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ANTI-LYSERGYL ANTIBODY: MEASUREMENT OF BINDING PARAMETERS IN IgG FRACTIONS*

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Abstract—Antibodies directed against lysergyl-poly-L-lysine were elicited in rabbits. A ligand binding assay was developed to monitor antibody activity. Results using this assay showed that immunoadsorption selects for the antibody population representing the average affinity in the serum with subsequent loss of high and low affinity subpopulations. Measurements of average intrinsic association constants (K_o) and heterogeneity indices (**a**) were made with IgG fractions of anti-lysergyl antisera and compared with purified antibody populations after immunoadsorption.

INTRODUCTION

Detection of narcotic and hallucinogenic drugs relies on a variety of assays. Bio-assays measuring antagonism of lysergic acid diethylamide (LSD) to serotonin induced contraction of rat uteri (Lanz *et al.*, 1955) have been used, but, because of background variability, are unsuitable for mass screening. Extraction of drugs from tissues and fluids with organic solvents and measurement of their characteristic fluorescence (Axelrod *et al.*, 1957; Gillespie, 1969; Miller and Maickel, 1970; Upshall and Wailling, 1972) have proven useful, but a high and variable non-specific fluorescence background presents problems. Immunological assays including complement fixation by anti-mescaline antibodies (Van Vunakis *et al.*, 1969) and hemagglutination by anti-morphine antibodies (Alder and Liu, 1971) provide adequate sensitivity. Radioimmunoassays have been developed involving binding of either a multivalent radioiodinated hapten–carrier conjugate or a radioactive monovalent hapten by a drug-specific anti-serum. Precipitation of the drug-antibody complex is achieved by either non-specific methods, such as ammonium sulfate precipitation (Peskar and Spector, 1973; Ringle and Herndon, 1972; Spector and Parker, 1970; and Wainer *et al.*, 1973), or specific precipitation utilizing anti-immunoglobulin sera (Peskar *et al.*, 1973 and Van Vunakis *et al.*, 1971). Although these assays are highly specific and sensitive for screening tests, the anti-drug sera, to date, have been poorly characterized. The average intrinsic association constants (K_o) and heterogeneity indices (**a**) of the various anti-drug preparations cited above are unknown. To characterize anti-drug antisera, we have developed means to study and measure the binding properties and heterogeneity of anti-lysergyl antibodies in gamma globulin fractions. Measurements derived from analyses of the gamma globulin fractions have been compared to antibodies purified by immunoadsorption.

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MATERIALS AND METHODS

Lysergyl-poly-L-lysine

Poly-L-Lysine (PLL) (Miles-Yeda Ltd., Lot No. 152, M.W. 85,000) was dissolved (10 mg/ml) in 0·10 *M* NaCl and to 10 ml of PLL was added 50 mg of D-lysergic acid (LSA) (Sigma Chemical Co.) dissolved in 2·0 ml pyridine. After the pH was adjusted to 7·0, 200 mg 1-ethyl-3(3-dimethylaminopropyl-carbodiimide hydrochloride (Pierce Chemical Co.) was added and allowed to react 24 hr at room temperature with stirring (shielded from light). Exhaustive dialysis against 0·05 *M* phosphate buffer, pH 8·0, was used to eliminate unconjugated reactants and by-products. Determination of LSA:lysine ratio was based on dry weight analysis and absorbance at 310 nm (E_M = 8300, Voss and Berger, 1972). Under the conditions described, an average of one mole of lysergic acid was conjugated per 10 mole of lysine or approximately 58 LSA residues per poly-lysine polymer. Figure 1 shows the absorption spectra of lysergyl-poly-lysine and lysergic acid.

DNP–PGG

Dinitrophenylated porcine gamma globulin (DNP–PGG) and dinitrophenylated bovine serum albumin (DNP–BSA) were prepared and characterized as described by Eisen (1964). The average number of DNP molecules conjugated per mole of protein was 59 and 24 respectively.

Succinylated KLH

Hemocyanin (KLH) isolated from the Keyhole limpet, *Megathura crenulata*, was obtained from Pacific Bio-Marine Supply Co., Venice, California, and purified according to the method of Campbell *et al.* (1963). To 60 mg KLH dissolved in 2·0 ml phosphate buffer, pH 7·0, was added 1 g succinic anhydride in small increments. The pH was maintained at 7·0 with the addition of 2·0 *N* NaOH. After addition of the anhydride over a 30-min period, the reaction was dialyzed against distilled water to remove free acid.

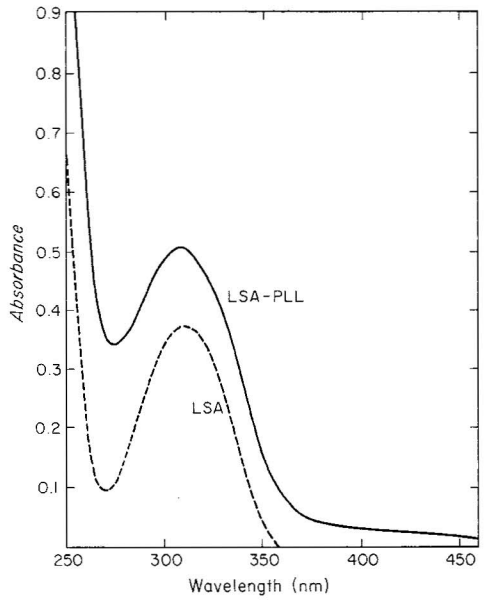

Fig. 1. Comparison of u.v. spectra of (-----) lysergic acid (LSA) (47 μM, 0·05 *M* PO_4, pH 8·0) and (———) lysergyl-poly-L-lysine (LSA–PLL) (100 μg 1 ml, 0·05 *M* PO_4, pH 6·5). Measurement made on Cary 14 spectrophotometer.

Preparation of antisera

Adult albino rabbits were immunized in the hind foot pads and intrascapularly with a total of 5 mg lysergyl-poly-L-lysine electrostatically complexed to 2 mg succinylated KLH and emulsified in an equal volume of complete Freund's adjuvant. A separate group of rabbits received a total of 5 mg of DNP_{50}PGG emulsified in an equal volume of complete Freund's adjuvant in a similar manner. Rabbits were boosted with equivalent amounts of immunogen, by the routes of immunization described above, at 35 days. Bleedings were obtained by cardiac puncture at 42, 49 and 56 days.

Serum fractionation

Lipoprotein was removed by dextran sulfate precipitation. Five milliliters of 5 per cent dextran sulfate 500 (Pharmacia Fine Chemicals) was added to 100 ml serum and stirred briefly at 4°C. $CaCl_2$ (9·0 ml, 11·1%) was added and stirring continued for 30 min. The lipoprotein–dextran sulfate precipitate was pelleted by centrifugation at 12,000 g for 10 min. An equal volume of saturated ammonium sulfate (SAS) was added to the delipified serum and allowed to incubate at 4°C for 16 hr. Precipitated protein was pelleted by centrifugation, resuspended in 100 ml of 0·02 *M* phosphate buffer, pH 8·0 and precipitated a second time in an equal volume of 80% SAS. The precipitate was pelleted, dissolved in a minimal volume of phosphate buffer and dialyzed exhaustively against 0·02 *M* phosphate. Resolution of the IgG fraction was accomplished by chromatography on DEAE-cellulose (2 × 18 cm) equilibrated in 0·02 *M* phosphate, pH 8·0. IgG was eluted in the void fraction with 0·02 *M* phosphate, pH 8·0.

Preparation of immunoadsorbants

DNP–BSA–BAC. Ten grams of bromoacetyl cellulose (BAC) were reacted with 3·0 g of DNP_{24} Bovine Serum albumin (DNP_{24}BSA) in 0·50 *M* acetate buffer pH 5·5 as described by Robbins *et al.* (1967) was modified by Gallagher and Voss (1969). Freshly prepared adsorbant was washed exhaustively in 8·0 *M* urea, 10% dioxane in 1·0 *N* acetic acid, and 6·0 *M* guanidine–HCl.

LSA–BSA–BAC. Ten grams of BAC were reacted with 3·0 g BSA in 0·50 *M* acetate buffer, pH 5·5. After incubation in 0·10 *M* bicarbonate, the conjugated cellulose was washed in 8·0 *M* urea and 0·1 *M* NaCl and suspended in an equal volume of 0·1 *M* NaCl. To the adsorbant was added 100 mg LSA dissolved in 5·0 ml pyridine. After adjusting the pH to 7·0, carbodiimide (1 g) was added and allowed to react 24 hr at room temperature protected from light. Exhaustive washing in 10% dioxane in 1 *N* acetic acid and 6 *M* guanidine–HCl preceded use in antibody purification studies.

Purification of antibody

To 25 ml of antisera was added 3·0 ml packed volume of the immunoadsorbant. Adsorption proceeded for 2 hr at room temperature under mild stirring conditions. Following 4–5 washes with the standard buffer, the immunoadsorbant was subjected to two incubations at 37°C for 1 hr, with 0·1 *M* N-CBZ–glycine (N-carbobenzoxy–glycine, Sigma Chemical Co.) in the standard buffer. Incubations or mock elutions were controls for temperature, buffer, time, and ionic conditions. All supernates were monitored at 278 nm and 360 nm for DNP, or 310 nm for LSA. Antibody was eluted with 10% dioxane in 1 *N* acetic acid (Hill, 1972) at room temperature for 30 min. Eluants were immediately dialyzed against cold standard buffer to minimize protein denaturation.

Antisera screening

Dialysis binding assay. Ability of antisera fractions to bind LSD ligand was measured by the retention of ^{3}H-LSD (New England Nuclear, Lot No. 692-030, Sp. Act. 3·6 Ci/mmole, stock solution was diluted to 1 mCi/ml). Dialysis of fractions against 0·05 *M* phosphate buffer, pH 8·0 (two 4-l. changes) for 24 hr at 4°C appeared optimal to remove unbound ^{3}H-LSD. Increased dialysis time or additional buffer

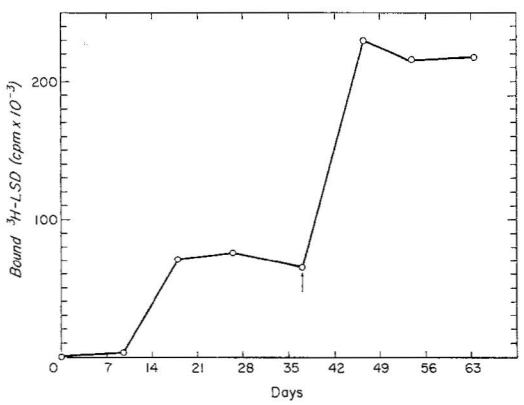

Fig. 2. Binding of ^{3}H-LSD (sp. act. 3·9 Ci/mmole) by rabbit antisera (50 μl) following immunization with lysergyl-poly-L-lysine complexed to succinylated KLH as measured by dialysis binding assay. Arrow indicates time of secondary immunization.

changes did not result in significant loss of bound ligand. These measurements were readily repeatable throughout the entire anti-lysergyl response (Fig. 2). To 1·0 ml of a serum fraction was added 10μl of ^{3}H-LSD. After incubation at 37°C for 1 hr, the sample was dialyzed against 0·05 *M* phosphate at 4°C, and an aliquot counted in a liquid scintillation spectrometer.

Radioimmunoassay. A radioimmunoassay was developed to screen sera and serum fractions for LSD binding. To a 1:10 dilution of sera in 0·05 *M* phosphate was added 10 μl (100,000 counts/min) ^{3}H-LSD and incubated 16 hr at 4°C. After sedimentation at 12,000 **g** for 10 min, followed were added and allowed to precipitate for 30 min at 4°C. After sedimentation at 12,000 **g** for 10 min. followed by a 50% SAS wash, the pellet was dissolved in 0·4 ml phosphate buffer and suspended in 8·0 ml toluene containing 0·4% PPO and 10% Biosolve BBS-3 (Beckman Instrument Co.). IgG fractions were screened in a similar manner with the exception that they were dissolved in 3·0% bovine serum albumin prior to the addition of ^{3}H-LSD to prevent non-specific precipitation of the label. Radioactivity determinations were performed in a Beckman LS-133 liquid scintillation spectrometer.

Equilibrium dialysis

Measurement of antibody binding affinity by equilibrium dialysis was performed as described by Eisen (1967). Samples (70 μl) of purified antibody or IgG fractions were dialyzed against 70 μl of increasing concentrations of ^{3}H-D-lysergic acid diethylamide (sp. act. 85,700 counts/min/nMole) or ^{3}H-ϵ-2,4-dinitrophenyl-L-lysine(sp.act. 27,500 counts/min/nMole) using lucite chambers (0·1 ml capacity/side). Antibody, IgG fractions and ligand were dissolved in 0·05 *M* phosphate buffer, pH 8·0. After 24 hr equilibration, 50-μl samples were removed from both sides of the chambers and counted.

To maximize ligand binding in equilibrium dialysis experiments, concentrations of IgG were increased to 8–10 mg/ml. Normal IgG controls at the same protein concentration and at each ligand concentration were included in all experiments to correct for possible osmotic effects.

Analog inhibition studies

Specificity of anti-lysergyl antibody was demonstrated by analog competition in equilibrium dialysis. To one side of the dialysis chamber was added 40 μl of ^{3}H-LSD at a concentration pre-determined to saturate 75 per cent of the antibody active sites. Varying concentrations of unlabeled LSD, LSA, tryptophan or indole acetic acid in 30 μl were added to the ligand side. Seventy microliters of the IgG fraction was added to the opposite well and allowed to equilibrate as described above.

Psychological assays

The effects of active immunization with LSA–PLL or passive administration of hyperimmune anti-lysergyl antibody upon mice under the influence of LSD were measured using the poke test (Cohen and Wakeley, 1968) and rearing test (Dandiya *et al.*, 1969). The poke test apparatus consisted of a 40 × 49 cm wooden platform containing 20 equally spaced holes (3 cm dia.) mounted on four legs, 48 cm in height. Individual mice were placed in the center of the grid and their exploratory movements recorded for 2 min. An ambulatory score was amassed for each mouse based on a total of the times a mouse poked his head into a hole, the number of squares explored and the frequency with which he looked over the edge of the platform. The rearing test is defined as the number of times a mouse stands on its hind legs during a 2-min period. Four groups of mice were utilized in this study. Group one (50 mice) served as the control group, receiving only an injection of saline prior to testing. Group two (15 mice) consisted of mice receiving 10μg LSD (100 μg/ml) intravenously (tail vein). Group three (15 mice) received 10 μg LSD, followed by passive administration of a hyperimmune IgG fraction μg/ml 15 mg/ml) of rabbit anti-lysergyl antibody. The amount of passive antibody (0·2 ml) was computed to provide an equivalent ^{3}H-LSD binding capacity to that of the actively immunized mice as measured in the radioimmunoassay. The final group (15 mice) contained mice intraperitoneally immunized with 500 μg LSA–PLL complexed to succinylated KLH as previously described. This group was boosted at 45 days and tested 14 days later with 10 μg LSD. All experiments involving LSD observed a waiting period of 15 min following drug administration to allow for drug action. The psychological tests were completed within 30 min following administration of the drug.

RESULTS

When the same antibody fraction was compared in the two binding assays, it was evident that the dialysis binding assay demonstrated almost equivalent drug binding as the radio-immunoassay but without the elevated background (Table 1) inherent in the latter. Since physical trapping of ^{3}H-LSD by ammonium sulfate precipitation resulted in elevated backgrounds, removal of unbound drug by dialysis is favored. All serum

Table 1. Comparative binding of ^{3}H-LSD ligand by anti-lysergyl antibodies in the dialysis binding assay and radioimmunoassay*

	Assay†,‡	
	Dialysis binding	Radioimmunoassay
Anti-lysergyl IgG fraction	131,128 3968	128,477 7482

* Representative data of a single experiment. Correlation between both assays was consistent in all experiments.
† Counts/min bound by 100 μg of protein.
‡ 5 × 10^5 counts/min ^{3}H-LSD (1·9 (1·9 Ci/m-mole) added.

Table 2. ^{3}H-LSD binding by normal rabbit serum fractions in the dialysis binding assay

Fraction*	CPM Bound (25-μl sample)
Whole serum	14,884
40% SAS† precipitate	455
40% SAS supernate	14,072
IgG fraction‡	284

* Samples resuspended to original serum volume.
† Saturated ammonium sulfate.
‡ Purified by DEAE anion chromatography, 0·02 *M* PO_4, pH 8·0 elution. Protein concentration adjusted to 10 mg/ml in 0·05 *M* PO_4, pH 8·0.

fractions were screened with the ligand dialysis assay. As shown by Winkelhake *et al.* (1973), non-immunoglobulin serum proteins bind the LSD ligand. Table 2 shows that drug binding by non-immunoglobulin fractions is diminished upon fractionation of the sera with ammonium sulfate and is further reduced upon resolution of the IgG fraction by DEAE chromatography. All sera were fractionated with ammonium sulfate, as previously described, to remove the source of nonspecific LSD binding. The kinetics of the immune response to the lysergyl-poly-L-lysine immunogen, as measured by the ligand binding assay, is shown in Fig. 2.

Purification of anti-lysergyl antibody

Pooled hyperimmune anti-lysergyl antisera were obtained from bleedings 7–21 days after the secondary immunization. Preliminary attempts at purification of the antibody from serum failed as a result of nonspecific trapping and retention of non-immunoglobulin serum proteins by the immunoadsorbant. Elutions of the immunoadsorbant with dioxane/acetic acid were performed since little protein was specifically eluted with lysergic acid at saturating concentration ($\sim$1 mg/ml, pH 8·0). Equilibrium dialysis measurements of such ligand elutions showed no binding of ^{3}H-LSD. Adsorption of nonspecific serum proteins was reduced by ammonium sulfate precipitation and anion exchange DEAE chromatography prior to immunoadsorption. Purity of eluted rabbit IgG was verified by immunoelectrophoresis against goat anti-rabbit serum and acrylamide disc gel electrophoresis in 6·0 *M* urea. Acrylamide disc gel electrophoresis indicated that adsorption of normal sera with LSA–BSA–BAC removed IgG as well as other serum proteins nonspecifically. Since normal IgG binding to the immunoadsorbant was significant, it could be expected that adsorption of IgG fractions would not result in pure anti-lysergyl antibody. Rather, dioxane/acetic acid elution would result in a mixture of antibody and normal IgG. While the inactive portion of the eluted IgG fraction might be considered 'low affinity' anti-lysergyl antibody it is functionally inactive as measured within the limits of equilibrium dialysis. Adsorbed normal IgG possessed no measurable ^{3}H-LSD binding. Based on this observation, enrichment for anti-lysergyl antibody, rather than purification was attempted. Immunoadsorption of the IgG fractions of hyperimmune sera was performed as described in Materials and Methods. The degree of enrichment for anti-lysergyl antibody was monitored by the ligand dialysis assay. Adsorbed IgG antiserum fractions demonstrated a 75–80 per cent decrease of ligand binding compared to unadsorbed IgG. Ligand binding of the eluted IgG indicated a 3–4-fold enrichment of antibody.

Modified equilibrium dialysis

To determine the binding characteristics of the unadsorbed antibodies, equilibrium dialysis experiments were performed with IgG fractions of hyperimmune anti-lysergyl antisera.

Since the concentration of specific anti-LSA antibodies in the IgG fraction was unknown, a preliminary plot of the data from equilibrium dialysis was required

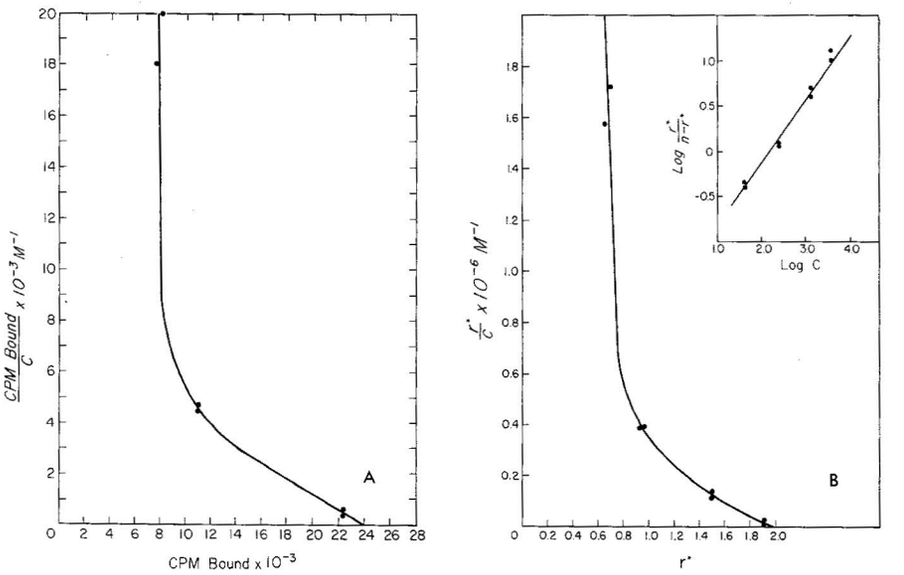

Fig. 3. Equilibrium dialysis of the IgG fraction of hyperimmune rabbit anti-lysergyl-poly-L-lysine antiserum. Equilibration at 4°C for 24 hr. Panel A: Binding of ^{3}H-LSD (sp. act. 85,700 counts/min/nMole) extrapolated to maximum binding (intercept) at infinite ligand concentration. Protein concentration 5·7 mg/ml (0·05 *M* pH 8·0). Panel B: Scatchard plot based on extrapolated value of maximum counts bound by antibody fraction at saturation levels of ligand, where $r = 2$. $^*r$ = (ligand bound/maximum ligand bound) × 2. Insert: Sips plot of data derived from equilibrium dialysis.

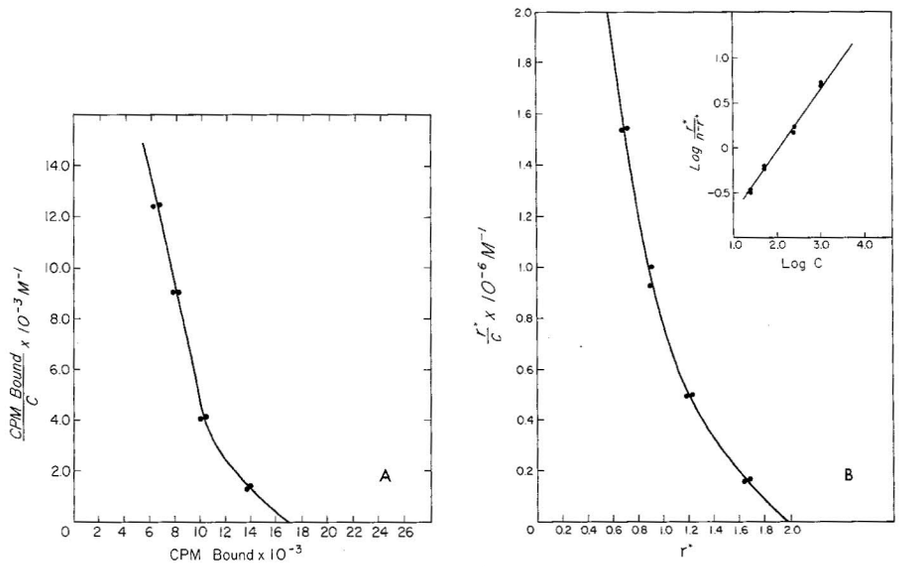

Fig. 4. Equilibrium dialysis of the enriched IgG fraction of hyperimmune anti-lysergyl-poly-L-lysine. Equilibration at 4°C for 24 hr. Panel A: Binding of ^{3}H-LSD (sp. act. 85,700 counts/min/n-mole) extrapolated to maximum binding (intercept) at infinite ligand concentration. Protein concentration 0·9 mg/ml (0·05 *M* PO_4, pH 8·0). Panel B: Scatchard plot based on extrapolated value of maximum counts bound by antibody fraction at saturation levels of ligand, where $r = 2$. $*r$ = (ligand bound/maximum ligand bound) × 2. Insert: Sips plot of data derived from equilibrium dialysis.

in order to calculate Scatchard (1949) and Sips (1948) plots. Figure 3(a) illustrated the preliminary plot showing that a plot of counts/min bound/ligand concentration versus counts/min bound can be extrapolated to estimate the maximum counts that would be bound at infinite ligand concentration. This maximum value (intercept) is equal to the active site saturation of the specific anti-LSD antibody population within the IgG fraction. Because the fraction tested is pure IgG, a valence of two applies at saturation, and the amount of antibody is equal to one half of the moles of ligand bound. Therefore, the moles of ligand bound at saturation can be used to determine r (moles ligand bound/mole antibody) at each point in the equilibrium dialysis, since:

(ligand bound/maximum ligand bound) × 2 = r.

Determination of r enabled the data to be used in Scatchard and Sips plots as shown in Fig. 3(b). The enriched population (immunoadsorption) of anti-lysergyl antibody (Fig. 4) demonstrated a K_o of $7{\cdot}7 \times 10^5$ M^{-1} and a heterogeneity index (**a**) of 0·699, while the IgG fraction from which it was obtained possessed a K_o of $3{\cdot}5 \times 10^5$ M^{-1} and **a** of 0·704. Experience in this laboratory for the last several years indicates that the error between duplicate or triplicate equilibrium dialysis experiments on the same antibody preparation is ±2–3X. This is accepted as the inherent error in the assay, but is of course dependent on protein concentration, specific activity of the ligand, and the K_o of the preparation. The amount of antibody present in the unadsorbed hyperimmune IgG pool was determined to be 0·853 mg/ml, or about 14 per cent of the total IgG fraction.

The validity of the preceding calculations to accurately measure the K_o and heterogeneity index (**a**) was verified by studying purified anti-2,4-dinitrophenyl (DNP) antibodies derived from measured IgG fractions. Purified anti-DNP antibody tested possessed a K_o of $2{\cdot}9 \times 10^6$ M^{-1} and **a** of 0·695. The IgG fraction exhibited a K_o of $2{\cdot}25 \times 10^6$ M^{-1} and **a** of 0·658. The amount of antibody present in the IgG fraction, estimated by the number of moles of ligand bound in equilibrium dialysis, compared within 13 per cent of the actual amounts of antibody purified (Table 3).

Analog inhibition studies

Figure 5 compares the structures of D-LSD and several indole analogs. Since rings A and B are com-

Table 3. Comparison of predicted and recovered anti-2,4-dinitrophenyl antibody from an immunoadsorption of IgG fraction*

Elution	Total mg recovered
Dioxane	8·36
Guanidine–HCl	9·06
Total	17·42
Predicted	19·98†

* Fifteen milliliters (7·7 mg/ml).

† Based on extrapolation of equilibrium dialysis (recovered/predicted = 0·87).

Table 5. Effects of active immunization with LSA–PLL or passive administration of rabbit anti-lysergyl antibody on mouse performance in the poke and rearing tests while under the influence of 10 μg of LSD

Poke test	Mean ± S.D.	Percent change*	Analysis of variance§ Difference of means**	Confidence interval
1. Normal	41·8 ± 11·5	—	2–1	23·3 ± 9·2††
2. + LSD	65·1 ± 16·1	55·7	3–1	1·4 ± 9·2
3. + LSD, + Ab†	43·2 ± 5·1	3·3	4–1	1·1 ± 9·2
4. Active immun‡ + LSD	42·9 ± 5·2	2·6	2–3	21·9 ± 11·4††
			3–4	0·3 ± 11·4
			2–4	22·2 ± 11·4††
Rearing test				
1. Normal	12·9 ± 5·5	—	2–1	10·0 ± 4·8††
2. + LSD	23·0 ± 7·3	78·3	3–1	4·3 ± 4·8
3. + LSD, + Ab†	17·3 ± 4·8	34·1	4–1	2·4 ± 4·8
4. Active immun.‡ + LSD	15·4 ± 5·3	19·4	2–3	5·7 ± 5·9
			3–4	1·9 ± 5·9
			2–4	7·6 ± 5·9††

* Compared to normal values (unimmunized).

† Passive anti-lysergyl antibody administered in amounts calculated to yield equivalent ^{3}H-LSD binding capacity present in actively immunized mice. Binding capacity determined by modified Farr Assay.

‡ Mice injected with 500 μg LSA–PLL/succKLH in CFA and boosted at 45 days. Fourteen days after boost, mice were tested in the poke and rearing tests.

§ Scheffe simultaneous confidence intervals (Scheffe, 1959).

** Comparison of groups.

†† Significant difference of means at 0·05 level.

mon to all compounds studied, the effect of the physiological concentration of tryptophan on the binding of anti-lysergyl antibody to LSD was of interest. As shown in Fig. 6, there is no measurable inhibition of anti-lysergyl antibody binding to LSD at a tryptophan concentration of less than 71 μM/L (10 nM added). This represents a 4–6-fold excess over the physiological concentration of the unbound tryptophan (McMenamy *et al.*, 1961). The indole analog, indole acetic acid, is a poorer inhibitor than tryptophan. All analogs were measured up to the limits of their solubility at physiological conditions. LSD and LSA demonstrate equivalent inhibition of ^{3}H-LSD binding at 50 per cent inhibition with 1·0 nmole, and gave measurable inhibition as low as 0·001 nmole.

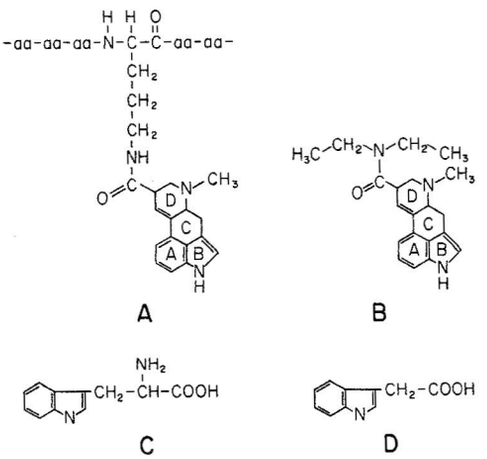

Fig. 5. Structures of (A) lysergyl conjugated polymer, (B) D-lysergic acid diethylamide, (C) tryptophan, and (D) indole-acetic acid. The letters assigned to the aromatic rings in A and B are based on accepted nomenclature (Baker *et al.*, 1972).

In vivo neutralization by anti-lysergyl antibodies

When measured independently, the indole alkaloids found in sera are poor inhibitors of the specific lysergyl-anti-lysergyl interaction (Van Vunakis *et al.*, 1971).

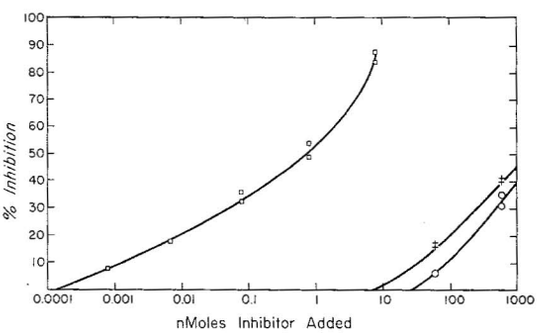

Fig. 6. Analog ligand inhibition of ^{3}H-LSD binding in equilibrium dialysis. ^{3}H-LSD concentration (sp. act. 85,700 counts/min/nmole) adjusted to saturate 75 per cent of available antibody sites. A protein concentration of 5·7 mg/ml (0·05 M PO_4, pH 8·0) was utilized throughout the experiment. Thirty microliters of (□—□) LSD/LSA, (○——○) indole-acetic acid, and (+——+) tryptophan in increasing concentrations dissolved in 0·05 M PO_4, pH 8·0 were added to the ligand side of the chambers. Equilibrated at 4° C for 24 hr.

Table 4. Comparison of the ^{3}H-LSD binding capacities of anti-lysergyl IgG fractions by the dialysis binding assay

Fraction	Ligand bound* $\times 10^{-6}$ counts/min	% Bound	Mg antibody protein	Total % protein
Anti-lysergyl IgG fraction	98·2	100·0	21·25†	100·0§
Purified anti-lysergyl antibody	40·2	40·9	6·54†	30·7
Adsorbed anti-lysergyl IgG fraction	27·2	27·7	11·51†	54·2
Normal IgG	0·36	0·36	——	
Unaccounted**	30·8	31·4	—	—
6·0 *M* Guanidine–HCl elution	—	—	3·2‡	15·1

* All values corrected to binding by 25 ml of original serum.
† Based on extrapolation in equilibrium dialysis.
‡ Based on absorbance at 278 nm ($E^{1\%} = 15$).
§ 100% value is based on equilibrium dialysis of anti-lysergyl IgG fraction.
** 100% minus total measurable binding.

The question arises if these indole compounds can act in concert to effectively inhibit anti-lysergyl antibody. To measure the extent to which this is possible an *in vivo* system measuring the neutralization of LSD by anti-lysergyl antibody was employed.

As shown in Table 5, administration of 10 μg LSD caused a 55·7 per cent increase in the ambulatory score as measured by the poke test. Passive administration of hyperimmune antibody or active immunization resulted in nearly total neutralization of the drug effect. Measurements utilizing the rearing test yielded similar data, although the degree of neutralization was not as complete. Differences between the two tests may reflect measurements of differing psychological parameters. However, both assays indicated that the anti-lysergyl antibody is able to interact with LSD without significant competition from other indole alkaloids in the serum.

DISCUSSION

To screen sera for the presence of anti-lysergyl antibody, two radioimmunoassays were developed with sufficient sensitivity to detect antibody in serum obtained 7 days after primary immunization. Since the actual ligand (LSD) concentration approaches 10^{-10} *M*, both the dialysis binding assay and the Modified Farr assay are biased for binding by high affinity antibodies which are present as early as 7 days after primary immunization and increase in concentration proportionately with time. While both assays possess equivalent sensitivity, the dialysis assay is characterized by a lower background. Higher backgrounds in the modified Farr assay result from nonspecific trapping of the label in the ammonium sulfate precipitate. Because only 1/100 of the ligand used in the dialysis binding assay is required in the modified Farr assay, it is the preferred assay for routine screening of serum fractions.

While these assays were designed to monitor antibody concentrations and specificity rather than to detect the drug in sera, the potential for drug screening is evident. A major consideration in drug detection must concern itself with the problems of protein bound vs free drug and the equilibrium between the two. Detection of the drug is probably limited to that of the free or unbound form. Total drug detection would require measurement of both free and bound, which is not possible with this assay.

Previous investigators (Werblin and Siskind, 1972) have measured antibody affinity and heterogeneity in 50 per cent saturated ammonium sulfate fractions of DNP–protein antisera. Measurements of affinity and heterogeneity do not preclude, however, the presence of non-immunoglobulin or multiple immunoglobulin class interaction. Potentially, significant levels of non IgG immunoglobulin may introduce variation in the measured K_o and heterogeneity index (**a**) of SAS serum fractions. For example, a minor population of IgM antibodies possessing a K_o of 10^{-4} to 10^{-5} M^{-1} (Voss and Eisen, 1968) throughout the immune response to a particular immunogen while the binding affinity of IgG continues to increase, could result in a bimodal representation of the binding data. In order to circumvent the possibility of nonspecific or multi-class interactions, DEAE fractionation of the 40 per cent SAS fraction was done to ensure only IgG participation in the binding studies and to validate the assumption of a valence of two. As shown in Table 2, normal serum possesses significant nonspecific binding of ^{3}H-LSD.

Determination of K_o and **a** of anti-DNP and anti-lysergyl antibody in both purified and IgG fractions compared favorably and substantiated the observation by Eisen and Siskind (1964) that purified antibody possesses binding properties representative of the antibody population present in serum. Although the K_o and **a** value determinations for purified antibody and antibody in IgG fractions closely compare (within

limits of error), some selectivity by immunoadsorption is evident. There appears to be a significant population of high affinity antibody that cannot be dissociated from the immunoadsorbant, as well as a distinct population of low affinity antibody which is not adsorbed by the immunoadsorbant. It is evident from Table 4, that while the purified anti-lysergyl antibody represents 30·7 per cent of the total anti-lysergyl antibody in the IgG fraction, it possesses 40·9 per cent of the binding capacity in the ligand dialysis assay. Examination of the once-adsorbed anti-lysergyl IgG fraction indicates that while only 27·2 per cent of the ligand binding remains, it represents over 50 per cent of the antibody in the unadsorbed IgG fraction. An additional 31·4 per cent of the binding capacity of the IgG fraction is accounted for by the 3·2 mg or 15 per cent more protein that is eluted only by 6·0 *M* guanidine–HCl From these data, the selective bias of the immunoadsorbant for the average affinity antibody is demonstratable. These studies represent the direct measurement of the K_o and **a** for hyperimmune anti-lysergyl-polylysine antibody as $3{\cdot}5 \times 10^5\ M^{-1}$ and 0·704 for an unpurified IgG fraction and $7{\cdot}7 \times 10^5\ M^{-1}$ and 0·699 for enriched IgG fraction.

Figure 5(a) shows the structure of LSD. Measurements of anti-lysergyl binding and analog inhibition studies indicate that rings C and D and the side groups comprise the immunodominant portion of the molecule. Inhibition of ^{3}H-LSD binding by either tryptophan or indoleacetic acid (which represent the A and B ring portion of the LSD molecule) is less than 50% at concentrations up to 1000 nmole/140 μl in equilibrium dialysis. Since immuno-drug screening in biological fluids competes with background indole alkaloid concentrations of approximately 10^{-6} *M* (McMenamy *et al.*, 1961), it is of utmost importance that these levels are not inhibitory to the anti-drug antibody assay. As shown in Fig. 6, the maximum concentration of unbound tryptophan (approximately 18 μM/L) found in normal sera is significantly below inhibition levels as measured in equilibrium dialysis.

Van Vunakis *et al.* (1972), compared the inhibitory ability of various indole alkaloids and demonstrated that LSD inhibited antigen binding 3·4-fold better than lysergic acid in a polyvalent radioimmunoassay. In Fig. 6 no measurable difference in inhibition of equilibrium dialysis was noted by either LSD or LSA. Recent reports (Inman *et al.*, 1973; Gopalkrishnan *et al.*, 1973) have indicated that measurement of antibody affinity with relatively large ligands, which tend to spatially fill the antibody active site (mol. wt 800–1000) yield relatively high binding constants (K_o). Since polyvalent antigens (Van Vunakis *et al.*, 1971) contain the same carrier linkage found in the immunogen, they more closely represent a 'total fit' characteristic of large hapten systems with high binding constants. In addition, haptens such as fluorescein (Lopatin and Voss, 1971) have been utilized to determine the effect of side group modification on ligand binding. Recent evidence (Voss and Gass, 1974) suggests that with increase in size of the antigenic determinant, there is increased specificity for the homologous antigen and decreasing tolerance for modifications in the immunodominant portion of the antigen. Figure 5(a) portrays a portion of the polyvalent test antigen (LSA–heteropolymer). Upon carbodiimide linkage of the LSA molecule to the lysine ϵ-amino groups of the polymer, the side group portion (i.e. LSA–NH–LYS) of the immunogen is recreated. Since this linkage presents a more complete antigenic determinant than does the ligand LSD (Fig. 7b), stronger binding is evident resulting in a more sensitive measurement due to the additional side group interactions. Ligand inhibition studies of antilysergyl antibodies reacting with hapten conjugates are able to differentiate between LSD and LSA because of the absence of the alkyl-amide moiety of the antigenic determinant. In contrast, assays such as equilibrium dialysis, utilizing LSD as the diffusible ligand, appear limited in sensitivity to side group differences since they do not test as great a portion of the antibody active site.

The radioimmunoassay (Berson and Yalow, 1970) represents an immunological tool to measure cross reaction. Since the sensitivity of the assay relies on highly diluted antisera and a radiolabeled hapten or antigen of high specific activity, the binding measurements are reflections of the high affinity molecules in the antibody population. The amount of labeled ligand added is small because of the high specific activity, and, therefore, the analogs are able to compete quite successfully at much lower concentration than alternative assays. Since the higher affinity populations may not, however, be representative of the entire complement of antibody, we chose to measure cross reaction of analogs in equilibrium dialysis, utilizing the full spectrum of antibody affinities. In addition, use of polyvalent antigens introduces an avidity factor which can amplify the binding of lower affinity antibody, an effect not measurable in equilibrium dialysis.

Finally it is apparent from *in vivo* drug neutralization studies that while the indole moiety is common to many low mol. wt compounds found in sera, as well as to the lysergyl group, the critical immunodominant determinant of the LSD molecule must reside in the C and D rings rather than in A and B. In the presence of indole alkaloids at concentrations normally found in sera, the anti-lysergyl antibody remains able to bind LSD indicating that the degree of inhibition is minimal.

In summary, the utilization of IgG fractions of antidrug sera enable measurements of affinity and heterogeneity without introduction of the selective biases of immunoadsorption and other purification techniques such as immunoprecipitation.

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