1-[1-14C]Naphthol and 2-[8-14C]naphthol were also examined in the cat. In the case of 1-naphthol, 89% of the dose (25 mg/kg injected intraperitoneally) was excreted as the sulphate and 1% as glucuronide in 24h and there was no evidence of a phosphate conjugate. In the case of 2-naphthol similarly administered, 57% of the dose was excreted in 24h as the sulphate of 2-naphthol, 15% as the sulphate of a dihydroxynaphthalene (probably 2,7-dihydroxynaphthalene) and 1% as 2-naphthylglucuronide, but there was no evidence of a phosphate conjugate.

The formation in vivo of the phosphates of foreign organic compounds is a reaction which has been rarely observed in the past. Di-(2-amino-1-naphthyl) hydrogen phosphate has been shown to be a metabolite of 2-naphthylamine in urine in dogs (Troll et al., 1959; Boyland et al., 1961) and in man (Troll et al., 1963). Troll & Belman (1967; cited by Irving, 1970) have also reported di-(2-hydroxylamino-1-naphthyl) hydrogen phosphate as a metabolite of 2-naphthylamine. 1-Naphthyl dihydrogen phosphate has been found to be a metabolite of 1-naphthol in houseflies, blowflies and New Zealand grass grubs (Binning et al., 1967). Monoethyl phosphate has been isolated from the liver of rats given large doses of ethanol intraperitoneally (Tomaszewski & Buckowicz, 1972). Heenan & Smith (1967) have found that p-nitrophenol and 1-naphthol form glucoside 6-phosphates in houseflies and blowflies, but in these conjugates the phosphate group is not attached directly to the phenol.

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Studies on the Conjugation of 4'-Hydroxyamphetamine in vitro

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One of the metabolites of amphetamine in a number of species is 4'-hydroxyamphetamine (Paredrine) (Dring et al., 1970) and this compound appears to be the immediate precursor of 4'-hydroxynorephedrine, the supposed false neurotransmitter produced during the metabolism of amphetamine. A study of the fate of 4'-hydroxyamphetamine (Capel, 1973; Sever et al., 1973) in a number of species has shown that it is metabolized mainly by conjugation of the phenolic hydroxyl group, but a species difference in the conjugation was found, the rat, guinea pig and pig conjugating it with glucuronic acid whereas man and four species of monkeys conjugated it with sulphate. The conjugation of 4'-hydroxyamphetamine has now been examined in vitro with tissue preparations.

By using 4'-hydroxy[14C]amphetamine free and conjugated 4'-hydroxyamphetamine could be readily estimated by solvent extraction. Incubation mixtures (3 ml) were brought to pH9.5 with 0.5M-borate buffer, pH9.5 (3 ml), and the free 4'-hydroxy-
amphetamine was extracted with butan-1-ol saturated with water (2×25ml). Conjugated 4'-hydroxyamphetamine was then assayed by scintillation counting for radioactivity of the aqueous phase which contained less than 1% of free 4'-hydroxyamphetamine.

For measuring the glucuronic acid conjugation of 4'-hydroxyamphetamine, the incubation mixture (3ml) contained the microsomal fraction from 100mg of liver prepared by the method of Miller & Dingell (1971), Tris buffer, pH8.4 (1.9ml), UDP-glucuronic acid (10μmol) and 4'-hydroxy[14C]amphetamine (661nmol; 2×10^4 d.p.m.). For measuring sulphate conjugation the incubation mixture (3ml) contained the 10000g supernatant from 100mg of liver, MgCl₂ (9μmol), Tris buffer, pH7.4 (1.8ml), ATP (20μmol), Na₂SO₄ (100μmol) and 4'-hydroxy[14C]amphetamine (661nmol; 2×10^4 d.p.m.). The reaction mixtures were incubated in air at 37°C for 30min.

Incubation of 4'-hydroxy[14C]amphetamine with UDP-glucuronic acid as above resulted in the formation of a metabolite (164nmol) which was not extracted by butan-1-ol. When the preparation was further incubated with β-glucuronidase (5000 units of Ketodase; Warner–Chilcott Laboratories, Morris Plains, N.J., U.S.A.) all the 14C could be extracted by butanol, but its release by β-glucuronidase could be prevented by the presence of saccharo-1,4-lactone (1 mM) in the incubation mixture, indicating that the conjugate was a glucuronide.

The formation of 4'-hydroxyamphetamine glucuronide was proportional to time up to 30min incubation and zero-order kinetics were approached when 661nmol of 4'-hydroxyamphetamine were used in the incubation. For the maximum rate of conjugation of 4'-hydroxyamphetamine, more than 20μmol of UDP-glucuronic acid were needed. For the conjugation of tetrahydrocortisone, Miller & Dingell (1971) had found that the maximum rate could be achieved with only 3μmol of UDP-glucuronic acid. In these experiments therefore sub-optimal conditions were used and under these conditions glucuronide formation at pH8.4 was 1.5 times higher than at pH7.4. Glucuronide formation with liver microsomal preparations from female rats was considerably greater than with those from males and when Triton X-100 (0.05%, v/v) was added to the incubation there was a fourfold enhancement of glucuronide synthesis.

The conjugation of 4'-hydroxyamphetamine with glucuronic acid occurred in microsomal preparations from liver only, since similar preparations from lung, brain, heart, kidney, intestine and spleen of female rats were inactive in this respect. The soluble fraction from these tissues including the liver did not conjugate 4'-hydroxyamphetamine with sulphate, although the same soluble fraction from liver actively conjugated 1-naphthol with sulphate.

Pretreatment of female rats with 4'-hydroxyamphetamine (50mg/kg, intraperitoneally, daily for 7 days) did not alter the extent of glucuronic acid conjugation of 4'-hydroxyamphetamine in vitro. With groups of five rats, the rate of glucuronide synthesis in controls was 133.5±26.5nmol/30min per 100mg of liver, whereas in the pretreated animals the value was 137.7±14.8. Under similar conditions the synthesis of sulphate conjugate was less than 1nmol in the preparations from control and pretreated animals. Livers from rats, which had been made tolerant to the anorectic action of amphetamine (by 5mg of (+)-amphetamine/kg, intraperitoneally, twice daily for 15 days), showed no alteration from normal rats in their ability to conjugate 4'-hydroxyamphetamine with glucuronic acid in vitro (treated 107.8±23.2nmol/30min per 100mg liver; control 117.6±32.0), and still showed no ability to form the sulphate of 4'-hydroxyamphetamine.

These results show that the conjugation of 4'-hydroxyamphetamine with glucuronic acid but not with sulphate in vitro in rats and certain other rodents reflects what happens during the conjugation of 4'-hydroxyamphetamine in the intact animals (Sever et al., 1973).

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Acrolein as a Possible Metabolite of Cyclophosphamide in Man

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It is generally accepted that cyclophosphamide, i.e. NN-di-2-chloroethyl-N'O-trimethyle
enephosphordiamide, produces therapeutic effects by undergoing metabolic activation.
Brock & Hohorst (1967) showed that activation of cyclophosphamide can be brought
about by rat liver microsomal preparations, in conjunction with NADPH and oxygen,
and they suggested that fission of cyclophosphamide in vivo gives rise to a substituted
3-aminopropionaldehyde. Consideration of this work and the observation of Alarcon
(1970) that acrolein is formed from aminoaldehydes derived from spermine led Alarcon
& Meienhofer (1971) to investigate whether acrolein is a metabolite of cyclophosph-
amide. They showed that formation of acrolein from cyclophosphamide occurs with
purely chemical oxidation systems and also when the drug is incubated with rat liver
microsomal preparations in the presence of cofactors. At this time we had shown that
administration of acrolein to rats leads to the excretion of 3-hydroxypropylmercapturic
acid, i.e. N-acetyl-S-(3-hydroxypropyl)-L-cysteine, in the urine (Kaye & Young, 1972;
Kaye, 1973), and it appeared to us that if cyclophosphamide gives rise to the formation
of acrolein in vivo then some of this latter compound may be converted into 3-hydroxy-
propylmercapturic acid.

Samples of urine from control rats and urine excreted by rats in the first 24h after
the subcutaneous injection of 1ml of 0.9% NaCl solution containing 20mg of cyclo-
phosphamide (Endoxana; WB Pharmaceuticals Ltd., Wembley, Middx., U.K.)
were subjected to descending paper chromatography overnight on Whatman 3MM
paper in four different solvent mixtures: A, butan-1-ol-water-acetic acid (12:5:3, by
vol.); B, propan-1-ol-water-NH₃ (sp.gr. 0.88) (80:20:1, by vol.); C, butan-1-ol-
pyridine-3m-NH₃ (4:3:3, by vol.); D, 2-methylpropan-2-ol-butan-2-one-water-
diethylamine (10:10:5:1, by vol.). 3-Hydroxypropylmercapturic acid, prepared as
described by Kaye et al. (1972), was run as a reference compound. Mercapturic acids
were detected on the chromatograms by using the platinum reagent of Toennies
& Kolb (1951) as modified by Barnsley et al. (1964).

In another experiment the urine excreted by 20 rats in the 24h after they had been in-
jected with cyclophosphamide was subjected to solvent extraction followed by ion-
exchange column chromatography and then, after methylation with diazomethane, it
was examined by g.l.c. (Kaye, 1973).

In a further experiment the tissues of two rats were labelled with 35S by feeding a diet
containing 5% of dried 35S-labelled yeast (Knight & Young, 1958). After 3 days the radio-
active diet was withdrawn, and one rat was injected with cyclophosphamide and the
other was used as a control. The urine of each animal was collected for 24h and subjected
to paper chromatography in solvent mixtures A, B, C and D. The dried chromatograms
were scanned for 35S-labelled compounds by means of a Packard model 7200 radio-
chromatogram scanner and then treated with the platinum reagent.

Two patients, Mrs. S. and Mr. W., who were receiving cyclophosphamide orally
(2 x 50mg and 3 x 50mg per day respectively), each provided a 24h collection of urine.
The urine was subjected to solvent extraction and ion-exchange column chromatography

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