SYNTHESIS, ISOLATION AND IMMUNOCHEMICAL CHARACTERIZATION OF N-(CARBOXYMETHYL-^{3}H)-D-LYSERGAMIDE

Jeffrey L. Winkelhake and Edward W. Voss, Jr.
Department of Microbiology, University of Illinois
at Urbana-Champaign, Urbana, Illinois 61801, U.S.A.
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SUMMARY

Covalent amide linkage of glycine-2-^{3}H to the non-hallucinogenic derivative, d-lysergic acid (LSA), by carbodiimide activation results in a lysergic acid analogue which has potential use in biochemical studies of the mode of action, metabolism and immunology of d-lysergic acid diethylamide (LSD). Optimal conditions for the reaction and isolation of the product, N-(carboxymethyl-^{3}H)-d-lysergamide, were determined. The product was analyzed by radioimmune assay and by equilibrium dialysis with highly specific antibody to the lysergyl moiety.

INTRODUCTION

The diethylamide group, benzene ring A and the N-6 nitrogen on lysergic acid are essential for the hallucinogenic properties of LSD (Figure 1) (1). However, the interrelationship between these structural features and the physiological effects of LSD are not known. While LSD:nucleic acid interactions (2,3) and LSD:protein interactions (4-8) have received considerable attention, information describing biochemical modes of action of the psychotomimetic drug remains conjecture (9).

In order to trace the biochemical events associated with LSD metabolism,

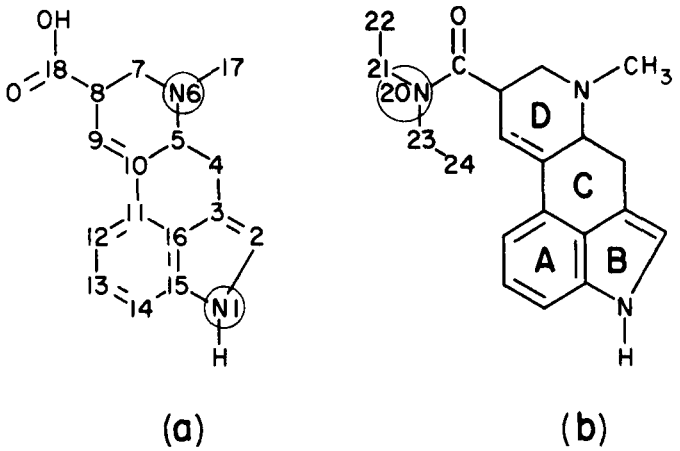

Figure 1. Diagramatic structure of : (a) d-lysergic acid (LSA) and (b) d-lysergic acid diethylamide (LSD). Designation of rings, A - D, and numbers, 1 - 24, is based on accepted nomenclature (25).

it is useful to have a radioactively labeled form of the drug. Generally labeled (^{3}H) LSD is difficult to synthesize by the classical Wilzbach reaction (10) because of acid lability of the indole ring. In addition, it is difficult to assess biochemical events associated with LSD metabolism utilizing generally labeled compounds.

In this paper we report the synthesis of a specifically labeled LSA analogue which has proven useful in studies of in vitro LSD:protein interactions. Glycine-2-^{3}H was linked by amide bond to the lysergyl moiety by means of a two-step, water soluble carbodiimide activated reaction resulting in N-(carboxymethyl-^{3}H)-d-lysergamide [(CM-^{3}H)-lysergamide, Figure 2]. Optimal conditions for the reaction and removal of by-products are described, as well as an immunochemical characterization of the isolated product. Applications of the product to biochemical studies relating molecular structure to biological functions of LSD are discussed.

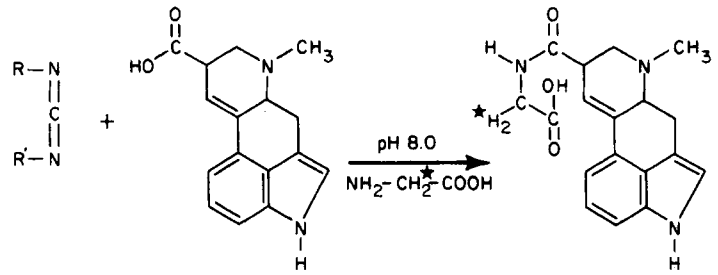

Figure 2. Scheme of carbodiimide activated amide linkage of glycine-2-^{3}H to d-lysergic acid. (★ indicates location of tritium label).

MATERIALS

The d isomer of lysergic acid (C-III grade II) was procured from Sigma Chemical Company, St. Louis, Mo., and d-LSD was a product of Sandoz Pharmaceuticals, Sandoz, Switzerland through the National Institute of Mental Health, NIH, Bethesda, Maryland. The 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (R,R'-carbodiimide) was obtained as a hydrochloride salt from Pierce Chemical Company, Rockford, Illinois. Glycine-2-^{3}H (6.9 Ci/mmol), d-LSD-(G)-^{3}H (1.9 Ci/mmol), "Aquasol" scintillation fluid and "Protosol" tissue solubilizer were purchased from New England Nuclear, Boston, Massachusetts. "Bio-Solv-BBS-3" and 2,5-diphenyloxazole (PPO) were obtained from Beckman Inst. Corporation, Fullerton, California. Sephadex G-10 was purchased from Pharmacia Fine Chemicals, Pistacaway, New Jersey. Silica gel chromatogram sheets with and without fluorescent indicator were obtained from Eastman Kodak Company, Rochester, New York. Highly specific antibody to the lysergyl moiety (rings A-D Figure 1) and normal rabbit immunoglobulin-G (IgG) were prepared as previously described (11,12).

METHODS

Lysergic acid (1.0 mg) and 10.0 mg R,R'-carbodiimide were dissolved in 1.5 ml distilled water in a light protected vessel. The mixture was stirred for 30 min. at room temperature, after which the pH was adjusted to 8.0 with 1.0N NaOH. Glycine-2-^{3}H (100 μCi) was added, and the reaction stirred for 20 hr. shielded from light. An LSD containing control reaction and mock carbodiimide reactions (absence of LSA, absence of glycine-2-^{3}H) were performed at equivalent molar concentrations of reactants.

After conjugation, each reaction mixture was streaked on silica gel sheets (without fluorescent indicator) and resolved by ascending chromatography in a chloroform:ethanol:ammonium hydroxide (16:10:2 v/v) solvent for 2 hr. Reaction products were detected by scanning the sheet with a short wavelength ultraviolet lamp (UV). The primary UV absorbing product was harvested from a segment of the sheet corresponding to an R_f of 0.16 by dissolution in 0.5 ml methanol:ammonium hydroxide (9:1 v/v).

The mixture of lysergyl products was further resolved by rechromatography on silica gel sheets in a chloroform:ethanol:glacial acetic acid (18:10:2 v/v) solvent for 6 hrs. A small duplicate chromatogram with fluorescent indicator served as control for detection and positioning of aromatic compounds by UV absorption. The R_f of (CM-^{3}H)-lysergamide was determined by comparing chromatograms of mock carbodiimide reactions (absence of LSA and absence of glycine-2-^{3}H) with chromatograms of the reaction mixture. Chromatograms were analyzed by UV absorption and cut into 0.5 cm strips for anlaysis of ^{3}H label content. Strips were placed directly into 8.0 ml scintillation fluid (10% Bio-solv-BBS-3, 0.5% PPO in toluene) and beta emission monitored in a Beckman LS-150 liquid scintillation spectrophotometer.

The labeled, UV absorbing compound was harvested from chromatograms (without fluorescent indicator) at an R_f of 0.30, dissolved from the silica

gel in 0.05M potassium phosphate, pH 8.0 buffer and stored at 0° protected from the light. Specific activity of the product was calculated using a molar extinction coefficient of 8300 at 310 nm (13).

Radiochemical Purity by Column Chromatography

One-tenth μCi of the labeled, isolated product (7.3 Ci/mmol by absorbancy at 310 nm), LSD -(G)-^{3}H, glycine-2-^{3}H and a complete carbodiimide reaction mixture were each applied to a 1.5 x 30 cm (I.D) Sephadex G-10 molecular sieve column equilibrated in distilled water. Fractions (0.5 ml) were collected into vials, and 8.0 ml scintillation fluid (20% Bio-solv-BBS-3, 0.7% PPO in toluene) was added for beta radiation analysis.

Immunochemical Characterization

To determine the quantity of labeled contaminants (e.g. glycine-2-^{3}H or polyglycine-2-^{3}H) in the isolated compound, an aliquot was tested against anti-lysergyl antibody in a radioimmunoassay based on the method of Farr (14,15) and previously described in detail (11). Briefly, 50 μl of the isolated compound was mixed with several concentrations of the antibody at 4° for 16 hrs. After incubation, an equal volume of cold, filtered, saturated ammonium sulfate was added to precipitate the antibody-ligand complex. The precipitate was pelleted (12,000 xg, 10 min.) and the pellet solubilized in 1.0 ml of 0.5M HCl, 1.0 ml "Protosol" and 6.0 ml "Aquasol" scintillation fluid for ^{3}H label analysis. Results were compared with antibody binding of LSD-(G)-^{3}H and glycine-2-^{3}H. In addition, all ligands were mixed with normal rabbit IgG at the same concentrations (μg/ml) as was used with anti-lysergyl antibody in order to detect non-specifically bound or trapped ligand in the ammonium sulfate precipitates.

To further establish the purity and molecular structure of the isolated, labeled compound, average intrinsic association constants (K_a) were determined for anti-lysergyl antibody utilizing LSD-(G)-^{3}H and the isolated compound in equilibrium dialysis experiments performed as pre-

viously described (16,17).

RESULTS

Mock glycine-2-^{3}H carbodiimide reactions (absence of LSD) yielded reaction by-products which remained at the origin during chromatography with alkaline solvent. Mock LSA carbodiimide reactions (absence of glycine-2-^{3}H) resulted in UV absorbing material with an R_f of 0.5 by thin layer chromatography in the alkaline solvent. Complete carbodiimide reactions, however, exhibited UV absorbing material and ^{3}H label at an R_f of 0.16. This material was harvested and re-chromatographed in acidic solvent. A duplicate chromatogram on fluorescent indicator silica gel afforded sensitive UV detection of unreacted LSA (R_f = 0.55), lysergyl hydrolytic products (R_f = 0.90) and a compound which contained ^{3}H label (R_f = 0.30). This labeled compound was not present on the chromatograms of carbodiimide reactions lacking glycine-2-^{3}H. Aromatic secondary amines are susceptible to carbodiimide catalyzed linkage only under limited conditions. For example, at 10 molar carbodiimide excess it is possible to substitute the ring nitrogen of deoxyguanosine-5'-phosphate but not the adenosine or cytosine moieties (18). Similarly, the indole nitrogen of LSD is not activated by 10-fold molar excess R,R'-carbodiimide since LSD carbodiimide reactions contained no UV absorbing material co-chromatographed with ^{3}H label. In addition, 1500-fold molar excess of LSA over glycine-2-^{3}H in the carbodiimide reaction would be predicted to establish conditions favoring linkage of the primary amine of glycine-2-^{3}H with the carboxyl group on LSA (Figure 1a).

Based on optical density at 310 nm, the isolated compound had a specific activity of 7.3 Ci/mmol. Since the initial specific activity of glycine-2-^{3}H was 6.8 Ci/mmol, the measured specific activity of the isolated compound was within 10% of what would be predicted for a 1:1

molar substitution of glycine-2-^{3}H to LSA. From these evidences, it was concluded that the isolated, labeled compound was (CM-^{3}H)-lysergamide.

Radiochemical Purity

The purity of the compound was assessed by column chromatography on Sephadex G-10 equilibrated in water, which excludes compounds of greater than approximately 700 daltons. Compounds less than 700 daltons are eluted sequentially by decreasing molecular weight and size. Figure 3 shows the elution profiles of (a) LSD-(G)-^{3}H and glycine-2-^{3}H, (b) the carbodiimide reaction mixture prior to thin layer silica gel chromatography and (c) the compound isolated by double thin layer silica gel chromatography. Comparison of relative peak sizes in Figure 3b indicates that the carbodiimide reaction yielded approximately 30% reaction product(s) of the approximate molecular size of LSD and 20% unreacted, labeled contaminants. By this criteria, less than 0.1% of the labeled contaminants seen in 3b could be detected in the isolated compound--Figure 3c.

Immunochemical Characterization

Anti-lysergyl antibody binding of labeled, isolated compound was compared with antibody binding of LSD-(G)-^{3}H and glycine-2-^{3}H in a radioimmune assay (summarized in Table I). While only 3-4% of the 30 pmol glycine-2-^{3}H added was trapped non-specifically in ammonium sulfate precipitated protein, 60% of the 90 pmol LSD-(G)-^{3}H and 90% of the 30 pmol (CM-^{3}H) lysergamide was specifically bound by anti-lysergyl antibody. These results indicated a lack of contamination of the isolated compound with non-lysergyl labeled products.

To further establish the absence of unlabeled LSA or lysergyl derived reaction products as well as potential ring alteration (rings A-D, Figure 1), anti-lysergyl antibody binding of (CM-^{3}H) lysergamide was compared with binding of LSD-(G)-^{3}H under equilibrium conditions for several ligand concentrations. Equilibrium dialysis affords a sensitive method for detecting

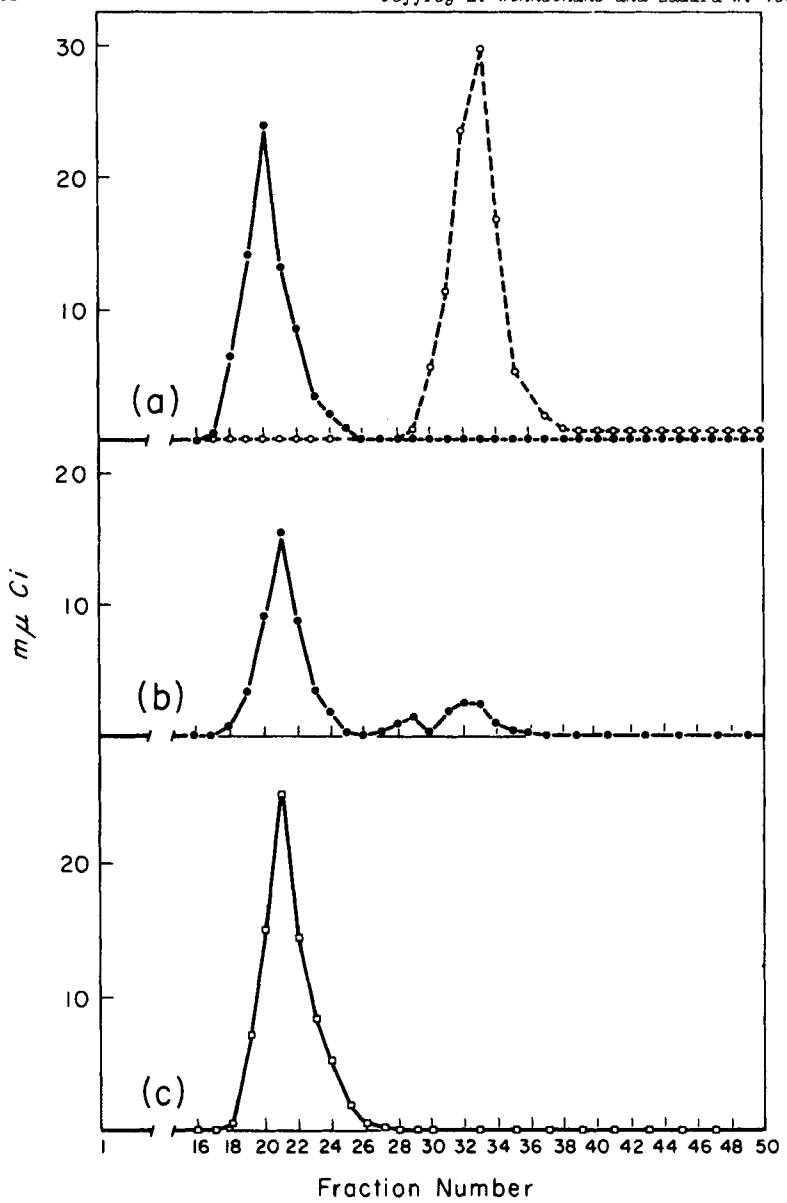

Figure 3. Sephadex G-10 molecular sieve chromatogram of : (a) LSD-(G)-^{3}H (•———•) and glycine-2-^{3}H (o- - - o), (b) the carbodiimide reaction mixture and (c) N-(carboxymethyl-^{3}H)-d-lysergamide. Compounds in (a) and (c) were isolated by thin layer chromatography prior to Sephadex G-10 analysis.

Table I. Comparative binding of glycine-2-^{3}H, LSD-(G)-^{3}H and N-(carboxymethyl-^{3}H)-d-lysergamide by anti-lysergyl antibody and normal rabbit IgG in a radioimmune assay.

Immunoglobulin added (µl)	Ligand tested	Percent Ligand Bound By :	
		Normal rabbit IgG	Anti-lysergyl antibody
	glycine-2-^{3}H	3.9 [a]	-- [b]
100	LSD-(G)-^{3}H	8.3	30.7
	N-(CM-^{3}H)-d-lysergamide	10.2	40.0
	glycine-2-^{3}H	3.1	--
200	LSD-(G)-^{3}H	9.1	51.3
	N-(CM-^{3}H)-d-lysergamide	10.1	81.2
	glycine-2-^{3}H	3.5	--
300	LSD-(G)-^{3}H	9.7	59.7
	N-(CM-^{3}H)-d-lysergamide	10.7	89.3

[a] Ligand concentrations were : glycine-2-^{3}H, 30 pmol ; LSD-(G)-^{3}H, 90 pmol ; N-(CM-^{3}H)-d-lysergamide, 30 pmol.

[b] All values were corrected for non-specific precipitation by normal rabbit IgG in control experiments.

minor alterations in the original haptenic immunogen (LSA) (19,20). Differences between binding parameters for LSD-(G)-^{3}H and the isolated, labeled compound would be predicted if the isolated compound contained either unlabeled, cross-reacting lysergyl products or if rings A-D (Figure 1) were altered in any way. Binding curves and association constants (K_o) of anti-lysergyl antibody for LSD-(G)-^{3}H and (CM-^{3}H) lysergamide are shown by Scatchard plots in Figure 4 (11,17,21). The average intrinsic association

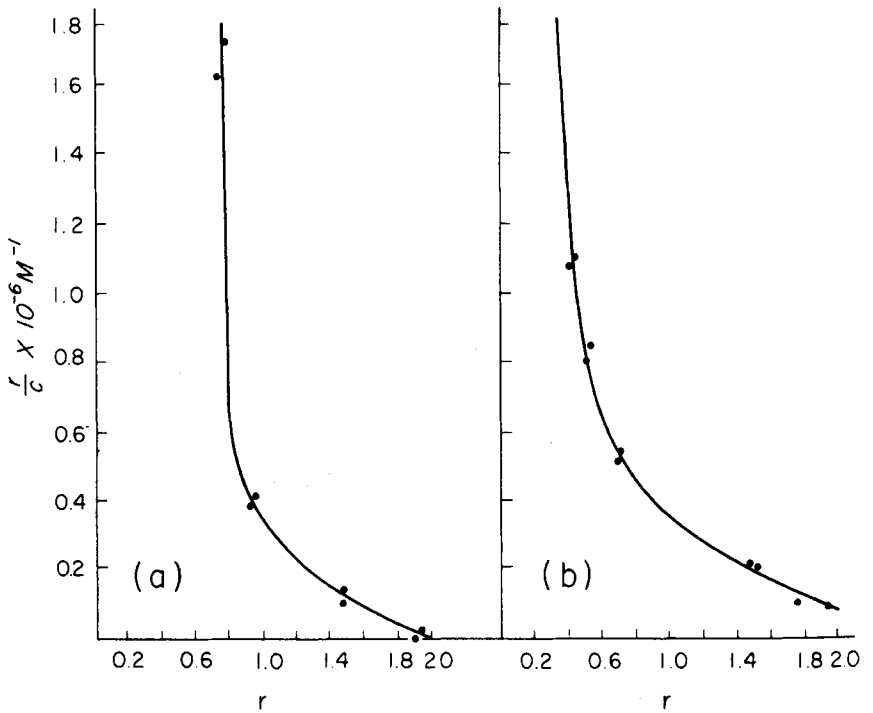

Figure 4. Scatchard plots of anti-lysergyl antibody binding in equilibrium dialysis to : (a) LSD-(G)-^{3}H, K_o = 3.5 x 10^{5} M^{-1}, (b) N-(CM-^{3}H)-d-lysergamide, K_o = 3.8 x 10^{5} M^{-1}. Symbols correspond to : r = moles of ligand bound per mole of antibody, C = concentration of free ligand,and K_o , the average intrinsic association constant, was determined as the reciprocal of C which caused saturation of one-half of the antibody active sites.

constant of the antibody for LSD-(G)-^{3}H was 3.5 x 10^5 M^{-1} and for (CM-^{3}H)-lysergamide was 3.8 x 10^5 M^{-1}.

DISCUSSION

The carbodiimide activated amide linkage of glycine-2-^{3}H to LSA results in a high yield of ^{3}H labeled product eluting from Sephadex G-10 molecular sieve columns at the same molecular size location as LSD (Figure 3). Isolation of the product by double thin layer chromatography on silica gel resulted in recovery of a compound containing less than 0.1% detectable labeled contaminants. At the molar concentrations used, the secondary amine in the indole portion of the lysergyl skeleton (rings A-D) was insensitive to water soluble carbodiimide activated amide linkage of glycine-2-^{3}H. This evidence and the specific activity measured for the isolated compound indicating a 1:1 molar ratio of glycine-2-^{3}H to LSA allowed for the molecular assignment of N-(carboxymethyl-^{3}H)-d-lysergamide for the isolated, labeled compound.

To establish the homogeneity of the compound and the absence of unreacted LSA or lysergyl by-products as well as the absence of unreacted glycine-2-^{3}H and polyglycyl reaction products, (CM-^{3}H)-lysergamide was analyzed immunochemically with highly specific antibody to the lysergyl moiety (11). It has been well established that minor differences in measured association constants for a given antibody population between cross-reacting ligands reflect differences in ligand structures (e.g. side groups) (19,20,22). Anti-lysergyl antibody utilized in this study was raised against LSA linked to lysine residues in a carrier protein and was shown to be highly specific for the lysergyl skeleton (rings A-D, Figure 1) (11). Anomalous side groups on the lysergyl moiety or an

alteration of the ring structure would be expected to significantly alter the K_o of the antibody for the altered ligand when compared with LSD-(G)-^{3}H. Similar measurable differences would be expected if an unlabeled, cross-reacting ligand (e.g. LSA) were present in (CM-^{3}H)-lysergamide. Because the original hapten was LSA, there would be no antibody specificity toward the glycyl moiety on the isolated compound or toward the diethylamide moiety on LSD-(G)-^{3}H. Based on the similarity in binding constants of the antibody for the two ligands (Figure 4) it can be concluded that there was little contamination of (CM-^{3}H) lysergamide with unlabeled LSA or lysergyl-like carbodiimide reaction by-products. Similarly, it can be concluded that there was no substitution of extraneous groups to or alteration of the basic lysergyl skeleton (rings A-D, Figure 1).

Specific antibody is gaining wide popularity in various clinical assays for LSD (23) as well as in experimental analysis of the biological modes of action of the drug (5-8,24). (CM-^{3}H)-lysergamide has been used in studies of in vitro LSD:protein interactions in our laboratory. The compound has yielded results analogous to those reported for LSD-(G)-^{3}H (7) in that it is found attached to protein during de novo protein biosynthesis by hyperimmune splenic lymphocytes. (CM-^{3}H)-lysergamide has an important advantage over generally labeled LSD in that the investigator knows the location of the tritium label. Such information is important in studies tracing biochemical phenomena directly involving the lysergyl moiety (e.g. drug metabolism). The compound may also be useful to neurochemists and pharmacologists interested in the role of the diethylamide side group of the LSD molecule. At present, it is not known if the new compound, N-(carboxymethyl-^{3}H)-d-lysergamide, is hallucinogenic.

REFERENCES

1. Kang, S. and Green, J. P. — Proc. Nat. Acad. Sci. (USA), 67 : 62 (1970).

2. Cohen, M. M., Hirschhorn, K. and Frosch, W. A. — New Eng. J. Med., 277 : 1043 (1967).

3. Dishotsky, N. I., Loughman, W. D., Mogar, R. E. and Lipscomb, W. R. — Science (Washington, D. C.), 172 : 431 (1971).

4. Carnegie, P. R., Smythies, J. R., Caspary, E. A. and Field, E. J. — Nature (London), 240 : 561 (1972).

5. Voss, E. W., Jr., Babb, J. R., Metzel, P. and Winkelhake, J. L. — Biochem. Biophys. Res. Commun., 50 : 950 (1973).

6. Voss, E. W., Jr., Metzel, P. and Winkelhake, J. L. — Molec. Pharmacol., 9 : 421 (1973).

7. Winkelhake, J. L., Voss, E. W., Jr.,and Lopatin D. E. — Molec. Pharmacol. 10 : 68 (1974).

8. Voss, E. W., Jr. and Winkelhake, J. L. — Proc. Nat. Acad. Sci. (USA), 71 : in press (1974).

9. Hoffman, A. in Drugs Affecting the Central Nervous System, A Burger, ed., Dekker, New York (1968) p. 169.

10. Wilzbach, J. J. — J. Amer. Chem. Soc., 79 : 1013 (1957).

11. Lopatin, D. E. and Voss, E. W., Jr. — Immunochem., in press- March 1974.

12. Weir, D. M. in Handbook of Experimental Immunology, F. A. Davis, Philadelphia (1967) pp. 25 - 36.

13. Voss, E. W., Jr. and Berger, B. B. — Psychopharmacologia, 26 : 140 (1972).

14. Farr, R. S. — J. Infect. Dis., 103 : 239 (1958).

15. Stupp, Y., Yoshida, T. and Paul, W. E. — J. Immunol., 103 : 625 (1969).

16. Voss, E. W., Jr., Corley, K. and Huang, L. H. — Immunochem., 6 : 631 (1969).

17. Kabat, E. A. and Mayer, M. M. in Experimental Immunochemistry, C. C. Thomas, Springfield, (1967) pp. 715 - 718.

18. Gilham, P. T. and Am, J. J. — J. Amer. Chem. Soc., 84 : 687 (1962).

19. Winkelhake, J. L. and Voss, E. W., Jr. — Biochem., 9 : 1845 (1970).

20. Pressman, D. and Grossberg, A. L. in The Structural Basis of Antibody Specificity, W. A. Benjamin, New York (1968).

21. Scatchard, G. — Ann. N. Y. Acad. Sci., 51 : 660 (1949).

22. Eisen, H. N. and Siskind, G. W. — Biochem., 3 : 996 (1964).

23. Taunton-Rigby, A., Sher, S. E. and Kelley, P. R. — Science (Washington, D. C.), 181 : 165 (1973).

24. Baker, R. W., Chothia, C., Pauling, P. and Weber, H. P. — Molec. Pharmacol., 9 : 23 (1973).