Metabolism of β -Naphthoxyethanol in the Rabbit

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After the development of the method of determining β -naphthoxyethanol described in the preceding paper (Spinks, 1950) the excretion in rabbit urine was examined. Since normal urine contains chloroform-soluble constituents which absorb light of wavelengths 272 and 325 $m\mu$, the technique adopted was to determine the normal daily excretion of such

Fig. 1. Absorption spectra of dark brown urine samples from horses receiving β -naphthoxyethanol (1 in 10 dilutions, 2 cm. cells).

constituents in terms of milligrams of apparent β -naphthoxyethanol, and then to give the drug and continue the daily estimations. In no experiment was any significant increase in the daily excretion observed, and it was concluded that the drug is wholly metabolized in the rabbit. The absence from the urine of a compound which is completely cleared from the blood within 30–60 min. after intravenous administration (Spinks, 1950) was noteworthy, and it was decided to study its metabolism. Further support for this study was provided by a symptom first observed in the horse by the author's colleague, Mr Parry. About one horse in three anaesthetized with β -naphthoxyethanol excreted dark urine, appearing greenish brown by transmitted light and brown by reflected light. Further darkening usually occurred on standing. The absorption spectra of all the samples examined (cf. Fig. 1) were non-specific, and tests for pathological amounts of urobilinogen, haematoporphyrin, uroporphyrin, coproporphyrin and bilirubin were negative. It was, therefore, thought likely that β -naphthol had been formed

from the drug, de-etherification being a well-known metabolic process (Williams, 1947). The administration of β -naphthol to dogs has been shown to result in darkening of the urine, ascribed to the formation *in vivo* of dihydroxynaphthalenes, which blacken rapidly in air (Lesnik & Nencki, 1886). If β -naphthol were a metabolite of the drug, it was obviously desirable to establish that it was not formed in large amount in view of its known toxicity. Since the difficulty of collecting total urine from the horse, and therefore of balancing metabolite excretion against drug intake, is great, it was decided to carry out the study in the rabbit. The administration of β -naphthoxyethanol to the rabbit leads to the excretion of brownish green urine, not usually, however, as dark as that from the horse.

EXPERIMENTAL

It was thought that the most probable metabolites were β -naphthol and other phenols; conjugated derivatives of these and of β -naphthoxyethanol; quinonoid compounds; and, possibly, β -naphthoxyacetic acid. Since all of these should show characteristic ultraviolet absorption spectra, the method selected was to extract urine and 'hydrolysed' urine at weakly basic and at acid pH, and to construct the absorption spectra of the extracts. Each of the suggested metabolites should have been traceable under one of these four sets of conditions.

Methods

Animals. The rabbits were chinchilla bucks weighing 2–3 kg.

Dosage. β -Naphthoxyethanol was administered as a 10% (w/v) dispersion.

Absorption spectra. All absorption spectra were constructed using the Beckman quartz spectrophotometer, model DU. Readings were taken at 2 $m\mu$. intervals in the ultraviolet, and at 4 $m\mu$. intervals in the visible region; 2 cm. cells were used throughout.

Melting points. All melting points are uncorrected.

Spectrophotometric study

In a typical experiment two rabbits received a total of 1.7 g. of β -naphthoxyethanol intraperitoneally. The combined 24 hr. urine totalled 222 ml.

Free non-acidic metabolites. Urine (1 ml.) was treated with 1 ml. of 0.2 M- Na_2HPO_4 and shaken for 5 min. with 40 ml. of

CHCl_3 . The extract was separated, dried over Na_2SO_4 and examined in the spectrophotometer. The spectrum (A, Fig. 2) was non-characteristic and showed no evidence of the presence of any naphthalene derivative. In this fraction would have appeared β -naphthoxyethanol, solvent-soluble phenolic compounds and quinones of low molecular weight. All could therefore be present only in negligible amount.

Fig. 2. Absorption spectra of: A, a chloroform extract of urine buffered to pH 8.5; B, a chloroform extract of urine acidified with HCl; C, a carbonate extract of B.

Fig. 3. Absorption spectra of authentic β -naphthoxyacetic acid: A, in chloroform (λ_{max} 262, 272, 282, 311, 318 and 325.5 $\text{m}\mu$; ϵ_{max} 4575, 4940, 3550, 1390, 1252 and 1770 respectively); B, in 1% (w/v) Na_2CO_3 (λ_{max} 262, 271, 280.5, 311.5 and 325 $\text{m}\mu$; ϵ_{max} 3880, 4220, 2930, 1200 and 1460 respectively).

Free acidic metabolites. Urine (0.5 ml.) was treated with 1 ml. of N-HCl and extracted with 40 ml. of CHCl_3 as described above. The absorption spectrum (B, Fig. 2) was very similar to that of β -naphthoxyethanol itself, suggesting the presence in the urine either of an acid of similar chromophoric structure, presumably β -naphthoxyacetic acid, or of a conjugated derivative of β -naphthoxyethanol, immediately hydrolysed on adding HCl. The former possibility was established by extracting fraction B with an equal volume of 1% Na_2CO_3 (w/v). The CHCl_3 residue was similar in spectrum

to A, but the spectrum of the carbonate extract (C) clearly indicated the presence of an acid. By comparing the extinction coefficients from spectra B and C with those of authentic β -naphthoxyacetic acid (prepared by the method of Spica, 1886) in the corresponding solvents (Fig. 3) it was found that approximately 25% of the drug could be accounted for as β -naphthoxyacetic acid.

Conjugated non-acidic metabolites. Urine (5 ml.) was treated with 5 ml. of N-HCl and heated at 100° for 1.5 hr. On cooling, 5 ml. of N-NaOH were added and the volume was adjusted to 20 ml. with distilled water. The filtered solution (4 ml.), equivalent to 1 ml. of the original urine, was treated with 4 ml. of 0.2M- Na_2HPO_4 and extracted with 40 ml. of CHCl_3 . The absorption spectrum (D, Fig. 4) showed

Fig. 4. Absorption spectra of: D, a chloroform extract of hydrolysed urine buffered to pH 8.5; E, the same as D, after washing with N-NaOH ; F, a carbonate extract of acids from a chloroform extract of acidified, hydrolysed urine.

some evidence of maxima at the same wavelengths as β -naphthoxyethanol. The extract was purified by removal of phenols with N-NaOH . It then gave spectrum E (Fig. 4), which showed maxima at 263, 272, 314 and 327.5 $\text{m}\mu$, and a marked inflexion at 282 $\text{m}\mu$, suggesting that β -naphthoxyethanol was present. The amount was difficult to assess because the spectrum was clearly affected by other absorbing materials, but it did not seem that more than 1–2% of the drug could be accounted for in this fraction (see p. 184).

The spectrum of the phenolic fraction was inferred by subtracting E from D. It was clearly due to a complex mixture of compounds, and no further spectrophotometric study was made until isolation had been attempted (see p. 184).

Conjugated acid metabolites. The hydrolysed and neutralized urine (1 ml.), equivalent to 0.25 ml. of original urine, was acidified with 1 ml. of N-HCl , and extracted with 40 ml. of CHCl_3 . The chloroform extract was shaken with an equal volume of 1% Na_2CO_3 (w/v), and the spectrum of the latter was constructed (F, Fig. 4). It was consistent with the presence in this fraction of the presumed β -naphthoxyacetic acid of the unhydrolysed urine, together with a mixture of other acids liberated by hydrolysis. The extinction coefficients did not suggest the formation by hydrolysis of further amounts of β -naphthoxyacetic acid.

Isolation experiments

The spectrophotometric study indicated the presence of β -naphthoxyacetic acid and conjugated β -naphthoxyethanol. These deductions were confirmed by isolation, and in addition β -naphthol was isolated as an azo derivative.

(I) *Isolation of β -naphthoxyacetic acid.* The main bulk of the urine (200 ml.) was acidified with an equal volume of N-HCl and extracted with 3×100 ml. of ether. The aqueous residue was retained for hydrolysis (see II below). The combined ether extracts were shaken with 3×30 ml. of 1% Na_2CO_3 (w/v) and the ether was discarded (cf. spectrum A). The combined carbonate extracts were acidified with conc. HCl . After standing overnight the dark, crystalline deposit was filtered, washed with water and dissolved in 25 ml. of boiling water. Charcoal (0.5 g., DY 3) was added, boiling continued for 5 min. and the charcoal removed by filtration. The pale yellow filtrate was evaporated to incipient cloudiness and allowed to stand overnight. The colourless plates were filtered, washed with distilled water and dried *in vacuo* over CaCl_2 . The yield was 130 mg., m.p. $151\text{--}153^\circ$; mixed with authentic β -naphthoxyacetic acid, m.p. $152\text{--}154^\circ$. One recrystallization from benzene gave rosettes (85 mg.), m.p. 154° . (Found C, 70.5; H 4.95. Calc. for $\text{C}_{13}\text{H}_{10}\text{O}_3$: C, 71.2; H, 4.95%). The mother liquors were not further examined.

(II) *Isolation of β -naphthoxyethanol.* The acid urine residue from (I) was heated for 1.5 hr. at 100° and allowed to cool. It was then extracted with 3×150 ml. of ether. The urine was discarded, and the combined ether fraction washed with 2×50 ml. of water, and then extracted with 3×20 ml. of 1% Na_2CO_3 . This extract did not give any crystalline product and was discarded. The ether residue was extracted with 3×20 ml. of 2N- NaOH and the combined aqueous fraction examined for phenolic metabolites (see III below). The ether fraction, now free from phenols and acids, was dried over Na_2SO_4 and the solvent removed on the steam bath. The tarry residue was dissolved in boiling light petroleum (b.p. $40\text{--}60^\circ$, 2 ml.) and the filtered solution allowed to stand overnight at room temperature. The precipitated solid weighed 8–10 mg. and melted at $62\text{--}68^\circ$. Recrystallization from light petroleum (b.p. $60\text{--}80^\circ$, 1 ml.) gave 6–7 mg. of almost pure β -naphthoxyethanol, m.p. $70\text{--}72^\circ$; mixed with an authentic specimen, m.p. $72\text{--}73^\circ$. The yield of crude material was equivalent to about 0.5% of the dose.

(III) *Isolation of β -naphthol as an azo derivative.* The NaOH fraction from (II) was acidified and extracted with 3×100 ml. of ether. The combined extracts were dried over Na_2SO_4 , and the solvent was removed on the steam bath. The residue (20 mg.) was tarry and resisted attempts at crystallization. It was therefore decided to convert the phenols to azo dyes and to attempt a separation by chromatography. The residue was dissolved in 1 ml. of 2N- NaOH and treated with a solution of naphthalene-1-diazonium chloride (from 150 mg. of recrystallized α -naphthylamine hydrochloride). The mixture was acidified and shaken vigorously with 100 ml. of CHCl_3 . The lower layer was separated, dried over Na_2SO_4 and passed through a column of activated alumina (7×1 cm.), the column being washed with 100 ml. of CHCl_3 to remove the last traces of dye (chromatogram 1). Some tarry impurities remained strongly adsorbed. The eluate gave absorption spectrum G of Fig. 5, which showed some similarity in position of the main maximum to that of 1-(naphthalene-1'-azo)-2-naphthol (prepared by the method

of Meldola & Hanes, 1894; K of Fig. 6). The volume was reduced to 10 ml. and the solution rechromatographed on alumina (14×2 cm., chromatogram 2). A faint bluish band and a faint orange band at the top of the column were discarded. The remaining bands, a weakly adsorbed broad pink

Fig. 5. Absorption spectra of chloroform solutions of mixed azo dyes from the conjugated phenolic fraction of the urine: G, total eluate from the first chromatogram; H, purple band from the second chromatogram; I, pink band from the second chromatogram.

Fig. 6. Absorption spectra of 1-(naphthalene-1'-azo)-2-naphthol in chloroform: K, authentic sample (λ_{max} , 512.5, ϵ_{max} , 17,600); J, sample from the conjugated phenolic fraction of urine.

band, and a more strongly adsorbed purple band were passed through the column by washing with 200 ml. of CHCl_3 and collected separately. The purple band gave spectrum H and the pink band spectrum I of Fig. 5. Both fractions were obviously mixtures, I, however, agreeing best with the spectrum of authentic 1-(naphthalene-1'-azo)-2-naphthol. This fraction was therefore evaporated to dryness, and the semi-crystalline residue dissolved in 10 ml. of benzene and chromatographed on alumina (14×2 cm.), the column being developed with benzene (chromatogram 3). Five main bands were observed as follows (in decreasing strength of adsorption): faint purple, purplish blue, orange, strong red, orange. The lower three bands were passed through the column by washing with 200 ml. of benzene, and collected separately. The red fraction was retained and the solvent removed to

give a crystalline residue (4.3 mg.). A trace dissolved in CHCl_3 gave a spectrum (*J* of Fig. 6) identical with that of authentic 1-(naphthalene-1'-azo)-2-naphthol. The remainder of the material was crystallized from ethanol CHCl_3 (70–30 (v/v), 0.5 ml.) to give needles (2.8 mg.) of 1-(naphthalene-1'-azo)-2-naphthol. (Found C, 80.3; H, 5.4. Calc. for $\text{C}_{20}\text{H}_{14}\text{ON}_2$: C, 80.4; H, 4.7 %); m.p. 227–228°, mixed with an authentic specimen, m.p. 227–228°. The yield was equivalent to about 0.1 % of the dose. The absence of β -naphthol from the free phenol fraction of the urine (and from the α -naphthylamine used) was readily demonstrated, no characteristic pink band being observed in the chromatograms.

DISCUSSION

β -Naphthoxyethanol is metabolized by the three routes shown:

Route I, to β -naphthoxyacetic acid, accounts for about 25 % of the drug (by spectrophotometric estimation); routes II and III account for about 0.5 and 0.1 % respectively (from amounts isolated). Each route represents a well-known metabolic reaction (Williams, 1947), and the main points of interest are the small amount of drug degraded by routes II and III, and the fact that about 75 % was not accounted for at all. Some of this may have been present as polyphenolic compounds (which could have remained in the aqueous phase under the conditions of extraction used) and their coloured oxidation products, but it is probable that much of the drug was either burned completely or converted to metabolites not specifically sought during these experiments, e.g. phthalic acid. The trace of β -naphthol isolated is of course negligible from the standpoint of toxicity. The most notable feature of the various degradative reactions, known and unknown, is their rapidity. Together they result in the reduction of blood concentrations of 5 mg. or more of the drug per 100 ml. to zero within half an hour to an hour.

satisfactorily by any chromatographic technique tried; normal horse urine gave a superficially similar mixture, but the presence of traces of β -naphthol in the experimental urine cannot be excluded.

SUMMARY

1. β -Naphthoxyethanol was not detected in the urine of rabbits receiving large doses intravenously or intraperitoneally.
2. Three metabolites have been identified as β -naphthoxyacetic acid (approx. 25 %), conjugated β -naphthoxyethanol (approx. 0.5 %) and conjugated β -naphthol (approx. 0.1 %).
3. It is suggested that part of the missing drug appears in the urine as coloured oxidation products of β -naphthol or other phenolic metabolites, but much must be degraded by unknown routes.
4. Traces of free and conjugated β -naphthoxyethanol and large amounts of β -naphthoxyacetic acid were detected in horse urine.

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