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THE METABOLISM OF 1-PHENYLAZO-2-NAPHTHOL IN THE RABBIT

J. J. CHILDS and D. B. CLAYSON

The Department of Experimental Pathology and Cancer Research,
The School of Medicine, Leeds

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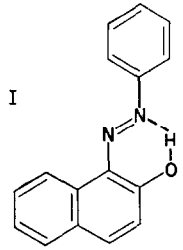
Abstract—The seven known monohydroxy-derivatives of 1-phenylazo-2-naphthol may be separated by thin film chromatography on silica plates by the use of two solvent systems.

The metabolites of 1-phenylazo-2-naphthol in the bile and urine of the rabbit have been examined and the following compounds identified using a combination of enzymatic, chromatographic and spectroscopic techniques:
glucuronides of 4′,6-dihydroxy-1-phenylazo-2-naphthol and 4′- and 6-hydroxy-1-phenylazo-2-naphthol were present in both bile and urine; the N-glucuronides of 1-phenylhydrazo-2-naphthol and 4′-hydroxy-1-phenylhydrazo-2-naphthol and also 1-amino-2-naphthyl hydrogen sulphate and 1-amino-2-naphthyl glucuronide were present in the urine. Less precise information has been obtained on the identity of certain other metabolites.

1-PHENYLAZO-2-NAPHTHOL (I) was formerly used to colour margarine. A number of its derivatives is still permitted to be used as food colours. 1-Phenylazo-2-naphthol itself



1-phenylazo-2-naphthol

is a mild carcinogen. Kirby and Peacock[1] found it to induce hepatomas after injection into stock mice, although Clayson *et al.*[2] were unable to obtain any tumours after feeding the chemical to mice of another stock or of the CBA strain. Bonser *et al.*[3] and Clayson and Bonser[4] showed that the implantation of 1-phenylazo-2-naphthol in a paraffin wax pellet into the lumen of the mouse bladder led to significantly more carcinomas than in mice which were implanted with pellets made from unadulterated paraffin wax. Furthermore, there were cancerous changes in the bladder epithelium of three of seven rabbits fed the chemical.[5, 6]

The metabolism of 1-phenylazo-2-naphthol is being investigated to ascertain whether it will help to elucidate the mode of carcinogenesis of the chemical. In a previous study, Daniel[7, 8] identified the following compounds in the urine of rabbits fed the dye: the glucuronides of 4′-hydroxy-1-phenylazo-2-naphthol, 4-hydroxyacetanilide and 1-amino-2-naphthol, the sulphate ester of 1-amino-2-naphthol and a sulphate ester which on hydrolysis yielded *p*-aminophenol. The purpose of this report is to indicate the complexity of the metabolism of 1-phenylazo-2-naphthol and to describe the identification of further metabolites.

MATERIALS AND METHODS

Chemicals

1-Phenylazo-2-naphthol was purified chromatographically by British Drug Houses Ltd. It ran as a single spot in all paper and thin film chromatographic systems tried. Hydroxy derivatives of 1-phenylazo-2-naphthol were prepared by standard methods and purified by recrystallization and, where necessary, by thin film chromatography. 1-Amino-2-naphthyl hydrogen sulphate, 1-acetamido-2-naphthyl glucuronide and solutions of 1-amino-2-naphthyl glucuronide were available in the laboratory.[9]

Spectra were determined with a Unicam automatic recording S.P.700 spectrophotometer (Table 1).

Collection of excreta. Rabbits were of mixed stock and of either sex, maintained on Diet S.G.1 (Oxo Ltd.) and water *ad libitum*. 1-Phenylazo-2-naphthol (about 150–350 mg/kg body wt.) or its 4′- or 6-hydroxyl derivatives were administered in suspension in arachis oil by stomach tube. Urine was collected in containers cooled with solid CO_2 to prevent decomposition of metabolites. Bile was obtained under Nembutal anaesthesia by inserting a polythene canula (1 mm bore, 1·5 mm external diameter) into the common bile duct. Periods of collection of bile of up to 8 hr were achieved after which the animal was killed. Urine and bile were stored in a deep freeze (—20°) until required.

Urine and bile were allowed to thaw at room temperature, the urine centrifuged to remove suspended matter, and both urine and bile partially saturated with $(NH_4)_2SO_4$ (5 g/10 ml fluid). The solutions were then extracted four times with volumes of ether, ethanol (3 : 1 v/v) approximately 1/3–1/2 the aqueous volume and the combined extracts dried over Na_2SO_4, and concentrated. These extracts were used in all the work to be described.

Chromatographic techniques. Extracts were chromatographed on Whatman No. 1 paper using either solvent a: *n*-butanol, ethanol, water, ammonia (s.g. 0·88) (6 : 3 : 2 : 1 v/v) or solvent b: *n*-butanol, acetic acid, water (12 : 3 : 5 v/v) for the best results. Because the individual spots, which were revealed by their colour, their fluorescence under u.v. light or by spraying with diazotised *m*-nitro-*o*-toluidine, often contained more than one metabolite, they have been given code numbers (Figs. 1 and 2).

2′-, 3′-, 4′-, 3-, 4-, 6- and 7-Hydroxy-1-phenylazo-2-naphthols may be separated on thin films of Keisel-gel G nach Stahl (E. Merck A.G.) (5- and 8-hydroxy-1-phenylazo-2-naphthol remain to be synthesized). 3′- and 4′-Hydroxy-1-phenylazo-2-naphthol could only be separated in one solvent, namely carbon tetrachloride–methanol (95 : 5 v/v). R_f values were not sufficiently reproducible to be quoted but the compounds run in the following order in benzene, methanol (97 : 3 v/v): 1-phenylazo-2-naphthol near the

TABLE 1. DETAILS OF SPECTRA OF 1-PHENYLAZO-2-NAPHTHOL (PAN), SOME MONOHYDROXY-DERIVATIVES AND 1-AMINO-2-NAPHTHYL POTASSIUM SULPHATE

Substance used for spectroscopy	Solvent	Numerator: wavelength of maximum absorption $10^{-3} \times cm^{-1}$ Denominator: Emax $\times 10^{-3}$									
PAN	Analar ethanol	P47·9 28·1	P43·5 38·0	S38·8 11·9	S37·6 10·9	S35·6 5·84	P32·0 7·33	P23·4 11·5	P20·8 15·1	S19·7 13·5	
PAN	Spectroscopically pure hexane	P48·5 32·8	P43·3 39·5	S37·4 10·4	P35·5 7·20	P32·6 7·32	S31·1 6·95	S28·0 I *ca.* 6·21	S23·0 −24·0 12·7	P21·1 13·4	S19·7 9·93
2′OHPAN	Spectrosol*	P48·7 *ca.* 38	S48·1 *ca.* 37	P43·2 24·0	S39·2 9·0	P38·2 9·0	S36·5 6·7	P31·6 5·3	S23·2 6·1	P19·5 14·8	S18·7 14·0
3′OHPAN	Analar ethanol	P47·6 40·0	P43·8 29·5	S37·6 I *ca.* 9·25	P31·6 6·87	S23·7 10·0	P20·5 13·2	S19·5 11·6			
4′OHPAN	Spectrosol*	P47·3 32·0	P43·6 28·0	S38·4 I 13·2	S37·3 I 10·8	S34·6 4·89	S27·6 8·06	P23·6 16·1	S22·7 I 15·5	P21·5 15·3	S18·6 5·29
4′OHPAN	0·1 N NaOH	S46·0 16·9	P41·0 21·9	S33·6 I *ca.* 8·19	P29·9 7·53	P27·6 8·99	P25·2 8·72	P19·6 7·40			
3OHPAN	Analar ethanol	P49·4 22·5	P43·0 39·4	S39·1 9·78	S37·6 8·72	S33·8 8·85	P32·5 9·25	P21·1 18·2			
4OHPAN	Analar ethanol	P48·8 25·4	S46·6 23·8	P41·5 27·2	S38·0 15·3	P31·6 5·68	S30·5 5·29	P22·0 24·6			
6OHPAN	Analar ethanol	S48·6 26·7	S47·2 28·8	P43·3 34·1	S37·0 I 9·51	S35·2 5·68	P29·7 10·0	P26·2 7·13	P19·3 14·5		
7OHPAN	Spectrosol*	P49·2 24·6	P42·6 40·4	S38·2 11·1	P33·4 10·7	P32·3 11·0	S23·6 13·5	P21·2 17·3			
1-Amino-2-naphthyl potassium sulphate	Distilled water	P47·0 41·3	P42·0 26·4	P33·0 4·99	S30·4 4·16						

All measurements were made using solutions of concentration 9 μg/ml or 10 μg/ml.
P — peak.
S — shoulder.
I — ill-defined shoulder.
* (British Drug Houses Ltd.)

N.B. Absorptions measured at wave numbers between 47,000 and 50,000 are subject to inaccuracy due to impurities.

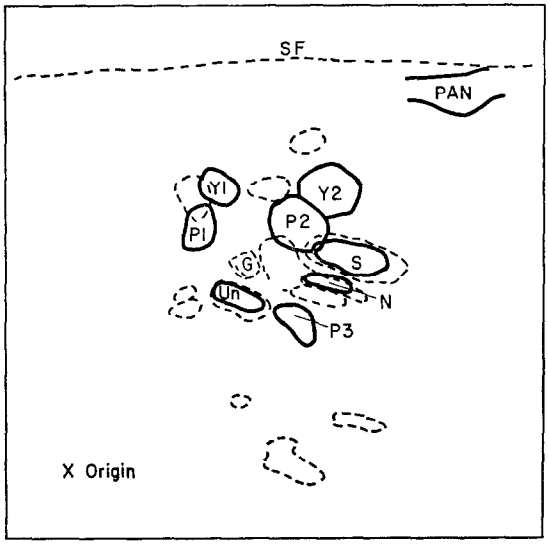


FIG. 1. Tracing of a Whatman No. 1 paper chromatogram of an ethanol–ether extract of urine of a rabbit fed 1-phenylazo-2-naphthol. First dimension solvent a; second dimension solvent b. SF= solvent front; - - - - - - - fluorescent under u.v. light; ——— coloured areas. See also Table 2. S — 1-amino-2-naphthyl hydrogen sulphate; G — 1-amino-2-naphthyl glucuronide; PAN — 1-phenylazo-2-naphthol; Un — unknown metabolite of PAN not discussed in the text; N — one of several normal constituents of rabbit urine (gives grey- brown colour if paper is sprayed with diazotised *m*-nitro-*o*-toluidine).

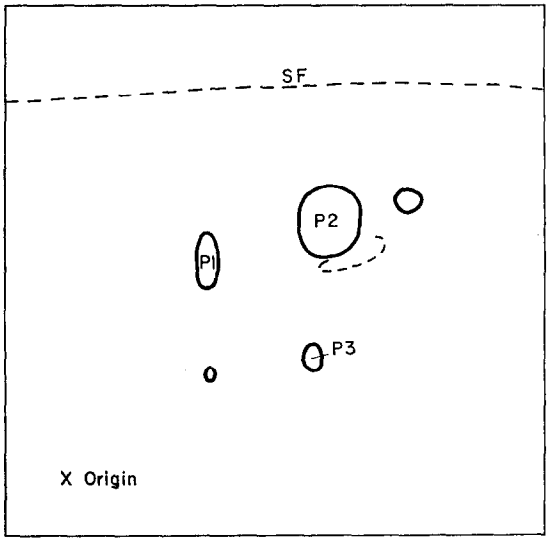


FIG. 2. Tracing of a Whatman No. 1 paper chromatogram of an ethanol, ether extract of bile of a rabbit fed 1-phenylazo-2-naphthol. First dimension solvent a; second dimension solvent b. SF— solvent front; - - - - - - - fluorescent under ultraviolet light; ——— coloured areas.

solvent front, then 2'-, 3-, 7-, 3'- and 4'- together, 6-, and finally 4-hydroxy-1-phenylazo-2-naphthol. In chloroform, 1-phenylazo-2-naphthol was followed by 2'-, then 3'-, 7-, 6-, 3'- and 4'- together, and finally 4-hydroxy-1-phenylazo-2-naphthol. In carbon tetrachloride, methanol (95 : 5 v/v), 1-phenylazo-2-naphthol was followed by 2'- and 3- together, then 4-, 7- and 3'- together, then 4'- and finally 6-hydroxy-1-phenylazo-2-naphthol.

Available dihydroxy-1-phenylazo-2-naphthols could be separated on silica plates using benzene, methanol (84 : 16 v/v) or chloroform, methanol (92 : 8 v/v).

Thin film cellulose plates were used for the rapid separation of metabolites with *n*-butanol, ethanol, water, ammonia (s.g. 0·88) (6 : 3 : 2 : 1 v/v). Some closely related metabolites could be separated on cellulose films using water or 10% ammonia as a solvent.

Separation of metabolites. Concentrated ether, ethanol extracts (10–20 ml) of urine (500–600 ml) were run through an alumina column (30 cm long, 2·5 cm in diameter) and the metabolites eluted with ether, ethanol (3 : 1 v/v) (about 150 ml), ethanol (about 200 ml), 50% aqueous ethanol (about 200 ml) and 0·1M citric acid, Na_2HPO_4, NaOH buffer pH 6 (about 500 ml) as described by Weisburger *et al.*,[10] for the separation of the metabolites of 2-acetamidofluorene. Non-polar components were eluted into the ether, ethanol; some of the 1-amino-2-naphthyl hydrogen sulphate into ethanol; the rest of the "sulphate" fraction into the 50% aqueous ethanol and the "glucuronide" fraction into the citrate, phosphate buffer.

The "sulphate" fraction was taken to dryness under reduced pressure or by freeze drying. An ethanolic solution of the residue was placed on a column (30 cm × 2·5 cm) made from celite (about 17 g) and Whatman diethylaminoethylcellulose (DEAEC) (about 17 g) suspended in ethanol. 4'-Hydroxy-1-phenylazo-2-naphthol and an unidentified compound were eluted into ethanol (about 150 ml) and a series of overlapping bands (P6 complex) into ethanol, 0·03M phosphate buffer (1 : 1 v/v) pH 6 (about 800 ml). Finally, ethanol, 0·1M phosphate buffer (1 : 1 v/v) pH 6 (about 650 ml) eluted two pink bands (P3 and P4).

The "glucuronide" fraction from the alumina column (600–800 ml) was treated with $(NH_4)_2SO_4$ (5 g per 10 ml) and extracted four times with equal volumes of ether, ethanol (3 : 1 v/v). The ether was removed under reduced pressure and the ethanolic extract placed on a DEAEC, celite column prepared as for the previous fraction. Ethanol (about 100 ml) extracted 1-phenylazo-2-naphthol and its 4'-hydroxy derivative. Ethanol, 0·03M phosphate buffer (1 : 1 v/v) pH 6 (800–1000 ml) eluted two well separated bands, namely (i) Y2 and P2, and (ii) Y1 and P1, and ethanol, 0·1M phosphate buffer (1 : 1 v/v) pH 6 (about 800 ml) eluted a third band, containing the two components of P5. Y2 and P2 were subsequently separated on Whatman 31 ET paper developed in *n*-butanol, acetic acid, water (12 : 3 : 5 v/v). Y1 and P1 were separated on thin film cellulose plates developed in 10% aqueous ammonia.

Action of enzymes on metabolites

Sulphatase. Limpet aryl-sulphatase (Sigma, Ltd.) was freed from β-glucuronidase by acidification to pH 2·25; the pH was then readjusted to pH 5. Solutions of metabolites were incubated at 37° with enzyme solution and a final concentration of 0·33M acetate buffer pH 5 for several hr. Enzyme inactivated by heating was used as a control.

β-D-Glucuronidase. Limpet glucuronidase prepared in the laboratory was stirred

with 0·5M acetate buffer pH 4 and centrifuged. The supernatant and solutions of metabolites were incubated at 37° for 12–16 hr with KH_2PO_4 (final concentration 0·025M) to inhibit sulphatase and benzyl penicillin to minimize bacterial action.

One set of controls contained enzyme inactivated by heating to 100° and another set contained active enzyme plus boiled saccharic acid solution containing 1·4-saccharolactone, a specific β-glucuronidase inhibitor.

The products of enzyme action were extracted into ether, or into ether–ethanol (3 : 1 v/v), and investigated chromatographically and spectroscopically.

RESULTS

Urine and bile from rabbits which had not received 1-phenylazo-2-naphthol were investigated chromatographically to determine which compounds were normally present in the extracts of the urine and bile. The general distribution of metabolites of 1-phenylazo-2-naphthol on paper chromatograms is indicated in Figs. 1 and 2, and Table 2.

1-*Amino*-2-*naphthyl hydrogen sulphate and* 1-*amino*-2-*naphthyl glucuronide* were identified in the urine by direct chromatographic comparisons of the spots formed on paper chromatograms with authentic standards. Both compounds gave practically colourless spots (R_f, sulphate 0·58 in solvent a, 0·53 in solvent b; glucuronide 0·45 in solvent a, 0·50 in solvent b) which became faintly yellow on ageing, both fluoresced under u.v. light and both gave a purple colour on spraying with *m*-nitro-*o*-toluidine. Daniel[8] described both compounds.

P1 gave a pink spot. It was separated into two major components by chromatography on thin cellulose films developed in 10 per cent aqueous ammonia. P1 was not attacked by arylsulphatase nor by cold M HCl but was decomposed by glucuronidase. The component of P1, P1B, present in the largest amount, gave rise to a product after treatment with glucuronidase which was chromatographically and spectroscopically identical to 4′,6-dihydroxy-1-phenylazo-2-naphthol. P1B is, therefore, a glucuronide of 4′,6-dihydroxy-1-phenylazo-2-naphthol but it is not known whether the glucuronide is attached to the 2, 4′ or 6 positions. The correctness of this identification is supported by the demonstration that P1B is present in the urine of rabbits treated with either 4′- or 6-hydroxy-1-phenylazo-2-naphthol.

P2 forms a pink spot which is believed to consist of at least two components, though it has not been found possible to separate these. On treatment with glucuronidase but not with sulphatase or cold M HCl, P2 gave two products which correspond chromatographically and spectroscopically to 4′- and 6-hydroxy-1-phenylazo-2-naphthol and a number of minor unidentified products. P2 is, therefore, believed to be a mixture of the glucuronides of 4′- and 6-hydroxy-1-phenylazo-2-naphthol.

The urine of rabbits fed with 4′-hydroxy-1-phenylazo-2-naphthol contained a metabolite corresponding to P2. This on treatment with glucuronidase gave only 4′-hydroxy-1-phenylazo-2-naphthol. Similarly, the urine of rabbits fed 6-hydroxy-1-phenylazo-2-naphthol gave a spot corresponding to P2 which only liberated this hydroxy derivative on treatment with glucuronidase.

P2 was also present in the bile of rabbits fed 1-phenylazo-2-naphthol and when treated with glucuronidase gave a mixture of 4′- and 6-hydroxy-1-phenylazo-2-naphthols. It, therefore, has a similar composition to the urinary metabolite.

TABLE 2. PROPERTIES OF EIGHT METABOLITES OF 1-PHENYLAZO-2-NAPHTHOL (PAN)

Metabolite	Colour of spot	Fluoresence under u.v. light	Colour after spraying with diazotised MNOT	Approx. R_f* on chromatogram in Fig. 1		Presence in urine of rabbit fed PAN	Presence in bile of rabbit fed PAN
				Solv. a	Solv. b		
P1	Pink	—	—	0·28	0·61	+	+
P2	Pink	—	—	0·49	0·61	+	+
P3	Pink	—	—	0·49	0·36	+	+
Y1	Yellow	—	Pink	0·31	0·70	+	—
Y2	Yellow-orange	—	Yellow-orange intensified	0·56	0·69	+	—
S	Faint yellow on ageing	+	Pink-purple	0·59	0·52	+	trace
G	Faint yellow on ageing	+	Pink-purple	0·38	0·51	+	—
Un	Faint yellow on ageing	+	Pink-purple	0·36	0·42	+	—

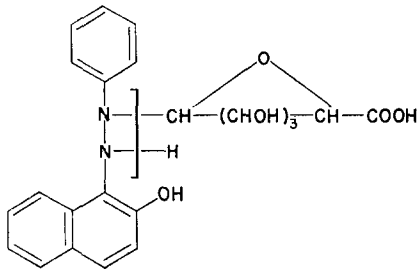
S — 1-amino-2-naphthyl hydrogen sulphate.
G — 1-amino-2-naphthyl glucuronide.
MNOT — *m*-nitro-*o*-toluidine.
* R_f values are somewhat variable.

Y1 was separated from P1 on thin films of cellulose developed in 10 per cent ammonia. It formed a yellow spot; it was attacked by glucuronidase and cold M HCl, but not by sulphatase, to give 4′-hydroxy-1-phenylazo-2-naphthol. Y1 was obtained crystalline by the addition of ether to a concentrated ethanolic solution but was not sufficiently stable for further investigation. Y1 was present in the urine of rabbits fed 4′- but not 6-hydroxy-1-phenylazo-2-naphthol. It is suggested that by analogy with Y2 (below) it is a glucuronide of 4′-hydroxy-1-phenylhydrazo-2-naphthol.

Y2 was similar to Y1 in so far as it was degraded by glucuronidase and cold M HCl but not by sulphatase. 1-Phenylazo-2-naphthol was the product. Y2 formed a faint orange spot which deepened in colour on acidification. Y2 was not present in the urine of rabbits fed either 4′- or 6-hydroxy-1-phenylazo-2-naphthol.

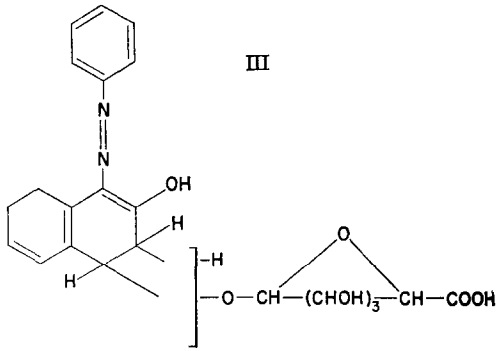
The most probable identification of Y2 is a glucuronide of 1-phenylhydrazo-2-naphthol (II) but the glucuronide of 1-phenylazo-3- or 4-hydroxy-3,4-dihydro-2-naphthol (III) cannot be excluded on the above evidence. The similarity between the

II



Glucuronide of 1-phenylhydrazo-2-naphthol

III



Glucuronide of 4-hydroxy-3,4-dihydro-2-naphthol

u.v. spectra of Y2 and 1-acetamido-2-naphthol (Fig. 3) suggests that an intact naphthalene ring is present in Y2. The correspondence of peaks and shoulders at 36,000 cm^{-1} and 34,600 cm^{-1} indicates similar polarization by the substituents in the 2-position while the shift in position of the peaks near 43,000–46,000 cm^{-1} and 30,000 cm^{-1} can be accounted for by the change in polarization on passing from the 1-acetylamino to the 1-hydrazo group. Formula III has a styrene configuration but the spectrum of

Y2 is markedly different from that of styrene. Styrene has a peak at 40,300 cm^{-1} which has an Emax of the order of twenty times the Emax of three other peaks at 36,600, 35,400 and 34,300 cm^{-1}.[11] Therefore it is concluded that Y2 is an *N*-glucuronide of 1-phenylhydrazo-2-naphthol.

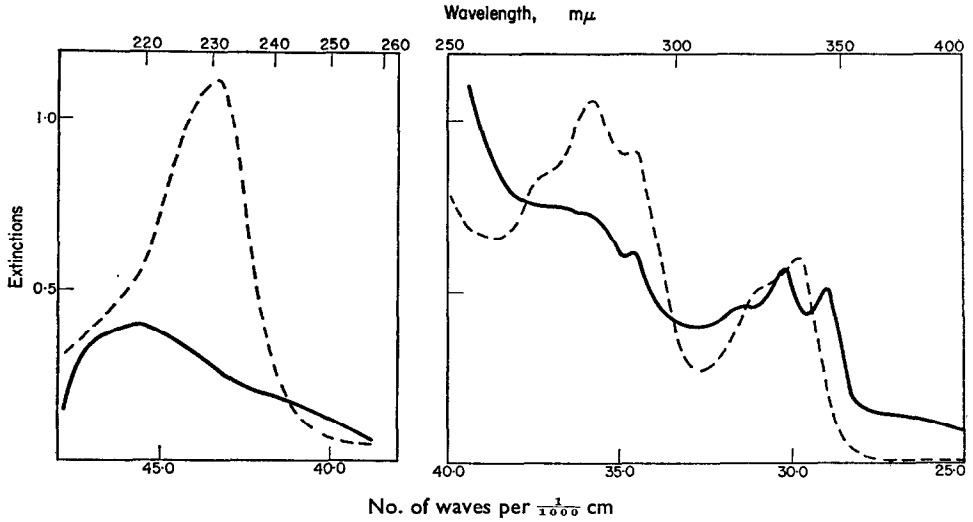


FIG. 3. Comparison of the spectrum of metabolite Y2 in ethanol (———) with that of a freshly prepared solution of 1-acetamido-2-naphthol in ethanol (– – – –). Ordinates = extinctions; abscissae top scale = wavelength in mμ, bottom scale = $10^{-3} \times cm^{-1}$.

The graphs in Fig. 3a (48,000–39,000 cm^{-1}) both represent 12·5 × dilutions of the graphs in Fig. 3b (40,000–25,000 cm^{-1}). Extinctions in Fig. 3b are those obtained for a concentration of 1-acetamido-2-naphthol = 40 μg/ml.

P3 forms a pink spot. It was stable to cold M HCl and to glucuronidase but was converted by arylsulphatase to a compound Px which, on further incubation at pH 5, even in the absence of the active enzyme, was converted into a less polar compound PFr. PFr did not separate from 4′,6-dihydroxyphenylazo-2-naphthol on thin films of silica developed in benzene, methanol (84 : 16 v/v) or chloroform, methanol (92 : 8 v/v). Its spectrum was very similar to that of 4′,6-dihydroxy-1-phenylazo-2-naphthol (Fig. 4). Differences at 48,000–49,000 cm^{-1} are probably due to impurities. A compound behaving chromatographically like P3 is present in the urine of rabbits treated with either 4′- or 6-hydroxy-1-phenylazo-2-naphthol. One explanation of these observations is that P3 is the sulphate ester of a dihydrodiol which is hydrolysed by arylsulphatase to the free dihydrodiol (Px) and this, in turn, yields one or more dihydroxy derivatives of 1-phenylazo-2-naphthol. P3 requires further investigation.

DISCUSSION

The present knowledge of the urinary and biliary metabolism of 1-phenylazo-2-naphthol in the rabbit is drawn together in Fig. 5. A number of metabolites remains to be investigated (P4, P5, P6) and no attempt has been made to determine whether further metabolites are formed which are not extracted by ether, ethanol from the urine or bile. Also traces of unidentified compounds are present in P1, P2 and Y1.

The reduction of 1-phenylazo-2-naphthol appears to occur in two stages: to 1-phenylhydrazo-2-naphthol and thence with fission of the azo group to derivatives of 1-amino-2-naphthol and aniline. Azobenzene has been reported to be metabolised to hydrazobenzene in the rat by Elson and Warren,[12] who isolated benzidine after acidification of the urine, and in the rabbit by Bray *et al.*,[13] who identified hydrazobenzene by paper chromatography. Unfortunately the glucuronides of the two hydrazo-compounds detected in this work have proved, so far, to be too unstable to permit their characterization in the solid state.

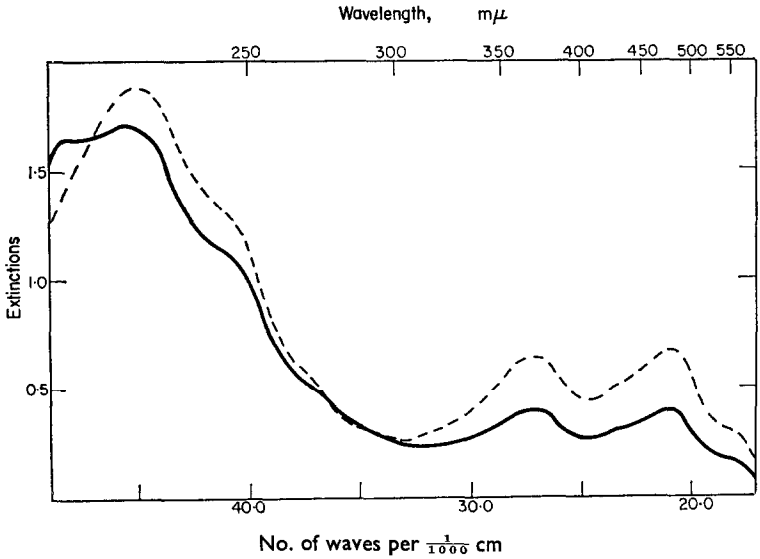


FIG. 4. Comparison of the spectrum of PFr (———) with that of 4′,6-dihydroxy-1-phenylazo-2-naphthol (– – – –). Ordinates = extinctions; abscissae top scale = wavelength in mμ, bottom scale = $10^{-3} \times cm^{-1}$.

Both compounds were purified on an alumina column, eluted, concentrated to dryness and taken up into Analar ethanol for spectroscopy.

1-Phenylazo-2-naphthol is hydroxylated by the rabbit in the 4′- and 6-positions and conjugated with glucuronic acid. Daniel[8] reported 4′-hydroxylation but failed to observe it in the 6-position. 4′-Hydroxylation of 2-phenylazo-1-naphthol-4- and 5-sulphonic acids has also been recorded (Barrett *et al.*[14]). The major dihydroxylated metabolite of 1-phenylazo-2-naphthol is substituted in the 4′- and 6-positions and has an intact azo-linkage.

To date no metabolite of 1-phenylazo-2-naphthol has been identified as the glucuronide of 6-hydroxy-1-phenylhydrazo-2-naphthol. It is possible that the hydrazo-group has an activating effect upon the 4′-position, leading to 4′-hydroxylation in preference to 6-hydroxylation, whereas the azo-group may inactivate the two rings it links to such an extent that hydroxylation also occurs in the 6-position (i.e. in the ring not directly attached to the azo-group).

Free 1-phenylazo-2-naphthol was found in urinary extracts but, under the carefully controlled conditions of collection of the urine, neither 4′- nor 6-hydroxy-1-phenylazo-2-naphthol was detected, although some of the 4′-derivative was formed during passage

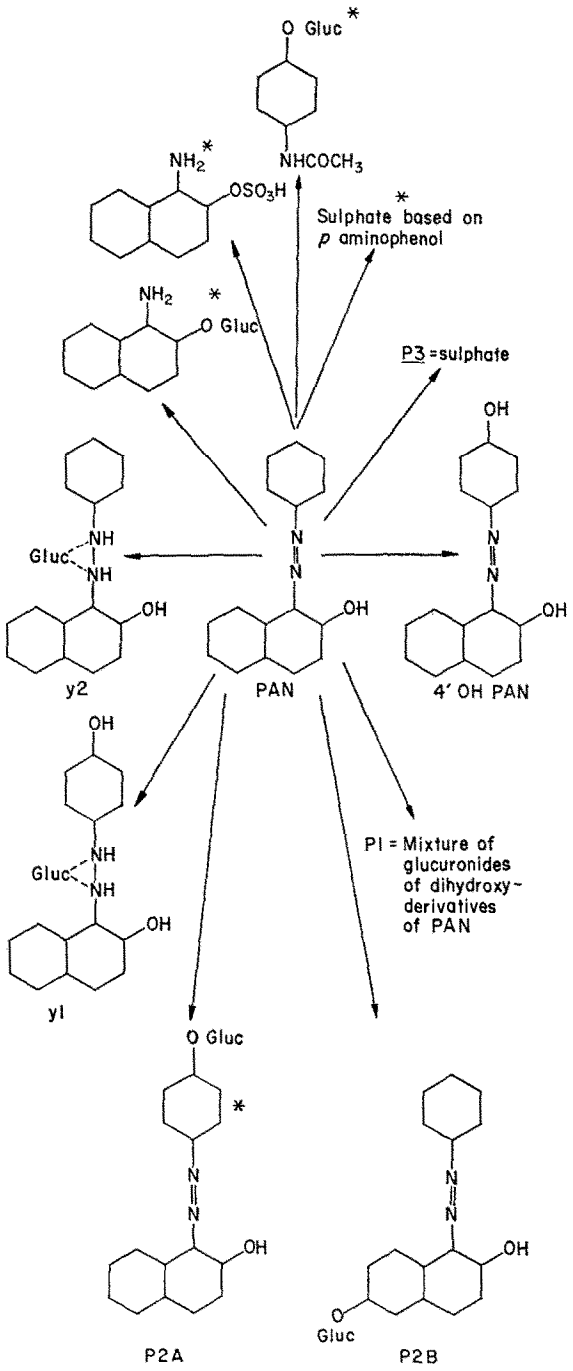


FIG. 5. Diagram summarizing the known pathways of metabolism of 1-phenylazo-2-naphthol (PAN).

* Detected by Daniel.[7, 8]

through the alumina and DEAEC columns. It is not possible to be sure whether the free 1-phenylazo-2-naphthol is actually secreted as such into the urine or whether it arises by the breakdown of Y2. If the latter is true, the failure to find free 4′-hydroxy-1-phenylazo-2-naphthol in the original urinary extract indicates that the glucuronide of 4′-hydroxy-1-phenylhydrazo-2-naphthol is more stable than that of 1-phenylhydrazo-2-naphthol.

Fewer metabolites find their way into the bile than into the urine. So far only compounds with an intact azo-group (P1, P2, P3) have been demonstrated in rabbit bile although all the metabolites described have been shown to be present in the urine. The distribution of excretion products between the urine and the bile requires further study. It is especially important as it may govern the site of concentration of carcinogenic metabolites and thus the site of election of tumours.

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