combined ether solution (about 2.5 liters) was subjected to mild alkali extraction (5% aqueous sodium carbonate). The alkaline phase was carefully neutralized with 10% aqueous hydrochloric acid, extracted with ether, and dried, and the solvent was removed *in vacuo*.

The resulting product (26 g) was then subjected to silica gel (130 g) column chromatography. The column was eluted with hexane–ether (4:1) followed by ether, acetone, and methanol. Fractions from the hexane–ether eluate, which showed two spots on TLC, were combined, and the solvent was removed *in vacuo*. The residue was treated with an excess of acetone, stirred, and filtered. The filtrate, on concentration *in vacuo*, gave a buff-colored precipitate, which was removed by filtration. On further concentration followed by cooling, the filtrate gave tiny yellow needles of gossypol, which were collected by filtration. More crystals were obtained from the mother liquor by repeating this step.

Crystallization from methylene chloride–petroleum ether yielded pure gossypol in long brilliant golden-yellow needles, mp 178–180°; this melting point was not depressed by mixing with an authentic sample[4]. Several melting points have been reported for gossypol: 184° (5), 199° (6), and 214° (7); gossypol exists in three crystalline modifications, each with a characteristic melting point.

All spectra obtained by UV, IR, PMR, and mass spectrometry agreed with literature data (8–10).

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COMMUNICATIONS

Isolation of Lysergide (LSD) with Agarose-Bound Antibodies to Lysergic Acid

Keyphrases □ Lysergide—isolation, agarose-bound antibodies to lysergic acid, column chromatography □ Antibodies—lysergide binding, coupled to agarose, isolation of lysergide

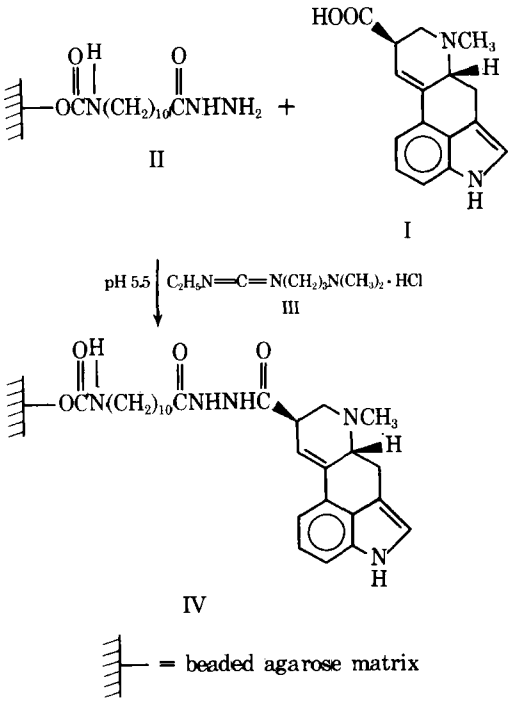
To the Editor:

Several research groups (1–3) have obtained antiserums specific to lysergide (D-lysergic acid diethylamide, LSD). These antiserums have been useful in the development of sensitive and specific radioimmunoassay procedures for lysergide in plasma and urine (2, 3).

We have now succeeded in purifying lysergide-binding antibodies from sheep antiserum, utilizing affinity chromatographic (4) columns prepared by covalent binding of D-lysergic acid to a suitable derivative of beaded agarose. Purified specific antibodies are themselves coupled to beaded agarose and subsequently utilized for the selective absorption of lysergide from solution. Elution of lysergide from the column is readily achieved.

D-Lysergic acid (I) was readily coupled to the long chain hydrazide derivative of beaded agarose (II) (5) (Scheme I). To 10 g of II was added 40 mg of D-lysergic acid dissolved in 32 ml of saline–phosphate buffer (0.15 *N* NaCl and 0.01 *M* phosphate, pH 7.4) containing 5% ethanol. Then 50 mg of water-soluble carbodiimide reagent (III) was added, and the pH was adjusted to 5.50. After stirring for 24 hr, the agarose was washed thoroughly with buffer and made up to a final volume of 20 ml. The washings were analyzed for uncoupled D-lysergic acid by UV absorbance at 308 nm and the resultant affinity adsorbent (IV) was calculated to contain 2.5 μmoles (0.67 mg) of D-lysergic acid/g.

To a column of IV (1.5 ml, 0.5 × 15 cm, packed in pH 7.4 buffer) was added 1.0 ml of undiluted lysergide antiserum obtained from sheep after immunization with a lysergic acid–human serum albumin conjugate (3). [This antiserum (10 μl) showed the ability to bind 50% of 1 ng of tritium-labeled lysergide at a dilution of 1:1000 (3).] One hour was allowed for equilibration prior to elution from the column. Both protein absorbance (280 nm) and ability to bind tritiated lysergide in the radioimmunoassay system were monitored.



Scheme I—Coupling of D-lysergic acid (I) to long chain hydrazide derivative of agarose (II)

The results (Fig. 1) show that the lysergide antibody was indeed retained; it could be eluted by the use of 0.1 *M* acetic acid, 0.01 *N* HCl, 6 *M* guanidine, lysergic acid, or lysergide. Fractions eluted with acid were neutralized with sodium hydroxide to pH 7.4 (phenol red) immediately upon collection. Other protein components of the serum, with no lysergide-binding ability, passed through the column in early fractions. In several experiments, it was determined that the protein content of the lysergide binding effluent was approximately 0.9 mg of protein/ml of original antiserum (6.0 nmoles/ml). As shown in Fig. 1, about two-thirds of this amount was eluted from the column with dilute hydrochloric acid; the remaining third required the use of 6 *M* guanidine hydrochloride (later removed by dialysis).

Microzone electrophoresis studies on material from fraction 11 (Fig. 1) indicated the presence of a faint band in the γ-globulin region and the absence of other protein bands characteristic of the whole antiserum components. This material was employed in subsequent studies.

For coupling of the agarose–hydrazide derivative (II) to the specific lysergide antibody, the hydrazide was first activated by conversion to the corresponding azide with nitrous acid (5). Three grams of the cold, freshly activated, azide derivative was then added to a cold solution of a mixture of 0.3 mg of antilysergide and 5 mg of bovine serum albumin carrier in phosphate buffer (pH 7.4). After 20 hr, the mixture was filtered and washed well with buffer. As a control preparation, 5 mg of bovine serum albumin alone was coupled to II under identical conditions. From subsequent calculations, it was determined that the coupled antilysergide content of the agarose was about 4.7×10^{-5} μmole/ml of agarose (sufficient

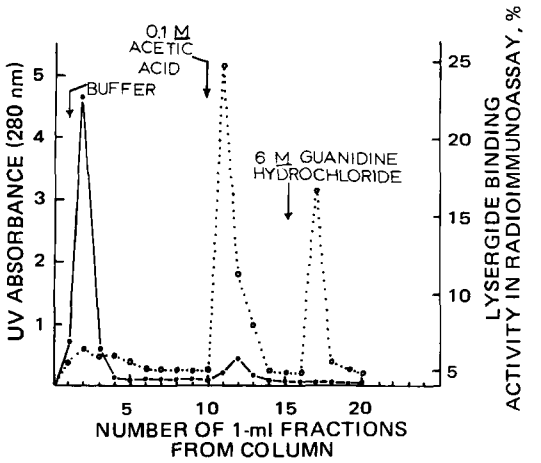


Figure 1—*Selective adsorption of specific lysergide antibodies from sheep antiserum by means of D-lysergic acid immobilized on beaded agarose (IV). Curve A (solid line) indicates protein retention by the affinity column as measured by UV spectral analysis of effluent fractions. About 8% of the protein was specifically retained. Curve B (dotted line) indicates that the major portion of the immunochemical binding ability of the effluent was contained in the fractions eluted with acid or guanidine hydrochloride. Bovine serum albumin (0.5%) was added to the elution buffer for stabilization of the antibody activity.*

to bind 30 ng of lysergide, with each divalent antibody binding 2 moles of lysergide).

To determine the selective retention of lysergide by the column, tritiated lysergide of specific activity 1.9 Ci/mmole was employed[1]. The radioactive lysergide (1 or 5 ng in a typical run) was placed in 1.0 ml of buffer on the column of adsorbent (1.5 ml as before) and allowed to equilibrate for 1 hr. Elution was accomplished with buffer or 0.01 *N* hydrochloric acid (Fig. 2). One-milliliter effluent fractions were counted *via* scintillation counting techniques, and the quantities of lysergide in each fraction were calculated.

Under these conditions, typical results indicated 64% retention of 1 ng of lysergide and 79% retention

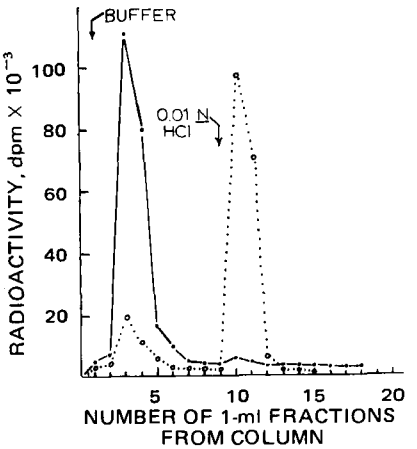


Figure 2—*Selective removal of tritiated lysergide from buffer by insolubilized antibodies. Curve A (solid line) indicates bovine serum albumin coupled to the matrix. Curve B (dotted line) indicates bovine serum albumin plus antibody coupled to the matrix.*

[1] New England Nuclear Corp., Boston, Mass.

of a 5-ng sample. Retained material was eluted from the column with 0.01 N hydrochloric acid. Under identical conditions, the column composed of coupled bovine serum albumin alone retained less than 1% of the radioactive material. Essentially identical results were obtained when ^{3}H-lysergide was added initially to the columns in pooled human plasma or human urine rather than buffer. Columns appeared to be equally effective upon at least one reuse after washing thoroughly with 0.01 N hydrochloric acid and then buffer.

Upon further quantitation and development, and perhaps with the use of a more specific antibody of higher titer, the described techniques might be utilized to advantage in conjunction with quantitative radioimmunoassay determinations of lysergide, either to isolate selectively very small quantities of lysergide far below the current sensitivity capabilities or to separate lysergide from tissue homogenates which may contain materials that render present assay methods inexact. Such methodology may be of importance in the study of lysergide levels in the brain and other organs.

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Determination of Protein Binding Parameters in Systems Involving Interaction between Sites

Keyphrases □ Protein binding—determination of parameters in systems involving interaction between sites, evaluation of method □ Binding, protein—parameters, interaction between sites, evaluation of method □ 1-Anilinonaphthalene-8-sulfonate—discussion of binding parameters

To the Editor:

A recent report by Ma *et al.* (1) utilized a log Q *versus* $\bar{\upsilon}$ plot to determine the protein binding parameters of 1-anilinonaphthalene-8-sulfonate in systems where interactions between binding sites were assumed to be occurring. As a result of this study, it is our feeling that the basic concepts underlying such systems and the application of the log Q *versus* $\bar{\upsilon}$ plot (2) to them should be reviewed. The intent of this communication is to ensure the proper use of the log Q *versus* $\bar{\upsilon}$ treatment by investigators encountering binding behavior similar to that reported by Ma *et al.* (1) for the interaction of 1-anilinonaphthalene-8-sulfonate with serum albumin.

The log Q *versus* $\bar{\upsilon}$ method applies to systems involving interactions between binding sites when n (number of) binding sites are initially all equivalent insofar as the binding of a drug molecule is concerned. As soon as one such molecule is bound, however, the affinity constant for a subsequent molecule's binding to a neighboring site is altered. The binding of successive molecules further alters the affinity constant for a remaining unoccupied site. Thus, in the case of n sites, the intrinsic binding constants $K_1, K_2, \ldots, K_n$ are not equal but may progressively increase or decrease. To calculate affinity constants under such conditions, a function Q is used:

$$Q = \bar{\upsilon}/(n - \bar{\upsilon})[D] \qquad \text{(Eq. 1)}$$

where $\bar{\upsilon}$ is the number of moles of drug bound per mole of protein at a free drug concentration $[D]$, with n representing the number of binding sites possible (2). A plot of log Q *versus* $\bar{\upsilon}$ is made, and the limiting values at $[D] \rightarrow 0$ and $[D] \rightarrow \infty$ give K_1 and K_n, respectively. Also, the limiting slopes, $d \ln Q/d\bar{\upsilon}$, at $\bar{\upsilon} \rightarrow 0$ and $\bar{\upsilon} \rightarrow n$ give $(n - 1)(K_2 - K_1)/nK_1$ and $(n - 1)(K_n - K_{n-1})/nK_{n-1}$, respectively (2). The log Q *versus* $\bar{\upsilon}$ plot is generally appropriate when K values are close (ratio of largest to smallest much less than 1000).

In using the log Q *versus* $\bar{\upsilon}$ plot, it should be noted that Eq. 1 implies that the value of n must be known or at least inferred with confidence in order to make a determination of the intrinsic binding constants. Ma *et al.* (1) suggested that n can be determined as that assumed value that best provides a linear log Q *versus* $\bar{\upsilon}$ plot. This usage is not proper, because the semilogarithmic plot need not approach linearity even when the value for n is known. As indicated by Eq. 1, the relationship between log Q and $\bar{\upsilon}$ is strictly linear only when the n binding constants are independent and equivalent. In such a situation, Q obviously is independent of free drug concentration and, indeed, is equal to the intrinsic binding constant. Thus, with independent and equivalent sites, a plot of log Q *versus* $\bar{\upsilon}$ is linear with a slope of zero.

When the binding sites are not independent, *i.e.*, when there is enhanced or inhibited binding of a small molecule to an available site, two situations may arise. In one case, the ratio of intrinsic binding constants, $K_{(s+1)}/K_{(s)}$, is a constant independent of s for all values of s from 1 to n (2). The constant ratio may be greater or less than unity, depending on whether interactions result in facilitated or inhibited binding. In the other system, the ratio of binding constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of s from 1 to n. Both systems will be considered.

In the first case, where a constant ratio of binding constants is present, a log Q *versus* $\bar{\upsilon}$ plot may have a region of linearity but the plot would not be expected