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THE METABOLISM OF 1-PHENYLAZO-2-NAPHTHOL IN THE RAT WITH REFERENCE TO THE ACTION OF THE INTESTINAL FLORA

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Abstract—1-Amino-2-naphthyl hydrogen sulphate, 1-amino-2-naphthyl glucuronide and the glucuronide of 1-phenylhydrazo-2-naphthol are present in the urine of rats treated with 1-phenylazo-2-naphthol. Both the bile and the urine contain the glucuronides of 4′—and 6-hydroxy-1-phenylazo-2-naphthol and 4′, 6-dihydroxy-1-phenylazo-2-naphthol. More unchanged 1-phenylazo-2-naphthol was found in the faeces after oral administration than after i.p. injection. Significantly more 1-amino-2-naphthyl hydrogen sulphate was present in the urine after i.p. injection than after feeding the dye. The dye has been shown to be reduced by incubation with intestinal contents or intestinal tissue, or with clones of bacteria isolated from the intestine of the rat.

KIRBY and Peacock[1] described the induction of hepatomas when mice of their stock were *injected* with a solution of 1-phenylazo-2-naphthol. Clayson, Lawson Santana and Bonser[2] failed to obtain tumours when this chemical was *fed* to mice of the CBA strain or of another stock. One explanation of this discrepancy is that the metabolism of the dye differs according to the route of administration. Barrett, Pitt, Ryan and Wright[3] showed that i.p. administered 2-phenylazo-1-naphthol-4- and-5-sulphonic acids are excreted by the rat as the 4′-hydroxy derivatives and their conjugates without the reduction of the azo linkage. Other workers have found that orally administered sulphonated[4, 5] or oil soluble[4, 6, 7] derivatives of 1-phenylazo-2-naphthol undergo reductive fission of the azo linkage. It was decided therefore to examine whether or not different routes of administration affected the metabolism of 1-phenylazo-2-naphthol in the rat.

MATERIALS AND METHODS

Wherever possible the materials and methods previously described by Childs and Clayson[7] have been used.

Animals. Albino rats, of either sex, of the Sheffield colony were fed Oxoid Diet 41B and water *ad libitum.* Specially purified 1-phenylazo-2-naphthol[7] (30 mg/rat, approx. 120–150 mg/kg body weight, in 2 ml arachis oil was administered by stomach tube or i.p. injection under light ether anaesthesia. Bile was collected by the method of Van Zyl,[8] the operation being performed under nembutal anaesthesia. The rats were housed singly in a Jencon's Metabowl to permit urine and faeces to be collected separately.

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Recovery of 1-*phenylazo-2-naphthol from faeces or urine.* This was carried out by an adaptation of the method described by Radomski.[5] In the estimation of faecal 1-phenylazo-2-naphthol, the optical density was read at 485 mμ and 420 mμ, i.e. at the absorption maxima of the dye and the faecal contaminants, respectively.

Urine was evaporated to dryness under reduced pressure and extracted directly with CCl_4. The amount of dye present was calculated directly from the optical density at 485 mμ.

Estimation of 1-*amino-2-naphthyl hydrogen sulphate in urine*

Aliquots of the ether, ethanol (3:1 v/v) extracts of the urine of rats, which had received 1-phenylazo-2-naphthol, were applied to Whatman No. 1 papers (10″ × 10″) and chromatographed in two dimensions in *n*-butanol, ethanol, water, ammonia [0·88] (6:3:2:1 v/v) and then in *n*-butanol, acetic acid, water (12:3:5 v/v).[7] The position of the 1-amino-2-naphthyl hydrogen sulphate was determined by its fluorescence under u.v. light. The spot was cut out and eluted with 50% aqueous ethanol (5 ml) and 0·5 ml diazotised *meta*-nitro-*ortho*-toluidine solution added (prepared from the amine (400 mg) in $\frac{N}{2}$ HCl (200 ml); to this solution (20 ml) was added $NaNO_2$ (20 mg) and, after 5 min, ammonium sulphamate (40 mg) and the product made up to 100 ml with distilled water). After standing for 30 min to develop the purple-red colour, the absorption was measured at 526 mμ. A calibration curve was prepared by chromatographing, eluting and developing standard amounts of 1-amino-2-naphthyl hydrogen sulphate in the same way (Fig. 1).

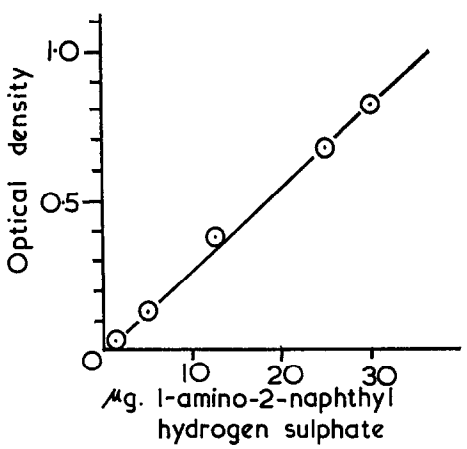

FIG. 1. Calibration graph for the estimation of 1-amino-2-naphthyl hydrogen sulphate at 526 mμ by method described in text.

Incubation of 1-*phenylazo-2-naphthol with intestinal contents*

Intestinal contents from the rat were washed into a small volume of saline (20 ml) and incubated at 37° with 1-phenylazo-2-naphthol (4 mg) in 5% aqueous Tween 80 (4 ml) with mechanical shaking. Similar suspensions were made with tissue minces in saline and the dye in Tween 80. The mixture was extracted with CCl_4 and the optical density determined at 485 mμ against a blank prepared from a similar incubation mixture to which no dye had been added.

The presence of free aniline was investigated by the use of the Böniger method[9] on steam distillates (10 ml) of incubation mixtures. The distillate was mixed with 0·2 M phosphate buffer (5 ml) pH 7·0 and 0·1% 1,2-naphthaquinone-4-sulphonic acid (sodium salt) (5 ml) added. The mixture was shaken with benzene (5 ml) and the spectrum of the extract compared with that prepared from aniline itself. Secondly, Brodie's method[10] was used. The steam distillate was acidified, diazotised with 0·2% $NaNO_2$ (1 ml) and coupled with 0·2% N-1-naphthylethylene diamine (1 ml) at pH 7. The colour obtained on acidification was compared spectroscopically with that from aniline.

Action of rat intestinal flora on 1-phenylazo-2-naphthol

The intestine of a male rat was removed under sterile conditions, and divided into ileum, caecum and colon with rectum. Samples of the contents of each part were cultured at 31° for 2 days in neutral broth and then transferred to McConkey's agar. A number of clones were isolated and their species determined. Each colony was cultured in neutral broth, 1-phenylazo-2-naphthol (600 μg) in 5% aqueous Tween 80 (1 ml) added, and the mixture incubated at 37° for 16 hr with mechanical shaking. The dye was extracted with CCl_4 and estimated spectrometrically.

RESULTS

The metabolism of 1-phenylazo-2-naphthol, following its oral administration to rats, was investigated by the techniques developed for the rabbit.[7] Although the fate of the chemical was similar in the two species, a number of differences was observed.

1-Amino-2-naphthyl hydrogen sulphate was present in the urine but not in the bile of treated rats. A visual comparison of the size and intensity of the spots of this metabolite on chromatograms indicated that much less of it was present in urine of rats than in urine of rabbits fed 1-phenylazo-2-naphthol. 1-Amino-2-naphthyl glucuronide was present in rat urine, but in bile there was only a faint spot corresponding to it in position and colour reactions, and this was not observed with every animal.

The P1 complex (which contains the glucuronide of 4′, 6-dihydroxy-1-phenylazo-2-naphthol,), the P2 complex (containing the glucuronides of 4′- and 6-hydroxy-1-phenylazo-2-naphthol) and the P3 complex (the sulphate ester of a labile intermediate which decomposes in solution to 4′, 6-dihydroxy-1-phenylazo-2-naphthol)[7] were all present in the urine and bile of the rat. The P1 complex, however, was only a minor constituent in the rat urine and could not be detected with certainty in every experiment. A glucuronide of 1-phenylhydrazo-2-naphthol (Y2) was present in rat urine. The presence of a glucuronide of 4′-hydroxy-1-phenylhydrazo-2-naphthol (Y1) was not established with certainty in either urine or bile. In addition to the metabolites already mentioned, rat bile contained six further coloured substances which gave rise to pink spots on paper chromatograms. These remain to be identified.

There were no qualitative differences in the metabolism of intact rats given 1-phenylazo-2-naphthol by stomach tube or into the peritoneum. However, i.p. injected rats which had a cannulated bile duct failed to excrete a detectable amount of 1-amino-2-naphthyl glucuronide into either the urine or the bile. The lack of qualitative differences in metabolism with different routes of administration of the dye led to the quantitive examination of certain metabolites.

1-Phenylazo-2-naphthol itself was estimated in faeces and urine by an adaptation of the method of Radomski.[6] Examination of the faeces of four rats which had not

received any dye gave a faecal interference value (C_F) of 3·0 which is similar to the value found by Radomski. After administration of 1-phenylazo-2-naphthol (30 mg) by stomach tube to each of three rats, 1·58 per cent (range 1·11–1·85 per cent) was recovered in the faeces excreted in the next 3 days. A further three rats were given the same dose of chemical into the peritoneum and 0·22 per cent (range:trace-0·32 per cent) was recovered during the three day collection of faeces. Urinary dye was measured in the same animals and was found to be 0·18 per cent (range: 0·07–0·35 per cent) after oral administration, and 0·14 per cent (range: 0·10–0·16 per cent) when given by the i.p. route. No special precautions were taken to prevent the degradation of the glucuronide of 1-phenylhydrazo-2-naphthol or its 4′-hydroxy derivative during these investigations and therefore it is likely that the urinary value for 1-phenyl-azo-2-naphthol or its 4′-hydroxy derivative during these investigations includes the decomposition products of these additional metabolites.

The estimation of urinary 1-amino-2-naphthyl hydrogen sulphate by quantitative paper chromatography demonstrated that appreciably more of this metabolite was present after intraperitoneal administration than if the dye was given by stomach tube. Six rats given the dye by mouth excreted 0·93 $\pm$ 0·34 per cent of the dose as the sulphate, whereas a further six animals given the same dose by the peritoneal route excreted 1·63 $\pm$ 0·35 per cent in this form. The difference is significant at the 0·01 per cent level (Student's *t* test).

Finally, it was decided to examine the action of the intestinal flora and minces of the intestinal wall, from each of four rats, on 1-phenylazo-2-naphthol. The dye dissolved in aqueous Tween 80 could be recovered quantitatively from normal saline suspension, but the recovery was sometimes lower when the dye was mixed with, but not incubated with, intestinal contents, intestinal minces or isolated bacteria suspended in culture medium. The estimations were commenced within 20–40 min of mixing. The recovery was practically quantitative in the case of the small intestinal wall, about 90 per cent with small or large intestinal contents and 80 per cent in the case of the large intestinal wall. No great emphasis can be placed on the absolute magnitude of these figures as no attempt was made to quantitate the amounts of intestinal material used. Clear evidence was obtained that incubation with the intestinal flora or minces led to further destruction of the dye. About 70 per cent of the remaining dye was destroyed by the mince from the small intestine and the contents of the large intestine, 50 per cent by small intestinal contents and 20 per cent by the large intestinal mince during 15 hr incubation at 37°.

Attempts to demonstrate the presence of 1-amino-2-naphthol, 1-amino-2-naphthyl hydrogen sulphate or 1-amino-2-naphthyl glucuronide in the incubates were unsuccessful. Aniline was shown to be present in the steam distillates of the incubates of mixed intestinal wall and contents by the spectroscopic examination of its product of reaction with 1,2-naphthoquinone-4-sulphonic acid (sodium salt)[9] (Fig. 2) or by diazotization and coupling with N-1-naphthylethylene diamine[10] and spectroscopic comparison with standards prepared from pure aniline (Fig. 3).

Nine clones of micro-organisms were isolated under sterile conditions from the rat intestine using neutral broth and McConkey's medium. Five of these were *E. coli*, two *A. aerogenes* and two *B. proteus*. This is similar to the more extensive observations of Vincent *et al.*[11] and Porter and Rettger (1940).[12] Mixing cultures of these organisms in neutral broth with 1-phenylazo-2-naphthol led to a loss of about 25 per cent of the

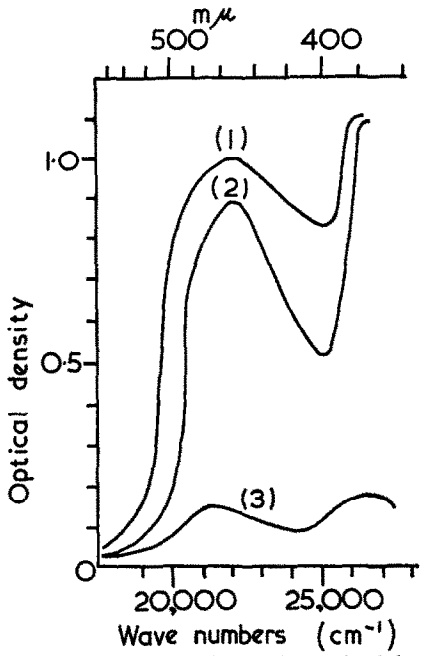

FIG. 2. Spectra obtained by reaction with 1,2-naphthoquinone-4-sulphonic acid of: (1) pure aniline; (2) steam distillate of an incubation mixture of 1-phenylazo-2-naphthol with mince of the *small* intestine and its contents from a rat, (3) steam distillate of an incubation mixture of 1-phenylazo-2-naphthol with mince of the *large* intestine and its contents from a rat.

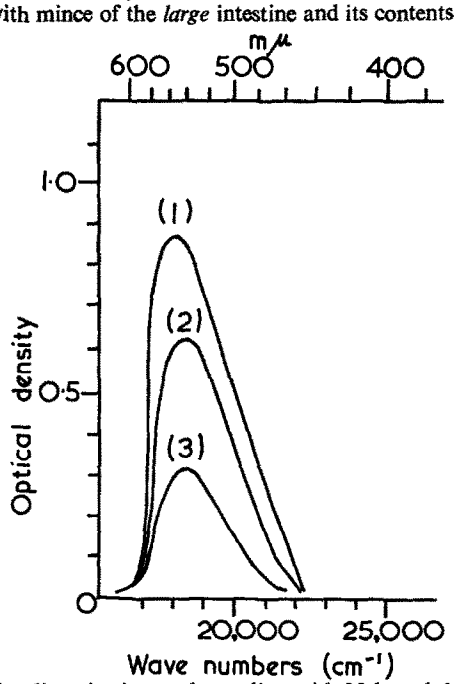

FIG. 3. Spectra obtaihed by diazotisation and coupling with N-1-naphthylethylene diamine of: (1) pure aniline; (2) steam distillate of an incubation mixture of 1-phenylazo-2-naphthol with mince of *small* intestine and contents from a rat; (3) steam distillate of an incubation mixture of 1-phenylazo-2-napthhol with mince of *large* intestine and contents from a rat.

dye (mean dye recovered: 77 per cent, range 69–86 per cent). Incubation at 31° for 16 hr led to the destruction of another 29 per cent of the dye. All the microorganisms examined were effective but not one of the three species was shown to be more efficient that the others under the conditions used.

DISCUSSON

1-Phenylazo-2-naphthol is metabolized by the rat and rabbit in similar ways. The principle differences established in this work are the relative paucity of 1-amino-2-naphthyl hydrogen sulphate and the complex which contains 4′, 6-dihydroxy-1-phenylazo-2-naphthol, and the relatively greater amount of 1-amino-2-naphthyl glucuronide in the urine of the rat; the difficulty in detecting the glucuronide of 4′-hydroxy-1-phenylhydrazo-2-naphthol in rat urine; and, the presence in rat bile of a number of coloured derivatives which were not detected in the rabbit.

Any differences in metabolism, after oral or i.p. administration of the dye to the rat, were of a quantitative nature with the surprising exception of the failure to detect 1-amino-2-naphthyl glucuronide in the urine of rats with a cannulated bile duct, after the i.p. injection of the dye. The demonstration that seven times as much 1-phenylazo-2-naphthol could be recovered from the faeces after oral administration than after i.p. injection, is probably a reflection of its incomplete absorption from the gut. The fact that more 1-amino-2-naphthyl hydrogen sulphate was present in the urine after injection than after feeding was unexpected in view of the demonstration by Radomski[6] that dyes of the nature of 1-phenylazo-2-naphthol are reduced at the azo linkage by the intestinal flora. The reductive fission of 1-phenyl-azo-2-naphthol itself by the intestinal contents or isolated bacteria has been established in the course of this work. The apparent paradox can probably be explained by the instability of free 1-amino-2-naphthol. This would be expected to lead to its rapid destruction in the intestinal tract rather than to its absorption, conjugation and urinary excretion. It is suggested that the urinary 1-amino-2-naphthol conjugates may be produced by the azo-reductase present in the rat liver[13] in combination with the conjugating enzymes. If this is correct, it follows that considerably less dye reaches the liver after oral administration than after i.p. injection, and this may have an important influence on its carcinogenicity.

The destruction of the dye after only a brief exposure at room temperature to the intestinal contents, intestinal minces or cultures of isolated intestinal bacteria is unlikely to be due to errors in sampling because there was practically quantitative recovery from suspensions in saline and because the reproducibility of recoveries from incubates with the same part of the intestinal tract was reasonable. The fact that this rapid destruction of the dye was of the same order of magnitude as the destruction induced by a further 16 hr incubation with the intestinal constituents suggests that there may be present in these biological extracts substances capable of reducing the dye by a non-enzymatic reaction. If this is true, it is pertinent to enquire whether the reduction of the azo-linkage during the incubation of the dye with the intestinal extracts is wholly a direct enzymatic process or is the result of the production of such reducing substances. Ryan and his associates[14, 15] report the failure of the rat to reduce i.p. injected phenylazopyrazolones (i.e. tartrazine and derivatives), although cultures of a *B. proteus* isolated from the intestinal flora of these rats were capable

of reducing tartrazine. It is note-worthy that washed bacterial cells required pre-incubation with the medium before they exhibited reducing activity.

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