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Comparative Inhibitory Action of D- and L-Tryptophan on the Effect of *d*-Lysergic Acid Diethylamide *in Vitro*

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SUMMARY

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The incorporation *in vitro* of *d*-[^{3}H]lysergic acid diethylamide into secreted protein from hyperimmune rabbit lymphoid cells was inhibited by both D- and L-tryptophan. The inhibition was specific for tryptophan isomers and analogues. On the basis of these findings a pathway was formulated to describe the preliminary steps culminating in the covalent incorporation of lysergic acid diethylamide into synthesized protein.

INTRODUCTION

Recent evidence (1, 2) has indicated that when *d*-lysergic acid diethylamide, an indole alkaloid, is incubated *in vitro* with antibody-producing cells, it interferes with tryptophan incorporation into immunoglobulin protein. Original studies (1) indicated that LSD,[1] tested over a 100-fold concentration range, specifically inhibited the incorporation of radioactively labeled tryptophan and resulted in the production and secretion of low molecular weight peptides. Further studies (2) showed that radioactively labeled LSD was directly incorporated into antibody protein. Although the incorporated LSD moiety has not been characterized, it has been isolated in minute quantities, and it appears that LSD is modified prior to incorporation (2). Since insufficient quantities of the incorporated LSD moiety are available for direct analyses, it is worthwhile to determine indirectly the mechanism or pathway of LSD incorporation.

Proposal of a modified LSD molecule is based on the premise that the diethylamide hallucinogen does not contain appropriate amino or α-carboxyl groups. Molecules, such as amino acids, bearing these groups are activated and participate in peptide linkage only when the groups are separated by a methyl moiety. Except for the secondary amine present in proline, α-amino and α-carboxyl groups must be in the primary configuration for formation of the planar amide bond. There are virtually no known examples in which consecutive amide linkages (end to end) are not planar (3). In order to elucidate the mechanism of the "LSD effect" *in vitro*, it is necessary to correlate present concepts of protein syn-

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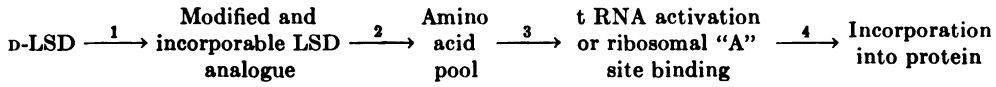
[1] The abbreviations used are: LSD, *d*-lysergic acid diethylamide; LSA, *d*-lysergic acid; IAA, indoleacetic acid.

thesis with existing observations that LSD inhibits tryptophan incorporation *in vitro* and is incorporated into proteins, and that on thin-layer chromatography the incorporated form of LSD derived from hydrolyzed proteins exhibited an R_F value different from those of native LSD and tryptophan. These facts suggest that D-LSD enters some metabolic enzymatic pathway and is modified preparatory to incorporation into a protein.

Assuming that enzymatic modification of the parent LSD molecule is the preliminary step in the pathway culminating in incorporation *de novo*, Scheme 1 is proposed to represent the possible events leading to the LSD effect.



SCHEME 1

Experiments reported here focus on several steps in this pathway. Competition between D- and L-stereoisomers of tryptophan and LSD for incorporation suggests that D-tryptophan inhibits the LSD effect *in vitro* whereas L-tryptophan eliminates the effect by dilution. Implications of these phenomena are discussed, and a mechanism of action in terms of the pathway is proposed.

MATERIALS AND METHODS

Preparation of immunogen. Fluorescein conjugated porcine γ-globulin was prepared as previously described (4). Based on a molecular weight for porcine γ-globulin of 150,000, 20–25 moles of fluorescein were covalently bound per mole of immunogen.

Immunization. Adult albino rabbits received a primary immunization with 5.0 mg of emulsified (complete Freund's adjuvant, Difco) fluorescyl-conjugated porcine γ-globulin intrascapularly and in the hind footpads. A second immunization (5.0 mg) was administered 4–6 days prior to death.

Antibody-producing cells. After a large blood sample had been obtained by cardiac puncture, rabbits were killed by intracardiac injection of 1.0 ml of sodium pentobarbital (Diamond Laboratories, Des Moines, Iowa). Techniques for preparing cell cultures have been reported in detail (1, 2). Briefly, spleens and/or popliteal lymph nodes were removed and immersed in minimal essential medium (Grand Island Biological Company). Lymphoid cells were teased (5) from the organs with a surgical blade and forceps. Cells released from the capsular structure were passed through a fine wire mesh filter and rinsed two to three times in Krebs-Ringer buffer (6).

Incubation cultures in vitro. After washing, 0.1-ml (packed volume) cell aliquots were incubated in 1.0 ml (final volumes) of minimal essential medium as previously described (1). Reaction protocols are summarized in Table 1. [^{3}H]Leucine (50 μl; 480 μmoles; specific activity, 52 Ci/mmole; Schwarz/Mann), [^{14}C]D-tryptophan (50 μl; 0.11 μmole; 22.6 mCi/mmole; New England Nuclear), [^{14}C]L-tryptophan (50 μl; 0.01 μmole; 45 mCi/mmole; New England Nuclear), and [^{3}H]LSD (10–20 μl; 1.9 Ci/mmole; New England Nuclear) were added to reactions as indicated.

Reactions A and B were control incubations demonstrating normal levels of radioactive incorporation and reference ^{3}H:^{14}C ratios. Since repeated experiments indicated that incorporation of [^{3}H]leucine was equivalent between reactions A and B, reaction A was not included in all experiments. To determine competition between LSD and labeled D- or L-tryptophan, unlabeled LSD was added to reaction C. [^{3}H]LSD was added to reaction D for competition with unlabeled D- or L-amino acids, measured as direct incorporation. Reactions were incubated for 4 hr at 37° in a CO_2 incubator (5% CO_2, 98% relative humidity).

Analysis of extracellular supernatant fractions. After incubation, the antibody-producing cells were pelleted by centrifugation, and the supernatant fraction was collected and dialyzed against frequent changes of 4-liter volumes of 0.05 M phosphate buffer, pH 8.0, for 72 hr at 5°. Precipitation of labeled material in 5% trichloracetic was

carried out as previously described (1). Nondialyzable labeled material was 95–100% precipitable in 5% trichloroacetic acid.

Intracellular pool. Cells were incubated with [^{3}H]LSD and incubated as described above. Pelleted cells were washed twice with buffer and then dissolved for 16 hr in 1.0 ml of tissue solubilizer (0.5 M Protosol, New England Nuclear). Aquasol (7.0 ml) was added, and samples were counted in the liquid scintillation counter.

Radioactivity measurements. Radioactive samples were solubilized in 8 ml of scintillation fluid (10% Beckman Bio-Solv BBS-3; 0.4% 2,5-diphenyloxazole in toluene) and counted on a Nuclear-Chicago Isocap 300 liquid scintillation counter.

Cell viability. Cell viability was determined by the trypan blue exclusion method (7).

Sephadex G-100 and G-200 chromatography. Sephadex G-100 and G-200 equilibrated in 0.10 M ammonium bicarbonate (pH 7.4) was packed to bed volumes of 50 × 2 cm and 78 × 2.5 cm, respectively. Labeled fractions (0.1–0.5 ml) in 1.0% sodium dodecyl sulfate were applied to the columns and eluted with 0.10 M ammonium bicarbonate. Fraction volume (2.2 ml) was monitored by a drop counter (American Optical) at 15 ml/hr. Fractions were dried in a vacuum oven (National Appliance) at 80° for 16 hr. To the dried residue was added 0.3 ml of distilled water, followed by agitation for 60 min at 37°. The dionized fractions were then solubilized in scintillation fluid and the radioactivity was measured.

Pronase digestion. Pronase digestion was carried out as previously described (8).

Fractionation of normal rabbit serum. To an aliquot of normal rabbit serum was added an equal volume of saturated ammonium sulfate. A precipitate was allowed to form at 4° for 16 hr and was collected by centrifugation at 10,000 rpm for 10 min. The supernatant fluid was dialyzed against 0.05 M phosphate buffer, pH 8.0, for 48 hr. To 1 ml of the dialyzed 50% saturated ammonium sulfate supernatant were added 10 μl of [^{3}H]LSD, and the mixture then was incubated at 37° for 1 hr. The reaction mixture was dialyzed against 8–16 liters of buffer, and the protein-bound [^{3}H]LSD was resolved on a Sephadex G-200 (78 × 2.5 cm, 0.10 M ammonium bicarbonate buffer, pH 8.0) column that had been calibrated with radioiodinated rabbit immunoglobulin and rabbit albumin markers. Column fractions (2.2 ml) were dried in a vacuum oven at 80° for 16 hr and resuspended in 0.5 ml of water. Each fraction was added to 8.0 ml of scintillation fluid (10% Bio-Solv in 2,5-diphenyloxazole–toluene) and counted.

Preparation of ^{125}I-labeled proteins. Proteins used as molecular weight markers were radioiodinated with ^{125}I by the chloramine T oxidation procedure described by Sonoda and Schlamowitz (9).

Preparation of immunogen and anti-lysergyl antibodies. Lysergyl immunogen and anti-lysergyl antibodies were prepared as described (10). Poly-L-lysine (Miles-Yeda, Ltd., lot 152, mol wt 85,000) was dissolved (10 mg/ml) in 0.10 M NaCl, and to 10 ml of this preparation 50 mg of *d*-lysergic acid (Sigma) dissolved in 2.0 ml of pyridine were added. Upon adjustment of the pH to 7.0, 200 mg of l-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (Pierce Chemical Company) were added and allowed to react with stirring at room temperature for 24 hr in the dark. After dialysis against a neutral buffer, the ratio of lysergic acid to lysine was determined by dry weight analysis and absorbance at 310 nm [E_M = 8300 (11)]. An average of 58 LSA residues were substituted per polylysine polymer. Adult albino rabbits were immunized in the hind footpads and intrascapularly with a total of 5 mg of lysergyl–poly-L-lysine complexed to 2 mg of succinylated keyhold limpet hemocyanin emulsified in complete Freund's adjuvant. Equivalent amounts of immunogen were administered 35 days after the primary immunization. Blood was obtained by cardiac puncture 42, 49, and 56 days after the latter injection.

Assay for reaction of anti-LSD antibodies with peptides. The low molecular weight, LSD-containing peptides are soluble in 50% ammonium sulfate. Since antibodies are insoluble at this concentration of ammonium sulfate. Since antibodies are insoluble at this concentration of ammonium sulfate, a binding assay was evident. To 1.0 ml of anti-LSD antibodies (γ-globulin fraction,

TABLE 1

Protocols for reaction in vitro

Reaction	Minimal essential medium (minus indicated amino acids)	LSD	Radioactive compounds	Amino acid or analogue inhibitor
A	Leucine	−	[^{3}H]Leucine	−
B	Leucine, tryptophan	−	[^{3}H]Leucine, [^{14}C]tryptophan	−
C	Leucine, tryptophan	+	[^{3}H]Leucine, [^{14}C]tryptophan	+
D	Tryptophan	−	[^{3}H]LSD	+[a]

[a] Present at a 10-fold higher molar concentration than tryptophan found in complete minimal essential medium.

5.7 mg/ml) were added 0.05 ml of labeled peptides and 1.0 ml of 3% bovine serum albumin. After incubation at 4° for 16 hr, 2.0 ml of saturated ammonium sulfate were added, followed by incubation for 30 min. The precipitate was collected by centrifugation at 10,000 rpm for 10 min, dissolved in water, and added to 8.0 ml of scintillation fluid (1 liter of toluene, 40 ml of Bio-Solv BBS-3, and 4 g of 2,5-diphenyloxazole).

Indoleacetic acid antigen. In a representative reaction, 40 mg of dicyclohexylcarbodiimide and 30 mg of indoleacetic acid were dissolved in 2 ml of dioxane, followed by a 30-min incubation at room temperature in the dark. The subsequent pale yellow precipitate was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant fraction was added dropwise to 3.0 ml of a rapidly stirred solution of bovine serum albumin (Armour) at 10 mg/ml in 0.05 M phosphate buffer, pH 8.0. After 5 hr the reaction was terminated by the addition of 1.0 ml of ethanolamine (16.3 M). The resultant precipitate was removed by centrifugation at 12,000 × *g* for 10 min and discarded. The supernatant fraction was exhaustively dialyzed to remove all remaining by-products and unreacted reagents. Indoleacetamide substitution was determined by dry weight analysis and absorbance at 278 nm (E_M = 5300) to be 17 residues/mole of protein ($IAA_{17}BSA$).

Adult albino rabbits were immunized in the hind footpads and intrascapularly with a total of 5 ml of immunogen in an equal volume of complete Freund's adjuvant. Booster infections, containing an equivalent amount of immunogen, were administered at 35 days, and blood was obtained by cardiac puncture 7–14 days later.

TABLE 2

Changes in [^{3}H]leucine to [^{14}C]tryptophan labeling ratio of secreted protein from rabbit anti-fluorescyl-producing cells upon incubation with LSD and in competition with D- or L-tryptophan

Protocols for the reaction *in vitro* are described in MATERIALS AND METHODS. Inhibitors were dissolved in minimal essential medium minus tryptophan and added to a final concentration of 100 μg/ml. ^{3}H:^{14}C ratios were calculated after correction for spillage.

Expt.	Reaction	LSD	Inhibitor	^{3}H:^{14}C
		μg/ml		
I	B	0		15.0
	C-1	1		23.7
	C-2	1	D-Tryptophan	13.9
	C-3	1	L-Tryptophan	75.0
II	B	0		3.9
	C-1	1		8.4
	C-2	1	D-Tryptophan	4.5
	C-3	1	L-Tryptophan	24.4

RESULTS

When LSD was incubated with rabbit anti-fluorescyl antibody-producing cells in the presence of [^{3}H]leucine and [^{14}C] tryptophan, tryptophan incorporation was inhibited (1). Inhibition was measured as the increase in ratio of [^{3}H]leucine to [^{14}C] tryptophan in reaction C, compared with controls (Table 1). This difference in ratios between cells incubated with and without LSD suggested that LSD interferred with tryptophan incorporation into protein *de novo* (Table 2). When D-tryptophan was

TABLE 3

Incorporation of [³H]LSD in vitro into secreted protein from rabbit anti-fluorescyl-producing cells and inhibition of incorporation by amino acids

Expt.	[³H]LSD	Inhibitor	Total radioactivity	Difference
	μg		*cpm 0.1 ml cells* (±%)	%
1	8.6		4.7×10^4	
2	8.6		3.4×10^4	
3	2.0		2.2×10^4 (±2)	
	2.0	L-Tryptophan	0.51×10^4 (±17)	−87
	2.0	D-Tryptophan	0.44×10^4 (±4)	−80
	2.0	L-Tyrosine	2.0×10^4 (±5)	−9
Normal cells	2.0		0.01×10^4 (±5)	
4	2.0		3.2×10^4 (±3)	
	2.0	L-Tryptophan	0.98×10^4 (±1)	−69
	2.0	D-Tyrosine	3.1×10^4 (±3)	−3
	2.0	D-Histidine	3.2×10^4 (±8)	
	2.0	L-Histidine	2.7×10^4	−17
	2.0	Puromycin	0.35×10^4	−89
5	2.5		0.97×10^4	
	2.5	L-Proline	1.1×10^4	+13

added to cells incubated with LSD, LSD interference was abolished and ratios returned to control levels. The addition of L-tryptophan, on the other hand, caused a significant increase in the ratio. These results corroborated experiments utilizing [³H] LSD as the only source of label (Table 3), in which both D- and L-tryptophan prevented incorporation. In all cases the labeled material was precipitable with trichloracetic acid and the tritium was not dialyzable or dissociated in 8 M urea, 6 M guanidine, or 10% dioxane in 1 N acetic acid. These observations, plus the release of label upon incubation with Pronase, suggested that LSD had been incorporated into protein covalently. Figure 1 further shows the kinetics of LSD incorporation into secretable protein during an 8-hr incubation period. Inhibition of [³H]LSD incorporation (Fig. 1 and Table 3) by puromycin suggested that LSD incorporation is an active process *de novo*. Analyses of intracellular material showed little or no labeled peptides, indicating that puromycin did not inhibit the secretion of labeled LSD peptides. Tyrosine, proline, and histidine did not inhibit incorporation of LSD (Table 3). Lysergic acid, indoleacetic acid, and tryptophan analogues (DL-7-azatryptophan and tryptazan) inhibited LSD incorporation in similar experiments (Table 4). Table 5 shows that LSD, LSA, indoleacetic acid, and tryptazan all caused increases in the ratios of [³H]leucine to [¹⁴C]tryptophan similar to those produced by L-tryptophan. The apparent significance

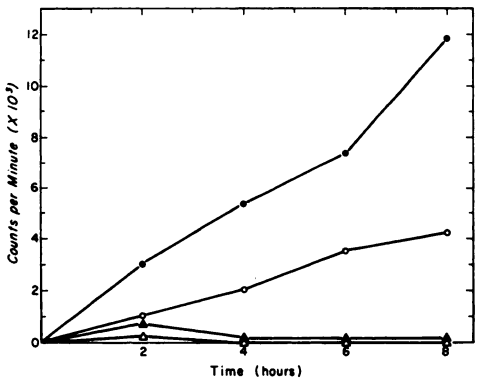


FIG. 1. *Incorporation of [³H]LSD and [³H]leucine in vitro with time*

Aliquots of the extracellular supernatant fraction were dialyzed and analyzed at the times indicated. Dialyzed extracellular material from hyperimmunized rabbit spleen cells was incubated with [³H]leucine (●——●) and [³H]LSD (○——○). The [³H]LSD material reacted with anti-lysergyl antibodies. Dialyzed material from the same cells was incubated with [³H]leucine and 100 μg/ml of puromycin (▲——▲) or with [³H]LSD and 100 μg/ml of puromycin (△——△).

TABLE 4

Effect of lysergic acid, indoleacetic acid, and tryptophan analogues on incorporation of [³H]-LSD into antibody protein in vitro

The indicated reagents were added at a concentration of 100 μg/ml of cells.

Expt.	[³H]LSD + reagent	Total radioactivity in extracellular protein	[³H]LSD incorporation
		cpm	%
1	Control (4 μg [³H]LSD)	4.5×10^4	
	LSA	1.1×10^4	−75
	D-Tryptophan	0.42×10^4	−91
	IAA	2.0×10^4	−55
	DL-7-Azatryptophan	2.4×10^4	−46
	Tryptazan	2.3×10^4	−49
2	Control (4 μg [³H]LSD)	4.6×10^4	
	D-Tryptophan	0.9×10^3	−81
	Tryptazan	2.1×10^4	−54

TABLE 5

Effect of lysergic acid, indoleacetic acid, and tryptazan on [³H]leucine and [¹⁴C]tryptophan incorporation

Expt.	Reagent added	Concentration	Change in ³H:¹⁴C ratio
		μg/ml/0.1 ml cells	%
1	None		
	LSD	4	+41
	LSA	10	+16
	LSA	100	+41
	IAA	10	
	IAA	100	−8
2	None		
	LSD	4	+178
	Tryptazan	40	+31

of these observations will be elaborated under DISCUSSION. None of the analogues adversely affected the viability of the cells during the incubation periods employed. Similarly, total incorporation of [³H]leucine was not significantly inhibited by the analogues.

Anti-LSD antibodies bound secreted peptides from both reactions C and D (Table 6). This finding is important, since it is evident that anti-LSD antibodies do not recognize tryptophyl side groups in peptides harvested from cells not subjected to LSD (reaction B, Table 6). Anti-IAA antibodies reacted weakly with peptides from reaction C, and this reaction was inhibited by 0.05 M LSA. Anti-IAA antibodies do not recognize tryptophyl side groups in peptides from reaction B.

LSD-induced increases in the ratio of [³H]leucine to [¹⁴C]tryptophan and the incorporation of [³H]LSD may be equivalent phenomena, since both are accompanied by secretion of low molecular weight components from the antibody-producing cells. Figure 2 shows that the secreted material from control reaction B (no LSD) corresponded in size to the 7 S immunoglobulin control as analyzed by gel filtration on Sephadex G-100. Material secreted from cells incubated with [³H]LSD had a low molecular weight, as did material secreted from cells incubated in the presence of unlabeled LSD (reaction C). Upon incubation with excess D- or L-tryptophan the secreted

TABLE 6

Binding of [³H]leucine- and [¹⁴C]tryptophan-labeled peptides from lymphoid cells incubated in the presence of LSD by anti-LSD antibodies

A total of 12,350 cpm were added to the reaction mixture. To the labeled peptide were added 1.0 ml of anti-LSD, 0.8 ml of 3% bovine serum albumin, and 0.2 ml of 0.05 M LSA.

Reaction	Antiserum	Activity bound	
		Ppt.	Inhibition by LSA
		cpm	*cpm*
C	Anti-LSD	10,790[a]	3,440
	Anti-IAA	5,310	3,100
	Normal serum	3,180	3,210
B	Anti-LSD	3,200[a]	3,220
	Anti-IAA	3,150	3,200
D	Anti-LSD	11,100[b]	3,190

[a] Total counts (i.e., ³H and ¹⁴C).

[b] Total counts refer to tritium label only.

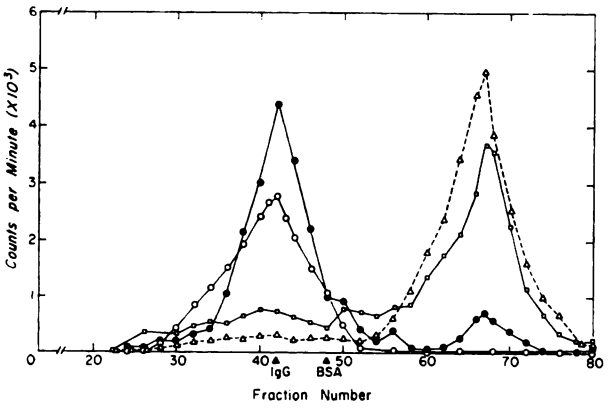


FIG. 2. *Sephadex G-100 chromatography of radioactively labeled extracellular fractions*

Columns (50 × 2 cm) were equilibrated in 0.10 M ammonium bicarbonate, pH 8.0. Fractions were made 1% in sodium dodecyl sulfate before being applied to the column. ●——●, dialyzed extracellular material from reaction B [the immunoglobulin (IgG) and BSA calibrations are indicated on the abscissa (▲)]; ○——○, dialyzed extracellular material from cells incubated with [^{3}H]LSD and inhibited by D-tryptophan; △- - -△, dialyzed extracellular material from cells incubated with [^{3}H]LSD; □——□, dialyzed extracellular material from cells incubated with [^{3}H]leucine and [^{14}C]tryptophan in the presence of unlabeled LSD and showing a ratio increase of 178%.

material corresponded to the molecular weight of immunoglobulin (Fig. 1). Only the low molecular weight fractions reacted with anti-LSD antibodies. In further control studies, [^{14}C]D-tryptophan was incubated with antibody-producing cells in minimal essential medium minus tryptophan to determine whether this optical isomer would be incorporated into protein. No incorporation was measured, as expected. Thus, although both D- and L-tryptophan affect the incorporation of LSD, they differ in their ability to be incorporated into secreted antibody protein.

Based on the finding that L-tryptophan inhibited the incorporation of LSD into protein, experiments were conducted to determine whether inhibitory levels of tryptophan are present in serum *in vivo*. Antibody-producing cells were incubated in minimal essential medium (minus tryptophan) and in the presence of 20% and 50% rabbit serum. These experiments produced an unexpected result (Table 7). Based on the cellular control (absence of serum), the tritium label in the extracellular material increased in direct proportion to the increase in serum concentration. In addition, a large proportion of the [^{3}H]LSD was firmly bound to a serum component in the absence of cells (reagent control). On Sephadex G-200 chromatography (Fig. 3) of the reagent control, [^{3}H]LSD remained bound to a serum component of a lower molecular weight than albumin. [^{3}H]LSD was dissociated (95%) from this protein upon dialysis against 6.0 M guanidine hydrochloride. After correction for nonspecific binding of [^{3}H]LSD

TABLE 7

Incorporation of [^{3}H]LSD into secreted protein in vitro from rabbit anti-fluorescyl-producing cells incubated with normal rabbit serum

Expt.	[^{3}H]LSD	Serum	Puromycin	Total radioactivity
	μg	%	μg	*cpm/0.1 ml cells*
A	4.2			2.2 × 10^5
	4.2	20		8.7 × 10^5
	4.2	50		18.0 × 10^5
B	2.0			0.48 × 10^5
	2.0		100	0.06 × 10^5
	2.0	50		4.8 × 10^5
	2.0	50	100	2.8 × 10^5
Reagent control	2.0[a]	50		3.1 × 10^5

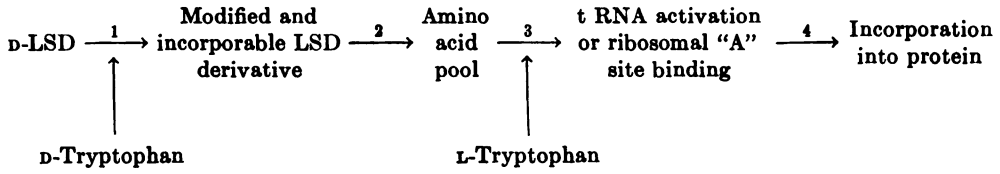
[a] [^{3}H]LSD was incubated with serum only (i.e., no cells).

to the serum component, however, incorporation of LSD *de novo* was also evident. All radioactivity found in the extracellular milieu, not accounted for by binding to serum protein, was inhibitable by puromycin and D-tryptophan. Thus the levels of free tryptophan in serum did not inhibit the LSD effect *in vitro* at drug concentrations of 2–4 μg/ml, indicating that LSD incorporation is feasible under physiological conditions.

Immune cells incubated with [^{3}H]LSD were compared for intracellular pool size with cells incubated separately with [^{3}H] LSD in competition with D-tryptophan, L-tryptophan, and L-histidine. All inhibitors were used at 10 times the molar concentration of [^{3}H]LSD. Within experimental error, there were no significant differences in the accumulation of tritium label after incubation of cells with and without inhibitors.

DISCUSSION

LSD interference with tryptophan incorporation can be explained by the direct incorporation of the hallucinogen (or a modified derivative) in the place of tryptophyl residues. The structure of this derivative can be estimated from the specificity of the antibody reactions (Table 6). Anti-LSD antibodies react only with the polypeptides secreted from cells incubated with LSD. Furthermore, anti-LSD antibodies do not recognize tryptophyl side chain residues, as evidenced by the lack of reaction with immunoglobulin molecules from reaction B. Similarly, anti-IAA antibodies do not recognize tryptophyl groups in native protein but do bind LSD [i.e., rings A and B (12)] constitutes part of the incorporated derivative. However, the LSD molecule has not been modified to tryptophan, since anti-LSD antibodies do not bind tryptophan (as a free ligand or as a side chain in proteins), and thus a significant portion of the C and/or D rings (12) is intact. We conclude that the LSD molecule is modified only moderately before being incorporated covalently into polypeptides. Interpretation is facilitated by the finding that LSD incorporation is inhibited by excess L- and D-tryptophan. Analysis of [^{3}H]leucine to [^{14}C]trytophan ratios (Table 2) indicates that the mechanisms of L- and D-tryptophan inhibition are not equivalent, although the final net effects (i.e., the secretion of 7 S antibody molecules) are similar. Competition of LSD with D-tryptophan results in apparent obliteration of the LSD effect *in vitro*, since ratios equal to the control were measured (reaction B). However, competition with L-tryptophan increased the ratio significantly. These effects by D- and L-tryptophan can be partially explained by restating the reaction presented under INTRODUCTION and denoting the steps where the isomers might exert their inhibitory effects (Scheme 2).



SCHEME 2

Results which show that D-tryptophan inhibited the change in [^{3}H]leucine to [^{14}C] tryptophan ratio (Table 2), and the direct incorporation of [^{3}H]LSD (Tables 3 and 4), are consistent with the hypothesis that the isomer inhibits modification of D-LSD to an incorporable form. Mechanistically, this implies the inhibition of an enzyme (hypothetically, *d*-lysergic acid demethylase) which modifies D-LSD to an incorporable form. Alternatively, D-tryptophan inhibition is consistent with inhibition of LSD transport at the membrane level (e.g., permease), although the similarity of intracellular pool sizes does not strongly support this concept. L-Tryptophan, tested in large excess, significantly reduced LSD incorpora-

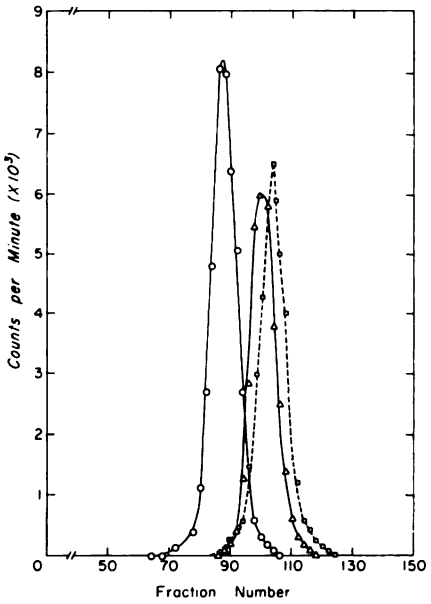


FIG. 3. *Sephadex G-200 chromatography of normal rabbit serum after incubation with [³H]LSD, precipitation in 50% ammonium sulfate, and extensive dialysis against buffer*

The column (78 × 2.5 cm) was equilibrated with 0.10 M ammonium bicarbonate buffer, pH 8.0. ^{125}I-labeled rabbit immunoglobulin (O——O) and ^{125}I-labeled rabbit albumin (△——△) were used as molecular weight control markers. □- - -□, protein fraction with bound [³H]LSD.

tion, and the secretion of 7 S protein resulted (Fig. 2). Since selected control amino acids, such as tyrosine, proline, and histidine, did not inhibit incorporation of LSD, the competitive effect appears to be tryptophan-specific. Competitive inhibition by the tryptophan analogues supports this specificity (Tables 4 and 5). Studies with LSA, IAA, and tryptophan analogues were designed to explore the significance of the common indole moiety, side groups, and stereospecificity. Table 4 shows that LSA inhibited (approximately 91%) LSD incorporation almost as efficiently as D-tryptophan when tested at equal concentrations. LSA also caused a significant increase in the ratio of [³H]leucine to [^{14}C]tryptophan (Table 5). However, the magnitude of the changes indicates that LSA is less efficient than LSD. For example, at 10 μg and 100 μg the ratio changed 16% and 41%, respectively, while LSD at 4 μg showed a 41% increase. This suggests that, if LSA affects protein synthesis by the same mechanism as LSD, the side group differences between the acidic carboxyl group (LSA) and the diethylamide group of LSD are significant. Preliminary studies indicate that the protein secreted from cells incubated with 100 μg of LSA has a low molecular weight. However, LSA competition with LSD would probably occur at both steps 1 and 3 in Scheme 2, since the amount of [³H]LSD incorporated is reduced in the presence of LSA and the [³H]leucine to [^{14}C]tryptophan ratios are elevated, as observed at the tryptophan amino acid pool level with L-tryptophan.

Experiments with IAA were designed to test the role of the indole group irrespective of stereospecificity. Results in Tables 4 and 5 showed that IAA decreased the incorporation of [³H]leucine relative to [^{14}C]tryptophan. This indicates that IAA inhibits LSD (pre-ribosome) from entering the amino acid pool, but cannot compete at the ribosome level. Therefore the logical level for IAA inhibition would be at either step 1 or 2 in the proposed pathway.

Studies with tryptophan analogues (DL-7-azatryptophan and tryptazan) complemented the indoleacetic acid studies. The analogues used can be incorporated into protein in lieu of tryptophan. Table 4 shows that both analogues inhibited the incorporation of [³H]LSD, and Table 5 shows that tryptazan caused an increase in the [³H]leucine to [^{14}C]tryptophan ratio. Thus these analogues may affect LSD incorporation at step 3 in the pathway, by entering the tryptophan amino acid pool.

The comparative inhibition of the LSD effect *in vitro* by D- and L-tryptophan and related analogues has enabled delineation of a multistep pathway which forms a working hypothesis for future experimentation. Further work should help to determine the physiological significance of these findings.

REFERENCES

1. Voss, E. W., Jr., Babb, J. E., Metzel, P. & Winkelhake, J. L. (1973) *Biochem. Biophys. Res. Commun.*, 50, 950–956.
2. Voss, E. W., Jr., Metzel, P. & Winkelhake, J. L. (1973) *Mol. Pharmacol.*, 9, 421–425.

3. Dickerson, R. E. & Geis, I. (1969) in *The Structure and Action of Proteins*, pp. 1–23, Harper & Row, New York.
4. Lopatin, D. E. & Voss, E. W., Jr. (1971) *Biochemistry*, **10**, 208–213.
5. Voss, E. W., Jr. & Bauer, D. C. (1967) *J. Biol. Chem.*, **242**, 4495–4500.
6. Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945) *Manometric Techniques*, p. 194, Burgess, Minneapolis.
7. Paul, J. (1970) in *Cell and Tissue Culture*, pp. 356–357, Williams & Wilkins, Baltimore.
8. Winkelhake, J. L. & Voss, E. W., Jr. (1970) *Biochemistry*, **9**, 1845–1853.
9. Sonoda, S. & Schlamowitz, M. (1970) *Immunochemistry*, **7**, 885–898.
10. Lopatin, D. E. & Voss, E. W., Jr. (1973) *Immunochemistry*, in press.
11. Voss, E. W., Jr. & Berger, B. B. (1972) *Psychopharmacologia*, **26**, 140–145.
12. Baker, R. W., Chothia, C., Pauling, P. & Weber, H. P. (1973) *Mol. Pharmacol.*, **9**, 23–32.