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THE METABOLISM OF 2-PHENYLAZO-1-NAPHTHOL-4- AND -5-SULPHONIC ACIDS IN THE RAT

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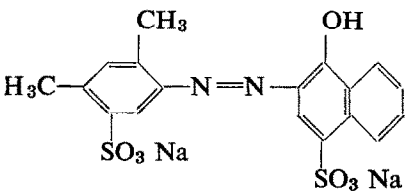
Abstract—After intraperitoneal injection of the title compounds into rats the major metabolite found in the urine was 2-(-4-hydroxyphenylazo-)-1-naphthol-4 or 5-sulphonate and the corresponding glucuronide. No reduction of the dye was found to take place. This was confirmed using 2-phenylazo-^{14}C-1-naphthol-5- sulphonate. Radioactivity was quantitatively excreted in the urine after 48 hr and was associated only with whole dye metabolites. Some enterophepatic circulation of the dyes took place but did not lead to reduction of the dyes in the gastro-intestinal tract. The reasons for the failure of the dye to be reduced *in vivo* are discussed. The hydroxylation of these compounds appears to be the first example of such a reaction *in vivo* with a non-lipid soluble molecule and has been shown to take place in the microsomal fraction of rat liver.

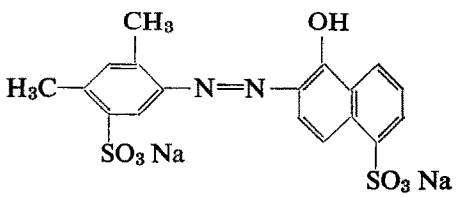
INTRODUCTION

The majority of red and orange dyes used to colour foods are substituted arylazonaphthols. Very little is known of the toxicity of these compounds[1] and their metabolism has only recently attracted any interest. Radomski[2] has studied the metabolism of two fat soluble arylazonaphthols in the rat. The excretion of a number of water soluble dyes of this type has been examined by Radomski and Mellinger[3] and by Daniel.[4] Lindstrom[5] has noted the metabolites from the aryl amines which could be produced by reduction of ponceau 3R (FD and C red No. 1). This dye has been found to produce liver tumours in rats.[6]

During a survey of the biliary excretion of water soluble azo dyes in the rat, Ryan and Wright[7] noted that about half of the dose of ponceau SX (I) and none of the dose of scarlet GN (II) was recovered. In following up this observation the biliary excretion of a number of related isomeric arylazonaphthols has been studied. Among these were sodium 2-phenylazo-naphthol-4 and 5-sulphonates (III and IV). The present paper is concerned with the nature of the unexpected metabolites of these dyes.



I



II

EXPERIMENTAL

(*a*) *Preparation of dyes.* The phenylazonaphthols were prepared by coupling diazotised aniline with the appropriate naphthol sulphonic acid in alkaline solution. The dyes were purified by recrystallisation from hot water.

Sodium 2-phenylazo-1-naphthol-4-sulphonate was obtained as a hydrate. Found: C, 52·5; H, 3·8; N, 8·1; S, 9·0%. $C_{16}H_{11}N_2O_4SNa.H_2O$ requires C, 52·2; H, 3·6; N, 7·6; S, 8·7%.

Sodium 2-phenylazo-1-naphthol-5-sulphonate was obtained as a hemihydrate. Found: C, 53·7; H, 3·6; N, 7·8; S, 9·0%; $C_{16}H_{11}N_2O_4SNa.\frac{1}{2}H_2O$ requires C, 53·3; H, 3·5; N, 8·0; S, 9·2%.

(*b*) *Preparation of metabolites.* The hydroxylated metabolites of the phenylazonaphthols were prepared by coupling diazotised *p*-acetoxyaniline[8] with the appropriate naphthol sulphonic acid in alkaline solution. The acetates were saponified by refluxing with a slight excess of 10% NaOH solution for 1 hr under N_2. Saturation of the dye solution with CO_2 precipitated the crude phenolic dye. Because of solubility, this was not purified further, but characterised as the S-benzyl-isothiouronium salt.

Sodium 2-(-4-acetoxyphenylazo)-1-naphthol-4-sulphonate was recrystallised from aqueous ethanol. Found: C, 50·8; H, 3.7; N, 6·6; S, 7·7%; $C_{18}H_{13}N_2O_6SNa.H_2O$ requires C, 50·7; H, 3·6; N, 6·6; S, 7·5%.

S-Benzylisothiouronium 2-(-4-hydroxyphenylazo)-1-naphthol-4- sulphonate was recrystallised from aqueous ethanol, m. 220–2°. Found: C, 55·5; H, 4·6; N, 10·8; S, 12·2%; $C_{23}H_{22}N_4O_5S_2$ requires C, 55·4; H, 4·5; N, 11·2; S, 12·9%.

Sodium 2-(-4-acetoxyphenylazo-)-1-naphthol-5-sulphonate was recrystallised from aqueous ethanol. Found: C, 50·7; H, 3·9; N, 6·4; S, 7·9%; $C_{18}H_{13}N_2O_6SNa.H_2O$ requires C, 50·7; H, 3·6; N, 6·6; S. 7·5%.

S-Benzylisothiouronium 2-(-4-hydroxyphenylazo)-1-naphthol-5-sulphonate was recrystallised from aqueous ethanol, m. 142–3°. Found: C, 55·6; H, 4·8; N, 10·9; S, 12·3%; $C_{23}H_{22}N_4O_5S_2$ requires C, 55.4; H, 4.5; N, 11.2; S, 12·9%.

(*c*) *Sodium 2-phenylazo-*^{14}*C-1-naphthol-5-sulphonate.* Aniline-^{14}C (93 mg, 10 μc) was mixed with conc.HCl (310 mg) and water (1 ml). The solution was cooled to 0° and $NaNO_2$ (69 mg) in water (1 ml) slowly added, maintaining the temperature at 0–2°. A solution of 1-naphthol-5-sulphonic acid (245 mg) and crystalline sodium acetate (136 mg) in the minimum volume of water was prepared and cooled to 0°. It was then added all at once to the diazonium salt solution. After 0·5 hr at 0° anhydrous Na_2CO_3 in water (1 ml) was added to the mixture which was then allowed to come to room temperature. The fine red precipitate was collected, washed and dried. After three recrystallisations from boiling water 205 mg of dye was obtained with a constant specific activity of 78,000 dis./m. per mg.

Metabolic experiments

Urinary metabolites were obtained from rats (250–300 g) given intraperitoneal injections of dye (5 mg) dissolved in water. Urine was collected for 24 hr. Biliary metabolites were obtained from rats with cannulated bile ducts after intravenous injection of dye (1 mg) in water. The rats were anaesthetised with urethane during bile collection (6 hr).

Isolation of metabolites

The urinary and biliary metabolites of the dyes were isolated by paper chromatography using the following solvent systems: A, *n*-butanol, acetic acid, water (4:1:5); B, ethyl acetate, pyridine, water (6:3:2); C, *n*-butanol saturated with 5% aqueous NH_3.

Whole urine or bile was streaked across Whatman No. 3 paper and chromatographed in solvent A. Each of the resulting bands (Table 1) were eluted with hot methanol and chromatographed separately on Whatman No. 3 paper with solvent A. The bands were collected and chromatographed once in solvent B and once in solvent C. The sequence was then repeated on acid-washed, solvent extracted paper (Macherey–Nagel MN 2214 ff) until the chromatograms showed no fluorescent bands under ultraviolet light and reproducible spectra were obtained (Table 1) from the dyes.

The biliary metabolites were not so rigorously purified and no spectra were recorded for them.

Reduction of metabolites

A sample of the major metabolite, band 2, was reduced with sodium dithionite. The solution was adjusted to pH 7–8 and extracted continuously with chloroform for 3 hr. Paper chromatography of the chloroform residue in *n*-butanol saturated with water showed a spot (R_F 0·7) identical with authentic *p*-aminophenol, after spraying with Ehrlich's reagent.

Hydrolysis of metabolites

Metabolites were hydrolysed either with 1N HCl at 100° for 1 hr or with β-glucuronidase according to Cox.[9]

Radioactive experiments

(*a*) *Determination of p-aminophenol in urine.* Two rats were given sodium 2-phenylazo-^{14}C-1- naphthol-5-sulphonate (5 mg) by intraperitoneal injection. *p*-Acetamidophenol (100 mg) was added to the 24 hr urine and the mixture hydrolysed with acid. The hydrolysate was adjusted to pH 8 and extracted continuously with chloroform (3 hr). Treatment of the chloroform residue with benzoyl chloride in pyridine gave, after several recrystallisations from benzene-petrol, the O, N dibenzoate of *p*-aminophenol, m. 230–3°. After each crystallisation the compound was counted at infinite thickness.

(*b*) *Urinary and biliary excretion of radioactivity.* Rats were given radioactive dye (1·6 mg/kg) by intraperitoneal and intravenous injection. Urine and bile was collected as described. Radioactivity was determined with planchettes prepared according to McCready.[10] Self absorption was corrected by using the active dye as an internal standard.

(*c*) *Assay of urinary metabolites.* Aliquots of the urine obtained above were chromatographed on solvent A. The coloured bands were eluted and the activity in each determined.

Microsomal hydroxylation of dyes

The dyes (7 μM) were incubated with microsomal suspension[11] (equivalent to 1 g of liver) to which was added nicotinamide (100 μM), NADP (0·8 μM), glucose 6-phosphate (30 μM) and $MgCl_2$ (50 μM). The final volume was made up to 7 ml with

tris buffer (0·1 M, pH 8·4). After shaking in air for 1 hr at 37°, trichloracetic acid was added (2 ml of 10%) and the dyes extracted into *n*-butanol and then passed into dilute $NaHCO_3$ solution. Aliquots of the aqueous solution were then spotted on Whatman No. 1 paper and chromatographed in solvent A against the synthetic dye and its hydroxylated derivative.

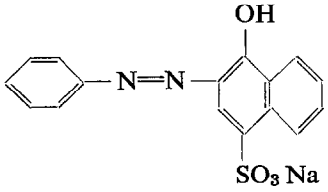
RESULTS AND DISCUSSION

After the i.p. injection of the 4- and 5-sulphonated phenylazonaphthols (III and IV) scarlet compounds were excreted in the urine of rats. Paper chromatography showed the presence of three coloured compounds. These had variable R_F values but travelled in the same relative positions in the solvent systems used (Table 1). The general similarity of the chromatographic behaviour of the metabolites of (III and IV) indicated that they differed only in the position of the sulphonate group and therefore the corresponding metabolites from each are discussed together.

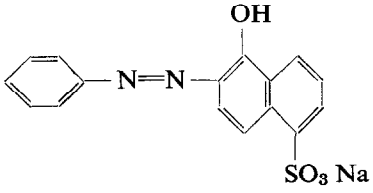
TABLE 1. R_F VALUES AND ULTRAVIOLET SPECTRA OF DYES AND METABOLITES

Compound	Approximate R_F values			max (mμ)
	Solvent A	Solvent B	Solvent C	
Sodium 2-phenylazo-1-naphthol-4-sulphonate	0·7	0·6	0·4	240, 305, 360, 500
Sodium 2-(-4-hydroxy-phenylazo-)-1-naphthol 4-sulphonate	0·5	0·4	0·2	230, 300, 515
Band 1	0·7	0·6	0·4	360, 500
Band 2	0·5	0·4	0·2	300, 515
Band 3	0·3	0·4	0	350, 495
Hydrolysed band 3	0·5	0·4	0·2	305, 515
Sodium 2-phenylazo-1-naphthol-5-sulphonate	0·6	0·5	0·4	230, 305, 360, 510
Sodium 2-(-4-hydroxy-phenylazo-)-1-naphthol-5-sulphonate	0·5	0·5	0·3	230, 360, 525
Band 1	0·6	0·5	0·4	300, 360, 510
Band 2	0·5	0·5	0·3	360, 520
Band 3	0·3	0·3	0·1	300, 360, 505
Hydrolysed band 3	0·5	0·5	0·3	300, 360, 520

Compound 1, the least polar of the urinary dyes, was unchanged starting material (III or IV) identified by paper chromatography and spectroscopy (Table 1). It was unaltered by acid hydrolysis.



III

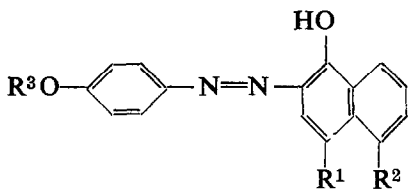


IV

Compound 2 was found to have a bathochromic shift of about 15 mμ in its long wavelength maximum (Table 1). This suggested that a hydroxyl group had been introduced at the free *para* position of the phenyl ring. This was confirmed by the identification of *p*-aminophenol among the reduction products of the metabolite. The hydroxylated dyes were synthesised and found to be identical with the metabolites, establishing (V; R^1 or $R^2 = SO_3Na$, $R^3 = H$) as the structures of compound 2.

Compound 3 was the most polar of the urinary dyes. In the alkaline solvent C it hardly shifted (Table 1) indicating the presence of an additional acidic group in the molecule. Its spectrum (Table 1) was very close to the parent dyes (III or IV). It seemed, therefore, that compound 3 was a conjugate of (V). Hydrolysis with either acid or β-glucuronidase[9] gave (V; R^1 or $R^2 = SO_3Na$, $R^3 = H$) showing compound 3 to be the glucuronide of compound 2.

The same compounds were excreted in rat bile after intravenous injection of (III) and (IV). In addition traces of a highly polar metabolite were found. This may have been the sulphate ester of the phenol (V) but it was not positively identified.



V

Acid hydrolysis of the urine and bile after dosage of (III) and (IV) gave no *p*-aminophenol. This was unexpected since it indicated that no reduction of the azo link of (III) or (IV) had taken place *in vivo*. This point was settled by dosing rats with sodium 2-phenylazo-^{14}C-1-naphthol-5-sulphonate. Addition of N-acetyl *p*-aminophenol to the urines followed by its re-isolation as the O, N dibenzoate incorporated no radioactivity into this compound. This confirmed that the dye had not been reduced *in vivo*.

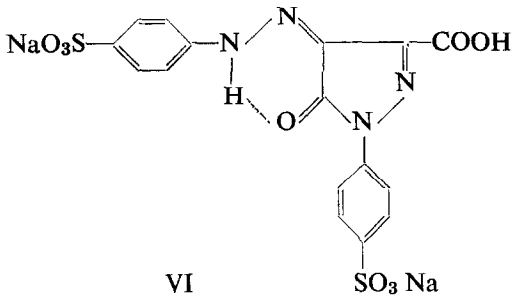
TABLE 2. RECOVERY OF RADIOACTIVITY FROM DOSES OF SODIUM 2-PHENYLAZO-^{14}C-1-NAPHTHOL-5-SULPHONATE

No. of rats	Route of administration	Time of experiment (hr)	Mean % dose excreted (± standard deviation)		
			Urine	Bile	Bladder contents
3	i.p.	24	61 (± 7)		
4	i.p.	48	101 (± 6)		
4	i.v.	6		15 (± 1·5)	
4	i.p.	6		14 (± 4)	11 (± 5)

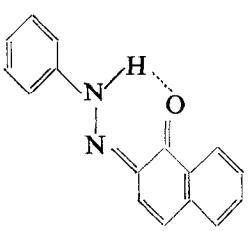
Additional evidence for this was obtained by a study of the quantitative excretion of radioactive (IV). The results are set out in Table 2. After 24 hr about 60% of the radioactivity was excreted in urine and the remainder appeared after 48 hr. Urine aliquots from 24 hr and 48 hr experiments were chromatographed and the coloured

bands eluted and counted. The total of the radioactivity in each dye component agreed with the total activity found in each urine sample. Only about 20% of the dose of dye was excreted unchanged. The remainder was either hydroxylated dye or its conjugates.

These results show that the rat is unable to reduce the azo link of (III) and (IV). We have found similar results for the pyrazolone dyes.[12] Neither tartrazine (VI) nor its unsulphonated analogue are reduced after intraperitoneal injection into rats. Structure (VI) which we have established for the pyrazolone dyes[13] is a keto-hydrazone stabilised by a strong intramolecular hydrogen bond. Vicinal arylazonaphthols are tautomeric mixtures in which (VII), analogous to (VI), predominates.[14] Such structures may be resistant to azo reductase.[15] Tartrazine is not reduced by active liver homogenates and the phenylazonaphthols (III) and (IV) are reduced very slowly[16] in agreement with this hypothesis. Azo dyes may not be as readily reduced by animals as previously believed. Radomski[3] has shown that water soluble azo dyes are reduced by the gastro-intestinal flora after oral dosage and we have found this takes place with tartrazine.[12] Even the microbiological reduction of azo dyes may be a slow reaction. Both unchanged dye and metabolites are excreted in bile after intravenous injection of (III) and (IV). About 15% of the dose of radioactive (IV) was excreted in bile after intravenous and intraperitoneal injection (Table 2). Taken with the urinary excretion results (Table 2), it is apparent that at least some enterohepatic circulation of the dyes takes place. These must be efficiently re-absorbed from the gut, after excretion in bile, avoiding reduction by the intestinal flora.



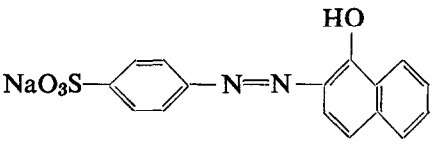
VI



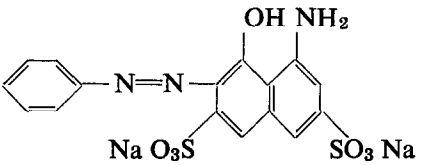
VII

The identification of the phenol (V; R^1 or $R^2 = SO_3Na$, $R^3 = H$) as the major metabolite of the dyes (III) and (IV) appears to be the first example of oxidative attack upon a sulphonated molecule *in vivo*. Hydroxylation of foreign organic compounds *in vivo* is a common reaction[17] and is catalysed by non-specific enzymes found in the microsomal fraction of liver.[18] The rate of hydroxylation seems to depend upon the lipid solubility of the substrate.[11, 19] Since sulphonation abolishes lipid solubility and considerably increases water solubility we were surprised to find that the microsomal fraction of rat liver is able to convert (III) and (IV) to (V; R^1 or $R^2 = SO_3Na$, $R^3 = H$) apparently in good yield. This result shows that the dyes (III) and (IV) are able to penetrate the hydrophobic membrane thought[11, 19] to surround the microsomal fragments. It is possible that the non-polar phenyl ring is able to pass through this lipid barrier and be oxidised. We have found that a number of dyes related to (III) and (IV) are oxidised in the unsulphonated ring,[20] However, only unchanged dye is

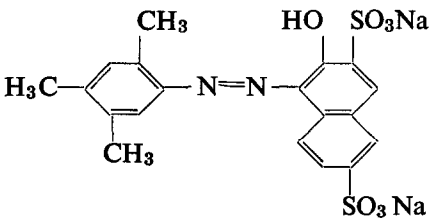
found in the urine of rats dosed with naphthalene fast orange (VIII), red 10B (IX) and the carcinogenic ponceau 3R (X). The reasons for the contrast between these dyes and the phenylazonaphthols (III) and (IV) is at present under investigation.



VIII



IX



X

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