*-methyl orange was not determined, but from paper chromatograms of urine and bile extracts it was apparent that it was almost entirely metabolized. 4-Methylamino-3′-sulphoazobenzene was the major constituent, with 4-amino-3′-sulphoazobenzene and *m*-methyl orange present in roughly equal amounts.

These results led us to examine the demethylating activity of various mouse liver fractions for methyl orange. The results are summarized in Table 3. The same metabolites were found as in the urine and bile of rats. They were identified by their chromatographic behaviour and u.v. spectra (Table 1). No attempt was made to measure the extent of demethylation quantitatively since the control experiments contained only unchanged starting material. In these experiments the amino compound appeared in trace amounts only and there was considerable unreacted starting material. However, from Table 3, it is apparent that the demethylation takes place in the microsomal fraction of liver and must, therefore, be brought about by the drug metabolizing enzymes.

TABLE 3. COMPOUNDS FORMED BY INCUBATION OF METHYL ORANGE IN MOUSE LIVER FRACTIONS

Liver fraction	4′-Sulpho-4-methylaminoazobenzene	4′-Sulpho-4-aminoazobenzene
9000 × *g* supernatant + co-factors	+	+
Control	—	—
Microsomal fraction + 100,000 *g* supernatant + co-factors	+	+
Control	—	—
Microsomal fraction + co-factors	+	+
Control	—	—

The demethylation of these compounds both *in vivo* and *in vitro* is interesting. Other workers have already pointed out a relationship between lipid solubility and drug metabolism.[2, 9, 10] It would therefore be expected that these lipid insoluble compounds would not be attacked by the microsomal enzymes. However, the results reported here, and our previous work,[3] cannot be explained on this basis.

In a study of the microsomal demethylation of a series of amines the results quoted by Gaudette and Brodie[2] show a five fold variation in the rates of demethylation of very lipid soluble amines. McMahon[9, 10] in some similar studies compared rates of demethylation and heptane solubility of a number of amines. He found rates varying by a factor of 3 while solubilities varied by a factor of up to 300. It seems, therefore, that lipid solubility is not a necessary condition for attack by the drug metabolizing enzymes. Mazel and Henderson[11] have also observed the demethylation of highly water soluble amines by liver microsomal preparations and arrived at similar conclusions. At present any explanation of these results is speculative. However, they do underline the necessity for a re-appraisal of the substrate requirements of these enzyme systems.

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