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STUDIES ON ENZYMATIC DEALKYLATION OF D-LYSERGIC ACID DIETHYLAMIDE (LSD)

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Abstract— New dealkylated metabolites, D-lysergic acid monoethylamide (LAE) and D-N^6-demethyl-lysergic acid diethylamide (norLSD), were formed by incubation of D-lysergic acid diethylamide (LSD) with rat liver 9000 *g* supernatant fractions. It was elucidated that these dealkylations were mediated by an NADPH and oxygen dependent enzyme in liver microsomes and were inhibited by SKF 525-A. Tranquillizing agents such as chlorpromazine, nitrazepam and meprobamate, and certain brain monoamines inhibited these enzymatic dealkylations of LSD. Species differences were investigated.

IT WAS shown by Axelrod *et al.*[1–3] that D-lysergic acid diethylamide (LSD) was transformed to 2-oxy-LSD by an enzyme in guinea-pig liver microsomes supplemented with NADPH and oxygen. Slaytor and Wright[4] found that administration of LSD to rats resulted in the biliary excretion of two metabolites which were β-glucuronides of a hydroxy-LSD and a hydroxy-isoLSD, hydroxylation occurring in the benzene ring. Szara[5] stated without proof that 13-hydroxy-LSD was formed by rat but not guinea-pig liver tissues.

We have now examined enzymatic attack at positions 6 and 8 of LSD, since an *N*-methyl group at the 6 and a side chain at the 8 position seem to play an important role in psychotomimetic action.[6] The present paper deals with dealkylation of LSD by animal liver preparations.

MATERIALS AND METHODS

Materials. LSD and D-lysergic acid monoethylamide (LAE) were prepared from D-lysergic acid by Garbrecht's method,[7] and D-N^6-demethyl-lysergic acid diethylamide (norLSD) was prepared from LSD by the method of Nakahara and Niwaguchi.[8] For the inhibition studies, the following substances were used: 5-hydroxytryptamine creatinine sulfate (5-HT), L-norepinephrine bitartarate (NE) and acetylcholine chloride (ACh) (Wako Pure Chemical Industries, Ltd.); chlorpromazine hydrochloride (Yoshitomi Pharmaceutical Industries, Ltd.); reserpine (CIBA Products Ltd.); meprobamate (Daiichi Seiyaku Co., Ltd.); nitrazepam (Sankyo Co., Ltd.); iproniazid phosphate (Tokyo Chemical Industry Co., Ltd.) SKF 525-A was kindly supplied by Professor Kitagawa, University of Chiba.

Preparation of subcellular fractions. Wistar male rats weighing 150–200 g, Hartley male guinea-pigs weighing 250–300 g or Albino male rabbits weighing 2·5–3·0 kg were stunned, exsanguinated, and the livers were quickly removed and chilled in isotonic KCl. The homogenate was prepared by hand in a glass homogenizer with a Teflon pestle using 2 ml of isotonic KCl per 1 g of liver. The 9000 *g* supernatant fraction was prepared by centrifugation of the homogenate for 20 min in a Marusan

50-B refrigerated centrifuge. This supernatant was then centrifuged at 105,000 *g* for 1 hr in a Marusan 50S-2 ultracentrifuge in order to prepare microsomal fraction, and the pellet obtained was washed with isotonic KCl, and recentrifuged at 105,000 *g* for 1 hr. The microsomal pellet was resuspended in isotonic KCl to give a concentration of 1 g of liver in 2 ml. All procedures were carried out at 0–4°. Microsomal protein was determined by the method of Lowry *et al.*[9]

Preparation of incubation mixture. A typical incubation mixture consisted of 3 ml of 9000 *g* supernatant fraction, 0·63 μmole of LSD, 0·2 μmoles of NAD or NADP, 100 μmoles of nicotinamide, 100 μmoles of $MgCl_2$, 0·5 ml of 0·8 M phosphate buffer (pH 7·4), and water to make a final volume of 5 ml. As a complete system 1 μmole of NADP was used. Incubations were conducted in 30 ml Erlenmeyer flasks for 2 hr, at 37°, with shaking.

Analytical procedures. The incubation was terminated by cooling to 0° and adding 3 ml of 1 N aqueous ammonia. Unchanged LSD and metabolites were extracted with $CHCl_3$. The extraction was repeated until the organic layer no longer showed fluorescence. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness *in vacuo*. The residue obtained was dissolved in 500 μl of $CHCl_3$, of which 5 μl was applied to determination of unchanged LSD and metabolites by quantitative *in situ* fluorometry on thin-layer chromatograms as previously reported.[10] Thin-layer chromatography (t.l.c.) was carried out on 250-μm layers of Silica Gel G (E. Merck). Solvent systems used for development were (A) MeOH–$CHCl_3$–*n*-hexane (1:4:2, v/v), (B) MeOH–$CHCl_3$ (1:4, v/v), and (C) acetone–$CHCl_3$ (4:1, v/v). Chromatograms were visualized under u.v. lamp (365 nm) and by spraying with Van Urk's *p*-dimethylaminobenzaldehyde reagent.[11] Metabolites obtained by a large scale experiment were separated, and purified by repeated column chromatography and preparative t.l.c. Column chromatography was carried out using neutral alumina as adsorbent and benzene–MeOH as eluting solvent. For the preparative t.l.c. the solvent systems (A) and (B) were used. T.l.c., u.v. spectrometry, fluorometry and mass spectrometry were carried out for identification of metabolites. Ultraviolet spectra were recorded in EtOH on a Hitachi Model EPS-3T spectrometer. Fluorescence spectra were obtained in EtOH with a Hitachi MPF-2 spectrophotofluorometer. Mass spectra were determined on a JEOL JMS-01SG double focusing mass spectrometer.

Inhibition studies. In all cases inhibitors were added to the complete system at the beginning of the incubation. The difference of the amounts of LSD unchanged with and without inhibitor was taken as a measure of inhibition.

RESULTS AND DISCUSSION

Metabolism of LSD by rat liver. Time-course studies showed that conversion of LSD ceased after 100–120 min (Fig. 1). Accordingly, subsequent incubations were carried out in 120 min.

Furthermore, on thin-layer chromatogram of the extracts of incubation mixture from 20 to 120 min, two spots (M_1 and M_2) which gave blue fluorescence under u.v. lamp and fast reaction with Van Urk reagent were observed besides the spot of unchanged LSD (Table 1.).

These facts suggested that LSD was metabolized by incubation in the complete system, with maximal activity occurring between pH 7·2 and 7·6. The requirements

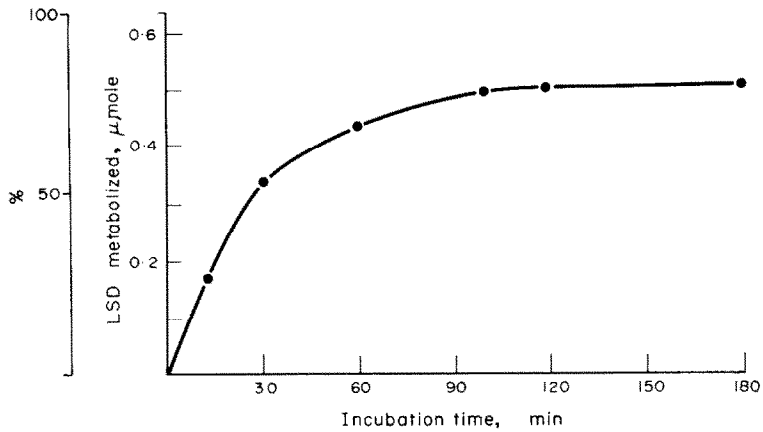

FIG. 1. Time course for the metabolism of LSD by rat liver 9000 *g* supernatant fraction. Incubation mixture contained 3 ml of 9000 *g* supernatant fraction, 0·63 μmole of LSD, 1 μmole of NADP, 100 μmoles of nicotinamide, 100 μmoles of $MgCl_2$, 0·5 ml of 0·8 M phosphate buffer (pH 7·4), and water to make a final volume of 5 ml.

TABLE 1. R_f VALUES OF THE EXTRACT OF INCUBATION MIXTURE ON THIN-LAYER CHROMATOGRAMS

Compound	Solvent system		
	(A)	(B)	(C)
LSD unchanged	0·47	0·73	0·36
M_1	0·38	0·61	0·20
M_2	0·21	0·50	0·11

for the enzymatic transformation of LSD were determined in rat liver 9000 *g* supernatant fraction and in microsomes. Maximal enzyme activity was obtained in the presence of 2 μmoles of NADP and nicotinamide with the supernatant fraction (Table 2).

When NAD was substituted for NADP, the activity was diminished. Incubation under the anaerobic conditions resulted in negligible activity. Oxygen was required for the enzymatic transformation of LSD. The substrate was little transformed with the heated extract. Little activity was observed in the microsomes, but when NADPH was added to the microsomes enzyme activity was restored. From the above facts it was concluded that LSD was converted by a drug metabolizing enzyme in rat liver microsomes.

Metabolites of LSD by rat liver 9000 g *supernatant fraction.* The fluorescent spot with the highest R_f on thin-layer chromatograms in any solvent was identified as LSD and it was concluded that the other two spots (M_1 and M_2) were metabolites of LSD (Table 1). Furthermore, the fluorescence observed under u.v. lamp indicated that the 9-10 double bond of the D-ring in these metabolites were intact and colour given by metabolites with Van Urk reagent shows that the 2 position in the indole ring is unsubstituted.

The $CHCl_3$ extract of incubation mixture obtained by a large scale experiment was

TABLE 2. REQUIREMENTS FOR METABOLISM OF LSD

Enzyme fraction	Cofactor (μmole)	LSD metabolized (μmole)	LSD metabolized (%)
9000 *g* supernatant fraction	NADP (2)	0·49	77·6
	NADP (1)	0·48	76·7
	NADP (0·5)	0·47	75·2
	none	0·38	60·4
	NAD (2)	0·41	65·5
	NAD (1)	0·40	63·5
	NAD (0·5)	0·39	62·5
	NADP (1), anaerobic	0·03	5·6
Heated extract	NADP (1)	0·03	5·6
Microsomes	NADPH (5)	0·39	62·5
	none	0·02	3·2

For the anaerobic experiments 30 ml flasks constructed like Thunberg tubes were used. The flasks were alternatively flushed with nitrogen and evacuated three times before adding the tissue preparation. The heated extract was prepared by subjecting the extract to 100° for 15 min. When the microsomes were substituted for the supernatant fraction, 5 μmoles of NADPH was added in five portions over a period of 60 min.

chromatographed on alumina. The first fluorescent fraction eluted with benzene–MeOH (99·8:0·2, v/v) gave unchanged LSD, the second eluted with benzene–MeOH (99·6:0·4, v/v), the metabolite M_1 corresponding to the R_f 0·38 spot in the solvent system (A), and the third eluted with benzene–MeOH (99·0:1·0, v/v), the metabolite M_2 corresponding to R_f 0·21 spot. The isolated metabolites M_1 and M_2 were purified by preparative t.l.c. Comparison of the data of M_1 and M_2 with those of authentic LAE and norLSD gave excellent agreement. Mass spectra are shown in Fig. 2.

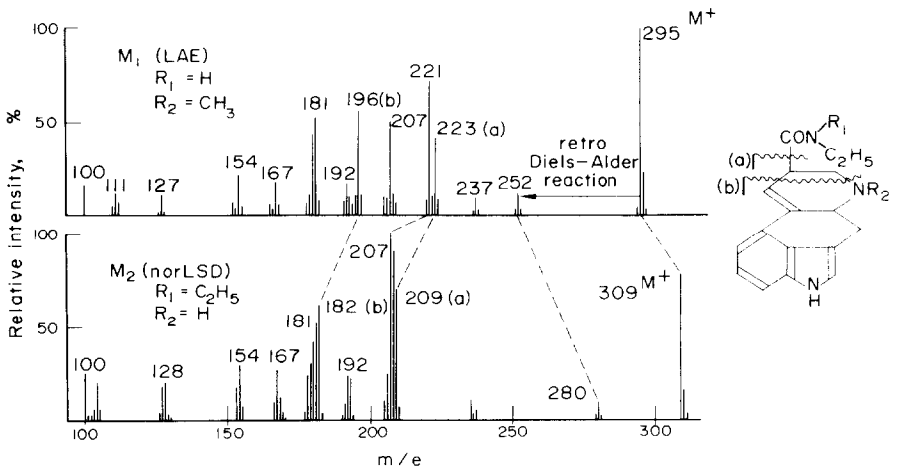

FIG. 2. Mass spectra of the metabolites M_1 (LAE) and M_2 (norLSD). Mass spectra were measured by a direct insertion probe technique: sample temperature, 150–200°; ion-chamber temperature, 100–200°; ionizing energy, 75 eV; ionizing current, 200 μA.

TABLE 3. METABOLITES OF LSD

Metabolite	Ultraviolet λ_{max}^{EtOH} (nm)	Fluorometry $\lambda_{max}^{Irr.}$ (nm)	Fluorometry $\lambda_{max}^{Fluor.}$ (nm)	Mass measurement for M$^+$ Found	Calcd.	Formula
M_1	312	324	400	295·171	295·168	$C_{18}H_{21}N_3O$
M_2	312	324	400	309·185	309·184	$C_{19}H_{23}N_3O$

From these data it was concluded that M_1 was LAE-formed by an enzymatic *N*-deethylation of a diethylamide radical in the side chain at the 8 position of LSD, and that M_2 was norLSD obtained by an enzymatic *N*-demethylation at the 6 position of LSD. It was considered that LSD was partially detoxicated by drug metabolizing enzyme, as it has been confirmed that hallucinogenic activity of LAE is 1/8–1/10 that of LSD.[6]

Species differences in metabolism of LSD. When both guinea-pig and rabbit liver 9000 *g* supernatant fractions were used for the incubation with the complete system, the same metabolites, LAE and norLSD, were formed. In any species the amount of LAE was much more than that of norLSD. The enzyme activity was rat > guinea-pig > rabbit as shown in Table 4.

TABLE 4. SPECIES DIFFERENCES

	Rat	Guinea pig	Rabbit
Enzyme activity* (nmole/mg protein/120 min)	27·3	25·4	21·7
Metabolites formed			
LAE (nmole/mg protein)	9·4	4·7	3·2
norLSD (nmole/mg protein)	1·4	0·6	0·8

* Enzyme activity was expressed as nmoles of LSD metabolized/mg microsomal protein/120 min.

Inhibition of the metabolism of LSD. SKF 525-A at a concentration of 1×10^{-3} M completely blocked the enzymatic dealkylation of LSD and the inhibition at a concentration 1×10^{-4} M was 77·8 per cent. The facts supported that LSD is transformed by NADPH-dependent microsomal enzyme. The effects of tranquillizers, monamines in the brain and monoamine oxidase inhibitor on the metabolism of LSD were examined. The results are shown in Fig. 3.

An antagonism between LSD and chlorpromazine has been confirmed in the field of behavioral science[12,13] and many investigators[14–16] have studied on biological interactions between LSD and tranquillizing agents. In this experiment chlorpromazine, nitrazepam and meprobamate inhibited markedly the enzymatic conversion of LSD, while little inhibition was observed by reserpine. Iproniazid inhibited moderately.

The facts that administration of LSD to animal increases 5-HT in the brain[17–21] and diminishes NE[22,23] suggest biological interactions between LSD and the chemical transmitters. In the experiment, 5-HT and NE inhibited the transformation of

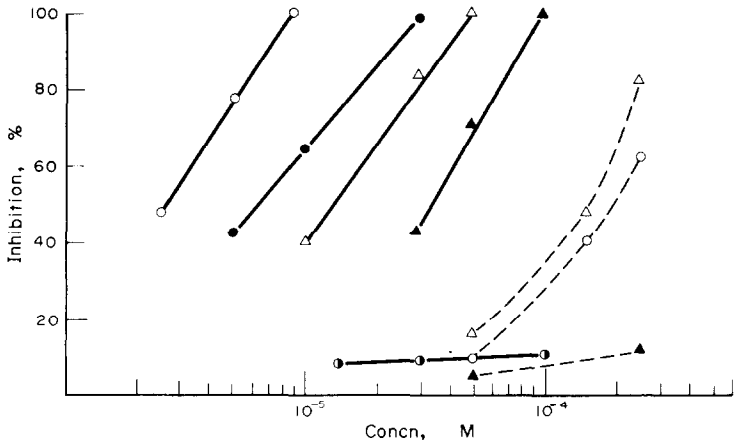

FIG. 3. Inhibitory effects of tranquillizing agents, monamines in the brain and monoamine oxidase inhibitor on the metabolism of LSD. (○——○) Chlorpromazine; (●——●) nitrazepam; (△- - -△) meprobamate; (◑——◑) reserpine; (△ - - - △) 5-hydroxytryptamine; (○ - - - ○) L-norepinephrine; (▲ - - - ▲) acetylcholine; (▲——▲) iproniazid.

LSD but ACh did little. These results obtained by use of a peripheral organ may be related to the interactions between LSD and some tranquillizers or transmitters in the brain.

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