J. Pharm. Pharmac., 1966, **18**, *Suppl.*, 52S–57S Received May 2, 1966

The stimulation of strychnine metabolism in rats by some anticonvulsant compounds

J. F. HOWES AND W. H. HUNTER

The metabolism of a series of 4-acetyl-1-naphthyl ethers has been examined in rats and in fractions of rat liver homogenate. The administration of the compounds to rats increases the rate of strychnine metabolism when the compounds used are metabolised by the microsomal fraction of liver homogenate.

THE stimulation of oxidative metabolism of foreign compounds by prior administration of drugs is now a well-recognised phenomenon (Gillette, 1963) and may influence the results of accepted pharmacological screening techniques. We wish to report the increase in strychnine metabolism produced by prior administration to rats of some anticonvulsant compounds (Hunter, Quinton, Sherman, Worthing & Boscott, 1964). We have also studied the oxidation of the compounds themselves *in vivo*, and *in vitro* using homogenate of rat liver and fractions derived therefrom by centrifuging.

Experimental

MATERIALS AND METHODS

The compounds were all prepared from 4-acetyl-1-naphthol as described by Hunter & others (1964) and are listed in Table 1 (R = 4-acetyl-1-naphthyl throughout). They were administered intraperitoneally, as suspensions in 1 ml arachis oil, at 250 mg/kg to male Wistar rats weighing about 300 g. Male rats were used because they metabolise strychnine more rapidly than do females (Kato, Chiesara & Vassanelli, 1962). Forty eight hr after this single dose the animals were killed, the livers were removed and covered with ice-cold isotonic potassium chloride solution. The livers were homogenised with 2 volumes of isotonic potassium chloride solution in a slow speed Waring blender (LO setting for 15 sec). The homogenates were centrifuged for 30 min at 10,000 *g* to remove nuclei, mitochondria and cell debris leaving the supernatant fraction. This supernatant fraction, consisting of microsomes and soluble fraction, was used to determine the rates of strychnine metabolism. In addition, the supernatant was further separated into a microsome fraction and a soluble fraction, used separately for some experiments as described below. The supernatant, prepared as described, was centrifuged 1 hr at 140,000 *g* at 0–4° and the soluble fraction removed. This soluble fraction was adjusted to pH 9·4 with glycine–sodium hydroxide buffer (Sorensen's glycine II) and to this was added NAD (2 μ mole/5 ml of solution).

The microsome pellet was resuspended in the original volume of phosphate buffer (pH 7·4) to give the microsome fraction.

From the Department of Pharmacy, Chelsea College of Science and Technology (University of London), Manresa Road, S.W.3.

TABLE 1. THE RATE OF METABOLISM OF STRYCHNINE BY SUPERNATANT FRACTION OF RAT LIVER (R = 4-ACETYL-1-NAPHTHYL)

Compound administered to rats		μMoles strychnine metabolised/g liver/hr
None		0·23 (0·22–0·24)
R–O·CH_2·CO·Me	I	0·62 (0·58–0·64)
R–O·CH_2·CHOH·Me	II	0·45 (0·44–0·47)
R–O·CH_2·CHOH·CH_2·Me	III	0·45 (0·40–0·48)
R–O·CH_2·CHOH·CH_2·O·$CHMe_2$	IV	0·53 (0·50–0·55)
R–O·CH_2·CH_2·OH	V	0·25 (0·22–0·28)
R–O·CH_2·CH_2·O·Me	VI	0·57 (0·55–0·60)

Compounds administered at 250 mg/kg i.p. in arachis oil.
Control animals received only arachis oil.
Each result represents the average of at least six animals.
Figures in brackets are the range of results.

METABOLISM OF STRYCHNINE *in vitro*

The supernatant (2 ml) was mixed with a solution (0·5 ml) containing nicotinamide (50 μmole), Mg^{++} (75 μmole), K^{+} (100 μmole) and strychnine sulphate (432 μg; 1 μmole). To this was added a reduced nicotinamide adenine dinucleotide phosphate (NADPH) generating system consisting of glucose-6-phosphate (20 μmole), NADP (0·4 μmole) and glucose-6-phosphate dehydrogenase (present in liver supernatant fraction). The mixed solution was made up to 5·0 ml with 0·05M "Tris" buffer to a final pH of 8·2. All pH values were checked using a Pye "Dynacap" pH meter and the solutions shaken (1 hr) at 37°. Aliquots (2 ml) of the incubation mixture were treated with sodium chloride (2 g), N sodium hydroxide (1 ml) and extracted with 1·5% v/v solution of isoamyl alcohol (25 ml) in heptane for 45 min. The solutions were centrifuged, the organic layer (20 ml) re-extracted with 0·1N hydrochloric acid (4 ml) and the absorbance of this acid extract was determined at 254 mμ and 278 mμ. This procedure gave strychnine recoveries corresponding to 98–102%. Chromatography on thin-layer plates showed that strychnine metabolites were not extracted with the strychnine.

METABOLISM OF THE COMPOUNDS *in vitro*

For the experiments on the *in vitro* metabolism of the compounds, colloidal suspensions were prepared by suspending the solid materials (12 μmole) in phosphate buffer (pH 7·4; 1·0 ml) and subjecting them to ultrasonic disintegration with an "Electrosonic" 80 Kc/sec ultrasonic generator for 1 min. Samples of the compounds prepared in this way were examined by thin-layer chromatography on silica-gel to verify that the procedure did not affect the compounds chemically. The colloidal suspensions (1 ml) were added to the appropriate liver fractions (5 ml) prepared as described above. When the supernatant fraction or microsome suspensions were used, the flasks were shaken for 1 hr at 37°. For the soluble fraction the flasks were incubated for 1 hr at 25°, without shaking. At the end of the incubation period, protein was precipitated by adding saturated barium hydroxide solution (2 ml) followed by zinc sulphate solution (2 ml, 20% w/v) and the precipitate was removed by centrifuging (5 min at 5,000 *g*). The clear solutions were decanted and

extracted with peroxide-free ether (3 × 5 ml); the extracts were evaporated *in vacuo* at room temperature and the residues examined by thin layer chromatography (Table 2).

TABLE 2. COLOUR REACTIONS AND Rf VALUES OF COMPOUNDS AND THEIR METABOLITES ON THIN-LAYER CHROMATOGRAMS (R = 4-ACETYL-1-NAPHTHYL)

Compound		Rf values in solvents A, B and C			Colour reactions with reagents			
		A	B	C	D	E	F	G
R–O·CH$_2$·CO·Me	I	0·90	0·75	0·70	g	—	—	y
R–O·CH$_2$·CHOH·Me	II	0·81	0·64	0·62	,,	—	—	—
R–O·CH$_2$·CHOH·CH$_2$·Me	III	0·83	0·68	0·64	,,	—	—	—
R–O·CH$_2$·CO·CH$_2$·Me		0·93	0·69	0·73	,,	—	—	y
R–O·CH$_2$·CHOH·CH$_2$·O·CHMe$_2$	IV	0·78	0·71	0·65	,,	—	—	—
R–O·CH$_2$·CH$_2$·OH	V	0·72	0·53	0·50	,,	—	—	—
R–O·CH$_2$·CH$_2$·O·Me	VI	0·81	0·67	0·67	,,	—	—	—
R–OH		0·67	0·75	0·61	g–b	y	o	—
R–O·CH$_2$·CO$_2$H		0·28	0·10	0·10	g	y	—	—
R–O·CH$_2$·CHOH·CH$_2$OH		0·34	0·35	0·20	,,	—	—	—
VII (Metabolite of III)		0·35	0·30	0·22	,,	—	—	—

Silica gel (Merck G) plates were used 250μ × 22 cm × 22 cm.
Solvents A Chloroform - methanol 9:1 v/v.
B Benzene - ethyl acetate 2:3 v/v.
C Chloroform - acetone 5:4 v/v.
Fresh solvent was used for each plate and allowed to run 10–12 cm.
Reagents: D Ferric chloride - perchloric acid.
E Acridine 0·1% w/v in absolute ethanol.
F Diazotised *p*-nitroaniline.
G 2,4-Dinitrophenylhydrazine 0·1% w/v in ethanol.
g—green; b—blue; y—yellow; o—orange.

The formaldehyde produced during the oxidative removal of the methyl group from 2-(4-acetyl-1-naphthoxy)ethyl methyl ether VI was determined by the method of Nash (1953) as modified by Cochin & Axelrod (1959).

METABOLISM OF THE COMPOUNDS *in vivo*

The fate *in vivo* of the compounds was examined by administering them to male rats as described above; control animals received arachis oil only. Urine was collected for 48 hr after dosage, from both treated and control animals. Pooled urine samples from pairs of rats receiving the same compound were extracted with n-butanol to remove the metabolites and, if present, unchanged compounds. The butanol was removed *in vacuo* at room temperature and small samples of the extracts examined by chromatography on silica gel plates (250 μ, Merck, silica gel G) using benzene-ethyl acetate (2:3 v/v) as developing solvent. The extracts were then hydrolysed by heating (1 hr) with 2N hydrochloric acid at 100°. (The use of bacterial β-glucuronidase was less successful). The hydrolysed materials were extracted with n-butanol or ether, and the metabolites separated by thin layer chromatography on silica gel plates (250 μ; Merck, silica gel G.). They were identified by comparing their chromatographic behaviour with that of authentic standards and by their colour reactions with ferric chloride-perchloric acid solution (20% w/w hydrated ferric chloride in perchloric acid), with 2,4-dinitrophenylhydrazine (0·1% w/v in ethanol), and with diazotised *p*-nitroaniline (Bray, Thorpe & White, 1950). A solution of acridine (0·1% w/v in absolute ethanol) was used to locate acidic metabolites (LeHongre, Tanner & Rentschler, 1957).

The colour reactions and Rf values of the compounds and their metabolic products are shown in Table 2. Acidic metabolites were further characterised by chromatography on pH gradient plates (Shellard & Alam, personal communication).

4-Acetyl-1-naphthol, when present as a metabolite was identified also by the characteristic bathochromic shift in the ultraviolet spectrum in basic solution (Fig. 1).

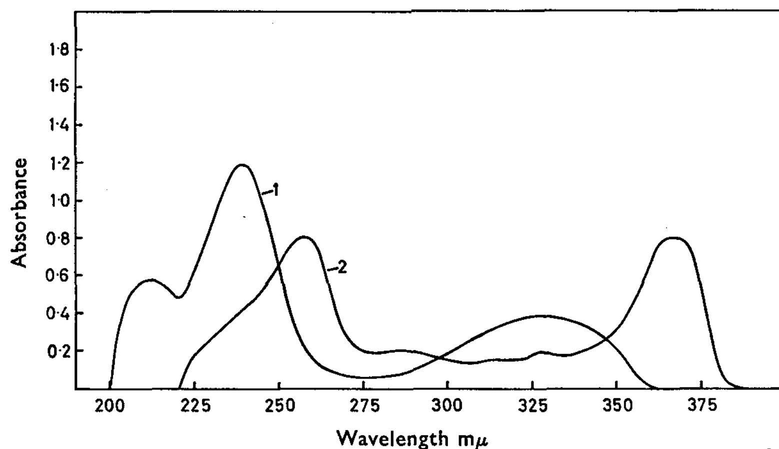

FIG. 1. Ultraviolet spectrum of 4-acetyl-1-naphthol. Curve 1. Solution of 5 μg/ml in 70% ethanol (Spectroscopic grade). Curve 2. The same solution with 1 drop of 1·0N NaOH added. On addition of acid, curve 1 is reproduced.

Results

The rates of strychnine metabolism in liver supernatant fraction from control rats and from treated rats are recorded in Table 1. With the exception of the primary alcohol (V) the compounds effectively stimulated

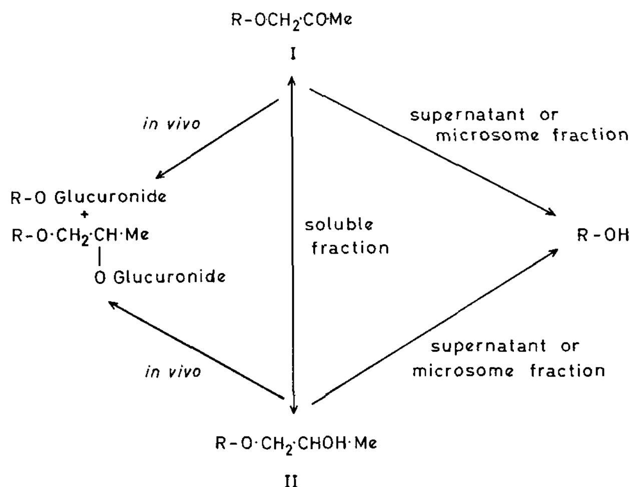

FIG. 2. Metabolic pathway for compounds I and II.

strychnine metabolism in rat liver. In Figs 2–4 are shown the products of the metabolism of the compounds used, *in vivo*, in liver supernatant fraction, in microsome suspensions and in the soluble fraction. In the series of compounds studied, the metabolic pathway *in vivo* could be demonstrated qualitatively at least, *in vitro*, by using a suitable fraction of liver homogenate. The *in vivo* metabolism of the ether (VI) led to

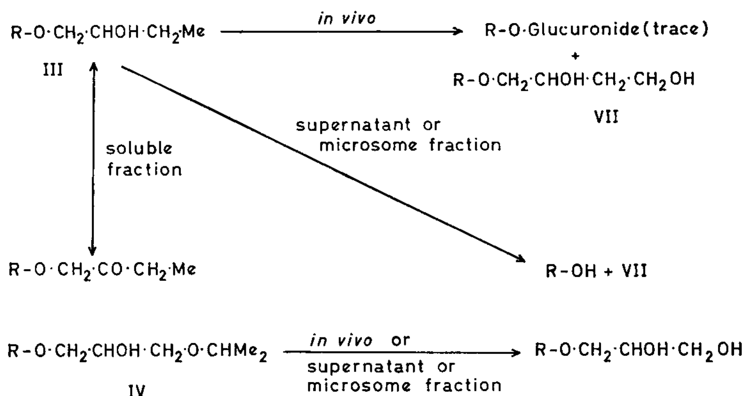

FIG. 3. Metabolic pathway for compounds III and IV.

excretion of the alcohol (V) as the glucuronide (VIII) and, when incubated with liver supernatant fraction or with a microsome suspension, the ether formed 0·33 μmoles of formaldehyde (0·30–0·38 in six experiments) per μmole of the compound. The ether was recovered unchanged after incubation with the soluble fraction of liver homogenate and no formaldehyde was formed.

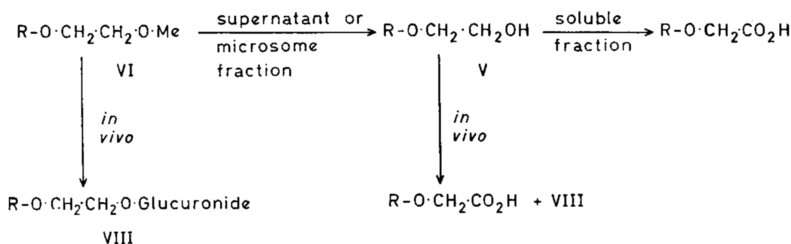

FIG. 4. Metabolic pathway for compounds V and VI.

Discussion

The effect of some anticonvulsant drugs upon the metabolism and toxicity of strychnine has been reported by Kato & others (1962). Oxidative metabolism of strychnine has been shown to occur in the microsomal fraction of liver from various species (Adamson & Fouts, 1959) and 2-hydroxystrychnine is a metabolite in rabbit liver (Tsukamoto, Oguri, Watabe & Yoshimura, 1964). All the compounds that stimulated strychnine metabolism were themselves oxidised by the microsome

fraction. The *in vivo* oxidation of the compounds led to metabolites identical with those produced *in vitro* by microsomes thus demonstrating the essential similarity of the two types of metabolism. Microsomal oxidation of these compounds in the rat could therefore be the process leading to increased rate of strychnine detoxication.

The primary alcohol (V) was the only compound whose *in vivo* metabolic pathway could be reproduced *in vitro* by the soluble fraction of liver homogenate. This alcohol was the only compound that failed to stimulate metabolism of strychnine. The conversion of the alcohol to the methyl ether (VI) produced a potent stimulator of strychnine metabolism from a compound that was initially inactive in this respect. Unlike the alcohol, the ether was oxidised only in the microsome fraction.

The results suggest that the long duration of anti-strychnine activity in this series (Hunter & others, 1964) could be explained by the increased rate of strychnine metabolism. In this series of compounds, only those members that are metabolised in the microsome fraction stimulated strychnine metabolism.

Acknowledgements. We thank Professor S. E. Dicker for the use of facilities for animal work, Dr. P. Rosenmund for a specimen of 2-hydroxy-strychnine and the Medical Research Council for a grant to J.F.H.

References

Adamson, R. H. & Fouts, J. R. (1959). *J. Pharmac. exp. Ther.*, **127**, 87–93.
Bray, H. G., Thorpe, W. V., & White, K. (1950). *Biochem. J.*, **46**, 271–276.
Cochin, J., & Axelrod, J. (1959). *J. Pharmac. exp. Ther.*, **125**, 105–108.
Gillette, J. R. (1963). *Fortschr. ArzneimittForsch.*, **6**, 11–74.
Hunter, W. H., Quinton, M., Sherman, P. H., Worthing, C. R., & Boscott, R. J. (1964). *J. mednl Chem.*, **7**, 167–174.
Kato, R., Chiesara, E., & Vassanelli, P. (1962). *Biochem. Pharmac.*, **11**, 913–922.
LeHongre, G., Tanner, H., & Rentschler, H. (1957). *Mitt. Geb. Lebensmittelunters. u. Hyg.*, **48**, 40–43.
Nash, T. (1953). *Biochem. J.*, **55**, 416–421.
Tsukamoto, H., Oguri, K., Watabe, T., & Yoshimura, H. (1964), *J. Biochem.*, **55**, 394–400.