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THE METABOLISM OF LYSERGIC ACID DI[^{14}C]ETHYLAMIDE ([^{14}C]LSD) IN THE ISOLATED PERFUSED RAT LIVER

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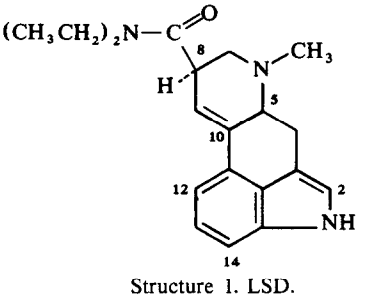
Abstract—Isolated female rat livers were perfused with a medium containing [^{14}C]LSD (1 mg and 0.13 μCi/g liver) and (+)-tartaric acid (15 mg). After 4.5 hr, the bile collected contained some 44% of the added radioactivity, the perfusate, 20% and the liver itself, 20%.

The radioactive compounds in the bile were identified as 14-hydroxy-LSD glucuronide (21% of the added ^{14}C), 13-hydroxy-LSD glucuronide (8%), 2-oxo-LSD (7%) and unchanged LSD (1%). Those in the pooled perfusate and homogenised liver were unchanged LSD (18%), 2-oxo-LSD (5%), a naphthostyril derivative of LSD (4%; probably derived from 2-oxo-LSD), nor-LSD (4%), hydroxy-LSD glucuronides (3%) and de-ethyl-LSD (2%).

Although (+)-lysergic acid diethylamide (LSD, Structure 1) has been of interest to both forensic science [1] and pharmacology for more than two decades, little is known about its metabolism. Previous workers have shown that, in mice and rats, LSD is almost entirely transformed into metabolites which are excreted mainly in the bile [2–4].

Axelrod *et al.* [5] reported that less than one per cent of the drug was excreted unchanged in urine or faeces by the rhesus monkey. Boyd [3] found four metabolites in rat bile, two of which were glucuronides. Further examination of these glucuronides led Slaytor and Wright [4] to suggest that they might be conjugates of 12-hydroxy-LSD and 12-hydroxy-iso-LSD.



Structure 1. LSD.

Axelrod *et al.* [5, 6] identified 2-oxo-LSD as an *in vitro* metabolite of LSD using guinea pig liver microsomes, and Szara [7] using rat liver microsomes found a metabolite which appeared to be 13-hydroxy-LSD. Recently Niwaguchi *et al.* [8] reported lysergic acid monoethylamide (de-ethyl-LSD) and nor-LSD (6-demethyl-LSD) as *in vitro* metabolites in rat, guinea pig and rabbit liver microsomes; however, these metabolites have not yet been shown to occur *in vivo*.

Since much of the metabolism of LSD is still uncertain we have re-investigated the fate of this hallucinogenic drug *in vivo* and by liver perfusion. The present paper describes the work with isolated perfused rat livers which were used in order to overcome the problem of toxicity, in particular to the central nervous system [9]. By using this technique, larger doses of the drug could be used and metabolized by the liver from a single animal.

Some of the results described in this and the following paper [10] have been briefly reported [11, 12].

MATERIALS AND METHODS

Compounds

(+)-Lysergic acid, m.p. 235–237°, $[\alpha]_D^{20}$ = approx. +40.5° (*c* 0.5 in pyridine), 5-hydroxyindole, m.p. 105–107°, 6-benzyloxyindole, m.p. 114–115°, and 7-benzyloxyindole, m.p. 69–71°, were purchased from the Sigma Chemical Company Ltd., Kingston-upon-Thames, Surrey, U.K.

(+)-*Lysergic acid diethylamide* (LSD) was prepared by amidation of lysergic acid [13]. A solution of the crude LSD, resulting from the amidation of 2 g of (+)-lysergic acid, in methanol (100 ml) and water (200 ml) was cooled to 0° and 1M-NH_4OH (100 ml) was added. After 3 hr at 0° the brown solid of LSD which had been deposited was filtered off, washed with water and dried overnight *in vacuo* over P_2O_5. The LSD was re-crystallised from diisopropyl ether to give LSD (1.31 g, 58%) of m.p. 81–83° and $[\alpha]_{589}^{20} = +16°$ (*c* 0.5 in pyridine, Perkin Elmer Model 141 Polarimeter) (Hofmann [14] gives +17°). The mass spectrum (Fig. 1a) and i.r. spectrum were identical with published spectra [15].

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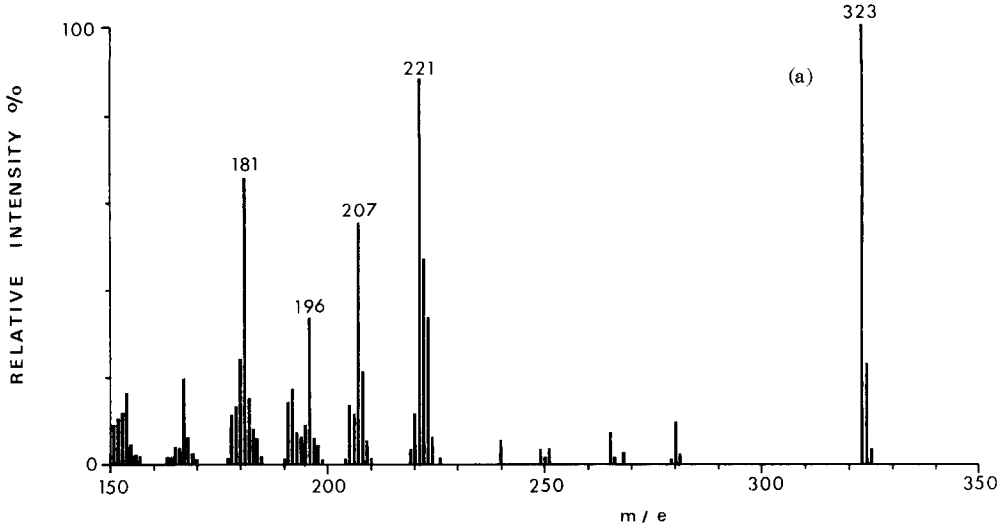

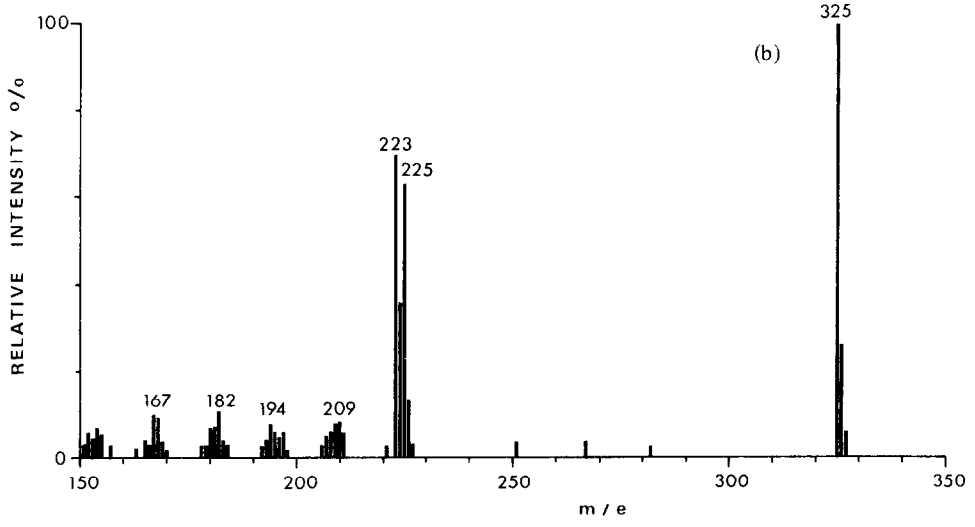

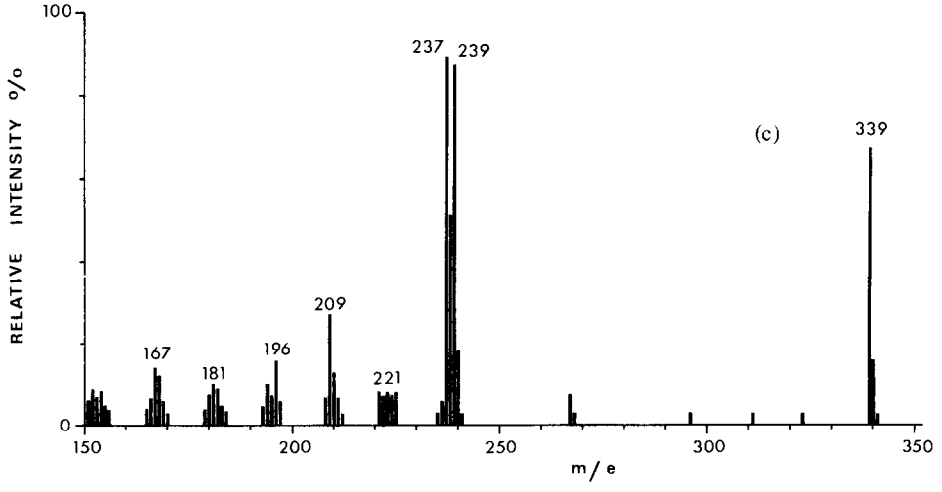

Fig. 1a–c.
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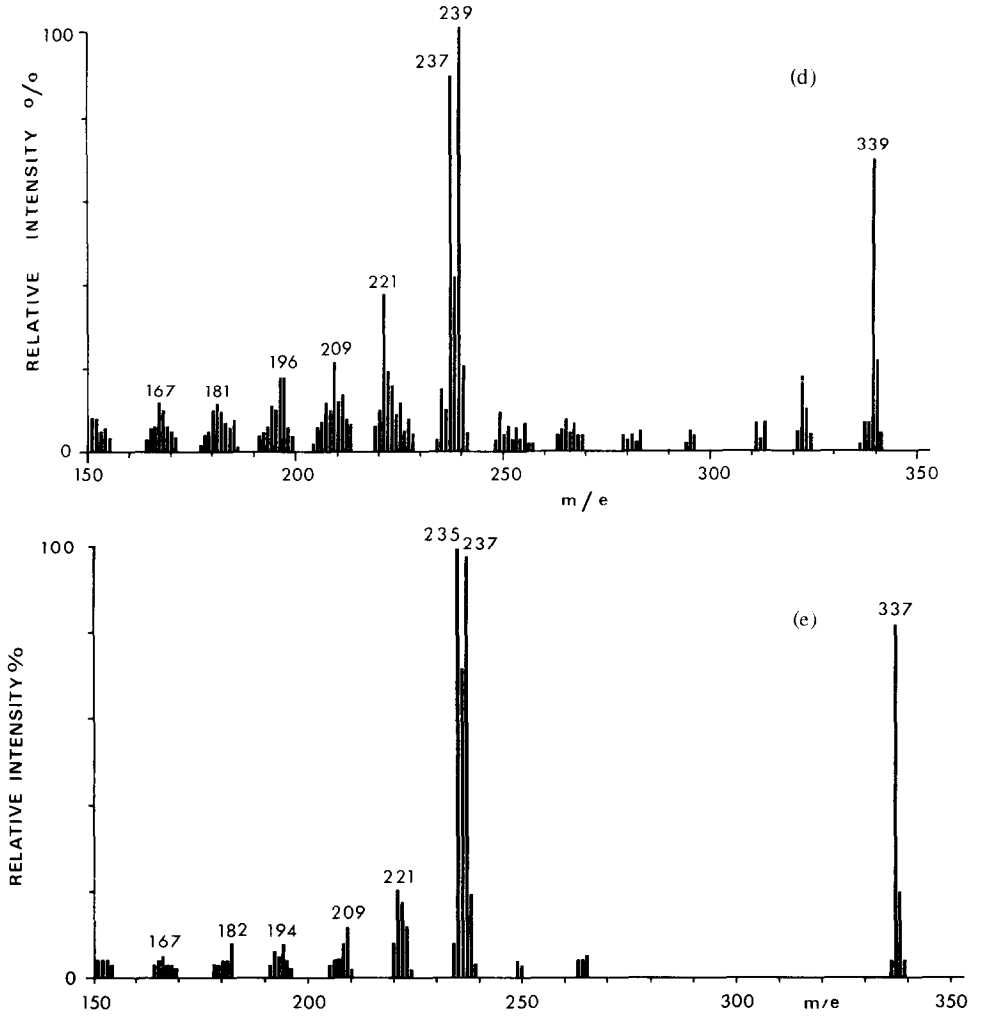

Fig. 1. Mass spectra of reference compounds and metabolites. Only ions with intensities ≥ 3% of the base peak are given. (a) LSD; (b) 2,3-dihydro-LSD; (c) synthetic 2-oxo-LSD; (d) metabolite R6 from bile of rats; (e) "aromatised" 2-oxo-LSD.

T.l.c. in all the solvent systems (see Chromatography) gave a single spot of the same R_f as an authentic sample (Sandoz, A.G., Basle, Switzerland).

(+)-Lysergic acid di[^{14}C]ethylamide ([^{14}C]*LSD*) was synthesised in this laboratory by Barnes [16].

(+)-Lysergic acid monoethylamide (de-ethyl-LSD) was also synthesised by the general method of Johnson *et al.* [13]. The amides in the mixture resulting from the reaction of 1 g of (+)-lysergic acid, namely (+)-lysergic acid monoethylamide and (+)-isolysergic acid monoethylamide were separated by t.l.c. in solvent system D (Table 1), eluted from the silica with methanol, evaporated to dryness *in vacuo* at 35–40° and redissolved in a minimum of diisopropyl ether (5 ml). Oxalic acid in diisopropyl ether was added to the solution to precipitate the respective oxalate salts. Both compounds chromatographed as single spots fluorescing blue under u.v. light (254 nm; Hanovia Chromatolite lamp, Slough, Bucks., U.K.) in all t.l.c. systems. The mass spectra of the two isomers were the same and were identical with the published mass spectrum [8].

6-Demethyl-(+)-lysergic acid diethylamide (nor-LSD). 6-Cyano-6-demethyl-LSD was prepared as described by Nakahara and Niwaguchi [17] and purified on t.l.c. in solvent system F (Table 1). The compound eluted with methanol from the silica gel had a mass spectrum identical with that published for 6-cyano-6-demethyl-LSD [18].

The 6-cyano compound was reduced to the nor-LSD as suggested for lysergic acid methyl ester by Fehr *et al.* [19]. The cyano compound after drying *in vacuo* at 35–40° was taken up in acetic acid (5 ml) and water (1 ml) and zinc dust (1 g, A.R. grade, Fisons Scientific Apparatus, Loughborough, Leics., U.K.) added. The mixture was boiled under reflux for 4 hr, cooled, filtered, diluted with water (5 ml) and adjusted to pH 9 with aq.NH_3(sp. gr. 0.88). The solution was saturated with NaCl and extracted with diethyl ether (4 × 10 ml) and the ether extract evaporated to dryness *in vacuo* at 35–40° to give nor-LSD which gave a single spot with a blue fluorescence under u.v. light, when chromatographed in all solvents in Table 1 and a mass spectrum identical with that published [18].

12-Hydroxy-(+)-lysergic acid diethylamide (12-hydroxy-LSD) was prepared by the method used by Stadler *et al.* [20] for the synthesis of 12-hydroxy-(+)-

Table 1. R_f of lysergic acid derivatives

Compound	Paper chromatography			Thin-layer chromatography			
	Solvent			Solvent			
	A	B	C	C	D	E	F
Lysergic acid	0.61	—	—	—	0.00	—	0.00
LSD	0.83	0.85	0.92	0.77	0.53	0.83	0.47
De-ethyl-LSD	0.72	0.72	0.90	0.76	0.37	0.75	0.29
De-ethyl-iso-LSD	0.83	0.85	0.92	—	0.58	0.83	0.61
Lumi-LSD	0.74	0.73	0.91	—	0.23	0.68	0.41
2-Oxo-LSD	0.75	0.72	0.85	0.76	0.50	0.83	0.26
Naphthostyril cpd.	0.91	0.95	0.91	0.76	0.72	0.91	0.58
2,3-Dihydro-LSD	—	—	—	0.62	0.40	—	0.17
12-Hydroxy-LSD	0.73	0.72	0.90	0.76	0.40	0.81	0.36
Nor-LSD	0.84	0.87	0.90	0.77	0.21	0.65	0.17

Whatman No. 1 paper was used for systems A and B and Whatman 3MM paper for system C. For t.l.c. aluminium sheets pre-coated with silica gel 60 F_{254} (0.2 mm; E. Merck A.G., Darmstadt, W. Germany) were used. Solvent systems: A, butan-1-ol–acetic acid–water (4:1:2, by vol.); B, butan-1-ol–formic acid–water (4:1:2, by vol.); C, propan-1-ol–aq.NH_3(sp. gr. 0.88)(7:3 v/v); D, $CHCl_3$–methanol (4:1 v/v); E, $CHCl_3$–methanol–water (5:5:1, by vol.); F, acetone–aq.NH_3 (sp. gr. 0.88)(100:1 v/v). The compounds were detected by their fluorescence under u.v. light and colour reaction with Van Urk reagent, 1% 4-dimethylaminobenzaldehye in 12M-HCl–ethanol (1:1 v/v)(Merck [41]).

lysergic acid propanolamide, with the exception that the zinc dust was added to the acidic solution. Reduction of LSD (0.8 g) gave a pale yellow powder (0.5 g) which was non-fluorescent under u.v. light and on t.l.c. gave two spots which both showed a slowly developing yellow colour with Van Urk reagent (see Table 1). The major spot (ca. 80%) gave a mass spectrum (Fig. 1b) with a molecular ion at *m/e* 325 which is consistent with the compound being 2,3-dihydro-LSD. Purification of the compound was found to be impractical without reducing the yield substantially, hence the impure 2,3-dihydro-LSD was used directly for the oxidation reaction.

The oxidation gave better yields on the small scale, therefore the preparation was repeated five times. 2,3-Dihydro-LSD (40 mg) was dissolved in acetone (2 ml) and potassium nitrosodisulphonate (300 mg; Rozantzev, [21]) was added, followed by water (2 ml) and the suspension shaken for 1 min and then shaken for a further minute after the addition of aq.1M-NH_3 (2 ml). All the solutions were maintained at 0° during the oxidation. Finally the solution was saturated with NaCl, extracted with diethyl ether (4 × 4 ml) which was then bulked, dried (Na_2SO_4), evaporated to dryness and purified by preparative t.l.c. in system F followed by system D. The methanol eluate of the silica gel was evaporated to dryness *in vacuo* at 35–40° to give 12-hydroxy-LSD (8.8 mg from 200 mg of the 2,3-dihydro-LSD). The compound gave a single spot on t.l.c., a positive phenolic test with diazotised 4-nitroaniline [22] and a mass spectrum consistent with a hydroxy-LSD (Fig. 2b) with a molecular ion at *m/e* 339. The proton magnetic resonance spectrum (in CD_3OD) showed in the aromatic region a quadruplet (6.61, 6.70, 6.95, 7.04 p.p.m.) which is typical of two ortho protons [23]. The coupling constant J_{ortho} was 8.55 Hz, supporting the supposition that the substitution was in the 12-position since the coupling constants for indole are $J_{5,6}$, 7.0 Hz and $J_{6,7}$, 8.0 Hz, the latter being analogous to the situation with 12-hydroxy-LSD [24].

2-Oxo-2,3-dihydro-(+)-lysergic acid diethylamide (2-oxo-LSD). The general method of Hinman and Bauman [25] for converting 3-substituted indoles to the corresponding oxindoles was used. LSD (55 mg) was dissolved in 95% *t*-butanol (10 ml) and *N*-bromosuccinimide (32 mg) was added to the stirred solution. The colour gradually turned yellow–orange over a period of 30 min and after a further 2.5 hr the volume was reduced to 5 ml by evaporation with a stream of N_2. The solid which deposited was filtered, washed with a little *t*-butanol and dried *in vacuo* over P_2O_5 to give pale orange crystals of 2-oxo-LSD (18 mg). The compound gave a single spot on t.l.c., had a weak blue fluorescence under u.v. light, and gave a slowly developing yellow colour with the Van Urk reagent. The mass spectrum (Fig. 1c) corresponded to the addition of one oxygen atom to the LSD molecule, having a parent ion of *m/e* 339, that is 16 mass units greater than the parent ion of LSD. The i.r. spectrum gave an additional peak over LSD corresponding to the new carbonyl group (5.84 μm) and was similar to the published spectrum [26]. The 2-oxo-LSD in methanol changed spontaneously in about 24–48 hr at room temperature to the naphthostyril compound described by Troxler and Hofmann [26]. This compound had a yellow fluorescence under u.v. light, gave a pale orange colour with the Van Urk reagent and its mass spectrum (Fig. 1e) showed a molecular ion at *m/e* 337, two mass units less than the molecular ion of 2-oxo-LSD.

10-Hydroxy-9, 10-dihydro-(+)-lysergic acid diethylamide (lumi-LSD). LSD (100 mg) in 10% (v/v) acetic acid was converted to lumi-LSD by the method of Blake *et al.* [27]. The crude product was subjected to preparative t.l.c. in solvent system D and the band of R_f 0.24 was eluted with methanol which was removed *in vacuo* at 35–40° to give lumi-LSD (30 mg; 28%).

The compound gave a single spot in systems E and F, was non-fluorescent, gave a final dark-blue colour with Van Urk reagent and a mass spectrum (Fig. 2a) with a molecular ion at *m/e* 341 which is consistent with the insertion of a molecule of water into the LSD nucleus.

Liver perfusion study

The perfusion fluid employed was that of Hems *et al.* [28] and 50 ml of it was used in each experiment. The perfusion apparatus was similar to that described by these authors except that oxygenation was carried out in a cylindrical reservoir (12 cm long × 6 cm wide × 6 cm deep) through which the medium flowed. The surface area of the medium was greatly increased by rotating (48 rev/min) sixteen equally spaced polytetrafluorethylene discs (5.5 cm dia.) partly immersed in the medium. The reservoir was covered so that the O_2/CO_2 gas mixture (95:5) could be passed over the surface of the medium.

Livers (9.0 ± 1.0 g) from female Wistar albino rats (200–220 g) were prepared surgically for perfusion according to the method of Hems *et al.* [28] with the exception that the liver was totally removed from the animal.

The [^{14}C]-LSD (1 mg LSD/g of liver) and (+)-tartaric acid (15 mg) were dissolved in 5 ml of Krebs and Henseleit [29] medium. The liver was allowed to reach a steady state (15 min) before the LSD solution was added to the perfusate in the reservoir oxygenator. Due to the vasoconstrictor action of LSD (Gant and Dyer, [30]), addition of the drug immediately reduced the flow of the perfusate through the liver from the usual 10–15 ml/min to 3–4 ml/min, but this returned to normal after 30–45 min. The flow rate did not alter in control experiments where LSD was excluded. Bile was collected at hourly intervals for 4.5 hr.

Separation of metabolites

Bile. This (2–5 ml) was passed at the rate of 1 ml/min through an XAD-2 polystyrene bead column (15 × 1 cm) which had been prepared according to Mulé *et al.* [31]. The column was washed with water (150 ml; 5 ml/min) to remove unabsorbed radioactivity (4–5% of total), allowed to run dry and then excess water removed by blowing N_2 through the column for 5 min. The adsorbed radioactivity was eluted with 200 ml of methanol which removed 85–90 per cent of the radioactivity originally present in the bile. The methanol was evaporated under reduced pressure at 35–40° and the solid taken up in the minimum of methanol and subjected to preparative t.l.c.

Perfusate and liver homogenate. The liver was homogenised with water (50 ml) in an Ultra-Turrax Homogeniser (Janke and Kunkel, G.G., Staufen i. Breisgau, W. Germany) and pooled with the respective perfusate. The mixture (approx. 100 ml) was divided into two halves. One half was adjusted to pH 10 with aq.NH_3 (sp. gr. 0.88) and extracted with $CHCl_3$ (2 × 100 ml). The other half was freeze-dried and then extracted exhaustively with methanol until about 95 per cent of the ^{14}C was removed. The extract was evaporated to dryness *in vacuo* at 35–40° and the residue was redissolved in a minimum of methanol for metabolite isolation and purification by t.l.c.

Chromatography. The R_f values and colour reactions of the compounds following paper and t.l.c. are given in Tables 1 and 2.

For the separation and purification of metabolites or

Table 2. R_f values of [^{14}C] metabolites from perfused rat liver

Metabolite	Paper chromatography			Thin-layer chromatography				Fluorescence under u.v.	Van Urk reagent
	Solvent			Solvent					
	A	B	C	C	D	E	F		
R1	—	—	0.00	—	0.00	0.00	0.00	n.d.	n.d.
R2	0.14	0.13	0.11	—	0.00	0.11	0.00	n.d.	n.d.
R3	0.22	0.25	0.25	—	0.00	0.24	0.00	n.d.	n.d.
R4	0.27	0.33	0.37	0.30	0.00	0.41	0.00	l.b.	l.b.
R5	0.33	0.40	0.47	0.34	0.00	0.49	0.00	d.b.	mauve→d.b.
R4 aglycone	0.65	0.72	0.83	0.73	0.39	0.79	0.40	l.b.	blue-green
R5 aglycone	—	—	—	0.66	0.34	0.78	0.30	d.b.	grey-blue
R6	0.80	0.73 } 0.86 }	0.83	0.62 } 0.75 }	0.30	0.63 } 0.73 }	0.00	l.b.	yellow
R7	0.72	0.72	0.90	0.76	0.37	0.75	0.29	d.b.	mauve→d.b.
R8	0.84	0.87	0.90	0.77	0.21	0.65	0.17	d.b.	mauve→d.b.
R9	—	—	—	—	0.72	—	0.58	n.d.	n.d.
R10	0.83	0.85	0.92	0.77	0.53	0.83	0.47	d.b.	mauve→d.b.
12-Methoxy-LSD	0.80	0.82	0.90	0.77	0.45	0.85	0.40	d.b.	mauve→d.b.
Methylated R4 aglycone	0.80	0.82	0.90	0.77	0.55	0.85	0.45	l.b.	blue-green
Methylated R5 aglycone	0.80	0.82	0.90	0.79	0.55	0.85	0.45	d.b.	grey-blue
12-Hydroxy-LSD	0.73	0.72	0.90	0.76	0.40	0.81	0.36	d.b.	d.b.

See Table 1 for details of paper and t.l.c. The final dark blue colours with Van Urk reagent appeared within 5 min of spraying. The yellow colour of metabolite R6 appeared in about 15 min.

n.d. = not detected.

l.b. = light blue.

d.b. = dark blue.

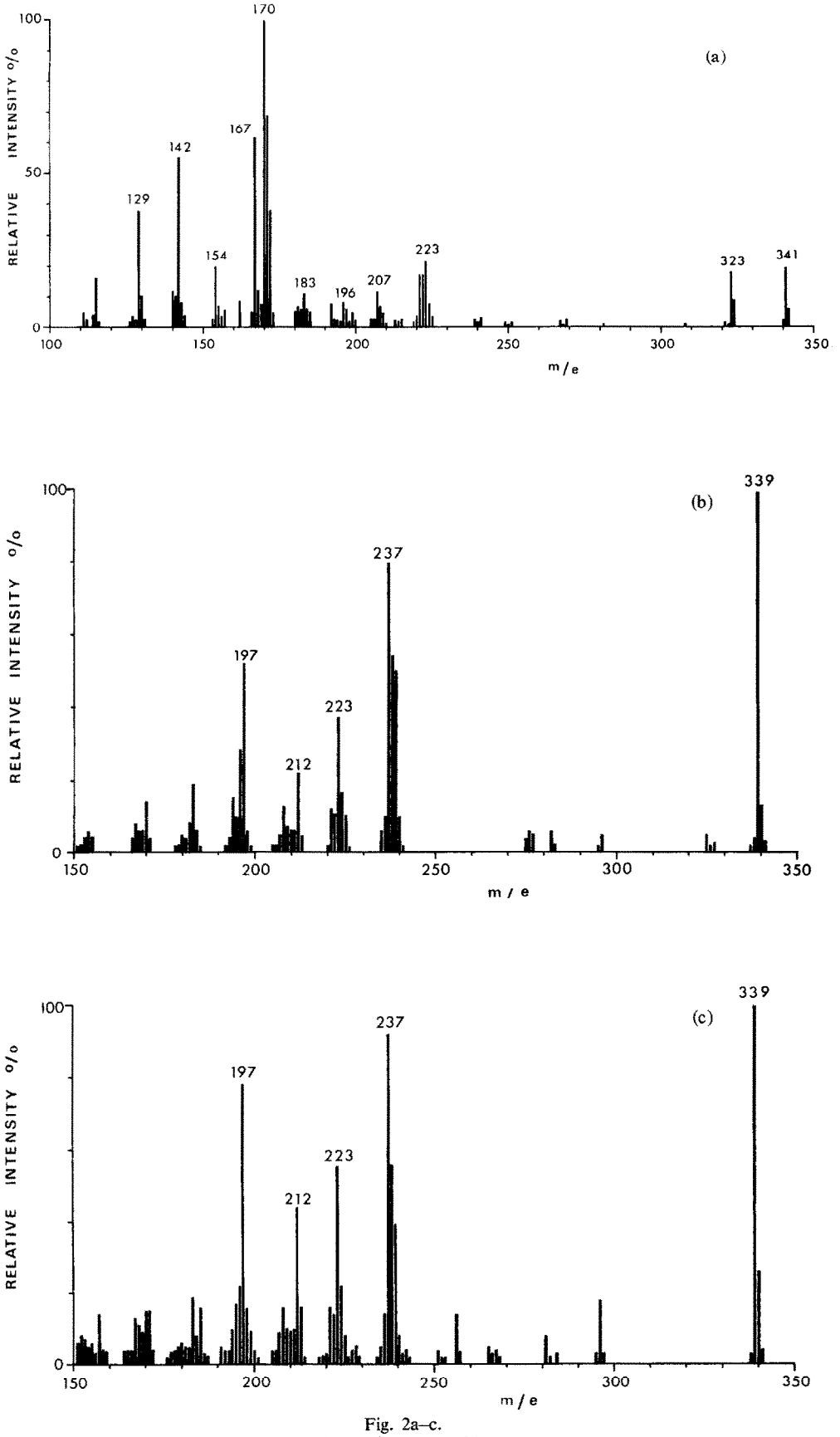

Fig. 2a–c.

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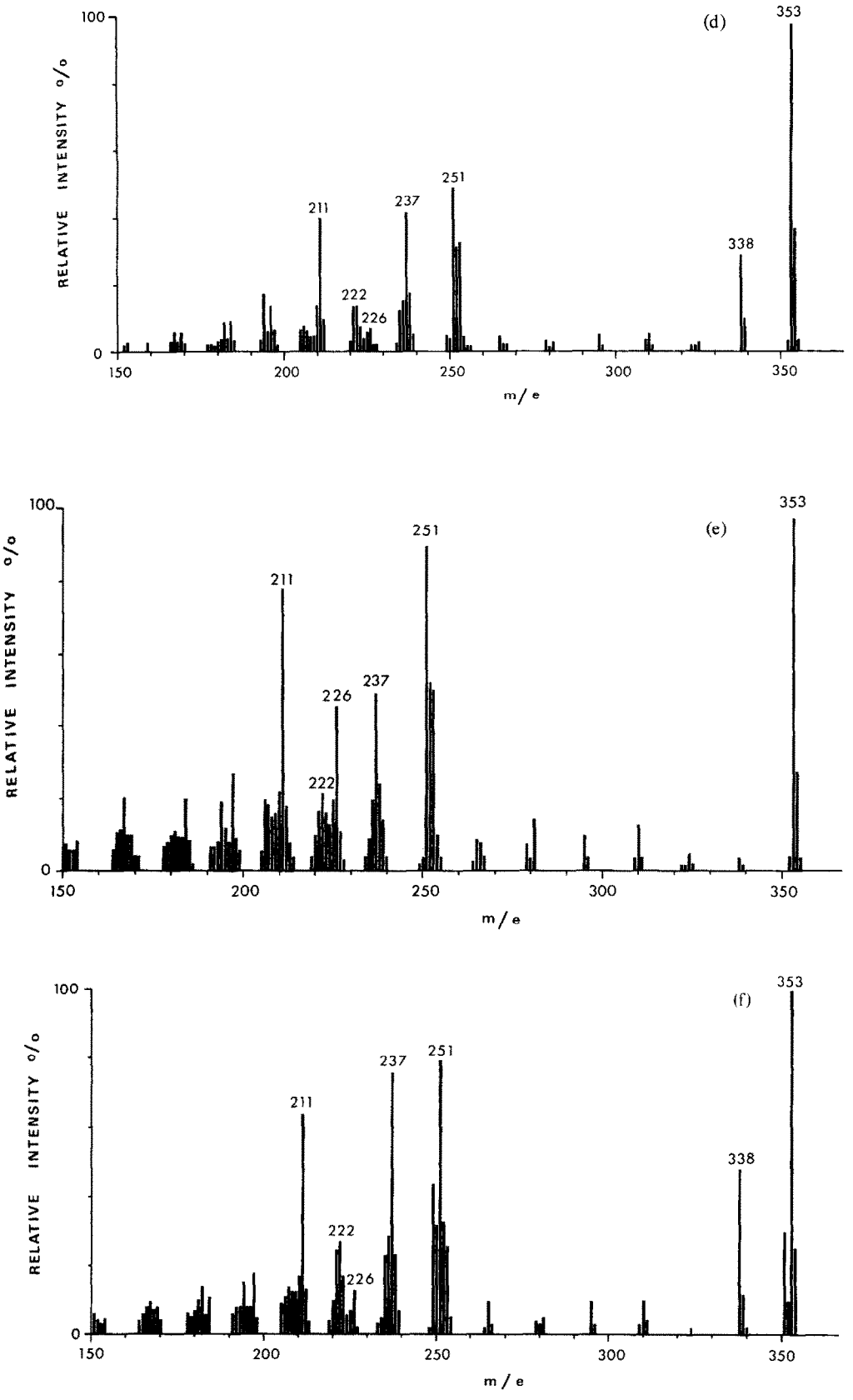

Fig. 2. Mass spectra of reference compounds and metabolites. See legend to Fig. 1. (a) Lumi-LSD; (b) 12-hydroxy-LSD; (c) aglycone of R4; (d) 12-methoxy-LSD; (e) methylated aglycone of R4; (f) methylated aglycone of R5.

the products of chemical syntheses, glass t.l.c. plates (20 × 5 or 20 cm) coated with silica gel HF_{254} (0.25 or 0.80 mm thick; E. Merck A.G., Darmstadt, W. Germany) activated by heating for 1 hr at 100° were used. These preparative silica gel plates were pre-eluted twice with methanol before use. The initial separation of metabolites was obtained by using solvent system C for the bile and D for the pooled perfusate and liver homogenate followed by either solvents E or F (Table 1).

Radiochemical techniques. The ^{14}C in the samples was determined using a Packard Tri-Carb liquid scintillation spectrometer (either model 3214 or 3320) and Bray's scintillator [32]. Bile (5 μl), perfusate (50 μl) and liver homogenate (100 μl) were counted directly at an efficiency of 67–80 per cent as determined by the channels ratio method. Radioactive areas on paper and t.l.c. were located by means of a Packard radiochromatogram scanner (Model 7200). Sections of the chromatograms (0.5 or 1.0 cm wide) were counted in the above scintillant in order to quantify the compounds. Thus the metabolites in bile were measured after separation in solvent system A, E or F and the methanolic extract of the pooled perfusate and liver homogenate in solvents D, E or F.

Enzyme hydrolyses. To bile (0.05 ml), 0.02*M*-sodium acetate buffer (pH 5, 1 ml) and either β-glucuronidase (1 ml; Ketodase, William R. Warner and Co. Ltd., Eastleigh, Hants., U.K.) or sulphatase (0.5 ml; Type H-2, Sigma Chemical Co.; this also contained β-glucuronidase) were added. Saccharo-1,4-lactone (2 mg; Sigma Chemical Co.) was added to the sulphatase preparation to inhibit the β-glucuronidase activity. The samples were incubated at 37° for up to 48 hr. Controls contained either boiled enzyme or saccharo-1,4-lactone. At the end of the hydrolysis period, methanol (25 ml) was added to the incubation mixture and the precipitated protein removed by filtration. The filtrate was evaporated to dryness *in vacuo* at 35–40° and the residue taken up in methanol (1 ml) for chromatography.

Methylation of phenolic compounds. The phenol (0.2–0.5 mg) was dissolved in methanol (1 ml) and methylated with diazomethane [33]. The extent of methylation was checked by t.l.c. in solvent systems D or F. Usually 20–60 min was required for near completion of the reaction, whereupon all the reaction mixture was subjected to t.l.c. in solvent D or F to separate the reaction product.

Spectra. I.r. spectra were recorded as either liquid paraffin mulls or KBr discs on a Perkin Elmer Infracord 137 Spectrophotometer. Mass spectra of compounds using the direct insertion technique were obtained on either a Varian MAT CH5 or an AEI MS902 mass spectrometer utilising a probe temperature of 120–200°, source temp. 220°, ionising energy of 70 eV and an ionising current of 300 μA. Proton magnetic resonance spectra were recorded on a Bruker HFX 90 Spectrometer with Fourier transform (Bruker Magnetics, Burlington, MA, U.S.A.).

Identification of metabolites

In bile. Samples of bile (containing approx. 10^4 d.p.m.) were chromatographed on paper in solvent systems A, B and C and on t.l.c. plates in system E. At least seven peaks (R1–R6 and LSD, see Table 3) were detected by the radiochromatogram scanner. After treatment with β-glucuronidase followed by paper and t.l.c. as above, the intensity of the peaks corresponding to R4 and R5 was greatly reduced, thus suggesting that they were glucuronides. No change was observed in either the controls or the sulphatase preparations. When hydrolysis of the bile was carried out for a short period (2–4 hr) and then followed immediately by t.l.c. in system D or F, the less polar aglycones of R4 and R5 were detectable under u.v. light and with Van Urk reagent (Table 4). But when the hydrolysis was carried out for a longer period (48 hr) the aglycone of R5 could not be seen at its known R_f value and it was therefore

Table 3. Quantitation of ^{14}C metabolites from rat livers perfused with [^{14}C]LSD

Metabolite	Probable Identity	Per cent of dose found as metabolites	
		In bile	In pooled homogenate and perfusate
R1	n.e.	2.0 (1.8–2.1)	0
R2	n.e.	1.9 (1.8–1.9)	0
R3	n.e.	1.4 (1.0–1.7)	0
R4	Glucuronide of 13-hydroxy-LSD	8.2 (7.0–9.0)	1.7 (0.5–2.5)
R5	Glucuronide of 14-hydroxy-LSD	20.9 (20.2–21.3)	1.6 (1.1–2.1)
R6	2-Oxo-LSD	7.4 (6.6–8.3)	5.0 (4.6–5.2)
R7	De-ethyl-LSD	0	2.1 (1.7–2.5)
R8	Nor-LSD	0	4.0 (3.3–4.6)
R9	Naphthostyril cpd. (see text)	0	4.1 (3.8–4.7)
R10	LSD	1.0 (0.8–1.2)	17.6 (14.7–21.1)
	Total of above:	42.8 (41.5–44.2)	36.1 (33.9–38.8) (Total 78.9)
	Total ^{14}C present:	44.2 (41.3–46.0)	39.2 (36.9–41.8) (Total 83.4)

To the Krebs and Henseleit medium [29] was added [^{14}C]LSD (1 mg and 0.13 μCi/g liver) and (+)-tartaric acid (15 mg). Average values for three experiments are given with ranges in parentheses and are expressed as a percentage of the dose of ^{14}C. Metabolites were determined by scintillation counting of paper or thin-layer chromatograms (see text).

n.e. = not examined (see text).

Table 4. Colour reactions of 5-, 6- and 7-hydroxyindole, 12-hydroxy-LSD and the aglycones from R4 and R5

Compound	Spray Reagent 1	2
5-Hydroxyindole	Purple	Brown
6-Hydroxyindole	Blue-green	Immediate red
7-Hydroxyindole	Grey-blue	Brown-orange
12-Hydroxy-LSD	Mauve→light blue→dark blue	Light brown
R4 aglycone	Immediate light blue→light blue-green	Immediate red
R5 aglycone	Mauve→dark blue→dark grey-blue	Yellow
LSD	Mauve→dark blue	Yellow

The following spray reagents were used: (1) Van Urk reagent (see Table 1); (2) diazotised sulphanilic acid in HCl; ten volumes of a 1% solution of sulphanilic acid in 1M-HCl were mixed at 0° with one volume of 5% (w/v) $NaNO_2$ solution. The excess nitrous acid was destroyed after 5 min by the addition of excess ammonium sulphamate [34]. The initial colour produced by a particular reagent is shown together with any subsequent major colour changes. The 6- and 7-hydroxyindoles were prepared by hydrogenation of the respective benzyloxyindoles [37].

concluded that this aglycone was unstable and decomposed during the longer incubation period.

Each of the two glucuronides, R4 and R5 was separated and purified from the bile by concentration with the XAD-2 resin (see Separation of Metabolites) and preparative chromatography in t.l.c. system C (see Chromatography), and was hydrolysed with β-glucuronidase for 3–4 hr. The aglycones were separated by t.l.c. in system F. The two aglycones appeared to be phenolic since they gave brownish-purple colours with diazotised 4-nitroaniline [22] on t.l.c. plates. The stable aglycone from R4 was subjected to mass spectrometry (Fig. 2c) and gave a molecular ion at *m/e* 339 suggesting a hydroxy-LSD. The mass spectrum was identical with that of 12-hydroxy-LSD (Fig. 2b) and the mass spectrum of methylated R4 aglycone with that of methylated 12-hydroxy-LSD (Fig. 2d and e), the latter two spectra both having molecular ions at *m/e* 353. The enzymic hydrolysis product of R5 was unstable and hence a recognisable mass spectrum could not be obtained. However, when the aglycone was treated with diazomethane immediately after isolation, a stable product, amenable to mass spectrometry was obtained. Its mass spectrum (Fig. 2f) was almost identical with that of the methylated derivatives of the aglycone of R4 and 12-hydroxy-LSD and there was a parent ion at *m/e* 353. The unstable aglycone of R5, therefore, was also a hydroxy-LSD. But since the synthetic and the two biosynthetic hydroxy-LSD derivatives had different chromatographic characteristics and gave different colour reactions (Tables 2 and 4), it was concluded that neither of the two aglycones could be 12-hydroxy-LSD. The only positions in LSD which could give rise to a phenol are 12, 13 and 14. Therefore it was concluded that metabolites R4 and R5 were glucuronic acid conjugates of 13- and 14-hydroxy-LSD, respectively, or vice versa.

To obtain further evidence as to the position of these hydroxyl groups, the colour reactions of the three hydroxy derivatives of LSD were compared with those of 5-, 6- and 7-hydroxyindole which correspond to 12-, 13- and 14-hydroxy-LSD respectively, (see Table 4). The results with the Van Urk reagent suggest that the R4 aglycone was 13-hydroxy-LSD and the R5 aglycone was 14-hydroxy-LSD. Furthermore, only 6-hydroxyindole and the aglycone of R4 gave an immediate bright red colour with diazotised sulphanilic acid which is a reagent specific for the detection of indolic compounds possessing a hydroxyl group in the 6-position [34].

The metabolite R6 gave a slowly developing yellow colour with Van Urk reagent suggestive of a product of either 2-substitution or pyrrole ring cleavage (see Heacock and Mahon [35]). This metabolite appeared to exist as two easily interconvertible forms since if the compound were separated on paper in system B or on t.l.c. in system C or E, it gave two spots which if eluted separately and re-run in the t.l.c. systems again gave the same two spots. The mass spectrum of R6 (Fig. 1d) had a molecular ion at *m/e* 339 indicative of the insertion of oxygen into the LSD nucleus. Comparison of the spectrum with the synthetic standards revealed that the cracking pattern was almost identical with that of 2-oxo-LSD. However, it was clear that neither of the two forms of R6 were identical with the synthetic 2-oxo-LSD since the R_f values, although similar, were not the same (Tables 1 and 2).

Compound R10 was unchanged LSD since its R_f values in all the chromatographic systems, the Van Urk colour reaction and the mass spectrum were the same as LSD.

The minor metabolites R1, R2 and R3 were present in amounts which made characterisation difficult but it was clear that they were very polar compared with the other metabolites judging from their R_f values and the fact that unlike all the other compounds they were not retained on a column of XAD-2 polystyrene beads. A comparison of chromatograms of fresh bile and of bile which had been stored at −20° for 6 months run on paper in system C, showed in the latter a large increase in peaks corresponding to R1 and R2 with a concomitant decrease in R6. However no significant changes were noted in the other peaks. It is therefore probable that R1 and R2 are decomposition products of R6.

In perfusate and liver homogenate. The methanolic extract of the freeze-dried pooled perfusate and liver homogenate (see Separation of Metabolites) was subjected to t.l.c. in systems D, E and F. In addition to metabolites already seen in bile (R4, 5, 6 and 10) radiochromatogram scanning also revealed three more

radioactive peaks, designated R7, 8 and 9. The areas corresponding to R1, 2 and 3 had very low levels of radioactivity (< 2 per cent of the dose) and were not examined further.

Extraction of the pooled perfusate and homogenate, made basic with aq.NH_3 (see Separation of Metabolites), showed that 30–40 per cent of the total radioactivity was extractable with $CHCl_3$. T.l.c. in solvent system D showed that only metabolites R6 (2–4 per cent of the total ^{14}C), R7 (1–2%), R8 (3–4%), R9 (2–3%) and R10 (20–30%) were present in the $CHCl_3$ extract. Further purification in system F gave compounds sufficiently pure for analysis. Thus R7 and 8 had the same u.v., fluorescence and colour reactions with Van Urk reagent as those associated with LSD itself. Mass spectrometry of R7 revealed a molecular ion of *m/e* 295 and a cracking pattern almost identical to authentic de-ethyl-LSD. The chromatographic characteristics were also the same as de-ethyl-LSD and, therefore, R7 was most probably de-ethyl-LSD. R8 had a mass spectrum with a molecular ion at *m/e* 309 and this together with the same criteria used in the identification of R7 showed the compound to be nor-LSD. Metabolite R9, which could not be purified sufficiently for mass spectral analysis, was less polar than LSD. It was thought to be the naphthostyril compound of Troxler and Hofmann [26] since the R_f values of the metabolite and the authentic naphthostyril compound (see synthesis of 2-oxo-LSD) were identical when co-chromatographed on t.l.c. plates in solvent systems A (R_f 0.71), D and F.

RESULTS

When rat livers were perfused with [^{14}C]LSD, the mean excretion of ^{14}C in the bile in 4.5 hr in 3 experiments was 44.2 per cent, the perfusate contained 19.5 per cent and the liver 19.7 per cent of the added radioactivity. Total recovery of ^{14}C in all the perfusion experiments was 87 per cent.

The major metabolite in the bile was the glucuronide of 14-hydroxy-LSD, accounting for almost a half of the total radioactivity excreted in the bile in the 4.5 hr. 13-Hydroxy-LSD glucuronide also appeared in the bile (8 per cent of the dose) as did 2-oxo-LSD (7%) and unchanged LSD (1%). In the pooled perfusate and liver homogenate an appreciable proportion was unchanged LSD (18%). The main transformation products were 2-oxo-LSD (5%), the naphthostyril compound (4%, see Identification of Metabolites) and nor-LSD (4%) whereas minor products were the hydroxy-LSD glucuronides and de-ethyl-LSD which each accounted for about 2% of the dose. Lumi-LSD was not detected.

DISCUSSION

In the past, work on the *in vivo* metabolism of LSD in the rat has been hindered by the high central nervous system toxicity of the drug and only doses of 1–3 mg/kg were considered safe [3, 4] since the intravenous LD_{50} in rats is only 16.5 mg/kg [9]. However, such low doses are not satisfactory for the isolation and identification of metabolites particularly when a number of metabolites is involved. By using isolated perfused liver preparations in this study, relatively larger amounts of LSD could be metabolised (equivalent to a dose of about 50 mg/kg *in vivo*) and thus the larger quantities of the metabolic products could be obtained in a small volume of bile. The excretion of ^{14}C in the bile became maximal in the second hour. The lower excretion rate in the first hour was probably caused by the high proportion of unchanged LSD which produced vasoconstriction and a subsequent low perfusate flow rate. The maximal rate of clearance was equivalent to 1.5–2.0 mg LSD/hr which was of the same order as that given by Boyd [3], namely 0.9 mg/hr.

The presence of two glucuronides as metabolites of LSD confirms the reports of Boyd [3] and Slaytor and Wright [4]. Although enzymic mechanisms exist for the hydroxylation of tryptophan to 5-hydroxytryptophan [36] and oxindole to 5-hydroxyoxindole [37] the corresponding LSD metabolite, 12-hydroxy-LSD was not detected. The suggestion made by Slaytor and Wright [4] that one of the metabolites was a glucuronide of 12-hydroxy-LSD was thus not confirmed. That 13-hydroxy-LSD (as a glucuronide) is a metabolite of LSD confirms the *in vitro* findings of Szara [7]. Hydroxylation of LSD in the 13-position might be expected from the reports that most indole compounds are hydroxylated, both *in vivo* and *in vitro*, at the corresponding 6-position [38, 42, 43]. However, hydroxylation in the 7-position (corresponding to the 14-position in LSD) has only been observed with a few indole derivatives [39] and this may be due to the unstable nature of such phenols. It is possible that hydroxylation of LSD at position 13 and 14 involves formation of an epoxide intermediate [40] which could rearrange to either 13- or 14-hydroxy-LSD, with the latter predominating.

Oxidation of the 2-position of LSD creates a new asymmetric centre at carbon 3 and thus two isomers of 2-oxo-LSD are possible. Since the metabolite 2-oxo-LSD (R6) and the synthetic 2-oxo-LSD had different chromatographic characteristics it would appear that they were either 3*R*-2-oxo-LSD and 3*S*-2-oxo-LSD respectively or vice versa. Axelrod *et al.* [5] reported the formation of 2-oxo-LSD by guinea pig microsomes, but no mention was made of its configuration. A metabolite detected in the pooled perfusate and liver homogenate was tentatively identified as the naphthostyril compound of Troxler and Hofmann [26]. This compound is probably not a metabolite but may have formed spontaneously from the 2-oxo-LSD during the extraction procedure (see the synthesis of 2-oxo-LSD). The aromatised products from the metabolite R6 and synthetic 2-oxo-LSD appear to be the same and this further supports the suggestion that the two 2-oxo-LSDs are probably isomeric at position 3. However the possibility that the metabolite and the synthetic compound are 5*R*-2-oxo-LSD and 5*S*-2-oxo-LSD respectively or vice versa cannot be totally ruled out.

The other routes of metabolism of LSD by the isolated liver are by demethylation at the 6 position to give nor-LSD and by de-ethylation of the side chain to give de-ethyl-LSD. These products have also been reported as *in vitro* (9000 *g* supernatant fraction) metabolites by Niwaguchi *et al.* [8]. That the recovery was less than 100 per cent is to be expected since de-ethylation would result in loss of label, presumably as CO_2.

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