THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 14, Issue of July 25, pp. 6679-6686, 1980
Printed in U.S.A.

d-[^{3}H]Lysergic Acid Diethylamide Binding to Serotonin Receptors in the Molluscan Nervous System

RELATIONSHIP TO SEROTONIN-SENSITIVE ADENYLATE CYCLASE*

(Received for publication, October 22, 1979, and in revised form, February 6, 1980)

Alan H. Drummond,‡ Franziska Bucher, and Irwin B. Levitan
From the Friedrich Miescher-Institut, P. O. Box 273, CH-4002 Basel, Switzerland

***d*-[^{3}H]Lysergic acid diethylamide (LSD) bound to both dopamine- and serotonin (5HT)-sensitive sites in a particulate fraction derived from the central nervous system of the snail *Helix pomatia*. Conditions were found which enabled the two sites to be studied independently. [^{3}H]LSD appeared to have a slightly higher affinity for dopamine-sensitive binding (K_d = 0.5 nM) than for 5HT-sensitive binding (K_d = 1.2 nM). A pharmacological analysis of the binding indicated that while dopamine- and 5HT-related agonists clearly discriminated between the two sites, putative antagonists showed little specificity for dopamine- or 5HT-sensitive binding. The pharmacology of 5HT-sensitive [^{3}H]LSD binding was studied in relation to a 5HT-sensitive adenylate cyclase present in a particulate fraction derived from the same tissue. There was a very good correlation between the abilities of a range of agents to act as agonists or antagonists in the 5HT-sensitive adenylate cyclase assay and their abilities to displace 5HT-sensitive [^{3}H]LSD binding ($r = 0.94$; $p < 0.001$). In particular, *d*-LSD and a number of neuroleptic drugs were inhibitory in both assays in a stereoselective manner. These data suggest that in molluscan tissues, 5HT-sensitive [^{3}H]LSD binding is related to the 5HT receptor which is coupled to adenylate cyclase.**

The identification of neurotransmitter receptors by radioligand-binding methods has become a valuable tool in the investigation of drug action (1). Before concluding that a particular ligand binding site does indeed represent a physiological neurotransmitter receptor, it is, however, important to establish that the profile of drug interaction with this binding site parallels that known to exist for receptor-mediated physiological responses (2). In the central nervous system, there are few suitable quantitative assays with which to compare binding data. One receptor-linked response which can be readily measured even in tissues as complex as the central nervous system is the stimulation of adenylate cyclase activity in homogenates or particulate fractions (for reviews, see Refs. 3 and 4). The molluscan nervous system affords certain advantages for such correlative studies: neurotransmitter-sensitive adenylate cyclase preparations have been reported (5–7), and in addition it is possible to monitor by intracellular recording the response of identified cells in the intact ganglia to known concentrations of perfused drugs.

This study describes the identification, using [^{3}H]LSD[1] binding, of putative dopamine and serotonin (5-hydroxytryptamine, 5HT) receptors in a particulate fraction derived from the CNS of the snail *Helix pomatia*, and defines conditions under which dopamine- and 5HT-sensitive [^{3}H]LSD binding can be studied independently. Both sites are characterized pharmacologically, and the drug sensitivity of the putative 5HT receptor binding is compared with that of the 5HT receptor which mediates the activation of adenylate cyclase in a preparation from the same tissue. The high correlation observed between the drug sensitivity of the two assays makes it very likely that the 5HT-sensitive [^{3}H]LSD binding measures the receptor which can couple to the enzyme adenylate cyclase. A portion of this work has been presented in preliminary form (8).

EXPERIMENTAL PROCEDURES

Materials—*d*-[^{3}H]Lysergic acid diethylamide (11.1 and 16.3 Ci/mmol) was from the Radiochemical Centre. It was stored at 4°C in absolute ethanol and diluted to the required concentration in 5.7 mM ascorbic acid immediately before use. The purity of the [^{3}H]LSD was checked at regular intervals using thin layer chromatography on silica gel (solvent systems as described below). The stability of the radioligand in the presence of the *Helix* nervous system particulate fraction was assessed by extraction of bound [^{3}H]LSD into 80% ethanol, 0.25% (w/v) ascorbic acid, followed by thin layer chromatography on silica gel using two solvent systems, chloroform:benzene:ethanol (4:2:1) or chloroform:ethanol:acetic acid (18:10:2). In both systems, both specifically and nonspecifically bound [^{3}H]LSD co-migrated with standard [^{3}H]LSD or unlabeled *d*-LSD.

[α-^{32}P]ATP (12 to 30 Ci/mmol) was from New England Nuclear. The following chemicals were obtained from Sigma Chemical Co.: serotonin creatinine sulfate, dopamine hydrochloride, tryptamine hydrochloride, epinine hydrochloride, 6-hydroxytryptamine creatinine sulfate, 5-methoxytryptamine, 5,6-dihydroxytryptamine creatine sulfate, noradrenaline hydrochloride, isoproterenol hydrochloride, 5-hydroxyindoleacetic acid, ergotamine tartrate, chlorpromazine hydrochloride, adenosine cyclic 3′:5′-monophosphate, ATP and GTP. *N*-Methyl-5-hydroxytryptamine oxalate and isobutyl-methylxanthine were from Aldrich, creatine kinase and creatine phosphate from Boehringer-Mannheim, and bufotenine hydrogen oxalate from Karl Roth. Apomorphine hydrochloride and the ergot derivatives (*d*-LSD and *l*-LSD tartrates, ergometrine bimaleate, dihydroergocryptine methanesulfonate, methysergide bimaleate, MPME methanesulfonate) were from Sandoz; the thioxanthene isomers (*cis*- and *trans*-flupenthixol; *cis*- and *trans*-chlorprothixene) from Lundbeck, clozapine from Wander; *d*- and *l*-butaclamol from Ayerst; spiroperidol, haloperidol, and pipamperone from Janssen; methergoline from Farmitalia; cyproheptadine from Merck, Sharp & Dohme; and cinanserin from Squibb. Dimethyl dopamine and coryneine (*N,N,N*-trimethyl-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland.

[1] The abbreviations used are : LSD, lysergic acid diethylamide; 5HT, 5-hydroxytryptamine (serotonin); CNS, central nervous system; DPI, (3,4-dihydroxyphenylamino)-2-imidazoline; S584, (3,4-dihydroxybenzyl)-4-(2-pyrimidinyl)piperazine; MPME, 8-(4-*p*-methoxyphenyl-1-piperazinylmethyl)-6-methylergolene.

dopamine) were gifts from Professor B. L. Ginsborg; piribedil and S584 from Dr. M. Schorderet; quipazine from Dr. P. Waldmeier; *d*- and *l*-propranolol from Professor M. Staehelin and 6,7-dihydroxytryptamine creatinine sulfate from Dr. A. Manian. DPI was from Boehringer-Ingelheim. All drugs were dissolved in 5.7 mM ascorbic acid.

Preparation of H. pomatia Particulate Fractions—*H. pomatia*, the Roman snail, obtained from R. Stein & Co., Lauingen, Federal Republic of Germany, were decapitated and the circumoesophageal ring of ganglia, which comprises its central nervous system, was removed. Ganglia (routinely from 100 snails which provide enough tissue for approximately 250 samples) were washed extensively in Buffer A (0.2 M sucrose, 2 mM Tris-HCl, pH 7.3) and then homogenized (60 mg wet weight/ml of Buffer A) using a Polytron (30 S; setting 5, slotted disintegrating head). The homogenate was centrifuged (750 × *g*; 15 min; 4°C) and the supernatant decanted. The pellet was resuspended in the same volume of ice-cold Buffer A and recentrifuged. The combined supernatants were centrifuged (15,000 × *g*; 30 min; 4°C) and the resulting pellet resuspended and washed once in Buffer B (Buffer A minus sucrose). After a further centrifugation, the pellet was resuspended in Buffer B using a polytron to a protein concentration of 1 to 2 mg/ml (approximately 300 mg original wet weight/ml of buffer) and stored at −70°C in 0.4-ml aliquots until use. Control experiments indicated that there was no difference between fresh and prefrozen membranes with respect to the amount of specific or nonspecific binding. Furthermore, the ability of dopamine, 5HT, or *d*-LSD to inhibit [^{3}H]LSD binding was the same with fresh and prefrozen membranes.

Measurement of Specific [^{3}H]LSD Binding—The binding reaction mixture contained membrane protein (20 to 100 μg/tube), 50 mM Tris-HCl, pH 7.3, 1.8 mM ascorbic acid, [^{3}H]LSD (routinely 2 nM final concentration), and various drugs as indicated in a total volume of 500 μl. After 10 min at 37°C, 5 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.3) was added and the mixture filtered immediately through a presoaked Whatman GFB glass fiber filter mounted on a suction apparatus. The filter was then washed four times, each with a 5-ml volume of the same buffer. Filtering and washing was complete within 5 to 10 s. Radioactivity bound to trapped particles on the filter was counted in a liquid scintillation counter using a Triton X-100-toluene-based scintillation fluid.

Nonspecific binding was defined as binding which was not displaced by a high concentration (10 μM) of unlabeled *d*-LSD. This concentration was chosen to estimate nonspecific binding since higher concentrations did not further inhibit binding. Specific binding is defined as total radioactivity bound minus nonspecific binding. Using [^{3}H]LSD concentrations of 2 nM or less, nonspecific binding comprised ≤10% of the total binding measured. Because of the low concentrations of membrane protein used in the assay (due both to limitations on the amount of tissue available, and to the advisibility of binding less than 2% of the radioligand added), routine experiments contained 150 to 400 cpm of specifically bound ligand/sample. None of the drugs or neurotransmitters used had effects on nonspecific [^{3}H]LSD binding.

5HT-sensitive [^{3}H]LSD binding (the S-site) was measured routinely using 1 to 2 nM [^{3}H]LSD in the presence of 200 μM dopamine (see below). Dopamine-sensitive binding (the D-site) was estimated using the same range of [^{3}H]LSD concentration, but in the presence of 100 μM 5HT.

Adenylate Cyclase Assay—Washed broken cell preparations for use in the adenylate cyclase assay were prepared from *Helix* circumesophageal ganglia as previously described (9). These preparations were generally freshly prepared prior to an experiment, since the 5HT sensitivity of adenylate cyclase declined upon freezing and thawing (see below and Ref. 9).

Incubations were performed in a volume of 40 μl which contained: Tris/maleate buffer, 25 mM (pH 8.0); $MgCl_2$, 5 mM; ascorbic acid, 0.5 mM; cyclic AMP, 1 mM; isobutylmethylxanthine, 1 mM; EGTA, 0.6 mM; ATP, 0.5 mM; [α-^{32}P]ATP, 1 to 2 × 10^6 cpm; creatine kinase, 80 units/ml; creatine phosphate, 20 mM, and (unless otherwise stated) GTP, 20 μM. After incubation for 2 min at 30°C, the reaction was terminated by addition of a "stopping solution" comprising 2% sodium dodecyl sulfate, 40 mM ATP, and 1 mM cyclic AMP. Cyclic [^{32}P]AMP formed was isolated by sequential chromatography on Dowex AG 50W-X2 and alumina as described by Salomon *et al.* (10). Values reported here are corrected to 100% cyclic AMP recovery.

Protein Determination—Protein was determined by the method of Schaffner and Weissmann (11) using bovine serum albumin as standard.

RESULTS

Discrimination between Dopamine- and 5HT-sensitive [^{3}H]LSD Binding—Preliminary experiments designed to optimize binding conditions demonstrated that the general characteristics of the binding process were as summarized in Table I. Note that the equilibrium dissociation constant (K_d) derived from kinetic experiments was very similar to that observed in equilibrium experiments. We have previously shown that, of a range of neurotransmitters tested, only 5HT and dopamine effectively competed for specific [^{3}H]LSD binding, and they competed at different subpopulations of binding sites (8). Thus, at concentrations below 100 μM, their inhibitory effects were additive. We used this discriminatory ability of 5HT and dopamine to derive conditions under which the ligand was specifically bound to either 5HT- or dopamine-sensitive acceptors. When the assay was conducted in the presence of 200 μM dopamine, only 5HT-sensitive binding could be measured (this, we called the S-site), in the presence of 100 μM 5HT, only dopamine-sensitive binding remained (the D-site) (Ref. 8 and data not shown).

The data presented in Fig. 1, plotted according to the method of Scatchard (12), indicate that [^{3}H]LSD has a higher affinity for the D-site than the S-site: in the absence of the monoamines, a curvilinear plot is found (Fig. 1*A*), from which two saturable sites are revealed when the data are treated by the method of Rosenthal (13) as modified by Pennock (14) (not shown). The high affinity site has a capacity of 540 fmol/mg of protein and a K_d of 0.55 nM. The low affinity site has a maximum binding capacity of 2.02 pmol/mg of protein and a K_d of 50 nM. Since our experiments were routinely carried out using a [^{3}H]LSD concentration of 2 nM or less, 85 to 90% of the membrane-bound radioactivity was located on the high affinity site, and the results presented here will refer exclusively to this site. In the presence of 200 μM dopamine (S-site conditions) the slope of the Scatchard plot is flatter and a K_d for LSD-binding to the S-site of 1.2 nM can be calculated (Fig. 1*B*). In the presence of 100 μM 5HT (D-site conditions), the slope becomes slightly steeper, giving a calculated K_d for D-site binding of 0.5 nM (Fig. 1*C*). Both of these values are slight underestimates of affinity since the small contribution of the low affinity [^{3}H]LSD binding site has been neglected. A summary of these data is presented in Fig. 1*D*, which emphasizes that the inhibitory effects of 5HT and dopamine depend on the concentration of [^{3}H]LSD used. Also, since the intercepts (extrapolated) on the abscissa are similar for [^{3}H]LSD binding to the D- or S-sites, these sites are present in *Helix* particulate fraction in approximately the same concentration (Fig. 1*D*).

TABLE I

General characteristics of [^{3}H]LSD binding to Helix particulate fraction: summary

pH optimum	6.2–7.3
Association rate constant (k_1)[a]	1.5×10^6 M^{-1} s^{-1}
Dissociation rate constant (k_{-1})[b]	1.11×10^{-3} s^{-1}
Equilibrium dissociation constant (K_d) derived from kinetic experiments[c]	0.74 nM
Equilibrium dissociation constant (K_d) derived from equilibrium experiments	0.50 nM

[a] Determined from a plot of $\log_e ([B_{eq}]/[B_{eq}] - [B])$ against time (where $[B_{eq}]$ is the amount of [^{3}H]LSD bound at equilibrium and $[B]$ is that bound at time (t)). The slope of this line represents the observed rate constant (k_{ob}) from which the second order rate constant (k_1) can be calculated using the equation $k_1 = (k_{ob} - k_{-1})/[[^3H]LSD]$ (see Ref. 48) (where k_{-1} is the dissociation rate constant, 1.11×10^{-3} s^{-1}, derived as described below).

[b] Determined from a plot of $\log_e [B]/[B_{eq}]$ against time. The slope of this line can be used to measure the dissociation rate constant.

[c] $K_d = k_{-1}/k_1$.

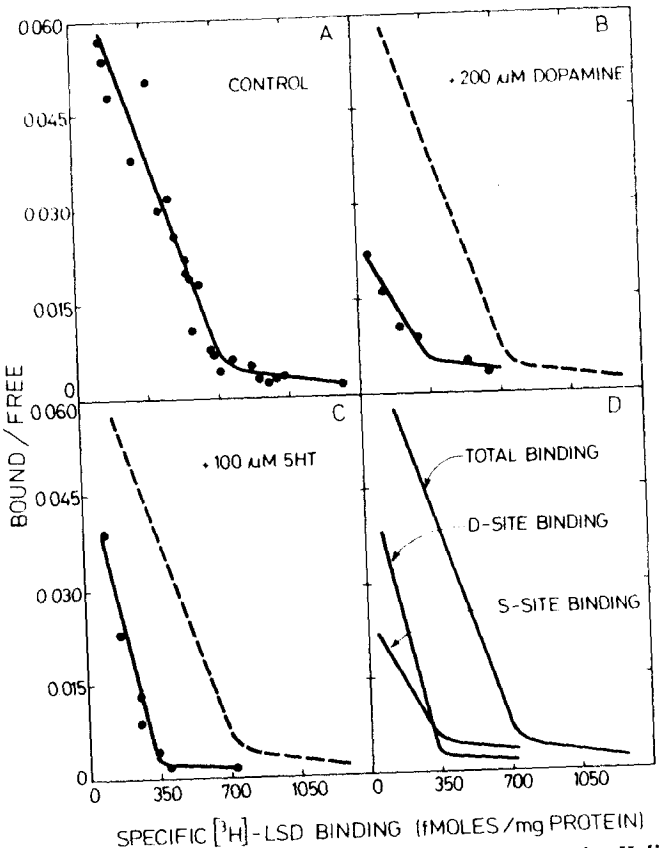

FIG. 1. **Scatchard plots of [^{3}H]LSD-binding data in *Helix* CNS.** *A*, control: ascorbic acid, Values are means of five experiments. *B*, in the presence of 200 μM dopamine. The *dashed line* represents the control curve from *A*. Values are means of two experiments. *C*, in the presence of 100 μM 5HT. The *dashed line* represents the control curve from *A*. Values are means of two experiments. *D*, cumulative graph for comparison of 5HT and dopamine effects.

Pharmacology of Dopamine- and 5HT-sensitive [^{3}H]LSD Binding—The affinities of a range of agonists and antagonists for both the D- and S-sites are shown in Table II. Certain dopamine- and 5HT-like agonists discriminated well between the two populations of binding sites. For example, the order of tryptamine derivatives in competing for S-site [^{3}H]LSD binding was rather different from that noted in the D-site studies. In particular, 5HT, *N*-methyl-5HT and 5-methoxytryptamine were considerably more potent (25- to 100-fold) against S-site binding (Fig. 2*A* and Table II, Column 2). Bufotenine, however, which was one of the more potent tryptamine derivatives against the S-site, retained its relatively high affinity against the dopamine-sensitive site. Removal of the ring hydroxyl (tryptamine) or alteration of its position (6HT) led to appreciable losses in S-site affinity (Fig. 2*A* and Table II). With the exception of apomorphine and S584, a metabolite of piribedil, dopamine derivatives were only weakly active against the S-site (Fig. 2*B* and Table II). The affinities of a range of antagonists for the S-site is shown in Column 2 of Table II. Notable here is the rather high affinity (≈1 nM) of some of the ergot derivatives which were tested (*e.g. d*-LSD and ergotamine) and the low affinity (>1 μM) of the butyrophenone group of compounds (spiroperidol, haloperidol and pipamperone).

In contrast to agonists, putative receptor antagonists discriminated poorly between the D- and S-sites (Table II). Of a range of over 20 antagonists tested, ergometrine was the only agent which showed marked selectivity for one or the other site (50-fold more potent against the D-site). The data in Fig. 3 demonstrate the interaction of a number of isomeric drugs with 5HT-sensitive [^{3}H]LSD binding. As was also the case for the D-site (Table II), *d*-LSD, *d*-butaclamol, and *cis*-flupenthixol were stereoselective inhibitors of S-site binding, with *cis*-chlorprothixene being much less stereoselective (Fig. 3).

5HT-sensitive Adenylate Cyclase Activity—Osborne (5) was the first to report the existence of 5HT- and dopamine-sensitive adenylate cyclases in homogenates of *H. pomatia* central ganglia. He found that dopamine and 5HT caused up

TABLE II

Effect of various agents on dopamine- and 5HT-sensitive [^{3}H]LSD binding

Helix ganglia particulate fractions were assayed with four to eight concentrations of drug in duplicate or triplicate. D-site and S-site binding was measured as described in the text. The concentrations of drugs required to inhibit binding by 50% (IC_{50} values) were calculated, and were converted to K_i values according to the equation $K_i = IC_{50}/(1 + c/K_d)$ (48, 49) where c is the concentration of radioactive ligand (usually 1 to 2 nM) and K_d is its dissociation constant (0.5 nM for D-site binding, and 1.2 nM for S-site binding, (see Ref. 8)).

Agent tested	Inhibition of [^{3}H]LSD binding (K_i)	
	D-site	S-site
	nM	
5HT-like agents		
5HT	>100,000	1,300
N-methyl-5HT	37,500	1,500
Bufotenine	2,400	2,100
Tryptamine	10,000	13,000
5-Methoxytryptamine	50,000	2,000
6HT	22,000	75,000
5,6-Dihydroxytryptamine	100,000	130,000
6,7-Dihydroxytryptamine	16,000	75,000
5-Hydroxyindole acetic acid	—[a]	—[a]
Dopamine-like agents		
Dopamine	10,000	>100,000
Epinine	4,000	77,000
N,N-Dimethyldopamine	7,000	30,000
Coryneine	—[a]	—[a]
Apomorphine	270	940
DPI	37,000	90,000
Piribedil	20,000	30,000
S584	760	3,000
Noradrenaline	54,000	Not tested
Isoproterenol	100,000	Not tested
Ergot alkaloids and derivatives		
d-LSD	0.5	1.2
l-LSD	10,000	12,000
Ergotamine	0.3	2
Ergometrine	0.8	40
Dihydroergocryptine	9.5	20
MPME	7	60
Methysergide	12	70
Methergoline	30	60
Neuroleptic drugs		
d-Butaclamol	4.6	14
l-Butaclamol	54,000	12,000
cis-Flupenthixol	410	240
trans-Flupenthixol	6,600	13,000
cis-chlorprothixene	150	130
trans-chlorprothixene	490	360
Chlorpromazine	130	600
Clozapine	100	350
Spiroperidol	2,000	6,200
Haloperidol	4,100	3,400
Pipamperone	20,000	17,000
5HT-antagonists and other agents		
Cyproheptadine	500	350
d-Propranolol	1,300	35,000
l-Propranolol	2,200	30,000
Cinanserin	Not tested	2,300
Quipazine	Not tested	32,000

[a] Too weakly active to measure accurately (K_i > 300,000 nM).

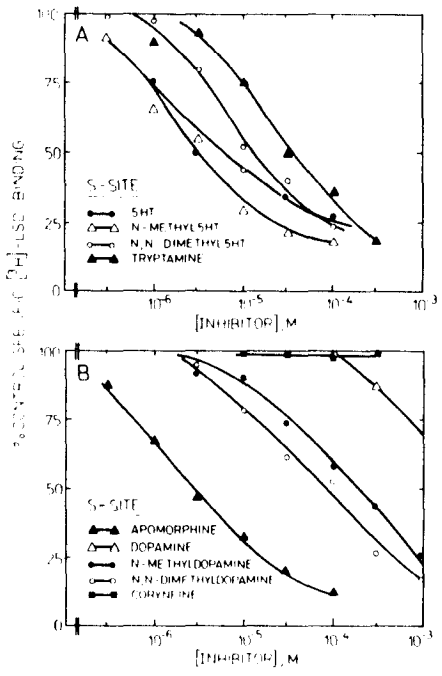

FIG. 2. **Inhibition of S-site [^{3}H]LSD binding by various agents.** Same as Fig. 1, except that 200 μM dopamine (in addition to the concentration indicated in the abscissa of *B*) was added to all tubes to abolish binding to the D-site and restrict it to the S-site. *A*, 5HT-related drugs; *B*, dopamine-related drugs. Values are means of two to four experiments each carried out in triplicate. The standard error of the mean was never greater than 8% of the mean.

to a 3-fold stimulation of the enzyme with EC_{50} values (concentration causing a half-maximal response) of 50 μM and 40 μM, respectively. Unfortunately, we have been unable to confirm the presence of a dopamine-sensitive cyclase in *Helix* homogenates or washed particulate fractions assayed under a variety of experimental conditions, including those described by Osborne (5) (not shown). Also, the stimulation of the enzyme by 5HT has never, in our hands, exceeded a doubling of the basal enzyme activity. The reasons for these discrepancies are unclear; neurotransmitter-sensitive adenylate cyclase preparations are, however, notoriously fickle.

The stimulation of *Helix* adenylate cyclase activity by 5HT in a washed particulate preparation was enhanced by the presence of GTP (Table III), which as previously reported (9)

TABLE III

Potentiation of 5HT-sensitive adenylate cyclase activity by GTP

Helix particulate fractions (25 μg/sample) were incubated for 2 min with 5HT or dopamine (10 μM) in the absence or presence of various GTP concentrations. Adenylate cyclase activity was measured as described in the text. The values in parentheses are the percentage increase in activity induced by 5HT or dopamine over the control level. Particulate fractions which had been stored at −70°C for several days were used for this experiment.

GTP concentration	Adenylate cyclase activity		
	Control	5HT	Dopamine
μM	*pmol cAMP formed/min/mg protein (means ± S.E.)*		
0	6.8 ± 0.4	7.7 ± 0.5 (13.2%)	7.0 ± 0.4 (2.9%)
1	18.4 ± 0.2	22.0 ± 0.9 (19.6%)	19.9 ± 2.5 (8.1%)
10	22.1 ± 0.7	29.5 ± 0.9 (33.5%)	22.5 ± 0.5 (1.8%)
100	22.4 ± 0.4	27.9 ± 0.8 (24.5%)	22.2 ± 0.6 (−0.9%)

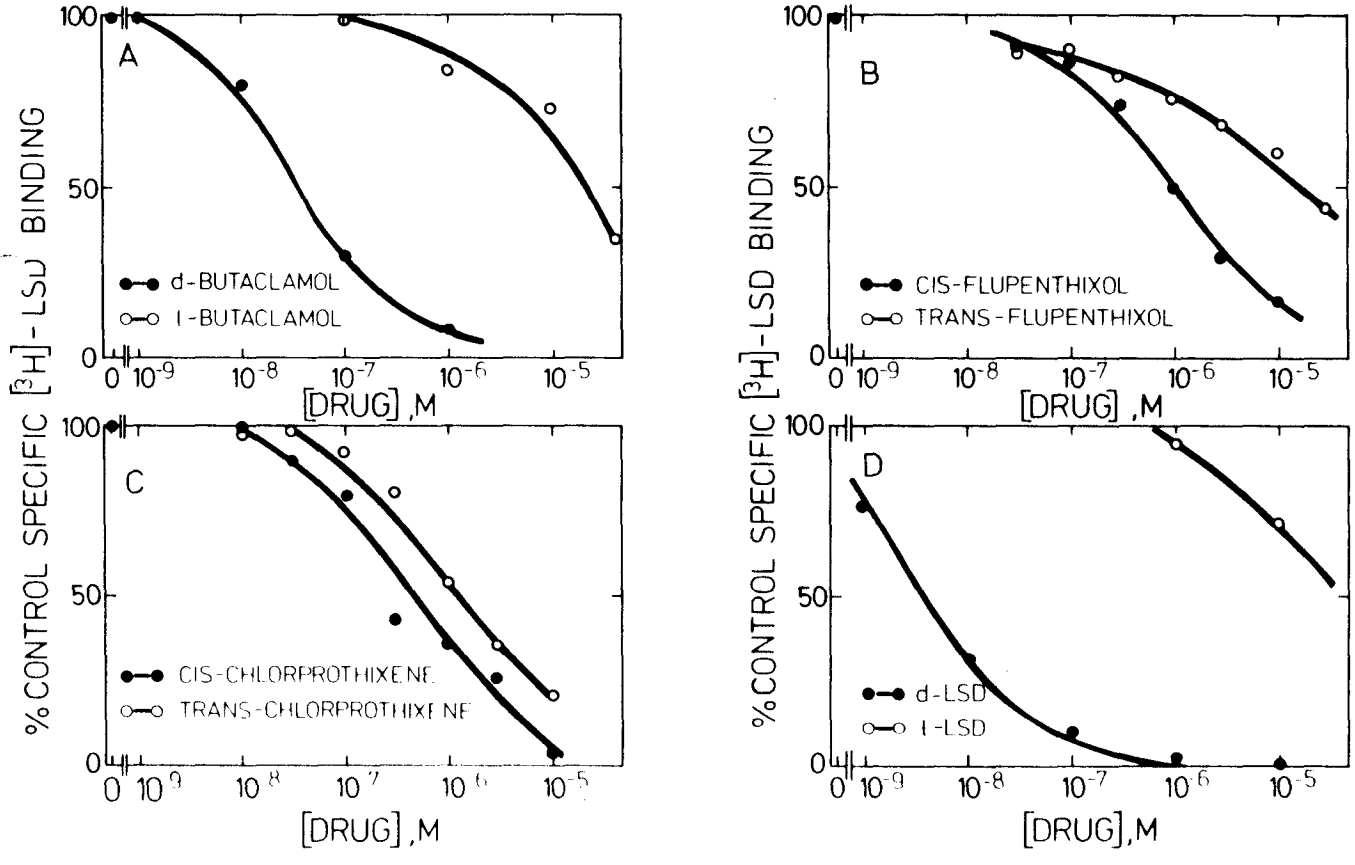

FIG. 3. **Inhibition of S-site [^{3}H]LSD binding by isomeric drugs.** Specific [^{3}H]LSD binding to the S-site was measured in the presence of 200 μM dopamine. Different concentrations of *d*- and *l*-butaclamol (*A*), *cis*- and *trans*-flupenthixol (*B*), *cis*- and *trans*-chlorprothixene (*C*), and *d*- and *l*-LSD (*D*) were added to the tubes to inhibit S-site binding. Values are means for two experiments each carried out in triplicate. The standard error of the mean was never greater than 6% of the mean.

caused a 3-fold stimulation by itself. The degree of stimulation elicited by 5HT (in the presence of 20 μM GTP) varied markedly depending upon the state of activation of the snails and the experimental conditions. If the particulate fractions were prepared from snails which had recently (1 to 2 days) been activated after long periods (more than 6 months) of inactivity (storage at 4°C), the stimulation of adenylate cyclase activity by 100 μM 5HT (a supramaximal concentration, see Fig. 4) was 80 ± 6% (n = 10, ± S.E.) over basal. Longer periods of activation (≥7 days) led to a particulate fraction whose adenylate cyclase activity was stimulated only 63 ± 5% (n = 9, ± S.E.) over basal. The greater degree of stimulation in the former situation was not related to a change in the basal activity of the preparation. If the fraction prepared from active snails was frozen at −70°C before use, the degree of stimulation by 5HT fell to 32 ± 1% (n = 8, ± S.E.) above basal (see Table III for example). This was not related to an alteration in the basal state of the enzyme. Accordingly, most of the experiments reported here were conducted on fractions freshly prepared from recently activated snails. Control studies (not shown) indicated that the affinity of the enzyme for 5HT or *d*-LSD was not dependent on the type of preparation used.

5HT stimulation of the enzyme was linear with time using incubations of less than 5-min duration. With longer periods, however, the degree of stimulation waned rapidly, such that by 10 min no appreciable 5HT sensitivity of adenylate cyclase could be measured (results not shown). All the experiments reported here were carried out using a 2-min incubation period, in order to minimize this deterioration, which also occurred during a preincubation conducted in the absence of added 5HT (not shown).

Of a range of 5HT-like agents tested, only its *N*-methyl and *N,N*-dimethyl (bufotenine) derivatives elicited a similar degree of adenylate cyclase activation (Table IV). A number of other agents (*e.g.* tryptamine, 5-methoxytryptamine, and 6HT) were partial agonists which could elicit maximal responses between 20 and 65% of that produced by 5HT itself (Figs. 4 and 5 and Table IV). The partial agonist nature of the action of tryptamine is well illustrated in Fig. 5, which demonstrates that tryptamine also partially antagonizes the stimulation of adenylate cyclase activity by 5HT. Bufotenine, *N*-methyl-5HT, and 5-methoxytryptamine evoked responses with EC_{50} values in the range of 1 to 6 μM, the other derivatives tested were at least 10-fold less potent. For example, removal

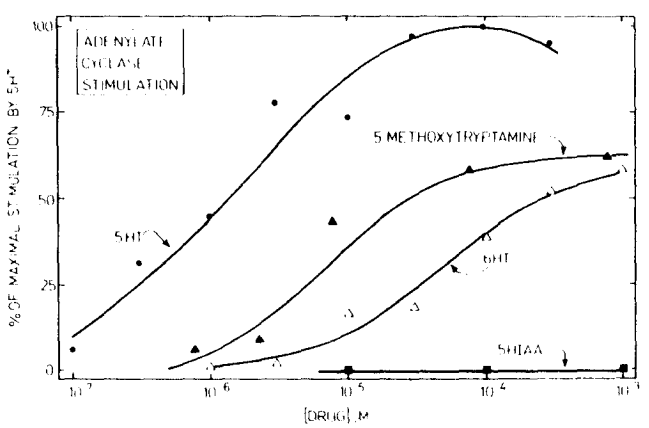

FIG. 4. **Stimulation of *Helix* CNS adenylate cyclase activity by 5HT and related agents.** Adenylate cyclase activity was measured in the presence of different concentrations of 5HT (●——●), 5-methoxytryptamine (▲——▲), 6HT (△——△), or 5-hydroxyindoleacetic acid (*5HIAA*) (■——■). Values are means for triplicate samples. The standard error of the mean was never greater than 9% of the mean.

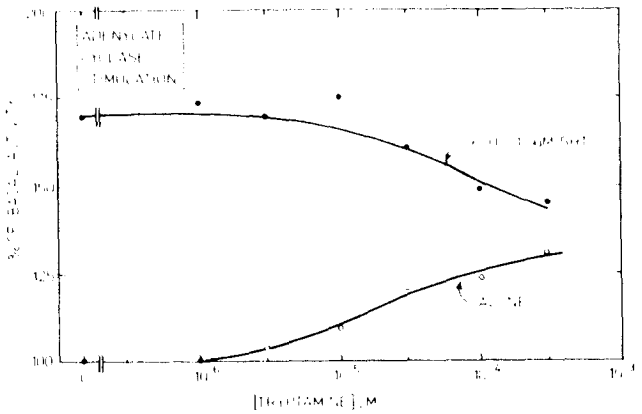

FIG. 5. **Effects of tryptamine on basal and 5HT-stimulated adenylate cyclase activity in *Helix* CNS.** Adenylate cyclase activity was measured in the presence of different concentration of tryptamine, in the absence (○——○) or presence (●——●) of 10 μM 5HT. Values are means for triplicate samples. The standard error of the mean was never greater than 7% of the mean.

TABLE IV

Effect of various agents on 5HT sensitive adenylate cyclase activity

Adenylate cyclase activity was measured using five to seven drug concentrations in triplicate. For agonists and partial agonists the EC_{50} value was the concentration required to give half-maximal activation of adenylate cyclase activity. For antagonists, activity was measured in the presence and absence of 10 μM 5HT to measure inhibition of the 5HT-sensitive component of adenylate cyclase. IC_{50} values were calculated according to the equation in Table II, where c is the 5HT concentration (10 μM) and K_d the EC_{50} value for 5HT (1.65 μM). Intrinsic activity was calculated as the maximal extent of stimulation produced by a given drug, relative to that produced by 5HT.

Agent tested	5HT-sensitive adenylate cyclase activity	
	EC_{50} or K_i	Intrinsic activity
	nM	
5HT and dopamine-like agents		
5HT	1,650	1.00
N-Methyl-5HT	1,550	1.00
Bufotenine	1,250	1.00
Tryptamine	18,300	0.36
5-Methoxytryptamine	6,200	0.63
6HT	26,000	0.37
5-Hydroxyindole acetic acid	—[a]	—[a]
Dopamine	>100,000	0.00
Apomorphine	12,000	0.00
Ergot alkaloids and derivatives		
d-LSD	10	0.00
l-LSD	>10,000	0.00
Ergotamine	47	0.00
Dihydroergocryptine	97	0.00
Neuroleptic drugs		
d-Butaclamol	21	0.00
l-Butaclamol	13,500	0.00
cis-Flupenthixol	100	0.00
trans-Flupenthixol	14,000	0.00
cis-Chlorprothixene	110	0.00
trans-Chlorprothixene	310	0.00
Spiroperidol	3,800	0.00
Pipamperone	9,500	0.00
Other		
Quipazine	4,000	0.00

[a] Too weakly active to measure accurately (EC_{50} or K_i > 300,000 nM).

of the ring hydroxyl group (tryptamine) or alteration of its position (6HT) led to 10- to 20-fold increases in the EC_{50} concentration for adenylate cyclase activation (see Figs. 4 and 5, and Table IV).

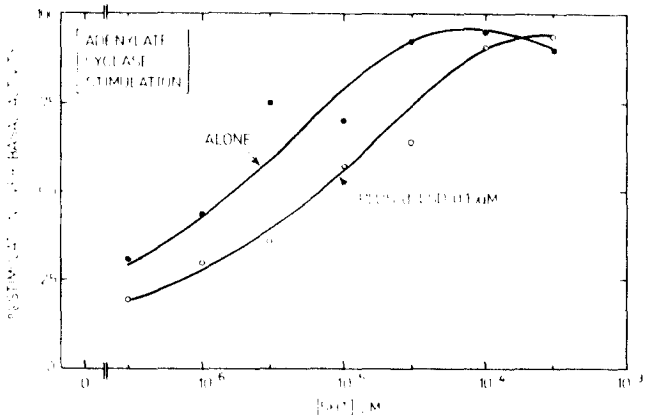

FIG. 6. **Effect of *d*-LSD on 5HT-stimulated adenylate cyclase activity.** Adenylate cyclase activity was measured in the presence of different concentrations of 5HT, in the absence (●——●) or presence (○——○) of 0.1 μM *d*-LSD. Because *d*-LSD inhibited basal adenylate cyclase activity by 10 to 15%, the stimulation by 5HT in the presence of *d*-LSD is expressed relative to this lowered basal activity.

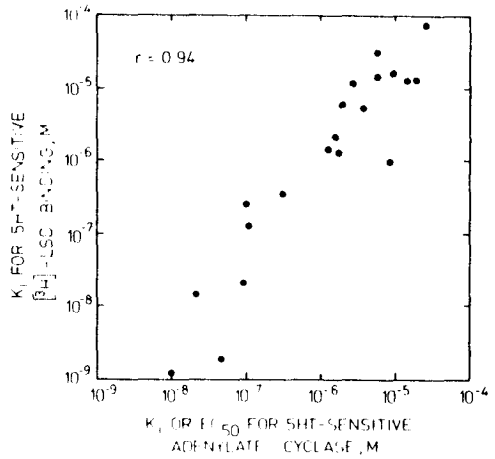

FIG. 7. **Comparison of drug-sensitivities of S-site [^{3}H]LSD binding and 5HT-sensitive adenylate cyclase.** K_i values for 5HT-sensitive [^{3}H]LSD binding of various drugs (values from Table II) were plotted against their K_i or EC_{50} values for 5HT-sensitive adenylate cyclase (values from Table IV). The correlation coefficient (r) was calculated by linear regression analysis.

d-LSD was a competitive antagonist of the 5HT-stimulated adenylate cyclase with a calculated K_i of 0.01 μM (Fig. 6). In contrast to our studies in the marine mollusc *Aplysia californica* (7), it did not exhibit partial agonist activity. Other ergot derivatives such as ergotamine and dihydroergocryptine were also potent antagonists (Table IV), although we did not determine the nature of the antagonism involved. As was noted with the [^{3}H]LSD-binding studies, the isomeric drugs tested were, to varying degrees, stereoselective 5HT antagonists: *d*-LSD, *d*-butaclamol, and *cis*-flupenthixol were > 1000-, > 600-, and 140-fold more active than their corresponding *l*- or *trans* isomers, and there was a small (but significant) degree of stereoselectivity for the *cis* isomer of chlorprothixene (Table IV). Many of the drugs tested also affected the basal activity of the enzyme. This was especially true for the lipid-soluble neuroleptic drugs such as chlorprothixene and flupenthixol (15), which inhibited the enzyme up to 35% at 100 μM in a nonstereospecific manner (data not shown). The data shown in Table IV relate purely to inhibition of the 5HT-sensitive component of the adenylate cyclase activity.

When the drug sensitivity of S-site [^{3}H]LSD binding and the 5HT-sensitive adenylate cyclase were compared (Fig. 7), a highly significant correlation was found ($r = 0.94, p < 0.001$), consistent with the hypothesis that 5HT-sensitive [^{3}H]LSD binding measures the physiological 5HT receptor which couples to the enzyme adenylate cyclase.

DISCUSSION

There are now a range of radioactive compounds which can be used to measure dopamine and 5HT receptors in mammalian nervous tissue. These include agonists and partial agonists such as [^{3}H]dopamine (16, 17), [^{3}H]5HT (18, 19), and [^{3}H]LSD (20–23), and antagonists such as [^{3}H]spiroperidol (24), [^{3}H]haloperidol (17, 22) and *cis*-[^{3}H]flupenthixol (25). The ligands presently available for measuring 5HT receptors are rather unsatisfactory: [^{3}H]LSD and [^{3}H]spiroperidol bind to dopamine receptors as well (26, 27), and [^{3}H]5HT, which can be used in the vertebrate CNS (18, 19), does not bind to high affinity receptors in the molluscan CNS.[2] In this study, and in the work reported in a preliminary communication (8), we show that [^{3}H]LSD binds to both dopamine and 5HT receptors in the molluscan nervous system, and have exploited this lack of specificity to characterize both receptors in detail. Unfortunately, we were unable to confirm the presence of a dopamine-sensitive adenylate cyclase in snail tissue (5), and because of the lack of an *in vitro* assay for the dopamine receptor, this discussion centers upon the 5HT receptor data.

The Pharmacology of Dopamine- and 5HT-sensitive [^{3}H]LSD Binding in the Molluscan CNS—In a previous paper (8), we described the separation of the total specific [^{3}H]LSD binding into the D-site (dopamine-sensitive) and S-site (5HT-sensitive) components by the addition of appropriate concentrations of dopamine or 5HT. Further justification of this method is provided by the pharmacological analysis presented in this paper. Only dopamine-like and 5HT-like agents could discriminate between the two sites, and of these, none appeared to have better specificity than the physiological agonists themselves (see Table II). For 5HT-related and, to a lesser extent, dopamine-related compounds, increased methyl substitutions on the primary amino group gave compounds which retained good receptor affinity but decreased specificity for the D- or S-sites. Thus, bufotenine and *N,N*-dimethyldopamine were much less selective than 5HT or dopamine themselves. Data from other receptor systems tend to support these findings: bufotenine and *N,N*-dimethyldopamine have been reported to have nicotinic cholinergic and α-adrenergic properties, respectively (28–30). We surveyed a wide range of putative 5HT and dopamine antagonists in an attempt to find other discriminating compounds, but with little success. The relative lack of specificity of these drugs for 5HT or dopamine receptors is in general agreement with data from the vertebrate CNS (27), although we failed to detect the relatively small degree of receptor specificity which has been reported for certain agents, *e.g.* pipamperone (a selective 5HT antagonist) and haloperidol (a dopamine antagonist) (27).

The Relationship of 5HT-sensitive [^{3}H]LSD Binding to 5HT Receptors in the Molluscan CNS—The bulk of evidence favoring the existence of 5HT receptors in the molluscan CNS stems from electrophysiological studies (for review see Ref. 31). Gerschenfeld and Paupardin-Tritsch have described six pharmacologically distinct 5HT-induced responses in *Helix aspersa* and in the marine mollusc *A. californica* (32). The receptors mediating three of these responses, termed A, B, and C, are inhibited by LSD and therefore may, in our studies, be measured by [^{3}H]LSD binding. Receptor C is, however, not sensitive to inhibition by bufotenine (32), and therefore can be excluded. The major difficulty in comparing our biochemical studies with the electrophysiological data is that the

[2] A. H. Drummond, unpublished work.

latter give no indication of the affinity of LSD for the physiological receptors. Bearing this reservation in mind, our tentative conclusion is that [^{3}H]LSD labels both the A-type receptor (which mediates an increase in Na^+-conductance) and the B-type receptor (mediating an increase in K^+-conductance).

The Relationship of 5HT-sensitive [^{3}H]LSD Binding in the Molluscan CNS to 5HT Receptors in the Vertebrate CNS—A number of different workers have analyzed [^{3}H]LSD binding to 5HT receptors in vertebrate brain (20–23). We have averaged their IC_{50} values for various drugs and compared them with *Helix* S-site [^{3}H]LSD binding (not shown). The correlation between the two sets of values is not particularly high ($r = 0.59$), however, this low value is in large part due to the low affinity of two drugs, pipamperone and spiroperidol, in the *Helix* binding assay (IC_{50} values in *Helix* 45 and 16.5 μM, respectively, and in rat brain 0.027 and 0.023 μM). When these drugs are omitted, the correlation coefficient rises to 0.90, which is highly significant ($p < 0.001$). It is unclear why these two agents should be so ineffective in *Helix*, but this may be related to our inability to detect specific [^{3}H]spiroperidol binding in molluscs.[2]

5HT-sensitive Adenylate Cyclase in the Molluscan CNS: Relationship to That Found in Other Systems—There are a number of examples in the literature of broken cell adenylate cyclase preparations which are stimulated by 5HT (5–7, 33–42). Their one common feature appears to be that the EC_{50} concentration of 5HT is in the range of 0.5 to 2 μM. The potency of other agonists and their degree of intrinsic activity tends to vary markedly. Thus tryptamine is a full agonist with a potency comparable to 5HT in some systems (34, 42) and a partial agonist with an approximately 10-fold lower potency than 5HT in others (40 and this paper, see Fig. 5). A major discrepancy between the present work and other 5HT-sensitive adenylate cyclases which have been studied is that *d*-LSD had no agonist activity on this preparation, although it is a partial agonist in the CNS of a related animal, the marine mollusc *A. californica* (7). We have no good explanation for this finding, although it is worth noting that in *Helix* *d*-LSD routinely lowered basal adenylate cyclase by 10 to 15% in a stereospecific manner (results not shown but see legend to Fig. 6, for example). This effect was not noted with other drugs (*e.g.* *d*- and *l*-butaclamol) and appears to be similar to the effects of 2-bromo-LSD on the basal activity of the *Fasciola hepatica* adenylate cyclase reported by Northup and Mansour (40). It remains possible that the partial agonist activity of *d*-LSD was not measured because the drug concomitantly reduced basal activity. In general, the potency of agonists in the *Helix* 5HT-sensitive adenylate cyclase is comparable with that reported by Northup and Mansour (40). It is obvious, however, that since the agonist affinity cannot be determined directly in any of the adenylate cyclase systems so far described because of uncertainties regarding agonist efficacy or spare receptor number, such disparities or correlations may be more apparent than real.

Such problems do not obtain with antagonists, however, and a more direct comparison is possible between the *Helix* cyclase and that originally reported in the vertebrate CNS by von Hungen *et al.* (34) and recently characterized in detail by Enjalbert *et al.* (43) and Hamon *et al.* (44). Clearly, the two systems are not identical: quipazine was totally inactive in the vertebrate preparation, but showed some, albeit weak, activity as a 5HT antagonist in *Helix*. Also, *cis*-flupenthixol was a substantially more potent antagonist in *Helix* than in rat brain (44). One common feature worth noting, however, is that spiroperidol was a rather poor inhibitor in both cases. This contrasts markedly with the high potency of spiroperidol as a 5HT antagonist in other non-adenylate cyclase-linked 5HT receptor systems (45, 46) suggesting perhaps that, as is the case with dopamine receptors (47), butyrophenone derivatives might be used to differentiate between multiple 5HT receptors.

5HT-sensitive [^{3}H]LSD Binding and Adenylate Cyclase in the Molluscan CNS—There was a high correlation between the *Helix* S-site [^{3}H]LSD-binding pharmacology and the sensitivity of the 5HT-sensitive adenylate cyclase to drugs (Fig. 7). These data make it very likely that in molluscs, 5HT-sensitive [^{3}H]LSD binding can be used to monitor the 5HT receptor which couples or is coupled to adenylate cyclase. Further evidence for such a thesis comes from our studies in *A. californica*, which demonstrate that 5HT-sensitive [^{3}H]-LSD binding and 5HT-sensitive adenylate cyclase have a similar distribution in a number of ganglia and muscles (7). To our knowledge, a similar study has not been carried out in the vertebrate CNS. It has been recognized, however, that multiple 5HT receptors probably exist there and that neither [^{3}H]5HT nor [^{3}H]spiroperidol are adequate ligands for the adenylate cyclase-linked 5HT receptors (44). It remains to be seen whether [^{3}H]LSD can be used for such a purpose in the vertebrate CNS.

Acknowledgments—We acknowledge with thanks the gifts of drugs by the various individuals specified under "Experimental Procedures" and by the following pharmaceutical companies: Sandoz, Lundbeck, Wander, Ayerst, Janssen, Farmitalia, Merck, Sharp & Dohme, Squibb, and Boehringer-Ingelheim.

REFERENCES

1. Yamamura, H. I., Enna, S. J., and Kuhar, M. J. (1978) *Neurotransmitter Receptor Binding*, Raven Press, New York
2. Paton, W. D. M., and Rang, H. P. (1965) *Proc. R. Soc. Lond. B Biol. Sci.* **163,** 1–44
3. Daly, J. (1975) in *Handbook of Psychopharmacology* (Iversen, L. L., Iversen, S. D., and Snyder, S. H., ed) Vol. 5, pp. 47–130, Plenum Press, New York
4. Nathanson, J. A. (1977) *Physiol. Rev.* **57,** 157–256
5. Osborne, N. N. (1977) *Experientia* **33,** 917–919
6. Levitan, I. B. (1978) *Brain Res.* **154,** 404–408
7. Drummond, A. H., Bucher, F., and Levitan, I. B. (1980) *Brain Res.* **184,** 163–177
8. Drummond, A. H., Bucher, F., and Levitan, I. B. (1978) *Nature* **272,** 368–370
9. Levitan, I. B., Bergstrom, E., and Simonet, M. (1978) *J. Neurochem.* **31,** 1353–1359
10. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548
11. Schaffner, W., and Weissmann, C. (1973) *Anal. Biochem.* **56,** 502–514
12. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
13. Rosenthal, H. E. (1967) *Anal. Biochem.* **20,** 525–532
14. Pennock, B. E. (1973) *Anal. Biochem.* **56,** 306–309
15. Norman, J. A., Drummond, A. H., and Moser, P. (1979) *Mol. Pharmacol.* **16,** 1089–1094
16. Burt, D. R., Enna, S. J., Creese, I., and Snyder, S. H. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 4655–4659
17. Seeman, P., Chau-Wong, M., Tedesco, J., and Wong, K. I. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 4376–4380
18. Bennett, J. P., and Snyder, S. H. (1976) *Mol. Pharmacol.* **12,** 373–389
19. Fillion, G., Fillion, M. P., Spirakis, C., Bahers, J. M., and Jacob, J. J. (1976) *Life Sci.* **18,** 65–74
20. Bennett, J. L., and Aghajanian, G. K. (1974) *Life Sci.* **15,** 1935–1944
21. Bennett, J. P., Jr., and Snyder, S. H. (1975) *Brain Res.* **94,** 523–544
22. Burt, D. R., Creese, I., and Snyder, S. H. (1976) *Mol. Pharmacol.* **12,** 800–812
23. Lovell, R. A., and Freedman, D. X. (1976) *Mol. Pharmacol.* **12,** 620–630
24. Fields, J. Z., Reisine, T. D., and Yamamura, H. I. (1977) *Brain Res.* **136,** 578–584

25. Hyttel, J. (1978) *Life Sci.* **23,** 551–556
26. Burt, D. R., Creese, I., and Snyder, S. H. (1976) *Mol. Pharmacol.* **12,** 631–638
27. Leysen, J. E., Niemegeers, C. J. E., Tollenaere, J. P., and Laduron, P. M. (1978) *Nature* **272,** 168–171
28. Haefely, W. (1974) *Naunyn-Schmiedebergs Arch. Pharmacol.* **281,** 145–165
29. Fozard, J. R., and Mobarok Ali, A. T. M. (1978) *Naunyn-Schmiedebergs Arch. Pharmacol.* **301,** 223–235
30. Kitzen, J. M., Ilhan, M., Cannon, J. G., and Long, J. P. (1976) *Eur. J. Pharmacol.* **38,** 365–372
31. Ascher, P., and Kehoe, J.-S. (1975) in *Handbook of Psychopharmacology* (Iversen, L. L., Iversen, S. D., and Snyder, S. H., eds) Vol. 4, pp. 265–310, Plenum Press, New York
32. Gerschenfeld, H. M., and Paupardin-Tritsch, D. (1974) *J. Physiol. (Lond.)* **243,** 427–456
33. Nathanson, J. A., and Greengard, P. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 797–801
34. Von Hungen, K., Roberts, S., and Hill, D. F. (1975) *Brain Res.* **84,** 257–267
35. Wollemann, M., and Rózsa, K. S. (1975) *Comp. Biochem. Physiol.* **51c,** 63–66
36. Higgins, W. J. (1977) *J. Cyclic Nucleotide Res.* **3,** 293–302
37. Enjalbert, A., Bourgoin, S., Hamon, M., Adrien, J., and Bockaert, J. (1978) *Mol. Pharmacol.* **14,** 2–10
38. Ahn, H. S., and Makman, M. H. (1978) *Brain Res.* **153,** 636–640
39. Ahn, H. S., and Makman, M. H. (1978) *Life Sci.* **23,** 507–512
40. Northup, J. K., and Mansour, T. E. (1978) *Mol. Pharmacol.* **14,** 804–819
41. Franquinet, R., Le Moigne, A., and Hanoune, J. (1978) *Biochim. Biophys. Acta* **539,** 88–97
42. MacDermot, J., Higashida, H., Wilson, S. P., Matsuzawa, H., Minna, J., and Nirenberg, M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1135–1139
43. Enjalbert, A., Hamon, M., Bourgoin, S., and Bockaert, J. (1978) *Mol. Pharmacol.* **14,** 11–23
44. Hamon, M., Nelson, D. L., Herbet, A., and Glowinski, J. (1979) in *Receptors for Neurotransmitters and Peptide Hormones* (Kuhar, M. J., Enna, S. J., and Pepen, G. C., eds) Raven Press, New York, in press
45. Boullin, D. J., and Glenton, P. A. M. (1978) *Br. J. Pharmacol.* **62,** 537–542
46. Graf, M., and Pletscher, A. (1979) *Br. J. Pharmacol.* **65,** 601–608
47. Kebabian, J. W., and Calne, D. B. (1979) *Nature* **277,** 93–96
48. U'Prichard, D. C., Bylund, D. B., and Snyder, S. H. (1978) *J. Biol. Chem.* **253,** 5090–5102
49. Cheng, Y., and Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108