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Enzymic formation of dehydrogenated and hydroxylated metabolites from lysergic acid diethylamide by rat liver microsomes

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1. The metabolism of lysergic acid diethylamide (LSD) was studied using rat liver microsomes, and two minor metabolites were obtained in addition to lysergic acid ethylamide and N^6-desmethyl-lysergic acid diethylamide (nor-LSD) which were reported previously.

2. One of the metabolites was identified as lysergic acid ethylvinylamide, apparently formed by dehydrogenation of a diethylamide group in the side chain at the 8-position, and the other as the phenol 13-hydroxy-LSD.

3. The formation of both lysergic acid ethylamide and lysergic acid ethylvinylamide was similarly induced by pretreatment of rats with either phenobarbitone sodium or 3-methylcholanthrene, while nor-LSD formation was induced only by phenobarbitone and that of 13-hydroxy-LSD only by methylcholanthrene.

Introduction

Lysergic acid diethylamide (LSD), a strong hallucinogenic compound, possesses a diethylamide group in the side chain at the 8-position, an *N*-methyl group at the 6-position and an indole ring, all of which are susceptible to metabolic attack.

In the previous paper (Niwaguchi, Inoue and Nakahara 1974), it was shown that LSD was transformed to lysergic acid ethylamide and N^6-desmethyl-lysergic acid diethylamide (nor-LSD) by liver microsomes supplemented with NADPH and oxygen. During a comparison of the metabolism of foreign compounds in mammals and micro-organisms, Ishii *et al.* (1980) found that LSD was transformed to lysergic acid ethylvinylamide, lysergic acid ethyl-2-hydroxyethylamide and lysergic acid ethylamide when *Streptomyces roseochromogenes* was used as the micro-organism. We then re-examined the metabolism of LSD using rat liver microsomes, and obtained lysergic acid ethylvinylamide and a phenolic derivative as minor metabolites in addition to lysergic acid ethylamide and nor-LSD.

This paper deals with the enzymic formation and the establishment of the structures of these minor metabolites, and also describes the effects of inducing agents on the formation of these metabolites of LSD *in vitro*.

Materials and methods

Materials

LSD was prepared from lysergic acid (Sigma Chemical Co. Ltd) by the method of Nakahara and Niwaguchi (1974) and the product recrystallized from benzene (m.p. 82–83°). The purity was checked by t.l.c., u.v. spectroscopy, i.r. spectroscopy and mass spectroscopy. Calcd. for $C_{20}H_{25}N_3O$: C, 74·3; H, 7·8; N, 13·0. Found: C, 74·4; H, 7·8; N, 13·0. Lysergic acid ethylvinylamide was obtained by microbial transformation of LSD as described previously (Ishii *et al.* 1980). NADPH, NADH and 3-

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methylcholanthrene were purchased from Sigma Chemical Co. Ltd, St Louis, USA. Phenobarbitone sodium was obtained from Sankyo Co. Ltd, Tokyo, Japan. All other chemicals and reagents were special grade.

Preparation of microsomes

Wistar male rats weighing 100–150 g were used and liver microsomes were prepared as described previously (Niwaguchi *et al.* 1974). In some experiments, rats were treated by intraperitoneal injection of phenobarbitone or 3-methylcholanthrene. Phenobarbitone sodium in physiological saline was given at a dose of 80 mg/kg once a day for 3 days. 3-Methylcholanthrene in corn oil was dosed at 40 mg/kg once a day for 3 days. The treated rats were killed by decapitation 24 h after the last injection, for preparation of liver microsomes.

Incubations

A complete incubation mixture consisted of 3·0 ml of microsomal suspension, 1·55 μmol LSD, 5 μmol NADPH, 100 μmol $MgCl_2$, 1 ml 0·4 M phosphate buffer (pH 7·4) and water to make a final vol. of 5·0 ml.

Incubations were conducted in air for 10 min at 37°C, with shaking. The incubation was terminated by cooling to 0°C and adding 3 ml of 1 M ammonia.

In order to isolate sufficient amounts of new metabolites to establish their structures, a large-scale experiment was performed using relatively large amounts of the substrate and cofactors under the same conditions as the complete system, except that the incubation was carried out for 30 min.

Analytical methods

Unchanged LSD and metabolites were extracted into chloroform (4 × 30 ml). The extracts were combined, dried over anhydr. Na_2SO_4 and evaporated to dryness *in vacuo*. The residue was dissolved in 250 μl of chloroform, and 10 μl was used for determination of metabolites by direct quantitative analysis on t.l.c. as described previously (Niwaguchi and Inoue 1976).

Protein was determined by the method of Lowry *et al.* (1951). T.l.c. was carried out on silica gel G plates (250 μm; E. Merck) and solvent systems used for development were (A) methanol–ethanol–chloroform (1 : 2 : 9, by vol.), (B) chloroform–acetone (1 : 4, v/v) and (C) diethylamine–chloroform (1 : 9, v/v). Chromatograms were visualized under an u.v. lamp (365 nm) and by spraying with the following reagents: (I) van Urk's *p*-dimethylaminobenzaldehyde reagent (van Urk 1929); (II) 0·1% 2,6-dichloroquinone-4-chloroimide in ethanol and 28% v/v ammonia (sp. gr. 0·90); (III) 0·5% diazotized sulphanilic acid and 1 M KOH (Kirchner 1967). U.v., fluorescence, and i.r. spectra were recorded on a Shimadzu model MPS–5000 spectrometer, a Shimadzu model RF–502 fluorometer and a JASCO model 701 G spectrometer, respectively. Mass spectra were obtained on a JEOL model JMS-OISG double focusing mass spectrometer. N.m.r. spectra were measured with a JEOL model JNM-FX200 (200 MHz) spectrometer for solution in deuteriochloroform with tetramethylsilane as internal standard. Melting points were observed on a microscope hot stage block and were uncorrected.

Isolation of metabolites

A chloroform extract obtained from a large-scale experiment was chromatographed on neutral alumina (activity grade II–III, E. Merck). The metabolites were eluted in the order, a new metabolite (M_1) with benzene–methanol (100 : 0·1, v/v), the unchanged LSD with benzene–methanol (100 : 0·5, v/v), lysergic acid ethylamide and nor-LSD with benzene–methanol (100 : 2, v/v), and another new metabolite (M_2) with benzene–methanol (100 : 5, v/v). The metabolite M_1 was purified by repeating column chromatography on the neutral alumina. For purification of the metabolite M_2, the residue obtained by removal of the eluting solvent from the last fraction was dissolved in a small quantity of chloroform, and the solution was chromatographed on a column of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). The elution was performed with chloroform and the fluorescent eluate was collected. The residue obtained by evaporation of chloroform was further purified by column chromatography on the neutral alumina.

Results and discussion

Formation of novel metabolites

From t.l.c. of the chloroform extract obtained from the complete incubation mixture, two spots, M_1 and M_2 were recognized in addition to the spots of the unchanged LSD, lysergic acid ethylamide and nor-LSD which were reported previously (Niwaguchi *et al.* 1974) (table 1). These two spots were considered to be metabolites of LSD from their colour reactions and fluorescence. It was confirmed that metabolites M_1 and M_2 were formed from LSD by NADPH- and oxygen-dependent enzyme systems in rat liver microsomes (table 2).

Table 1. Thin-layer chromatography of LSD and its metabolites.

Compound	R_F value in solvent system			Colour of fluorescence	Colour reagent		
	A	B	C		I	II	III
LSD	0·73	0·36	0·38	blue	blue-violet	—	—
Lysergic acid ethylamide	0·53	0·19	0·21	blue	blue-violet	—	—
Nor-LSD	0·36	0·05	0·21	blue	blue-violet	—	—
Metabolite M_1	0·84	0·55	0·56	blue	blue-violet	—	—
Metabolite M_2	0·65	0·26	0·07	light-blue	blue	blue	orange

Developing solvent systems and colour reagents are described in the text.

Table 2. Requirement of oxygen and NADPH for formation of metabolites M_1 and M_2.

Incubation system	Metabolite formed (nmol/10 min per mg protein)	
	M_1	M_2
Microsomes with NADPH (complete system)	0·62 ± 0·08	1·10 ± 0·12
Complete system under anaerobic condition	0	0·03 ± 0·02
Heated microsomes with NADPH	0	0
Microsomes with NADH	0·12 ± 0·02	0·20 ± 0·12

Each value is the average ± S.D. of 5 rats.

Identification of metabolite M_1

The metabolite M_1 was isolated as colourless needles, m.p. 86–88°C, by column chromatography as described in *Methods*. It showed an absorption maximum at 312 nm, a fluorescence maximum at 400 nm, excitation 314 nm, in ethanol, and its i.r. spectrum showed characteristic bands at ν_{max}^{KBr} 1660 cm^{-1} (C=C) and 1625 cm^{-1} (amide). The mass spectrum of M_1 showed a parent ion peak at *m/e* 321, and the principal ion peaks of fragments including the indole skeleton were the same as those of LSD (Inoue, Nakahara and Niwaguchi 1972) except for peaks at *m/e* 278 produced by the retro Diels–Alder reaction at the D ring and at *m/e* 295 formed by elimination of C_2H_2 from the parent ion. The high resolution mass spectrum of M_1 indicated a parent ion peak at *m/e* 321·183 corresponding to the molecular formula $C_{20}H_{23}N_3O$, 321·184.

Comparison of the properties of M_1 with those of authentic lysergic acid ethylvinylamide gave excellent agreement. Consequently, it was concluded that M_1 was lysergic acid ethylvinylamide which was apparently formed by an enzymic dehydrogenation of an ethyl group in the diethylamide substituent at the 8-position of LSD.

Dehydrogenation of an alkyl group is not common in the metabolism of foreign compounds. Desaturation of fatty acids caused by an enzyme system in liver microsomes is well known (Oshino *et al.* 1966). However, the possibility that this desaturating enzyme catalysed the transformation of LSD to the ethylvinylamide was ruled out by an inhibition experiment using potassium cyanide; the formation of lysergic acid ethylvinylamide was unaffected by addition of 10^{-3} M KCN to the complete system. Moreover, the fact that NADH was not such an effective electron donor as NADPH for the formation of the ethylvinylamide (table 2) also excluded the participation of the enzyme system for desaturation of fatty acids. It has been

generally accepted that in a process of oxidative de-ethylation of an ethylamide radical, the initial oxygenation by the microsomal mixed-function oxidase system might occur at an α-carbon atom adjacent to a nitrogen atom, producing a labile intermediate which is converted to a de-ethylated metabolite. Therefore, in the biotransformation of a diethylamide radical at the 8-position of LSD, it is considered possible that an intermediate, lysergic acid ethyl-1-hydroxyethylamide would be initially formed, and spontaneously yield lysergic acid ethylamide by loss of a proton of the hydroxyl group of the intermediate and lysergic acid ethylvinylamide as a minor metabolite by loss of a β-proton.

Identification of metabolite M_2

The metabolite M_2 was isolated as colourless needles, m.p. 145°C (decomp.), by repeated column chromatography as described in *Methods*. M_2 gave a slightly different colour with the reagent (I) on t.l.c. from that given by LSD, and reacted positively with the phenolic reagents, as shown in table 1. It showed absorption maxima at 304 and 340 nm in ethanol, the latter maximum being shifted to 368 nm in ethanolic 1% w/v aq. NaOH. It gave a fluorescence maximum at 429 nm with excitation at 305 or 342 nm, and its i.r. spectrum gave ν_{max}^{KBr} 3400 and 1150 cm^{-1} (OH). The mass spectrum of M_2 showed a parent ion peak at *m/e* 339, and principal ion peaks of fragments including the indole skeleton indicated an increase of 16 mass units over the corresponding ion peaks of LSD (Inoue *et al.* 1972) as shown in figure 1.

The high-resolution mass spectrum of M_2 indicated a parent ion peak at *m/e* 339·194 corresponding to the molecular formula $C_{20}H_{25}N_3O_2$, 339·195. From the above data, it was deduced that the metabolite M_2 was a hydroxy-LSD formed by oxidation of the benzene ring. Therefore, methylation of M_2 was undertaken. Excess diazomethane in diethyl ether was added to M_2 in ethanol and the mixture was allowed to stand overnight at 4°C. The product obtained had an R_F value of 0·78 in solvent (A), light-blue fluorescence under an u.v. lamp, blue coloration with the reagent (I), and no coloration with the reagents (II) and (III) on t.l.c. It showed absorption maxima at 300 and 330 nm in ethanol, and the latter maximum was not shifted in ethanolic alkaline solution, in contrast to that of M_2. Its mass spectrum showed a parent ion peak at *m/e* 353 and principal ion peaks of fragments including the indole skeleton showed an increase of 30 mass units over the corresponding ion

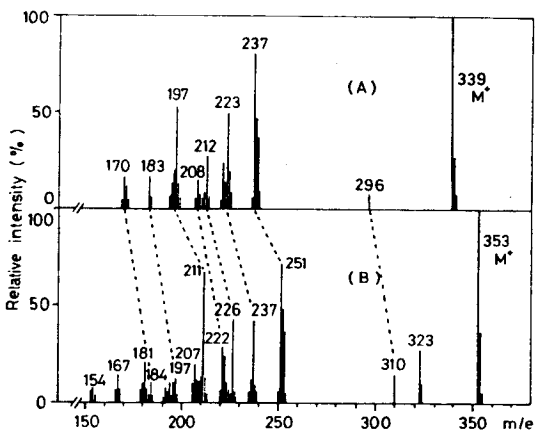

Figure 1. Mass spectra of (A) metabolite M_2 and (B) its methylated product.

peaks of LSD (figure 1). These facts indicated that a phenolic OH in the molecule of M_2 had been methylated to methoxy-LSD. Furthermore, the n.m.r. spectrum of M_2 exhibited four proton signals in the aromatic region (figure 2), among which a pair of doublet signals at δ 6·70 and 6·73 p.p.m. were attributable to 12- and 14-protons, respectively, or vice versa as shown by the *meta*-coupling constant ($J = 1{\cdot}0$ Hz), whereas the doublet signals having an *ortho*-coupling constant ($J = 6$–9 Hz) in the parent compound were not revealed. This spectral evidence showed that a proton at the 13-position of LSD was substituted in the metabolite.

Until now only a few hydroxylated metabolites of LSD have been reported. Axelrod *et al.* (1956, 1957) showed that 2-oxy-LSD was formed by guinea-pig liver microsomes supplemented with oxygen and NADPH, by comparing the metabolite with synthetic 2-oxy-LSD (Freter, Axelrod and Witkop 1957). Slaytor and Wright (1962) presumed that 12-hydroxy-LSD and 12-hydroxy-iso-LSD were obtained from rat bile, by analogy with the formation of 12-hydroxy-ergometrine in the metabolism of ergometrine; Szara (1963) reported that a hydroxyl group of a metabolite formed by rat liver microsomal system was probably at the 13-position from the fact that the absorption peak of the diazotized sulphanilic acid product of the metabolite was identical with that of 6-hydroxyindole, and recently Siddik *et al.* (1975) suggested that the phenolic metabolites obtained from rat urine and faeces were 13- and 14-hydroxy-LSD, since the metabolites were different from authentic 12-hydroxy-LSD (Stadler *et al.* 1964) in chromatographic characteristics. In our experiment using the rat liver microsomal system, however, it was verified by n.m.r. spectroscopy that the metabolite M_2 was 13-hydroxy-LSD.

Effects of inducing agents on the formation of each metabolite

The inducing effects of phenobarbitone pretreatment of rats on the formation of each metabolite of LSD were different from those of 3-methylcholanthrene pretreatment (table 3). Formation of lysergic acid ethylamide and lysergic acid

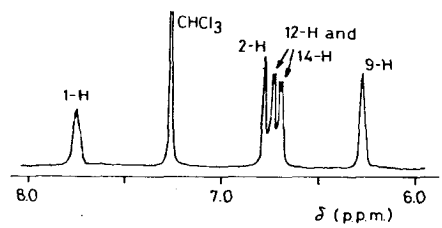

Figure 2. N.m.r. spectrum of metabolite M_2.

Table 3. Effects of inducing agents on formation of metabolites of LSD.

Treatment	Metabolite formed (nmol/10 min per mg protein)			
	Lysergic acid ethylamide	Lysergic acid ethylvinylamide	Nor-LSD	13-Hydroxy-LSD
None	3·12 ± 0·51	0·62 ± 0·08	4·64 ± 0·37	1·10 ± 0·12
Phenobarbitone	4·54 ± 0·42*	0·92 ± 0·10*	6·65 ± 0·36*	1·13 ± 0·15
3-Methylcholanthrene	4·49 ± 0·36*	0·95 ± 0·11*	4·61 ± 0·32	1·60 ± 0·16*

Each value is the average ± S.D. of 5 rats.
* $P < 0{\cdot}005$ versus the amounts of metabolites obtained from microsomes from untreated rats.

ethylvinylamide was similarly induced by either phenobarbitone or methylcholanthrene pretreatment. The formation of nor-LSD was induced only by phenobarbitone pretreatment, on the other hand the formation of 13-hydroxy-LSD was markedly enhanced by methylcholanthrene pretreatment but not induced by phenobarbitone.

From the above facts, it appears that the metabolites are formed from LSD by at least three enzyme systems, which promote attack on a diethylamide group at the 8-position, an *N*-methyl group at the 6-position and an aromatic proton at the 13-position, respectively.

Metabolism of LSD by rat liver microsomes is summarized in figure 3. The fact that LSD is thus metabolized by at least three types of enzyme system seems to be further evidence in support of the multiplicity of cytochrome P-450 enzymes.

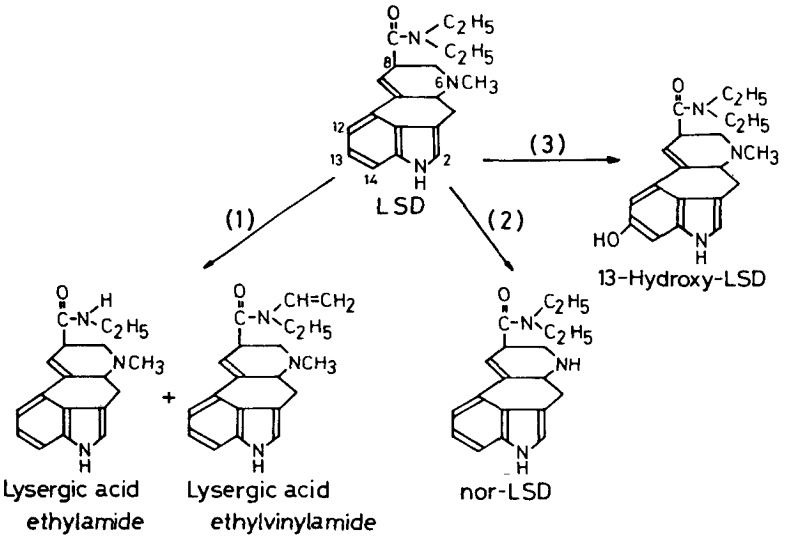

Figure 3. Metabolism of lysergic acid diethylamide by rat liver microsomes. Route (1) was enhanced by either phenobarbitone or 3-methylcholanthrene pretreatment of rats, (2) by phenobarbitone and (3) by 3-methylcholanthrene.

References

Axelrod, J., Brady, R. O., Witkop, B., and Evarts, E. V., 1956, *Nature, Lond.*, **178,** 143.

Axelrod, J., Brady, R. O., Witkop, B., and Evarts, E. V., 1957, *Ann. N.Y. Acad. Sci.*, **66,** 435.

Freter, K., Axelrod, J., and Witkop, B., 1957, *J. Am. chem. Soc.*, **79,** 3191.

Inoue, T., Nakahara, Y., Niwaguchi, T., 1972, *Chem. Pharm. Bull. (Tokyo)*, **20,** 409.

Ishii, H., Niwaguchi, T., Nakahara, Y., and Hayashi, M., 1980, *J. chem. Soc., Perkin I* (submitted).

Kirchner, J. G., 1967, In *Technique of Organic Chemistry*, vol. 12, edited by E. S. Perry and A. Weissberger, (New York: Interscience Publishers), p. 174.

Lowry, O. H., Rosebrough, N. J., Farr, A. H., and Randall, R. J., 1951, *J. biol. Chem.*, **193,** 263.

Nakahara, Y., and Niwaguchi, T., 1974, *Yakugaku Zasshi*, **94,** 407.

Niwaguchi, T., Inoue, T., and Nakahara, Y., 1974, *Biochem. Pharmac.*, **23,** 1073.

Niwaguchi, T., and Inoue, T., 1976, *J. Chromatogr.*, **121,** 165.

Oshino, N., Imai, Y., and Sato, R., 1966, *Biochim. Biophys. Acta*, **128,** 13.

Siddik, Z. H., Barnes, R. D., Dring, L. G., Smith, R. L., and Williams, R. T., 1975, *Biochem. Soc. Trans.*, **3,** 290.

Slaytor, M. B., and Wright, S. E., 1962, *J. med. pharm. Chem.*, **5,** 483.

Stadler, P. A., Frey, A. J., Troxler, F., and Hofmann, A., 1964, *Helv. Chim. Acta*, **47,** 756.

Szara, S., 1963, *Life Sci.*, **2,** 662.

van Urk, H. W., 1929, *Pharm. Weekblad*, **66,** 473.