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Immunochemical Characterization of a Lysergyl Derivative Incorporated into Protein

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SUMMARY

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A derivative of *d*-lysergic acid diethylamide (LSD), lysergyl$_{der}$, previously shown to be covalently coupled to secreted low molecular weight peptides, was shown during incubation *in vitro* of immune lymphoid cells with LSD to possess structural features closely resembling the parent molecule. Binding studies of LSD and lysergyl$_{der}$ ligands with antibodies directed against both lysergic acid and lysergyl$_{der}$ suggested that both antibodies possessed similar specificity. [^{3}H]LSD and [^{3}H]lysergyl$_{der}$ were bound by anti-lysergyl antibody with average intrinsic association constants (K_0) of 3.5×10^5 M^{-1} and 7.8×10^5 M^{-1}, respectively. Both ligands were bound by anti-lysergyl$_{der}$ antibody with K_0 values of $1\text{-}2 \times 10^5$ M^{-1}. Single-point equilibrium dialysis experiments indicated that both antibody populations bound the derivative in preference to LSD. Mild alkaline hydrolysis of LSD was shown to generate a molecular species which was bound by anti-lysergyl$_{der}$ antibody with the same energy as lysergyl$_{der}$. Alkaline hydrolysis had no effect on lysergyl$_{der}$. These data suggest generation of a demethylated derivative of LSD during incubation of LSD *in vitro*.

INTRODUCTION

Rabbit anti-fluorescyl antibody producing lymphoid cells incubated *in vitro* with lysergic acid diethylamide secreted peptides rather than the 7 S immunoglobulin molecule (1). These low molecular weight peptides, intrinsically labeled with [^{14}C]L-tryptophan and [^{3}H]L-leucine in the presence of LSD,[1] exhibited a higher ^{3}H:^{14}C ratio than labeled 7 S molecules secreted by cells not exposed to LSD (1). Increased ratio changes have been attributed to interference with [^{14}C]L-tryptophan incorporation by LSD. This change was accompanied by a concurrent loss of measurable anti-fluorescyl antibody activity and ammonium sulfate precipitability of labeled peptides (1-3). Upon addition of excess L-tryptophan the "LSD effect *in vitro*" was reversible. Studies revealed that LSD is incorporated in place of tryptophan, resulting in termination of peptide synthesis (1, 4). Subsequent experiments indicated that cells incubated in the presence of [^{3}H]LSD as the only radioactive source secreted intrinsically labeled peptides (4). Covalent linkage of the derivative to newly synthesized protein was demonstrated by precipitability of labeled peptides with tri-

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[1] The abbreviations used are: LSD, *d*-lysergic acid diethylamide; lysergyl$_{der}$, a derivative therefrom.

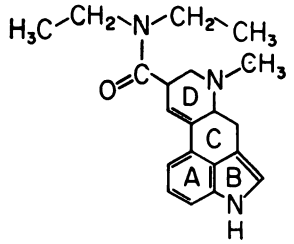

FIG. 1. *Structural model of LSD with molecular configuration determined by Baker et al. (5)*

chloracetic acid and the nondissociability of radioactive label by dialysis against denaturing agents or high concentrations of lysergyl ligand (4). This suggested incorporation *de novo* of [^{3}H]LSD or a tritiated derivative (lysergyl$_{der}$).

Since the hallucinogenic indole alkaloid LSD lacks an α-amino or carboxyl group (Fig. 1), considered necessary for charging by the appropriate tRNA for addition to growing polypeptide chains by peptidyltransferases, a dilemma was evident. Therefore it was proposed that the LSD molecule undergoes structural modification prior to incorporation into protein (3). Preliminary examination of the reactivity of lysergyl-containing peptides with anti-lysergyl antibody (2) indicated that they contain a significant portion of the LSD moiety. Assuming that the incorporated lysergyl moiety (lysergyl$_{der}$) represents the modified derivative, attempts were made to isolate the incorporated product by alkaline hydrolysis of the peptides or by resolving intracellular amino acid pools (4). While both approaches indicated the presence of an LSD-related molecule, neither method yielded adequate quantities of the compound for direct chemical analysis.

An alternative approach to ascertaining the degree of structural modification of the LSD molecule preparatory to incorporation into protein was to elicit and determine the specificity of antibody to lysergyl$_{der}$-containing peptides. This approach utilized isolated lysergyl$_{der}$ peptides as hapten-carrier immunogen. Antibody elicited by the host upon immunization with the peptides would support previous evidence for the covalent coupling of lysergyl$_{der}$ to protein, as well as provide a valuable reagent for immunochemical characterization of the derivative. However, such an approach to the problem necessitated elimination of anti-protein or anti-peptide immunological reactivity. It was determined that LSD also interferes with the synthesis of IgA molecules synthesized by MOPC-315 murine plasmacytoma cells.[2] The IgA molecule normally secreted by the MOPC-315 plasmacytoma cells is homogeneous and possesses anti-nitrophenyl activity. In the presence of LSD MOPC-315 cells produce lysergyl peptides which share only one common antigenic determinant, lysergyl$_{der}$, with rabbit lysergyl$_{der}$ peptides, permitting comparison of the lysergyl$_{der}$ portions without interference by antibodies elicited to the peptide carrier.

This study reports the employment of antibodies elicited to the MOPC-315 lysergyl peptides to characterize the derivative of LSD incorporated into rabbit peptides.

MATERIALS AND METHODS

Antibody-producing cells. Albino rabbits were immunized with a fluorescein-conjugated porcine γ-globulin as previously described (6). After a blood sample had been obtained by cardiac puncture, rabbits were killed by carbon dioxide asphyxiation. The preparation of splenic and/or popliteal lymph node cell cultures has been previously reported (1). Briefly, organs surgically removed were immersed in minimum essential medium (Grand Island Biological), and cells were teased free of tissue with a surgical blade and forceps. Cells were passed through a fine-gauge, stainless-steel wire mesh and washed several times in minimal essential medium. MOPC-315 murine plasmacytoma cells were obtained surgically 3 weeks after cell transfer *in vivo* and were treated in a similar manner.

Incubation cultures in vitro. After washing, 0.10-ml (packed volume) cell aliquots were incubated in 1.0 ml (final volume) of minimal essential medium (minus tryptophan and leucine) as previously described (1). Experiments utilized three reactions. The first reaction served as a source of intrinsically

[2] J. L. Winkelhake, E. W. Voss, Jr., and N. E. Cobb, manuscript in preparation.

labeled, secreted, unaltered protein. To supplement the medium, 50 μl of [^{3}H]leucine (480 μmoles; specific activity, 52 Ci/mmole; Schwarz/Mann) and 10 μg of unlabeled tryptophan were added. The second reaction served to generate lysergyl$_{der}$ peptides. Twenty-five microliters of [^{3}H]LSD (0.013 μmoles; 1.9 Ci/mmole; New England Nuclear) were added to the reaction, which was supplemented with 50 μg of L-leucine. These mixtures were incubated for 4 hr at 37° under 5% CO_2, 98% relative humidity. A third reaction, containing 50 μl of reconstituted [^{3}H]amino acid mixture (Schwarz/Mann) and 10 μg of LSD (Sandoz), was incubated under similar conditions, but after 1 hr the cells were pelleted by centrifugation, suspended in fresh medium and incubated for 16 hr.

Anti-lysergyl antisera. Anti-lysergyl antibodies were prepared as previously described (7). Rabbits were immunized in the hind foot pads and intrascapularly with a total of 5 mg of lysergyl–poly-L-lysine complexed to 2 mg of succinylated keyhole limpet hemocyanin emulsified in complete Freund's adjuvant. Booster doses, containing an equivalent amount of immunogen, were administered 35 days following the primary stimulation. Bleedings were obtained weekly by cardiac puncture.

Anti-lysergyl$_{der}$ peptide antisera. Extracellular supernatant fluids from LSD cell incubations of rabbit or murine plasmacytoma cells *in vitro* (1.0 ml) were dialyzed against two changes of 0.05 M phosphate buffer, pH 8.0, at 4° over 24 hr to remove free LSD. All peptide preparations within a species were pooled to provide protein concentrations sufficient for immunization. Rabbits were immunized in the hind foot pads and intrascapularly with 1.0 ml of the dialyzed supernatant fraction emulsified in complete Freund's adjuvant. After 35 days an equivalent amount of immunogen was given. Bleedings were taken weekly thereafter. All antisera were resolved to IgG fractions by ammonium sulfate precipitation and DEAE-cellulose anion exchange chromatography (7). Antilysergyl reactivity was monitored by radioimmunoassay as described below.

As a control, in a companion experiment, the requirement for immunogenicity of covalent linkage of LSD or lysergyl$_{der}$ to protein was examined. It had previously been demonstrated that a non-immunoglobulin protein in normal serum interacts ionically with LSD at high affinity (2). [^{3}H]LSD was bound (to this protein) by incubating 1.0 ml of normal mouse serum with 0.013 μmole of [^{3}H]LSD (equivalent to cell incorporation experiments) for 4 hr at 37°. The incubation supernatant fraction was dialyzed against phosphate buffer as described previously. Immunization and bleedings were coordinated as outlined.

Protease digestion. To 1.0 ml of labeled lysergyl$_{der}$ peptides was added 0.1–0.2 ml of Enzite-Agarose-protease (Miles-Seravac) in 0.2 ml of Tris buffer, pH 8.0, and 0.02 ml of 0.02 M $CaCl_2$ and the peptides were digested for 2 hr at room temperature.

Alkaline hydrolysis. Labeled lysergyl$_{der}$ peptides and [^{3}H]LSD were hydrolyzed in 4.25 N NaOH by the method of Hugli and Moore (8). Washed, partially hydrolyzed potato starch was used as an antioxidant to preserve the indole moiety. After hydrolysis at 110° for 16 hr the hydrolysate was neutralized and analyzed by ascending silica gel chromatography using chloroform–ethanol–glacial acetic acid (18:10:2 by volume).

Equilibrium dialysis. Seventy-microliter samples of IgG fractions of antisera were dialyzed against 70 μl of various concentrations of [^{3}H]*d*-LSD, using Lucite chambers, as previously described (6). Each measurement was made in duplicate, and two complete determinations were made for each antibody fraction. Samples were analyzed in a Beckman LS-133 liquid scintillation spectrometer. Average intrinsic association constants (K_0) were determined from a Scatchard plot of the data (9). Antibody concentrations were determined by extrapolation to ligand bound at saturating ligand concentrations (6). Heterogeneity indices (a) were derived from Sips plots (10).

Comparative binding radioimmunoassay. The relative affinities of lysergyl$_{der}$ derived from protease-digested peptides and [^{3}H]-LSD for anti-lysergyl and anti-lysergyl$_{der}$ peptide antibody were determined by a modification of the technique of Stupp *et al.*

(11). To a series of tubes containing 0.9 ml (0.5%) of bovine serum albumin were added 1.0 mg of an IgG fraction of either anti-lysergyl, anti-lysergyl$_{der}$, or normal rabbit serum (100 ml) and increasing concentrations of either [^{3}H]lysergyl$_{der}$ (protease-digested) or [^{3}H]LSD (specific activity, 1.0 Ci/mmole). Following incubation at 4° for 16 hr, an equal volume of cold (4°) saturated ammonium sulfate was added to each tube to precipitate immunoglobulins. After 30 min at 4° the precipitate was pelleted by centrifugation at 12,000 × *g* for 10 min. Pellets, washed twice in 50% saturated ammonium sulfate, were solubilized in 0.4 ml of H_2O, followed by the addition of 0.4 ml of Protosol (Beckman Instrument Company) and 5.0 ml of Aquasol (Beckman) and radiolabel analyses.

RESULTS

To characterize the incorporated lysergyl derivative, anti-lysergyl$_{der}$ antiserum was prepared against lysergyl$_{der}$ peptides secreted from incubations of rabbit spleen and MOPC-315 cells with LSD *in vitro*. Anti-lysergyl activity was monitored with the radioimmunoassay described in MATERIALS AND METHODS. Relative to other time points, the bleedings taken 21 days after the booster immunization yielded maximum ligand binding and were utilized throughout these studies. As previously shown, unfractionated normal serum contains a non-immunoglobulin constituent which binds LSD ligand with high affinity (2). To eliminate this component, all sera were resolved to IgG fractions prior to binding studies.

Equilibrium dialysis measurements of [^{3}H]LSD binding by both anti-lysergyl$_{der}$–MOPC-315 peptide antibody and anti-lysergyl$_{der}$–rabbit peptide antibody are shown in Fig. 2. Anti-lysergyl$_{der}$–MOPC-315 peptide antibody bound [^{3}H]LSD with an average intrinsic association constant (K_0) of 8–10 × 10^5 M^{-1} and a heterogeneity index

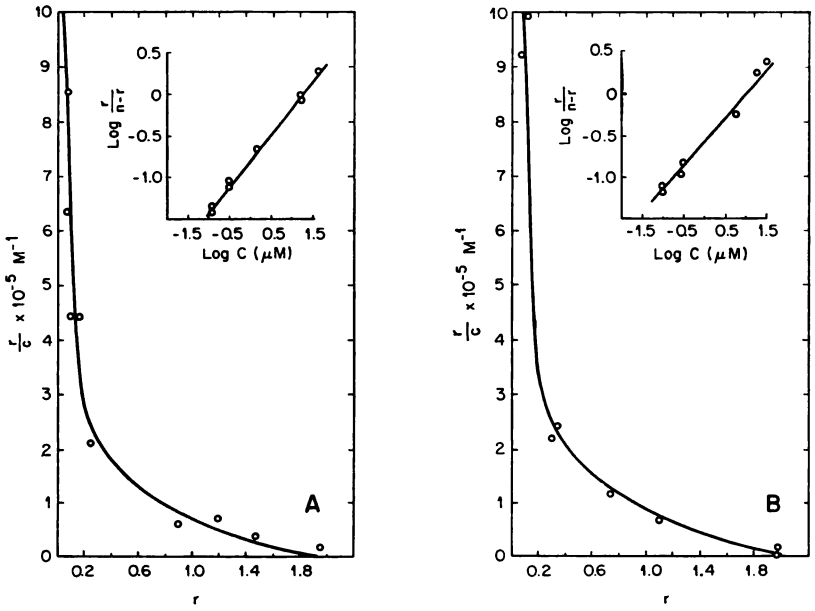

FIG. 2. *Equilibrium dialysis measurements of IgG fractions of hyperimmune rabbit anti-lysergyl$_{der}$–MOPC-315 peptide (A) and rabbit anti-lysergyl$_{der}$ rabbit peptide antisera (B) against [^{3}H]LSD*

The binding of [^{3}H]LSD (specific activity, 85,700 cpm/nmole) was extrapolated to the maximum counts bound by the antibody at saturating levels of ligand, when r = 2. Insets are Sips plots of data derived from equilibrium dialysis. The protein concentration was 8 mg/ml in 0.05 M phosphate buffer, pH 8.0. Equilibrium dialysis was conducted at 4° for 24 hr. Binding parameters are: r = moles of ligand bound per mole of antibody; c = free ligand concentration (moles per liter) after equilibrium was attained; n = antibody valence.

(a) of 0.74. Anti-lysergyl$_{der}$–rabbit peptide antibody bound [^{3}H]LSD with a K_0 of 9–10 $\times$ 10^4 M^{-1} and an a value of 0.68. Thus both murine and rabbit lysergyl$_{der}$ peptides elicited antibodies with specificity for the [^{3}H]LSD ligand.

Immunization of rabbits with [^{3}H]LSD bound noncovalently to a high molecular weight normal serum protein failed to elicit anti-lysergyl activity. This reinforced the concept that covalent linkage is required for immunogenicity (12).

With knowledge of the binding constant for the interaction between anti-lysergyl$_{der}$ antibodies and LSD, it was possible to determine the relative binding properties of the antisera for lysergyl$_{der}$. Because the antisera were raised by injection of lysergyl$_{der}$ peptides, elicited antibodies may possess specificity directed to the peptide portion; thus a potential problem of anti-carrier antibody existed. Since murine (MOPC-315) proteins do not cross-react immunologically with rabbit, murine peptides were utilized to generate anti-lysergyl$_{der}$ antibody, while labeled lysergyl derivative was prepared in the rabbit system for all binding studies. Thus the experimental design tended to negate peptide carrier effects. In addition, large multiring aromatic haptens elicit antibodies specific for the haptenic group with minimal recognition of side group effects.[3]

Since it was not possible to harvest sufficient quantities of free lysergyl$_{der}$ from intracellular pools to perform equilibrium dialysis binding studies, it was necessary to utilize isolated lysergyl$_{der}$ peptides as a ligand. To reduce peptide components to a minimum and create a freely diffusible ligand, the peptides were digested with unsolubilized Enzite-Agarose-protease. Implicit in these experiments is the assumption that lysergyl$_{der}$ was of approximately the same specific activity as the original LSD molecule (i.e., 1.9 Ci/mmole). The finding that lysergyl$_{der}$ peptides were reactive with antilysergyl antibody in the radioimmunoassay suggested significant homology between lysergyl$_{der}$ and LSD. This assumption was supported by previous findings that anti-hapten antibody will react only with closely related analogues of the homologous ligand (13, 14). As previously shown in our laboratory (7) and by Van Vunakis *et al.* (16), recognition of the lysergyl moiety by anti-lysergyl antibody is quite specific.

Saturating levels of lysergyl$_{der}$ ligand could not be obtained for direct measurement of K_0 for the derivative. (K_0 is determined at the point where a given free ligand concentration causes half-saturation of the antibody-active sites.) Since the K_0 values of both anti-lysergyl$_{der}$ and anti-lysergyl antibody were determined for LSD, a comparative binding study was possible. The radioimmunoassay was used to measure the binding of anti-lysergyl$_{der}$–MOPC-315 peptide antibody and anti-lysergyl antibody for [^{3}H]lysergyl$_{der}$ ligand (Fig. 3). Binding data are displayed in the form of Sips plots as log $r/(n - r)$ vs. log c. The relative horizontal displacement of the plot describing the reaction of antibody with the lysergyl$_{der}$ ligand relative to LSD ligand revealed differences in K_0 for the two ligands. Figure 3A compares binding of [^{3}H]LSD and [^{3}H]lysergyl$_{der}$ rabbit peptides (protease-digested) by anti-lysergyl antibody. As previously reported (6), anti-lysergyl antibody possessed a K_0 of 3.5 $\times$ 10^5 M^{-1} for [^{3}H]LSD. As shown in Fig. 3A, 2.25 times more lysergyl$_{der}$ than LSD was bound by the antibody. Anti-lysergyl antibody bound the derivative with a K_0 of approximately 7.8 $\times$ 10^5 M^{-1}. Figure 3B represents binding of the same two ligands by anti-lysergyl$_{der}$–MOPC-315 peptide antibody. A 1.7-fold increase in binding of lysergyl$_{der}$ over that of LSD is evident. This result permits the determination of a K_0 of approximately 1.6 $\times$ 10^5 M^{-1} for the anti-lysergyl$_{der}$ antibody population.

Comparisons of ligand binding were carried out in single-point equilibrium dialysis studies. Tritium-labeled, digested lysergyl$_{der}$ rabbit peptides were allowed to react with IgG fractions of anti-lysergyl and anti-lysergyl$_{der}$–MOPC-315 peptide antibody in equilibrium dialysis experiments. Binding of [^{3}H]lysergyl$_{der}$ by these two antibody populations was compared with binding of [^{3}H]-LSD at equal molar ligand concentrations (based on equal counts per minute). Table 1

[3] E. W. Voss, Jr., and Z. I. Gass, manuscript in preparation.

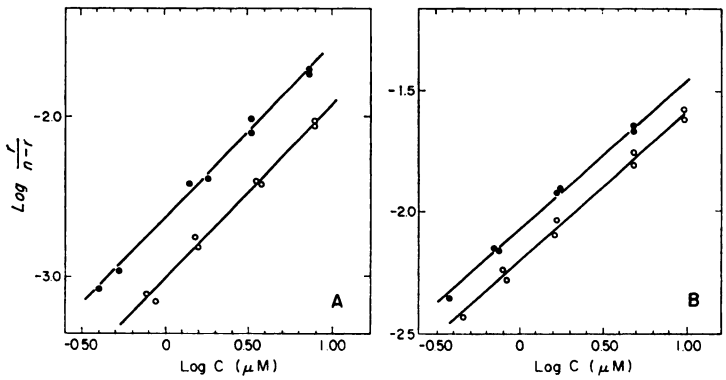

FIG. 3. *Ligand binding measurements of IgG fractions of hyperimmune rabbit anti-lysergyl-poly-L-lysine (A) and rabbit anti-lysergyl$_{der}$-MOPC-315 peptide antisera (B) determined in a modified radioimmunoassay using saturated ammonium sulfate to precipitate immunoglobulin fraction*

Increasing concentrations of [^{3}H]LSD (○——○) and [^{3}H]lysergyl$_{der}$ (●——●) (specific activity, 1.9 Ci/mmole) were added to 100 μl aliquots of antiserum IgG fractions at concentrations of 8 mg/ml in 0.05 M phosphate buffer, pH 8.0. *r*, *c*, and *n* are defined in the legend to Fig. 2.

shows that rabbit anti-lysergyl$_{der}$–MOPC-315 peptide antibody bound protease-digested lysergyl$_{der}$ peptides significantly better than [^{3}H]LSD. Both reactions were inhibitable by unlabeled LSD. Quantitative differences in binding between the two ligands show that anti-lysergyl$_{der}$ antibody is very specific for the lysergyl$_{der}$ homologous system. Anti-lysergyl antibody also bound the derivative better than LSD; however, the difference was not as great as with anti-lysergyl$_{der}$.

In an attempt to obtain lysergyl$_{der}$ ligand free of protein, [^{3}H]lysergyl$_{der}$ peptides were subjected to mild alkaline hydrolysis. This procedure was originally developed for high recovery of tryptophan prior to amino acid analysis (8). Thin-layer chromatograms of the products of alkaline hydrolysis are shown in Fig. 4.

After both [^{3}H]LSD and [^{3}H]lysergyl$_{der}$ peptides had been subjected to alkaline hydrolysis, the products were compared with unhydrolyzed reactants in single-point equilibrium dialysis experiments against anti-lysergyl$_{der}$–MOPC-315 peptide antibody. Although there was no change in antibody binding of lysergyl$_{der}$ after hydrolysis (Table 2), there was a 35% increase in binding of hydrolyzed [^{3}H]LSD, to within 5% of lysergyl$_{der}$. These data suggest that whereas lysergyl$_{der}$ is unaffected, LSD is subject to modification by alkaline hydrolysis (i.e., lysergyl$_{der}$ had already undergone modification during incorporation into protein). Results further suggest that the hydrolytic product of LSD closely resembles lysergyl$_{der}$. Since it was possible that amino acid residues remained bound to lysergyl$_{der}$ after hydrolysis, cells were incubated with a mixture of labeled essential amino acids and unlabeled

TABLE 1

Comparison of [^{3}H]LSD and [^{3}H]lysergyl$_{der}$ binding by rabbit IgG fractions of hyperimmune anti-lysergyl and anti-lysergyl$_{der}$ MOPC-315 antisera in single-point equilibrium dialysis experiments

IgG fraction[a] and ligand[b]	Radioactivity bound[c]
	cpm
Anti-lysergyl$_{der}$-MOPC	
[^{3}H]Lysergyl$_{der}$ peptides[d]	2,578 ± 52
[^{3}H]LSD	717 ± 21
Anti-lysergyl	
[^{3}H]Lysergyl$_{der}$ peptides[d]	12,678 ± 98
[^{3}H]LSD	11,006 ± 86
Normal IgG	
[^{3}H]Lysergyl$_{der}$ peptides[d]	0 ± 14
[^{3}H]LSD	0 ± 16

[a] At a concentration of 8 mg/ml in 0.05 M phosphate buffer, pH 8.0.

[b] Tested at the highest concentrations available.

[c] Mean of three experiments ± standard deviation.

[d] Protease-digested.

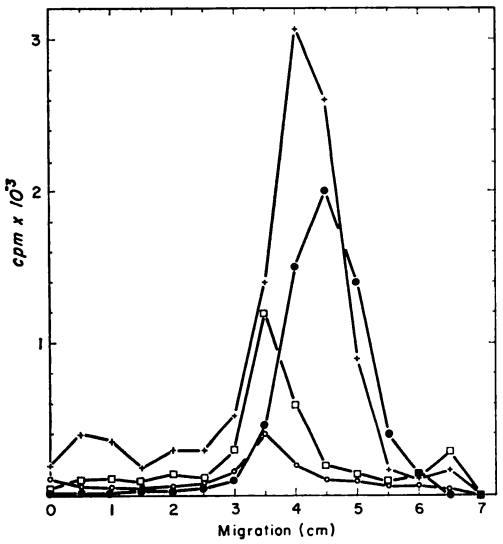

FIG. 4. *Silica gel thin-layer chromatography of [³H]LSD and [³H]lysergyl$_{der}$ peptide hydrolysate* ●——●, [³H]LSD; +——+, protease-hydrolyzed [³H]lysergyl$_{der}$ peptides; □——□, alkaline-hydrolyzed [³H]LSD; ○——○, alkaline-hydrolyzed [³H]lysergyl$_{der}$ peptides. Chromatography was performed in the ascending fashion in chloroform–ethanol–acetic acid (18:10:2 by volume). Origin, 0 cm; solvent front, 7 cm. R_F values of the major peaks: [³H]LSD, 0.66; protease-digested [³H]lysergyl$_{der}$ peptides, 0.60; alkaline-hydrolyzed [³H]lysergyl$_{der}$ peptides, 0.50; alkaline-hydrolyzed [³H]LSD, 0.50.

LSD to generate uniformly labeled peptides. After alkaline hydrolysis of the labeled peptides the products were subjected to binding by antibody in equilibrium dialysis. No ³H label (i.e., amino acids) was bound. In addition, thin-layer chromatography of [³H]lysergyl$_{der}$ peptides did not reveal labeled species other than free amino acids. Both observations indicated that alkaline hydrolysis removed all amino acid residues and that the R_F determined for the derivative was accurate.

DISCUSSION

To show that a derivative of LSD is the structural analogue incorporated into proteins synthesized *de novo*, immunochemical characterization was undertaken. Studies were based on the premise that the derivative would be bound by antibodies directed against both LSD and lysergyl$_{der}$. The latter were produced by injection of lysergyl$_{der}$ peptides. Elicitation of antibodies by injection of a monosubstituted protein (LSD peptide) was puzzling, since hapten immunogenicity correlates directly with the density of covalently conjugated groups to the carrier. However, lysergyl$_{der}$ peptides have been observed to aggregate readily.[4] For example, dissociating agents are required to resolve the monomeric form on molecular sieve chromatography. This tendency to aggregate possibly confers the polyvalency required for immunogenicity of the lysergyl$_{der}$ peptides.

Antibodies of both specificities (i.e., anti-LSD anti-lysergyl$_{der}$) were allowed to react with lysergyl$_{der}$, which was derived by hydrolysis of peptides produced by incubation *in vitro* of LSD with antibody-producing cells. However, measurement of the average intrinsic association constant (K_0) of an antibody for a ligand requires ligand concentrations sufficient to saturate 50% of the antibody sites (100–200 μM). Inability to harvest such concentrations of lysergyl$_{der}$ required an alternative method of comparing antisera based on characteristics of ligand

TABLE 2

Comparison of ligand binding by rabbit IgG fractions of hyperimmune anti-lysergyl$_{der}$–MOPC-315 antisera:[a] single-point equilibrium dialysis experiments after alkaline hydrolysis of [³H]LSD and [³H]lysergyl$_{der}$ ligands

Ligand[b]	Radioactivity bound[c]
	cpm
[³H]LSD	956 ± 23
[³H]LSD, alkaline hydrolysis	1296 ± 38
[³H]Lysergyl$_{der}$ peptides, protease-digested	1375 ± 36
[³H]Lysergyl$_{der}$ peptides, protease-digested, alkaline hydrolysis	1348 ± 41

[a] At a concentration of 8 mg/ml in 0.05 M phosphate buffer, pH 8.0.

[b] All ligands were adjusted to equal counts per minute after hydrolysis.

[c] Mean of three experiments ± standard deviation.

[4] Unpublished observations.

binding plots. The Sips plot displays antibody binding data as $\log r/(n - r)$ vs $\log c$. Since the K_0 of the antibody may be computed on the horizontal axis of the plot at the point where $\log r/(n - r) = 0$ ($r = \frac{1}{2}n$), the horizontal displacement of a second parallel line on such a plot correlates with the difference in K_0. Thus it was reasoned that if the K_0 of an antibody preparation could be accurately measured with the reference ligand [^{3}H]LSD, the K_0 of lysergyl$_{der}$ could be estimated even though sufficient concentrations were not available. The K_0 of antibody for lysergyl$_{der}$ would be reflected in the displacement of the binding curve on the abscissa relative to the reference ligand. Inherent in these assumptions was the fact that the heterogeneity index (derived from the slope) must be approximately equal to yield valid comparisons. Figures 2 and 3 show the results of experiments which relate to these concepts.

The average intrinsic association constants (K_0) of two antibody preparations for LSD were independently measured. Anti-lysergyl antibody possessed a K_0 of 3.5×10^5 M^{-1} and $a = 0.70$, and anti-lysergyl$_{der}$–MOPC-315 peptide antibody, a K_0 of $8\text{–}10 \times 10^5$ M^{-1} and $a = 0.74$ (Fig. 2).

The experimental design for assessing lysergyl$_{der}$ characteristics employed rabbit anti-lysergyl$_{der}$–MOPC-315 peptide antibody with rabbit [^{3}H]lysergyl$_{der}$. This system eliminated potential carrier (peptide) cross-reactivity and permitted direct measurement of antibody-lysergyl$_{der}$ interaction. These measurements provided the standard ligand curves plotted in Fig. 3. Reactivity of anti-lysergyl antibody with lysergyl$_{der}$ peptides suggests that the aromatic skeleton of the LSD molecule remained largely intact after incorporation into protein. Anti-indoleacetic acid antibody does not bind tryptophyl residues in native protein, but reacts with lysergyl$_{der}$ peptides, suggesting that the indole portion (rings A and B) of the molecule is intact and is accessible to the antibody by projection from the protein backbone (16). These observations preclude incorporation of only portions of the LSD molecule in the form of tryptophan or other amino acids.

Lysergyl$_{der}$ ligand binding experiments were based on the assumption that the [^{3}H]LSD molecule is not extensively modified prior to incorporation. Specificity of hapten-antibody interactions has been carefully documented (13). Minor modifications of haptenic structure, such as alteration of side groups or changes from *ortho* to *para* linkages, may result in a decrease in K_0 of 1000-fold or more (14). Since lysergyl$_{der}$ peptides were reactive with anti-lysergyl antibody and the original LSD molecule possessed a general tritium label, it was reasoned that the specific activities of the derivative and LSD were the same.

Ligand binding measurements were performed with protease-digested [^{3}H]lysergyl$_{der}$ rabbit peptides, assigning a specific activity of 1.9 Ci/mmole to the derivative molecule. These experiments (Fig. 3) revealed that anti-lysergyl antibody bound the derivative better than LSD by a factor of 2.25. A factor of 2 is the generally accepted value for experimental variation in K_0 measurements by equilibrium dialysis. Measurement of binding by anti-lysergyl$_{der}$ antibody also revealed greater binding of the derivative, but within the 2-fold error limit. Accuracy of these measurements was substantiated by similarities in slopes of the binding data.

Subsequent experiments utilized single-point equilibrium dialysis measurements to compare relative binding of LSD and lysergyl$_{der}$ by both antibody preparations. Table 1 indicates that anti-lysergyl$_{der}$–MOPC-315 peptide antibody bound the lysergyl$_{der}$ hydrolysate 3.6-fold better than LSD. The derivative was bound 1.15-fold better by the anti-lysergyl antibody. Thus the consistent difference cited in these experiments suggested a significantly higher K_0.

Lysergyl$_{der}$ was subjected to alkaline hydrolysis to free the lysergyl derivative of all protein (or amino acids). Comparison of the binding of alkaline-hydrolyzed derivative with that derived from protease digestion revealed no change in binding properties. Binding of alkaline-hydrolyzed LSD, however, was increased by 35% over unhydrolyzed LSD (Table 2). The binding data suggest that alkaline hydrolysis of the LSD molecule generates a relatively more homologous ligand. The major product from alkaline

hydrolysis of LSD is 6-nor-LSD (demethylated form) (17). Since hydrolysis of lysergyl$_{der}$ resulted in no change in binding, it appears also that similar modification had already taken place during processing *in vitro* and that the molecule was resistant to further modification.[5]

Studies by La Du *et al.* (18) and Axelrod *et al.* (19) indicated that a variety of pharmacologically active drugs are rapidly metabolized by enzymes associated with liver microsomal fractions. One such action is *N*-demethylation. LSD was demonstrated to be rapidly metabolized to a variety of products. Mild alkaline hydrolysis as utilized in these experiments has been shown by Fehr *et al.* (17) to generate an *N*-demethylated lysergyl product. Cumulatively these data suggest that lysergyl$_{der}$ is an *N*-demethylated product of LSD, the result of an enzyme system found *in vivo* capable of processing a wide variety of pharmacologically active drugs (18). *N*-Demethylation of the LSD molecule at position 6 results in the generation of a secondary amide as with the amino acid proline. Subsequent amide linkage of the secondary amide in 6-nor-LSD (demethylated form) to the carboxyl end of the growing peptide chain (perhaps mediated by a peptidyltransferase) would result in lysergyl$_{der}$ peptides. Since the proposed lysergyl$_{der}$ has no α-carboxyl group necessary for continual amide linkage (back to back), protein chain elongation would be terminated. Such a hypothesis is consistent with these experimental findings.

Final structural analysis of the incorporated lysergyl$_{der}$ and its linkage to the protein will depend on generation of sufficient quantities of the compound. Such quantities are at present unobtainable from cell experiments *in vitro*.

[5] While there exists the potential argument that a second (minor) lysergyl species is produced by alkaline hydrolysis, reacting with energy equal to lysergyl$_{der}$, earlier discussion of antibody specificity and effects of side group modification tend to rule this out.

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