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THE METABOLISM OF PRONETHALOL

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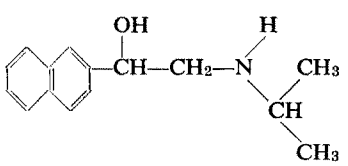
Abstract—Pronethalol is metabolised by two main pathways, side chain oxidation, and ring hydroxylation and conjugation. Five metabolites have been identified by comparison with synthetic substances. Four of these, 2-amino-1-(2-naphthyl)ethanol, 2-naphthylglycollic acid, 2-naphthylglyoxylic acid, and 2-naphthoic acid are formed by degradation of the isopropylaminoethanol side chain. The fifth, the 7-hydroxy-analogue of pronethalol, is partly present in the free form, but the major amount is present as a glucuronide in which the glucuronic acid residue is attached to the phenolic hydroxyl group.

In several species of animals, and in humans, the differences in the major metabolites are of a quantitative rather than a qualitative nature. We have been unable to determine the nature of the proximate carcinogen responsible for producing thymic tumours in mice, but it is unlikely to be one of the major metabolites.

The 7-hydroxy-analogue of pronethalol is about five times more potent than pronethalol as a β-adrenergic blocking agent and is probably responsible for some, but not all, the pharmacological activity of pronethalol.

PRONETHALOL ('Alderlin')†, racemic 2-isopropylamino-1-(2-naphthyl)ethanol,[1] was shown by Black and Stephenson[2] in 1962 to block specifically cardiac and other β-adrenergic receptors in laboratory animals, and has since been investigated clinically.[3–7] In 1964, Alcock and Bond[8] described the production in mice of thymic tumours and other sarcomata associated with the administration of pronethalol, which had not appeared to be carcinogenic to rats, guinea pigs or dogs, and gave a preliminary account of the metabolism of pronethalol. A more detailed account of its metabolism is given here.



Pronethalol.

METHODS

Thin-layer chromatography

Fluorescent Silica gel plates were used with solvent systems (a) *n*-butanol:acetic acid:water, 40:10:5; and (b) ethanol:ammonia (0·880):water, 40:2:2·5.

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† The term' Alderlin' is a registered trade mark of Imperial Chemical Industries Ltd.

Paper chromatography

Papers (Whatman No. 1) were developed with butanol:acetic acid:water, 50:5:45, by the descending method.

Paper electrophoresis

^{14}C-metabolites in urine were separated during 30 min at 4 kV using 0·05 M NaH_2PO_4. Transverse (2 cm) strips of the electrophoretogram were cut out and the radioactivity was determined in toluene phosphor (system (a)).

Radioactive counting

A Packard liquid scintillation spectrometer was used. The phosphor systems were: (a) 1000 ml toluene, 4 g 2,5-diphenyloxazole, and 0·1 g 1,4-*bis*-2-(5-phenyloxazolyl)benzene; (b) 1000 ml toluene, 10 g 2,5-diphenyloxazole, and 0·2 g 1,4-*bis*-2-(5-phenyloxazolyl)benzene; (c) 1000 ml dioxane, 100·4 g naphthalene, 10·0 g 2,5-diphenyloxazole, and 0·25 g of 1,4-*bis*-2-(5-phenyloxazolyl)benzene. An internal standard of ^{14}C-benzoic acid in toluene was used to determine counting efficiency.

Conductivity measurements

A Mullard A.C. Conductivity Bridge was used.

Preparation of ^{14}C-pronethalol

^{14}C-Pronethalol synthesised* from 2-naphthoic acid (^{14}COOH) had specific activity 0·91 μc/mg.

ISOLATION AND IDENTIFICATION OF METABOLITES OF PRONETHALOL

Acid metabolites

^{14}C-Pronethalol was dosed subcutaneously at 50 mg/kg to guinea pigs, rabbits, or rats. 24 Hr urines were acidified, extracted with chloroform, and then the acids were extracted back into sodium bicarbonate solution. This solution was acidified and re-extracted with chloroform. The dried chloroform solution was concentrated and cooled. The solid which separated was isolated by filtration, the filtrate being retained. The solid was crystallised from ethanol-water and then chloroform to give 2-naphthylglycollic acid as colourless plates, m.p. and mixed m.p. 158°. (Found: C, 71·5; H, 5·1%; $C_{12}H_{10}O_3$ requires C, 71·4; H, 4·95%). The i.r. spectrum was identical with that of 2-naphthylglycollic acid. The material was not optically active (conc. 1·0 in ethanol).

The retained filtrate was subjected to counter current distribution. Two runs of 40 transfers were carried out, one at pH 6·0 in phosphate buffer, the other at pH 3·3 in citrate buffer, both with chloroform as the stationary phase. Tubes 8–20 of the pH 6·0 run yielded 2-naphthoic acid as colourless needles, m.p. and mixed m.p. 185°, from aqueous ethanol. The i.r. spectrum was identical with that of 2-naphthoic acid. Tubes 30–40 of the pH 6 run were combined and run at pH 3·3; tubes 21–36 contained 2-naphthylglycollic acid. 250 mg of 2-naphthylglycollic acid was recovered in all from 31. guinea pig urine.

* The synthesis, by Mr. J. Burns, will be reported elsewhere.

2-Naphthylglyoxylic acid was isolated from rabbit urine as follows. Urine from rabbits dosed subcutaneously with ^{14}C-pronethalol at 50 mg/kg was acidified and extracted with chloroform. The extract was concentrated and the solid 2-naphthylglycollic acid was removed by filtration. The filtrate was subjected to counter current separation (50 transfers; citrate buffer, pH 3·3, against chloroform). Tubes 25–37, which contained a mixture of 2-naphthylglycollic acid, 2-naphthylglyoxylic acid, and β-naphthoic acid, were combined and the solvent was evaporated. The residual solid was dissolved in ether and extracted with saturated sodium bisulphite solution. The extract was acidified and extracted with chloroform. The chloroform extract furnished a yellow gum. Infra-red spectroscopy and TLC using solvent system (a) con firmed that the gum was largely 2-naphthylglyoxylic acid (R_f 0·62).

Amphoteric metabolites

Urine from mice dosed subcutaneously with ^{14}C-pronethalol at 100 mg/kg (50 mg/kg for rats) was acidified (pH 5) and then passed through a column of anion exchange resin de-acidite FF (52–100 mesh, 1·0–1·5 water regain) in the hydroxide form. The column was eluted with 1N acetic acid and fractions of eluate containing radioactivity were combined and passed through a column of cation exchange resin Zeokarb 225 (52–100 mesh, 8% DVB) in the hydrogen form. The radioactive fractions eluted with 1N ammonium hydroxide were freeze dried and the residue was extracted with methanol. A buff coloured solid was precipitated by adding excess ethyl acetate. It was hygroscopic, gave a positive naphthoresorcinol test indicative of a glucuronide, and its i-r spectrum was characteristic of an ammonium salt of a polyhydroxy compound.

Solids obtained from mouse and from rat urine were examined by TLC. R_f values are given.

System (a)	System (b)
Mouse: 0.11 (major); 0.27 (minor) Rat: 0·11 (major); 0·27 (minor)	Mouse: 0.15 (major); 0·05 and 0·38 (minor) Rat: 0·16 (major); 0·05 (minor).

Acid hydrolysis of mouse glucuronide. Crude glucuronide (0·2 g) was refluxed with 2N hydrochloric acid (10 ml) under nitrogen for 40 min. The solution was extracted with ether at pH 2, 10, and 8. The gum (25 mg) obtained from the pH 8 extract was triturated with cold ethyl acetate-light petroleum (b.p. 40–60°) and the solid which separated was crystallised from ethyl acetate-light petroleum (b.p. 40–60°). 1-(7-Hydroxy-2-naphthyl)-2-isopropylaminoethanol (6·8 mg) m.p. and mixed m.p. 163–4°, was obtained. The m.p. was depressed to 140–145° by 1-(6-hydroxy-2-naphthyl)-2-isopropylaminoethanol, m.p. 170–171°. The u.v. spectrum was identical with that of 1-(7-hydroxy-2-naphthyl)-2-isopropylaminoethanol. (Found: C, 72·9; H, 7.8. $C_{15}H_{19}NO_2$ (mol. wt. 245) required: C, 73·5; H, 7·75%).

Enzymic hydrolysis of the glucuronides

(a) Mouse or rat glucuronide in 0·2 M phosphate buffer (pH 6·8) was incubated at

37° for 20 hr with a β-glucuronidase preparation from *E. coli*. The mixture was examined by TLC.

System (a)	System (b)
Mouse: 0·38 (major); 0·2 (faint) Rat: 0·38 (major); 0·1 (faint)	Mouse: 0·73 (major); 0·05 and 0·41 (minor) Rat: 0·73 (major); 0·07 and 0·44 (minor)

7-Hydroxy-analogue of pronethalol: 0·38, 7-Hydroxy-analogue of pronethalol: 0·73

(b) Glucuronide in 0·5 M acetate buffer (pH 4·6) was incubated under nitrogen with a β-glucuronidase preparation from *Helix pomatia* at 37° for 3 hr. The pH was adjusted to 8·5 with sodium bicarbonate and the mixture was extracted with chloroform. The extract was dried and evaporated, and the residue examined by TLC.

System (a)	System (b)
Mouse: 0·45 (major); 0·80 (faint)	Mouse: 0·68 (major); 0·79 (faint)

7-Hydroxy-analogue of pronethalol: 0·45, 7-Hydroxy-analogue of pronethalol: 0·68

The 7-hydroxy-analogue of pronethalol and the major spot in each case gave an orange-pink colour with diazotised sulphanilic acid; the faint spot did not.

Rat glucuronide gave similar results but human glucuronide gave only the spot for the 7-hydroxy-analogue of pronethalol.

Further purification of glucuronides

Freeze dried eluate from the Zeocarb column was applied to a column of Sephadex G25 as a solution in 1% sodium chloride. This column was eluted with water and the fractions with high glucuronide[9] content, absorption at 280 mμ, and low conductivity were bulked and freeze dried.

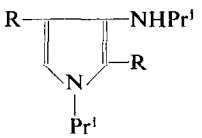
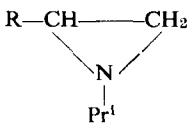
Solid from rat urine prepared in this way was chromatographed on paper. Four spots were observed, R_f 0·26 (major), R_f 0·68 (minor), and R_f 0·16 and 0·80 (faint). The spots of R_f 0·26 and 0·68 were eluted and incubated with β-glucuronidase (*Helix pomatia*). The u.v. spectrum of the hydrolysate of the major spot was identical with that of the synthetic 7-hydroxy-analogue of pronethalol and showed the characteristic shift with alkali. Identification was confirmed by TLC. This evidence supports the view that the major constituent of the amphoteric material is the glucuronide of the 7-hydroxy-analogue of pronethalol.

The ultraviolet spectrum of the material obtained from the hydrolysis of the minor spot of R_f 0·68 was naphthalenic in character and did not shift under alkaline conditions. This unknown minor metabolite could also be isolated by heptane extraction of the β-glucuronidase hydrolysate from crude mouse or rat glucuronide. It was basic and non-phenolic. TLC examination has eliminated certain possible structures.

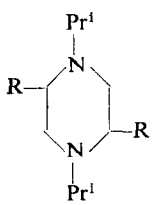
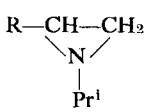
R = 2-Naphthyl*; R_f values given.

* The synthesis of these compounds will be reported in a chemical publication.

Solvent system (b)

Unknown	0·77
$RCH(OH)CH_2NHPr^i$	6·07
$RCH(OH)CH_2NH_2$	0·52
	0·76 Characteristic yellow-green fluorescence.
	0·76

Solvent system (a)

Unknown	0·81
$RCOCH_2NHPr^i$	0·52
$RCOCH_2NH_2$	0·55 Pink spot
	0·64
	0·79

It is not the ethyleneimine[10] related to pronethalol. After acid hydrolysis under conditions known to hydrolyse the ethyleneimine to pronethalol, it was recovered unchanged, R_f 0·77 in solvent system (b).

Basic metabolites

24-hr urine from guinea pigs dosed subcutaneously with ^{14}C-pronethalol (50 mg/kg) was acidified (pH 2) and extracted with chloroform to remove acid metabolites. It was extracted again at pH 11·5 with chloroform, and the concentrated extract was subjected to counter-current distribution (40 transfers) between chloroform and 0·1 M phosphate buffer (pH 6·5). Tubes were examined for radioactivity (phosphor system (c) and absorption at 283 mμ. From the peak of activity in tubes 16–30 pronethalol was isolated m.p. and mixed m.p. 106°. The remaining activity was in tubes 36–40 which contained the 7-hydroxy-analogue of pronethalol.

Estimation of pronethalol[11] *and its 7-hydroxy-analogue*

The sample (2 ml) was shaken with 1 ml 1 N sodium hydroxide and 12 ml heptane. 10 ml of the heptane extract containing pronethalol was then extracted with 2 ml

0·1 N hydrochloric acid and the fluorescence of the hydrochloric acid layer was read on an Aminco Bowman spectrophotofluorimeter at 340 mμ using an activating wavelength of 285 mμ. To 2 ml of the aqueous layer remaining from the heptane extraction was added 0·66 ml 1 N hydrochloric acid, 2 ml phosphate buffer (pH 8·0; 0·5 M), and 2 g sodium chloride, and the mixture was extracted with 10 ml amyl alcohol. 8 ml of the extract and 8 ml heptane was extracted with 2 ml acetic acid (0·2M). The fluorescence of the acetic acid layer, containing the 7-hydroxy-analogue of pronethalol, was read at 377 mμ using an activating wavelength of 295 mμ. Standard curves were prepared for mixtures of the two compounds.

Alternatively, 0·25 ml of β-glucuronidase hydrolysate, 0·1 g sodium chloride, and 1·75 ml borate buffer (pH 8·5; 0·5 M), was extracted with 8 ml ethyl acetate. 7 ml of the extract was shaken with 7 ml heptane and 1·25 ml acetic acid (0·1 M). 1 ml of the acetic acid layer was treated with 1 ml of 2% sodium carbonate in 0·1 N sodium hydroxide, 0·2 ml Folin and Ciocalteu reagent (diluted three times), and after 30 min the colour was read in a Hilger Uvispek spectrophotometer at 750 mμ using 4 cm microcells.

Tissue distribution of pronethalol and its metabolites

Female mice were dosed orally at 10 mg/kg with ^{14}C-pronethalol. Six were killed immediately and another six at intervals up to 96 hr. Various organs (Table 1) were

TABLE 1. PERCENTAGES OF RADIOACTIVITY IN TISSUES REMOVED FROM MICE AT VARIOUS INTERVALS OF TIME. THE RADIOACTIVE DOSE WAS CALCULATED FROM THE AMOUNT OF RADIOACTIVITY FOUND IN THE ANIMALS KILLED IMMEDIATELY

	Hours after dosing					
Tissue	0	¾	2½	5	24	96
Gut	96	60	58	50	7	2·5
Lung	0·5	0·6	0·4	0·2	0·1	0·1
Kidney	0·7	2·0	0·8	0·8	0·3	0·3
Heart	0·2	0·2	0·2	0·1	0·1	0·05
Liver	0·8	12·5	4·7	4·5	1·3	0·8
Spleen	0·2	0·4	0·3	0·2	0·1	0·1
Thymus	0·04	0·07	0·04	0·04	0·04	0·08
Brain	0·5	0·7	0·2	0·2	0·3	0·4
Remaining tissues	0·6	1·2	0·9	0·8	0·6	0·3

removed immediately after death and homogenised in water. 2 ml of homogenate dried at 50° *in vacuo* was digested overnight at 60° with 3 ml of 1 M Hyamine solution, and then 5 ml absolute alcohol, 10 ml phosphor system (b), and 0·3 ml of conc. hydrochloric acid were added. The mixture was counted for radioactivity and the result corrected for background and colour quenching. The total radioactivity in the animals killed immediately was assumed to be the same as the amount administered.

Estimation of pronethalol and its metabolites in blood

^{14}C-Pronethalol (200 mg, 6·17 μc) was administered orally to female patients. Blood taken before and after dosing was centrifuged and then 0·5 ml serum was digested overnight with 3 ml Hyamine solution at 38°. 10 ml of phosphor system (b) and

0·5 ml conc. hydrochloric acid were added and the solution was counted. Pronethalol and its 7-hydroxy-analogue were estimated by the method described previously. Acid metabolites were estimated by chloroform extraction of the acidified serum and extraction back into phosphate buffer (pH 7·4; 0·1 M), an aliquot of which was counted using phosphor system (b).

Estimation of pronethalol in urine

Urine was collected for 24 hr from rats, mice and rabbits dosed orally with pronethalol at 10 mg/kg. Unchanged pronethalol was estimated fluorimetrically. Urine buffered with acetate (pH 4·5; 0·5 M) was treated with β-glucuronidase (*Helix pomatia*) for 4 hr at 37° and then liquots of the hydrolysate were analysed for pronethalol.

IN VITRO STUDIES

Preparation and fractionation of liver homogenate

Animals were killed, exsanguinated, and their livers were homogenised in two volumes of 0·32 M cold sucrose using a Potter–Elvehjem homogeniser. The homogenate was centrifuged at 10,000 *g* for 30 min and the supernatant containing the microsomal and soluble fraction was used for most of the experiments.

Enzymic degradation of pronethalol

5·0 ml 10,000 *g* supernatant from female guinea pig liver, 0·5 μmole $NADPH_2$ and 3·7 ml 0·5 M Tris buffer pH 8·25 were incubated at 37° in an atmosphere of 95% O_2, 5% CO_2 with 1 ml of pronethalol solution containing 200 μg. 0·15 μmole of $NADPH_2$ was added at 10–15 min intervals throughout the reaction. 1 ml samples were used for pronethalol estimations. Tris buffers giving final pH values from 6·6 to 8·6 were used to determine the pH optimum for the disappearance of pronethalol. Pronethalol solutions containing 50–100 μg (final concentration 5–100 μg/ml) were used to study the effect of substrate concentration on reaction velocity.

To demonstrate that the primary amine is a metabolite, 1 ml samples were withdrawn at intervals and shaken with 1 ml of 1 N NaOH and 10 ml of benzene. The benzene extract was taken to dryness and the residue dissolved in 0·1 ml of methanol. 5 μl of this solution was examined by TLC using solvent system (b). A spot of R_f 0·52 was observed; the R_f values of pronethalol and the primary amine were 0·67 and 0·52 respectively.

Appearance of acid metabolites

4·0 ml of liver preparation, 0·5 μmole $NADPH_2$, 1·0 ml of phosphate buffer (pH 7·4; 0·5 M), and 1·0 ml of pronethalol solution containing 0·5 mg per ml (0·23 μc per mg) were shaken at 37° in an atmosphere of 95% O_2, 5% CO_2. 0·5 ml samples were shaken with 0·5 ml 1 N HCl and 5 ml of chloroform and then the chloroform layer was back extracted into 2·0 ml of either phosphate (pH 7·4; 0·2 M) or Tris HCl buffer (pH 8·25; 0·1 M). An aliquot of buffer extract was counted using phsophor system (c).

Ring hydroxylation

6·0 ml 10,000 *g* supernatant (from Netherland Dwarf doe rabbit liver), 2·7 ml Tris buffer (pH 8·25; 0·5 M), 0·5 μmole in 0·3 ml $NADPH_2$, and 1 mg acetanilide (or

pronethalol) in 1 ml were incubated at 37° in a 95% O_2, 5% CO_2 mixture. 1 ml samples were withdrawn, 0·5 g sodium chloride was added, and the *p*-hydroxyacetanilide was extracted into 10 ml ether : isoamyl alcohol (1 : 1), and then back extracted into 1·5 ml 0·1 N sodium hydroxide. The *p*-hydroxyacetanilide (or 7-hydroxy-analogue of pronethalol) was estimated using the Folin and Ciocalteu reagent.

RESULTS

Tissue distribution of pronethalol and its metabolites

Table 1 shows the percentage of the radioactive dose found in mouse tissues after oral dosing with ^{14}C-pronethalol. The liver, and to a lesser extent the kidney, contained the greatest quantities of pronethalol and its metabolites. Slight radioactivity in the tissues of animals killed immediately was probably due to absorption of the compound in the interval between dosing the animals and removing the tissues.

Excretion of metabolites

(a) *Animals.* Assay of the excreta from the rat, mouse, guinea pig and rabbit, following an oral or subcutaneous dose of 10 mg/kg of ^{14}C-pronethalol, showed that radioactivity occurred mostly in the urine. No radioactivity was detected in the expired CO_2. Table 2 shows the excretion of radioactivity from mice. Although only 70% of

TABLE 2. THE PERCENTAGE OF ADMINISTERED RADIOACTIVITY RECOVERED IN URINE AND FAECES OF THREE FEMALE MICE DOSED WITH ^{14}C-PRONETHALOL AT 10 MG/KG

Time (hr)	Urine	Faeces	Total
0–24	57	5	62
24–48	3	3	6
48–72	1	1	2
Total	61	9	70

TABLE 3. THE CUMULATIVE PERCENTAGE OF THE ADMINISTERED RADIOACTIVITY IN INDIVIDUAL URINE SAMPLES FROM PATIENTS DOSED WITH ^{14}C-PRONETHALOL. THE 48 AND 72 HR VALUES WERE FOR BULKED SAMPLES

Patient 1		Patient 2		Patient 3	
Time of passing of urine (hr)	Cumulative % of dose	Time of passing of urine (hr)	Cumulative % of dose	Time of passing of urine (hr)	Cumulative % of dose
2·3	9	2·4	32	1·9	18
4·0	20	3·8	46	3·7	41
6·5	34	6·5	64	6·4	59
8·2	42	8·5	72	8·2	67
9·5	46	9·6	74	9·3	69
12·5	54	12·6	82	10·6	74
17·0	64	21·3	94	12·4	77
21·2	72	23·6	96	17·4	85
48·0	89	48·0	103	21·6	89
72·0	94	72·0	105	48·0	98
				72·0	100

the dose was accounted for, excretion of radioactivity had dropped to a very low level on the second and third days. The radioactivity in the faeces fell less quickly over the three days than that in the urine, suggesting that biliary excretion of metabolites was occurring.

(b) *Humans.* ^{14}C-Pronethalol (200 mg containing 6·17 μC) was dosed orally to three female patients and urine, faeces, and blood samples, were collected. Table 3 shows the percentage of the radioactivity appearing in urine samples during 72 hr. Most of the radioactivity appeared during the first 24 hr.

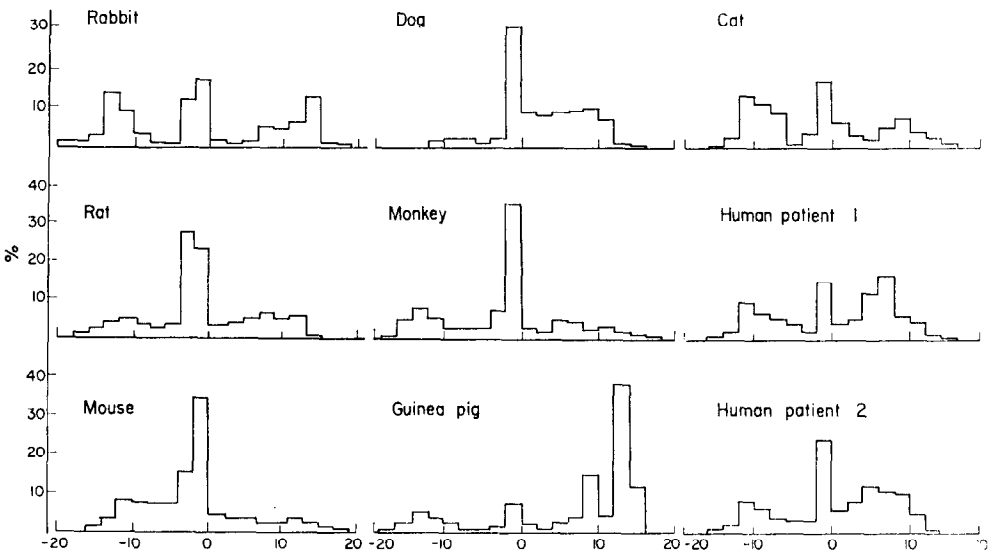


FIG. 1. Paper electrophoresis of urine of animals dosed subcutaneously and two patients dosed orally with ^{14}C-pronethalol. Urine was applied at the origin (O) and run for 30 min at 4 kV in 0·05 M NaH_2PO_4. Values represent the percentage of the total activity on each 2 cm strip.

TABLE 4. THE PROPORTION OF PRONETHALOL METABOLISED BY THE TWO MAIN ROUTES IN VARIOUS SPECIES. CALCULATED FROM THE RESULTS OF PAPER ELECTROPHORESIS

Species	% of drug metabolised to basic and amphoteric metabolites	% of drug metabolised to acidic metabolites
Rabbit	65	35
Dog	54	46
Cat	69	31
Rat	71, 67	29, 33
Monkey	70	30
Mouse	85, 86	15, 14
Guinea pig	23	77
Human		
Patient 1	53	47
Patient 2	56	44
Patient 3	76	24

Separation of urinary metabolites by paper electrophoresis

Fig. 1 shows the results of electrophoresis of the urine of 8 species including man. In all cases basic, amphoteric, and acidic metabolites were present. The relative proportions of these metabolite groups (Table 4) varied considerably from the guinea pig where acidic metabolites accounted for 77 per cent of the total, to the mouse where the amphoteric metabolites accounted for about 85 per cent. Other species including man vary between these extremes.

Identification of metabolites in urine

(i) *Acidic metabolites.* Fig. 2 shows the results of two countercurrent separations of acidic metabolites from the urine of guinea pigs dosed with ^{14}C-pronethalol at

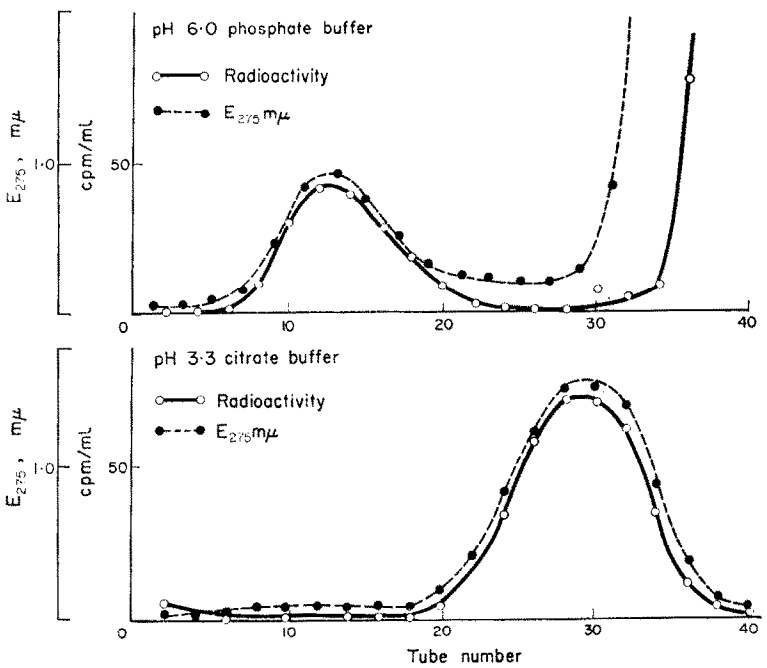


FIG. 2. The countercurrent separation of two acidic metabolites from the urine of guinea pigs dosed ^{14}C-pronethalol. Chloroform was the stationary phase in both cases. Tubes 30–40 of the first separation at pH6 were used for the second separation at pH 3·3.

50 mg/kg subcutaneously. Two major metabolites, 2-naphthylglycollic and 2-naphthoic acid, have been isolated from guinea pig, rat, and rabbit urine, and identified by TLC in human urine. 2-Naphthylglyoxylic acid, a minor metabolite has been identified in rat, rabbit, and human urine.

(ii) *Amphoteric metabolites.* The amphoteric metabolites appear to be glucuronides. In addition to the major and minor spots recorded on TLC examination of the crude glucuronides there were other faint ones. Further purification of the crude glucuronides eliminated some of these impurities and concentrated others. The glucuronides as isolated were partly in the form of their ammonium salts. The major glucuronide is that of the 7-hydroxy-analogue of pronethalol, and this has been isolated in crude form

from mouse, rat and human urine. Rat and mouse glucuronide which had been purified on a Sephadex G25 column were treated with β-glucuronidase (*Helix pomatia*). The amount of 7-hydroxy-analogue of pronethalol liberated indicated that the solid contained at least 91 per cent of the glucuronide of this compound.

A minor metabolite, possibly a glucuronide, was concentrated during further purification of rat and mouse glucuronides. Hydrolysis by β-glucuronidase gave a spot running faster than the 7-hydroxy-analogue of pronethalol in both solvent systems. The identity of this material, which is basic and non-phenolic, remains unknown, but a few possibilities have been eliminated by TLC. It is not an ethyleneimine.

(iii) *Basic metabolites.* In the guinea pig these are unchanged pronethalol and the 7-hydroxy-analogue of pronethalol (unconjugated). Less than 1·5 per cent of pronethalol, (fluorimetric estimation) is present in the urine of mouse, rat, rabbit and guinea pig. After treating the urine with β-glucuronidase, slightly higher levels of pronethalol were found. For example mouse, rat, and rabbit urine before treatment contained 1·5, 0·3, and 0·7 per cent of free pronethalol respectively, whereas after β-glucuronidase treatment the levels were 1·7, 0·6 and 1·5 per cent.

Pronethalol and certain metabolites in blood

Table 5 shows the levels of pronethalol and certain metabolites in the serum of female patients dosed orally with 200 mg of ^{14}C-pronethalol. After 1 hr pronethalol accounted for about 3·5 per cent of the total activity in the serum. Concentrations of the 7-hydroxy-analogue of pronethalol are noticably higher than those of pronethalol.

In vitro *metabolism of pronethalol by liver enzymes*

Many drugs are known to be metabolised by enzymes in liver microsomes requiring both $NADPH_2$ and oxygen.[12] The pH optimum for degradation of pronethalol by a guinea pig liver system (Fig. 3) was 8, and the Michaelis constant was 70 μM

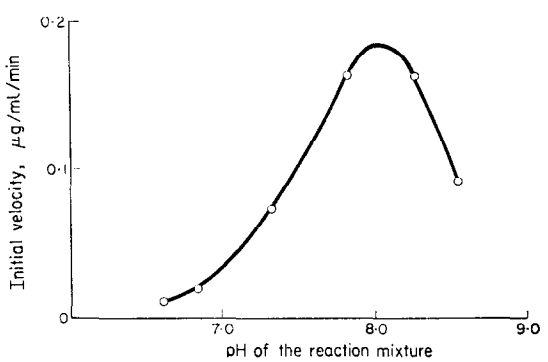


FIG. 3. The pH dependence of the rate of disappearance of pronethalol from a guinea pig liver supernatant preparation.

(Fig. 4). Oxidation of the pronethalol side chain has been followed by estimating acidic metabolites formed in the presence of guinea pig liver homogenate and a 10,000 *g* supernatant fraction (Fig. 5). Whole homogenate and 10,000 *g* supernatant showed similar activity and $NADPH_2$ was necessary. Mebanazine, ('Actomol')* a

* The term 'Actomol' is a registered trade mark of Imperial Chemical Industries Ltd.

TABLE 5. THE LEVELS OF PRONETHALOL AND CERTAIN METABOLITES IN SERUM OF THREE FEMALE PATIENTS FOLLOWING A SINGLE DOSE OF 200 MG OF ^{14}C-PRONETHALOL

Time (hr)	Patient 1				Patient 2				Patient 3		
	Total metabolites	Pronethalol	7-Hydroxy analogue of pronethalol	Acids	Total metabolites	Pronethalol	7-Hydroxy analogue of pronethalol	Acids	Total metabolites	Pronethalol	7-Hydroxy analogue of pronethalol
½	4·4	0·1	0·4	1·0	7·2	0·2	0·75	—	1·1	0·1	0·6
1	9·2	0·3	0·3	3·2	8·8	0·3	0·9	2·25	5·3	0·2	0·65
2	8·6	0·15	0·9	1·7	7·4	0·35	5·2(?)	—	4·8	0·2	1·15
4	7·3	0·1	1·2	0·9	5·3	0·25	0·65	—	3·3	0·05	1·65

All levels expressed in μg per ml of serum. Total metabolites were calculated as if they had the same molecular weight as pronethalol. Acids were calculated assuming that they were of the same molecular weight as 2-naphthylcollic acid.

monoamine oxidase inhibitor,[13] completely inhibited oxidation of the side chain at 2×10^{-4} M. Whole homogenates and 10,000 *g* supernatants from rat, mouse, and guinea pig livers, differed widely in their ability to oxidise the pronethalol side chain (Fig. 6). Guinea pig liver was most active with rat and mouse very much less, in accordance with what would be predicted by comparing the percentages of acidic metabolites in the urine of these species (Table 4). The rate of disappearance of pro-

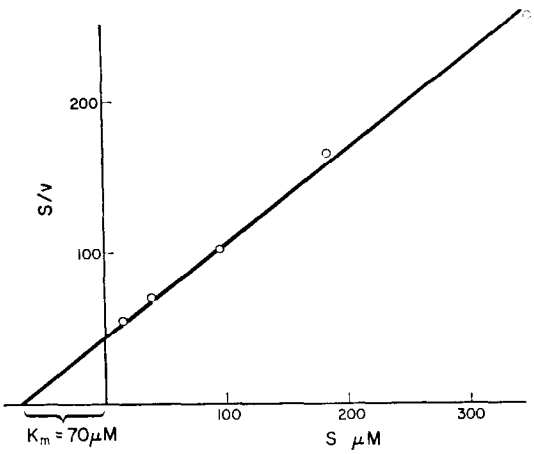


FIG. 4. Plot of S against S/v (S = substrate concentration, v = initial reaction velocity) to determine the Michaelis constant (K_m) for the degradation of pronethalol by a guinea pig liver supernatant preparation.

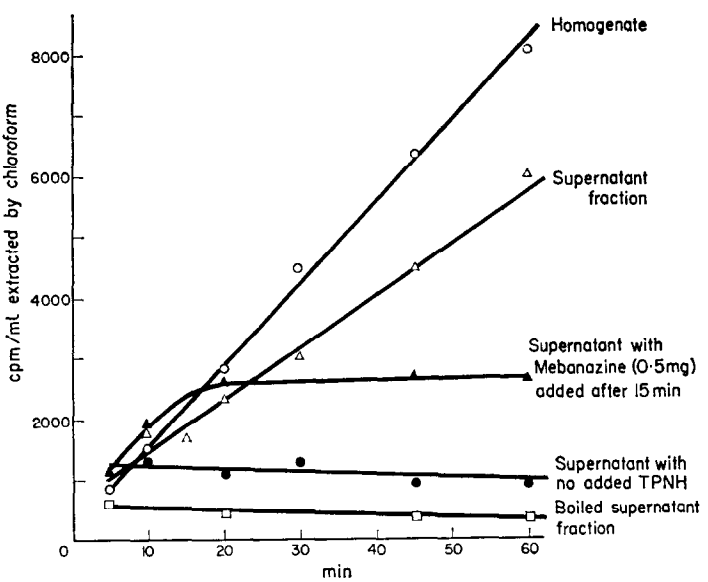


FIG. 5. The formation of ^{14}C-acidic metabolites from ^{14}C-pronethalol by homogenate and 10,000 *g* supernatant fraction from guinea pig liver.

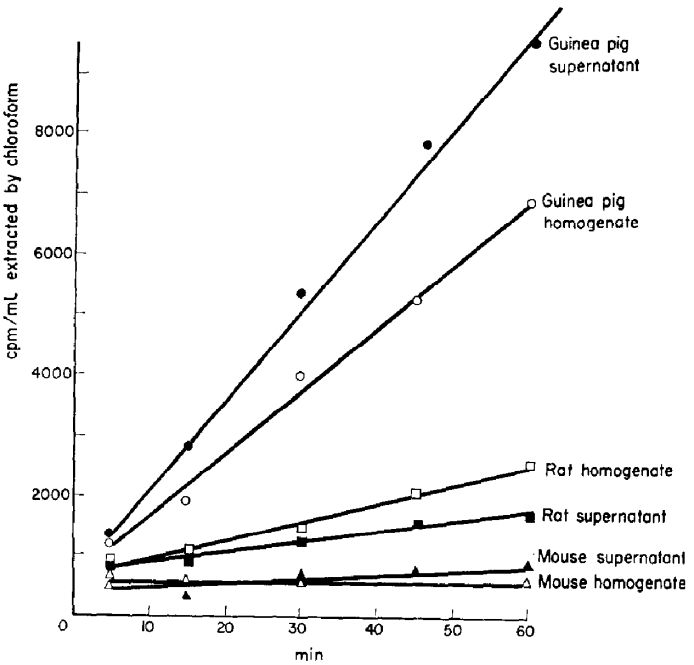


FIG. 6. The formation of ^{14}C-acidic metabolites from pronethalol by liver homogenates and 10,000 *g* supernatant fractions from the rat, mouse and guinea pig.

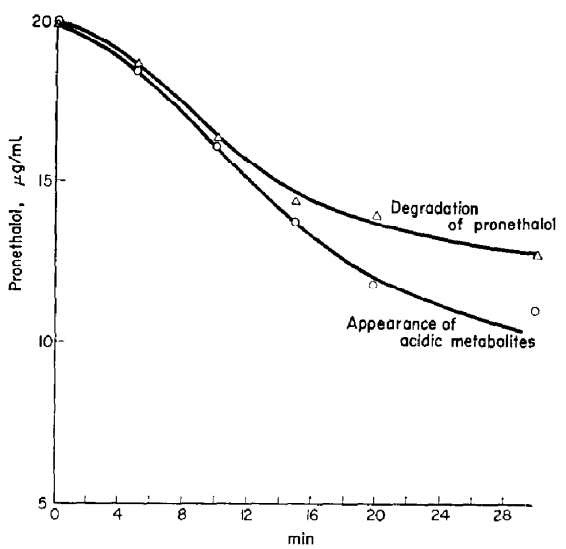


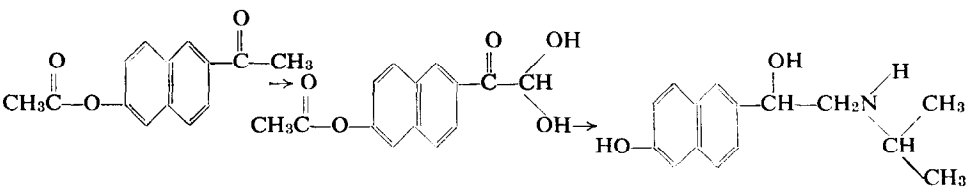
FIG. 7. The degradation of ^{14}C-pronethalol and the production of acidic metabolites by a 10,000 *g* supernatant fraction from rabbit liver. (The counts extracted from an acid solution ware converted to an equivalent amount of pronethalol and this amount was then subtracted from the initial level of pronethalol).

nethalol and the rate of appearance of acidic metabolites have been compared using rabbit liver and found to be almost identical (Fig. 7), suggesting that *in vitro* pronethalol was disappearing largely by side chain oxidation. Attempts to demonstrate ring hydroxylation of pronethalol *in vitro* using rabbit liver preparations failed, although acetanilide was readily hydroxylated in this system.[14]

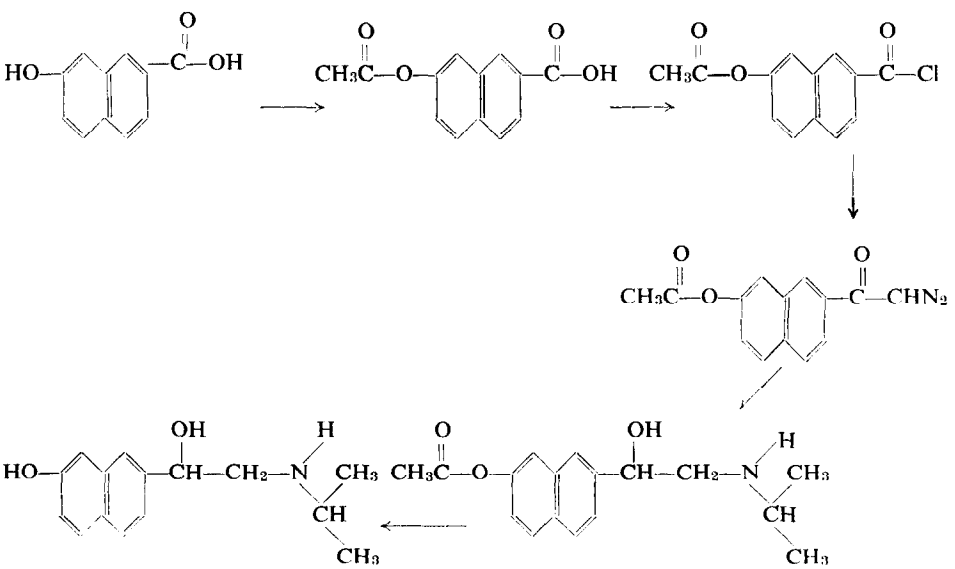
The amount of the primary amine, which is a metabolite of pronethalol *in vitro*, is greatest after about 20 min incubation.

Synthesis of the 6- *and* 7-*hydroxy-analogues of pronethalol*

The 6-hydroxy-analogue[15] was synthesised from 6-acetoxy-2-acetylnaphthalene[16] by the general method of Fodor and Kovács.[17] Deacetylation of the 6-acetoxy group occurred during hydrogenation of the glyoxal hydrate in the presence of isopropylamine.



The 7-hydroxy-analogue[15] was synthesised from 7-hydroxy-2-naphthoic acid[18] by the route:



6-Acetoxy-2-naphthylglyoxal hydrate. A solution of 1·9 g 6-acetoxy-2-acetylnaphthalene in 25 ml acetic acid and 0·98 g selenium dioxide were heated at 100° for 2 hr and then heated under reflux for 1 hr. The cooled mixture was filtered and the filtrate was poured on to ice. The solid which separated was crystallised from water to give 6-acetoxy-2-naphthylglyoxal hydrate as colourless needles, m.p. 94–95°. (Found: C, 64·5; H 4·4%. $C_{14}H_{12}O_5$ (mol. wt. 260) required: C, 64·6; H 4·6%).

1-(6-*Hydroxy*-2-*naphthyl*)-2-*isopropylaminoethanol.* A solution of 650 mg 6-acetoxy-2-naphthylglyoxal hydrate in 18 ml ethanol and 6 ml isopropylamine was hydrogenated in the presence of platinum at atmospheric pressure and room temperature. The solvents were evaporated and the residual gum was shaken with 10 ml 1 N hydrochloric acid and 25 ml ether. The layers were separated and the pH of the aqueous layer was adjusted to 11 by the addition of 2 N sodium hydroxide. The mixture was extracted with ether and the extract was washed with water and dried ($MgSO_4$). The extract was evaporated and the residual gum was triturated with light petroleum (b.p. 40–60°). The solid thus obtained was crystallised from ethyl acetate and gave 1-(6-hydroxy-2-naphthyl)-2- isopropylaminoethanol, m.p. 170–171°. (Found: C, 73·1; H 7·7; N 5·9%. $C_{15}H_{19}NO_2$ (mol. wt. 245) required: C, 73·5; H, 7·75; N 5·7%).

U.V. spectrum (methanol): λ_{max} = 258 mμ (log = 3·62 INF)); 276 (3·79); 285 (3·62 INF); 324 (3·27); 3·35 (3·33).

(methanol + (N/10) NaOH) λ_{max} = 276 mμ (INF), 286, 293, 352.

7-*Acetoxy*-2-*naphthoic acid.* A solution of 20·3 g 7-hydroxy-2-naphthoic acid in 20 ml pyridine and 125 ml acetic anhydride was kept at room temperature for 18 hr and then poured into a mixture of ice and water. The solid which separated was crystallised from methanol. 7-Acetoxy-2-naphthoic acid was obtained as colourless plates, m.p. 209–210°, in yield of 20·7 g. (Found: C, 67·7; H, 4·5%. $C_{13}H_{10}O_4$ (mol. wt. 230) required: C, 67·8; H, 4·35%).

7-*Acetoxy*-2-*naphthoyl chloride.* 20·2 g 7-Acetoxy-2-naphthoic acid, 20 g phosphorus pentachloride, and 500 ml light petroleum (b.p. 60–80°) were heated under reflux for 2 hr and then the hot mixture was filtered. The filtrate deposited 7-acetoxy-2-naphthoyl chloride as colourless needles, m.p. 126°, in a yield of 18 g. (Found: C, 62·8; H, 3·8; Cl, 13·7%. $C_{13}H_9O_3Cl$ (mol. wt. 248·5) required: C, 62·7; H, 3·6; Cl, 14·3%).

7-*Acetoxy*-2-*diazoacetylnaphthalene.* A solution of 18 g 7-acetoxy-2-naphthoyl chloride in 200 ml benzene was slowly added to an excess of diazomethane in 750 ml ether at below 10°. The solution was kept below 10° for 18 hr and then the excess of diazomethane and the solvents were evaporated. The residual solid was crystallised from ether. 7-Acetoxy-2-diazoacetylnaphthalene (containing a trace of 7-acetoxy-2-chloroacetylnaphthalene) was obtained as yellow needles, m.p. 127–28°, in yield of 13 g. (Found: C, 65·8; H, 4·1; N, 10·3%. $C_{14}H_{10}N_2O_3$ (mol. wt. 254) required: C, 66·1; H, 3·9; N, 11·0%).

1-(7-*Acetoxy*-2-*naphthyl*)-2-*isopropylaminoethanol.* A solution of 3 g 7-acetoxy-2-diazoacetylnaphthalene in 100 ml acetone and 10 ml ethanol was hydrogenated in the presence of 300 mg platinum at atmospheric pressure and room temperature. The solvents were evaporated and the residual gum was stirred with 50 ml ether. Ethereal hydrogen chloride was added and the solid which separated was crystallised from methanol-ethyl acetate to give 1-(7-acetoxy-2-naphthyl)-2-isopropylaminoethanol hydrochloride, m.p. 212°, in a yield of 0·77 g. (Found: C, 63·2; H, 6·8; N, 4·5% $C_{17}H_{22}ClNO_3$ (mol. wt. 323·5) required: C, 63·1;H, 6·8; N, 4·3%).

1-(7-*Hydroxy*-2-*naphthyl*)-2-*isopropylaminoethanol.* A solution of 245 mg 1-(7-acetoxy-2-naphthyl)-2-isopropylaminoethanol hydrochloride in 10ml methanol and 5 ml 1 N sodium hydroxide was heated under reflux for 1½ hr and then the methanol was evaporated. The pH of the solution was adjusted to 11 and the mixture was extracted with ether. The ether extract was washed with water, dried ($MgSO_4$),

evaporated, and the residual gum was triturated with light petroleum (b.p. 40–60°). The solid thus obtained was crystallised from ethyl acetate to give 1-(7-hydroxy-2-naphthyl)-2-isopropylaminoethanol, m.p. 165°. (Found: C, 73·5; H, 7·8; N, 5·5%. $C_{15}H_{19}NO_2$ (mol. wt. 245) required: C, 73·5; H, 7·75; N, 5·7%).

U.V. spectrum (methanol): $\lambda_{max} = 258$ mμ (log $\epsilon = 3{\cdot}55$ INF); 266 (3·68); 276 (3·66); 287 (3·48 INF); 321 (3·31); 331 (3·36).

(methanol + (N/10) NaOH); $\lambda_{max} = 278$ mμ, 295, 286, 354.

DISCUSSSION

Pronethalol is rapidly metabolised and the metabolites are largely excreted in the urine following an oral or subcutaneous dose. The major urinary metabolites have been identified and these fall on two metabolic pathways (Fig. 8). The first identified metabolite in the side chain oxidation pathway is the primary amine, the formation of which has only been demonstrated *in vitro*. The primary amine is presumably acted upon by monoamine oxidase followed by an aldehyde dehydrogenase to give 2-naphthyl-glycollic acid, the first metabolite along this pathway to appear in the urine. Involvement of the enzyme monoamine oxidase is suggested by the inhibition of acid production when mebanazine, a monoamine oxidase inhibitor is added. Dehydrogenation of 2-naphthylglycollic acid would give 2-naphthylglyoxylic acid which by oxidative decarboxylation would give 2-naphthoic acid.

The 7-hydroxy-analogue of pronethalol is the first metabolite on the ring hydroxylation and conjugation pathway. This hydroxy-analogue is partly excreted as such but is mainly converted to a glucuronide at the ring hydroxyl group. The structure of the 7-hydroxy-analogue was proved by an unambiguous synthesis.

Paper electrophoresis studies give a quantitative measure of the utilisation of the two pathways. Because the amount of free pronethalol is low, the amphoteric and neutral fractions can be considered to constitute the products of the ring hydroxylation and conjugation pathway. The acidic fraction arises from the side chain oxidation pathway. Table 4 shows the percentage of drug metabolised by the two pathways in eight species. In most species ring hydroxylation and conjugation is the major pathway. Only in the guinea pig is side chain oxidation more prominent than ring hydroxylation and conjugation. In rabbit, cat, rat, and monkey, roughly twice as much pronethalol is metabolised by ring hydroxylation and conjugation as by side chain oxidation. These results probably reflect the relative activities in the livers of various species of the enzymes responsible for the first steps of the two pathways. These are *N*-dealkylation in one case and ring hydroxylation in the other. Various reports support this view. Thus, the guinea pig has been shown to demethylate *N*-methyl compounds more readily than the rat,[19] while the rat hydroxylates aromatic ring systems more readily than the guinea pig.[20, 21] The cat excretes the greatest proportion of unconjugated base (Fig. 1) which agrees with the observation[22] that this species is relatively poor at forming glucuronides. The results on three patients indicate that humans do not differ markedly from animals in their metabolism of pronethalol. The main metabolites are the same as in other species.

The nature of the metabolites of pronethalol, noradrenaline,[23] adrenaline,[24] isoprenaline,[25] and D.C.I.,[27] can be rationalised as follows. Noradrenaline and adrenaline are largely converted by *O*-methyl transferase to 3-*O*-methyl ethers which are then partly conjugated; a small amount of dealkylation and deamination occurs leading to

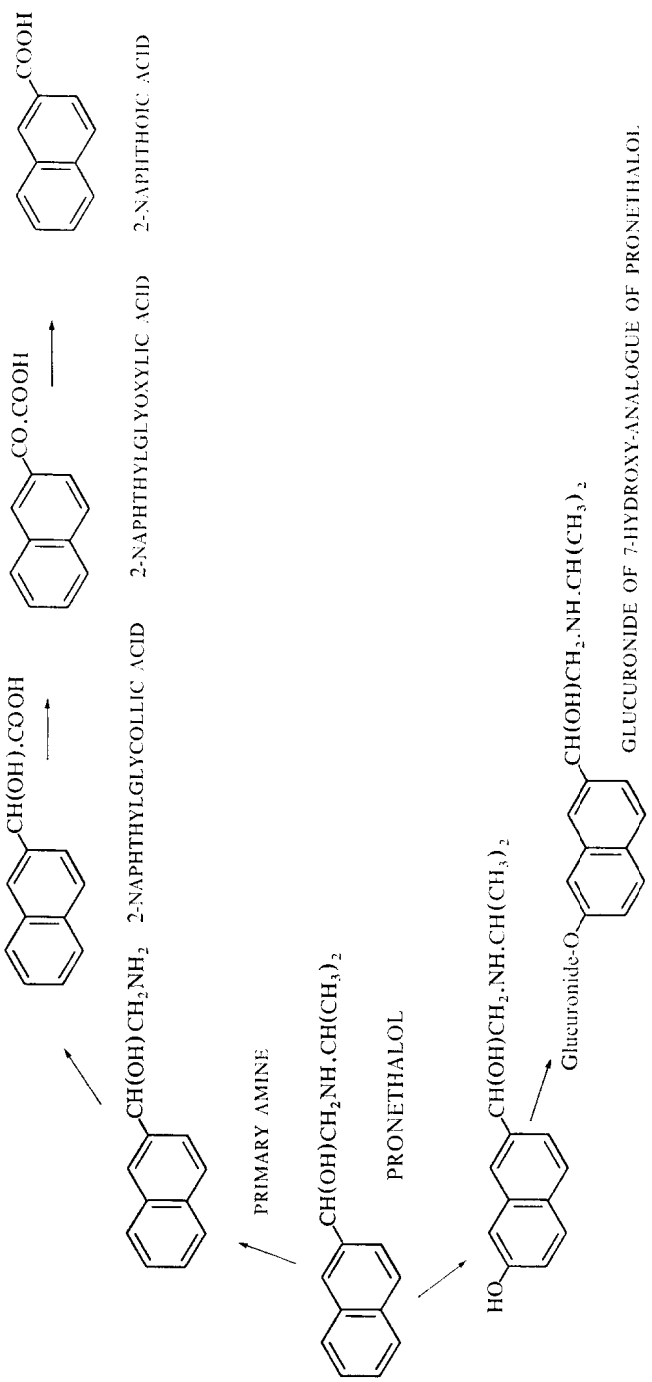


FIG. 8. Scheme for the metabolism of pronethalol

3,4-dihydroxymandelic acid and 4-hydroxy-3-methoxymandelic acid. Isoprenaline is converted to 3-*O*-methyl ether which is then partly conjugated but no acidic products are observed. This suggests that *N*-deisopropylation is relatively more difficult than *N*-demethylation[26,28] and therefore does not occur when there are other more readily available pathways. In the case of D.C.I. there is no phenolic hydroxyl group available for conjugation and so *N*-deisopropylation does occur, leading eventually by side chain oxidation to 3,4-dichloromandelic acid. No phenolic metabolites of D.C.I. have been found but conjugates of D.C.I. itself, possibly a sulphate ester and/or a glucuronide on the side chain hydroxyl group, have been demonstrated. Such conjugates were considered to arise because large amounts of D.C.I. were administered. They could however be formed as a last resort is side chain oxidation was too slow and ring hydroxylation could not be achieved. The naphthalene ring of pronethalol is probably more susceptible to hydroxylation than the dichlorophenyl ring of D.C.I. and so hydroxylation and conjugation offer an alternative pathway to side chain oxidation. Thus it seems reasonable to expect that conjugation of the side chain hydroxyl group of pronethalol would be a relatively minor route unless dosing was at such a level as to swamp the other two pathways.

It has been suggested that the ethyleneimine[10] related to pronethalol might be the proximate carcinogen responsible for the production of thymic tumours in mice, and some circumstantial evidence has been presented in support of this idea. We have not been able to show that the ethyleneimine is a metabolite of pronethalol nor have we demonstrated conclusively that a possible precursor of the ethyleneimine, such as an *O*-sulphate or *O*-glucuronide conjugate on the side chain hydroxyl group, is a metabolite. Such a conjugate could only be a very minor metabolite as less than 1 per cent of pronethalol is present in the urine in a conjugated form. Although we have been unable to determine the nature of the proximate carcinogen in the mouse, we have shown by comparison with other species, that it is unlikely to be a major metabolite.

Finally, it has been shown by our colleagues, Dr. J. W. Black and Dr. R. G. Shanks, that the 7-hydroxy-analogue of pronethalol is about five times more potent than pronethalol in its ability to antagonise isoprenaline-induced tachycardia in the cat. The method has been reported elsewhere.[11] The levels of the 7-hydroxy-analogue in serum are noticeably higher than those of pronethalol and no doubt some of the pharmacological action of pronethalol can be attributed to this metabolite. However, it is not solely responsible for the β-blocking action of pronethalol because β-blockade is immediately evident when pronethalol is injected into the cat and because β-blockade can be demonstrated *in vitro* using isolated guinea pig atrial strips.

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REFERENCES

1. J. S. Stephenson, U.K. Patent No. 909,357.
2. J. W. Black and J. S. Stephenson, *Lancet* **ii**, 311 (1962).
3. A. C. Dornhorst and B. F. Robinson, *Lancet* **ii**, 314 (1962).
4. T. R. E. Pilkington, R. D. Lowe, B. F. Robinson and E. Titterington, *Lancet* **ii**, 316 (1962).
5. G. A. O. Alleyne, C. J. Dickinson, A. C. Dornhorst, R. M. Fulton, K. G. Green, I. D. Hill, P. Hurst, D. R. Lawrence, T. Pilkington, B. N. C. Prichard, B. Robinson and M. L. Rosenheim, *Br. Med. J.* **ii**, 1226 (1963).
6. J. P. P. Stock and N. Dale, *Br. Med. J.* **ii**, 1230 (1963).
7. M. Johnstone, *Br. J. Anaesth.* **36**, 224 (1964).

8. S. J. ALCOCK and P. A. BOND, *Proc. Eur. Soc. Drug Tox.* IV, 30 (1964).
9. I. NIR, *Anal. Biochem.* **8**, 20 (1964).
10. R. HOWE, *Nature, Lond.* **207**, 594 (1965).
11. J. W. BLACK, W. A. M. DUNCAN and R. G. SHANKS, *Br. J. Pharmac. Chemother* **25**, 577, (1965).
12. J. AXELROD, *J. Pharmac. exp. Ther.* **110**, 315 (1954).
13. S. J. G. GILMOUR, *Br. J. Psychiat.* **111**, 899 (1965).
14. C. MITOMA, H. S. POSNER, H. C. REITZ and S. UDENFRIEND, *Archs Biochem. Biophys.* **61**, 431 (1956).
15. P. A. BOND and R. HOWE, U.K. 1,059,968.
16. G. C. JOSHI and N. M. SHAH, *J. Indian chem. Soc.* **29**, 255 (1952).
17. G. FODOR and O. KOVÁCS, *J. Am. chem. Soc.* **71**, 1045 (1949).
18. C. BUTLER and F. A. ROYLE, *J. chem. Soc.* **123**, 1649 (1923).
19. J. AXELROD, *J. Pharmac. exp. Ther.* **114**, 430 (1955).
20. J. AXELROD, *J. Pharmac. exp. Ther.* **110**, 315 (1954).
21. S. SZARA, *Life Sci.* **9**, 662 (1963).
22. D. ROBINSON and R. T. WILLIAMS, *Biochem. J.* **68**, 23p (1958).
23. L. G. WHITBY, J. AXELROD and H. WEIL-MALHERBE, *J. Pharmac. exp. Ther.* **132**, 193 (1961).
24. J. AXELROD, H. WEIL-MALHERBE, and R. TOMCHICK, *J. Pharmac. exp. Ther.* **127**, 251 (1959).
25. G. HERTTING, *Biochem. Pharmac.* **13**, 1119 (1964).
26. S. E. MAYER, *J. Pharmac. exp. Ther.* **135**, 204 (1962).
27. H. BLASCHKO, *Pharmac. Rev.* **4**, 415 (1952).
28. G. A. ALLES and E. V. HEEGAARD, *J. biol. Chem.* **147**, 487 (1943).