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Adenylate Cyclase from *Fasciola hepatica*

1. Ligand Specificity of Adenylate Cyclase-Coupled Serotonin Receptors

JOHN K. NORTHUP[1] AND TAG E. MANSOUR[2]

Department of Pharmacology, Stanford University School of Medicine, Stanford, California 94305

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SUMMARY

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Cell-free particles from the liver fluke *Fasciola hepatica* contain a highly active serotonin stimulated adenylate cyclase. Serotonin (5-HT) stimulates this enzyme 25 to 30-fold over basal activity to activities of 0.80 ± 0.10 nmoles/min·mg protein. Histamine, dopamine, octopamine, epinephrine, and carbachol failed to activate this adenylate cyclase. The kinetics of activation by 5-HT showed apparent negative cooperativity with a Hill coefficient of 0.7. The lower apparent affinity half-maximal activation by 5-HT occurred at 2.1 ± 0.3 μM. Any substitutions on the 5-HT molecule reduced apparent affinity and degree of activation. Apparent affinity and intrinsic activity of indoleamines decreased with decreasing structural similarity to 5-HT. All indoleamines tested compete for the serotonin receptor. Derivatives of lysergic acid also activate this adenylate cyclase, with very high apparent affinity. D-lysergic acid diethylamide (LSD) was the most potent derivative activating maximally about 25% of 5-HT stimulated activity. Half-maximal activation by D-LSD occurred at 40 nM. Activation by LSD was totally stereospecific, with the L-isomer inactive even at 1 mM. Both D- and L-LSD antagonized 5-HT stimulation, but the L-isomer had a 500-fold decreased affinity. 2-bromo LSD (BOL) competitively antagonized 5-HT activation as shown by Schild analysis. BOL also directly inhibited basal (nonactivated) adenylate cyclase. The direct inhibition and antagonism of 5-HT by BOL both involved a single population of receptor sites with the same inhibition constant. This result suggests that BOL inhibits adenylate cyclase by interacting with the 5-HT site and that BOL has a "negative" efficacy for this receptor. The evidence supports a single class of adenylate cyclase regulated only by serotonin receptors.

INTRODUCTION

Adenylate cyclases have proved to be excellent model systems because these enzymes retain their sensitivity to hormone stimulation in membrane preparations. The β-adrenergic receptor, in particular, has been studied in considerable detail through the actions of β-adrenergic agents on adenylate cyclase (1). In a system analogous to

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[2] To whom correspondence should be addressed.

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the β-adrenergic receptor in mammalian liver, a serotonin (5-HT)[3]-sensitive adenylate cyclase has been implicated as a regulator of carbohydrate metabolism of the trematode parasite, *Fasciola hepatica* (2–5). The indoleamine stimulates cAMP accumulation in intact flukes and it activates adenylate cyclase in cell-free particulate fractions from these organisms (4, 6). This adenylate cyclase, then, may be considered as a model serotonin receptor system. The liver fluke adenylate cyclase is of particular interest since few other tissues have been found to possess 5-HT stimulated adenylate cyclases. 5-HT has been reported to stimulate adenylate cyclase in cockroach thoracic ganglia (7), but the physiological significance of this system is unknown. Serotonin has also been reported to increase cyclic AMP in the blowfly salivary gland, probably as part of the biochemical mechanism for serotonin stimu- these systems, and in the liver fluke, lysergic acid diethylamide (LSD) interacts with high affinity with the serotonin receptor. In addition to effects on carbohydrate metabolism, 5-HT and LSD are potent stimulators of fluke motility (10). Furthermore, the rank order potencies of LSD derivatives in stimulating fluke motility correlate remarkably well with the hallucinogenic potency in man (11). Thus, the fluke serotonin receptors may be directly relevant to understanding the molecular actions of LSD in the mammalian central nervous system. This has been a problem of considerable interest since the initial suggestion that LSD, in producing its psychotomimetic effects, might act through central 5-HT receptors (12). Smooth muscle serotonin receptors have not provided good models for studying this question as they are pharmacologically different from the receptors in the central nervous system (13, 14). Direct ligand binding studies in membranes from mammalian central nervous system did not fully elucidate the nature of the receptors because these membrane systems are not known to be coupled to a measurable response to 5-HT (15–18). A serotonin-regulated adenylate cyclase, then, may provide an important model system for understanding the molecular mechanisms of serotonin action.

This investigation is an extension of our studies on the nature of adenylate cyclase from *F. hepatica* and its properties as a model serotonin receptor system. We will report on the interactions of indoleamines, lysergic acid derivatives, and various other related agents with these receptors as assayed by adenylate cyclase activity. We will also report on the "negative" efficacy of a certain ligand for the receptor and discuss the implications of this finding on the mechanism of 5-HT activation of adenylate cyclase.

MATERIALS AND METHODS

Cell-free particles from liver flukes were prepared by a minor modification of the previously reported method (4, 6). Intact flukes were blotted dry, weighed, and frozen on Wollenberger clamps chilled in dry ice. Frozen flukes were then pulverized in a mortar chilled in dry ice, and the powder was used for the initial homogenization. This procedure produced a much more uniform homogenate than was obtained previously (4, 6) and allowed storage of frozen flukes in dry ice for several weeks prior to preparation. The sucrose solutions used contained 5 mM dithiothreitol and 1 mM EDTA. Undiluted particles were stored in liquid nitrogen and thawed immediately prior to use. The particles were then diluted with a solution containing 0.25 M sucrose, 50 mM glycyl-glycine pH 7.5, 5 mM dithiothreitol, and 1 mM EDTA and rehomogenized by several passes with a motor driven Teflon homogenizer. Particles stored for several months in liquid nitrogen had adenylate cyclase activity identical to unfrozen particles used immediately.

Adenylate cyclase was assayed by the method of Salomon *et al.* (19). Reactions were initiated by addition of particles to the reaction mixture containing final concentrations of 0.1 M sucrose, 50 mM glycylglycine pH 7.5, 5 mM phosphocreatine, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 or 0.1 mM Na_2ATP with about 1-2 μ

[3] Abbreviations used are as follows: 5-HT, 5-hydroxytryptamine; LSD, lysergic acid diethylamide; and BOL, 2-bromo-D-lysergic acid diethylamide.

Ci[α^{32}P]ATP per 0.25 ml assay, 5 or 2 mM $MgCl_2$, 40 U/ml creatine phosphokinase, about 1 mg/ml fluke protein, and test substances at indicated concentrations in a final volume of 0.25 ml. Incubations were carried out at 37°C for up to 10 minutes. The reaction was terminated by addition of 0.25 ml reagent containing 2% sodium dodecyl sulfate pH 7.4, 10 mM NaEDTA, 10 mM Na_2ATP, and 1 mM cAMP. [^{3}H]cAMP (about 10,000 cpm) was added as a standard and the cyclic nucleotide was separated by column chromatography. Under these conditions adenylate cyclase velocity was linear with protein (0.12–1.6 mg/ml) and with time up to 10 minutes. Zero time and boiled enzyme blanks gave about 20 cpm/μCi [$^{32}\alpha$P]ATP added, which was less than 15% of the least active sample. Recovery of [^{3}H]cAMP through the chromatography steps averaged about 55%. All values are reported as the average of duplicate incubations which agreed within 5%. Each experiment was repeated at least twice.

Materials. The following compounds were purchased from Sigma Chemical Company: Rabbit skeletal muscle creatine phosphokinase; ATP sodium salt, Sigma grade; 5-hydroxydimethyltryptamine; 5-methoxydimethyltryptamine monooxalate hydrate; and N,N-dimethyltryptamine. N-methyltryptamine; 5-methoxy-N,N-dimethyltryptamine; 5-hydroxy-N-methyltryptamine; and 3-isobutyl-1-methylxanthine were products of Aldrich. Compounds obtained from Calbiochem were 5-HT creatine sulfate, B grade; 5-methoxytryptamine, A grade; and tryptamine HCl A grade. [^{3}H]adenosine cyclic-3',5-monophosphate (21.7 Ci/mMole) was a product of Schwarz-Mann, and [α^{32}P]-ATP (50–150 Ci/mMole) was obtained from Amersham or New England Nuclear Company. D-lysergic acid diethylamide; 2-bromo-D-lysergic acid diethylamide, D-lysergic acid ethylamide, L-lysergic acid diethylamide; D-lysergic acid dimethylamide; D-lysergic acid morpholide; D-lysergic acid pyrrolidide; 1-methyl-D-lysergic acid diethylamide; 1-methyl-D-lysergic acid butanolamide; ergonovine; and D-lysergate were products of Sandoz and were obtained fom the National Institute of Mental Health. Tetrahydroharman; 12-hydroxytetrahydroharman; 1-methyl-N,N-dimethyl tryptophol; and N-acetyl-5-hydroxytryptamine were generous gifts from Dr. Jack Barchas, Stanford. All other materials were reagent grade obtained from various sources.

RESULTS

Kinetics of activation of adenylate cyclase by serotonin. As previously reported (4, 6) serotonin markedly stimulates the activity of particulate adenylate cyclase from the fluke. However, under the conditions used in the experiments reported here, the maximal activation was 0.80 ± 0.10 nmoles cAMP/min·mg (mean ± SD of 13 experiments). This value is considerably greater than that previously reported. The half-maximally effective concentration (K_A) of 5-HT was 2.1 ± 0.3 μM (mean ± SD of 13 experiments), a value considerably less than was found before. Two factors account for the marked sensitivity of these enzyme preparations. 1. Effective phosphodiesterase inhibition was achieved with 0.5 mM isobutyl methylxanthine (20) used in these assays rather than 10 mM caffeine used previously. 2. The presence of 100 μM GTP in the assay markedly increased the V_{max} for serotonin and D-LSD (21).

Because of the comparatively high activity of the fluke adenylate cyclase and the sensitivity of the assay method, data for activation by very low concentrations of 5-HT were reliably determined. Experiments in which very broad ranges of concentration of 5-HT (10^4) were studied have shown apparent negative cooperativity in the kinetics of activation of adenylate cyclase. This is illustrated in the Lineweaver-Burk plot of enzyme activation as a function of 5-HT concentration shown in Fig. 1. These data fit a concave downward curve characteristic of negative cooperativity or heterogeneity of enzyme. While this might be interpreted as two classes of 5-HT stimulated adenylate cyclase with K_A values of 0.14 and 2.6 μM for 5-HT, the Hill plot for these data, inset Fig. 1, suggests a single class of negatively cooperative 5-HT sites. The Hill coefficient for these data is 0.7, a value significantly less than 1.0.

Substrate kinetics of the enzyme show no

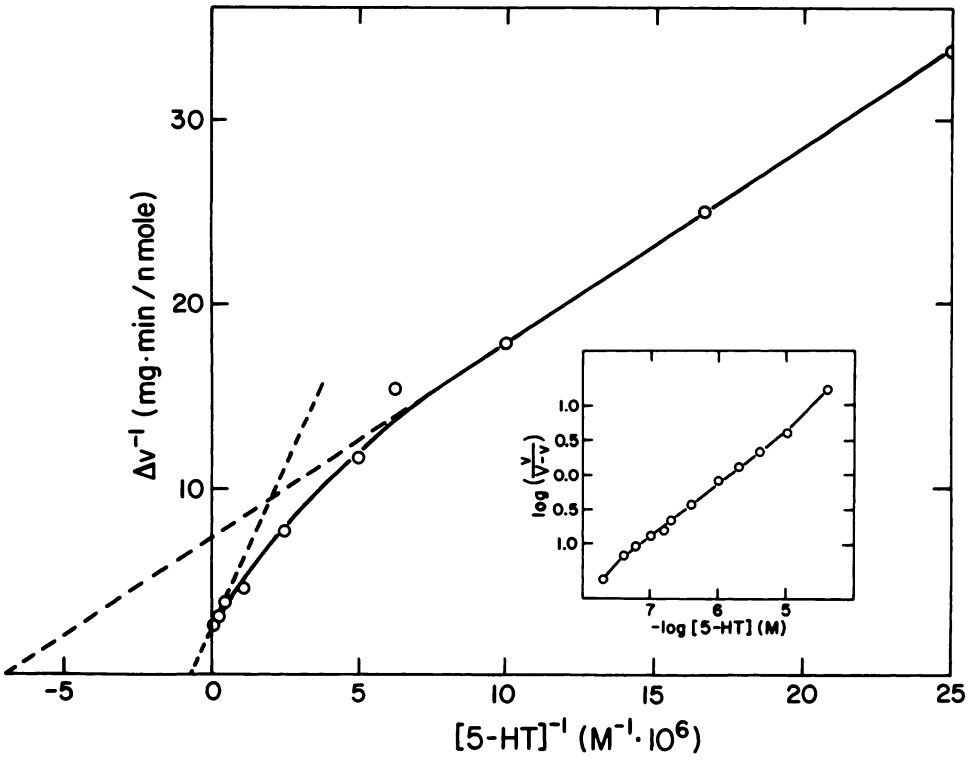

FIG. 1. *Lineweaver-Burk plot of 5-HT activation*

Serotonin activation of adenylate cyclase was determined by adding serotonin at concentrations that varied from 0.02–100 μM to adenylate cyclase reaction mixtures containing 0.1 mm ATP and 0.1 mm GTP and assaying adenylate cyclase as described in METHODS. Basal velocity was subtracted from all values and the Lineweaver-Burk inverse plot fitted by eye. Serotonin concentrations greater than 4 μM have been omitted for clarity, but they also fit well on the higher K_A line drawn here. These data yield two K_A values—0.14 and 2.6 μM. The inset of this figure shows the Hill plot of this data. V_{max} was determined by linear regression of the Woolf inverse for the concentrations >1 μM 5-HT (V_{max} = 0.48 nmole/mg·min, $r^2 > 0.99$), and the fraction of this V_{max} was used for the Hill plot. Log [v/(Vm-v)] versus log [5-HT] was fitted by eye.

evidence of heterogeneity of enzymes. Fig. 2 is a Lineweaver-Burk plot of ATP concentration versus adenylate cyclase velocity for basal (non-activated) and 5-HT, D-LSD or NaF activated enzyme. In all cases the enzyme appears to have Michaelis-Menten kinetics with a K_m between 90–150 μM. The apparent differences between K_m values in this experiment are not significant. The K_m value for ATP determined for 5-HT activated enzyme was 140 ± 18 μM (mean ± SD of four experiments), the K_m for NaF activated was 112 ± 30 μM (mean ± SD of four experiments), and the K_m for basal enzyme was 147 ± 33 μM; (mean ± SD of four experiments). These data are consistent with a single class of adenylate cyclase with V_{max} increased to different degrees by different activators. These data show no evidence for cooperativity of the substrate site.

In all the studies reported below the ATP concentration was held constant and changes in adenylate cyclase velocity (presumably V_{max}) were used to study the regulation of enzyme activity by the serotonin receptor.

Kinetics of activation by serotonin and LSD analogs. Simple substitutions on the 5-HT molecule alter the efficacies of analogs as agonists of the serotonin receptor. Fig. 3 shows saturation curves for analogs of 5-HT as activators of adenylate cyclase. These results confirm our initial observa-

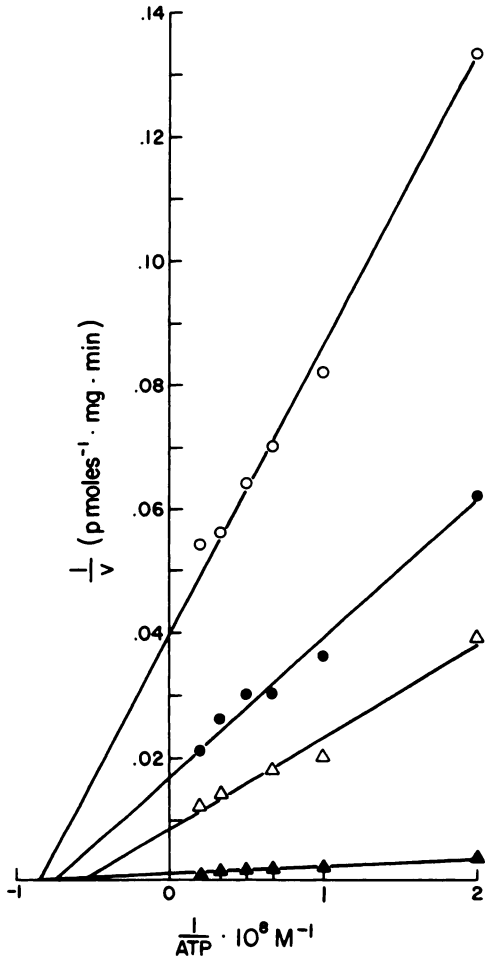

FIG. 2. *Lineweaver-Burk plot of ATP saturation curves*

The substrate kinetics of adenylate cyclase were determined by adding the indicated concentrations of ATP to reaction mixtures containing only [α^{32}P]ATP (1 μM) with 5 mm $MgCl_2$ and activators as indicated—basal (○), 1 μM D-LSD (●), 10 μM 5-HT (△), and 5 mM NaF (▲). Adenylate cyclase was determined as described in METHODS. Lineweaver-Burk inverse plots were determined by linear regression (r^2 > 0.99 for all lines).

tions that both 5-hydroxyl and free ethylamino groups are essential for agonists of the receptor. Compounds lacking the 5-hydroxy group, tryptamine, N-methyltryptamine, N,N-dimethyltryptamine, and gramine, are much poorer agonists and have decreased affinity compared with their 5-hydroxy congeners. Similarly, the 5-methoxy compounds, 5-methoxytryptamine and 5-methoxy-N,N dimethyltryptamine are poorer agonists. Substitution on the ethylamine have similar effects in both the hydroxylated and non-hydroxy analogs. A single methyl substitution as in 5-hydroxy-N-methyltryptamine and N-methyltryptamine decreases both efficacy and affinity. The dimethyl analogs have greatly reduced efficacy. The compound lacking both 5-hydroxyl and free amino groups, N,N-dimethyltryptamine is almost devoid of activity as an agonist. Apparent affinity does not vary greatly among these indoleamines. Even the least potent, 5-methoxytryptamine, has a K_A of 20 μM, while that of 5-HT is 2 μM.

Two lines of evidence suggest that all of these compounds activate adenylate cyclase through a common class of serotonin receptor. First, competition between antagonists or partial agonists and serotonin can be shown. Fig. 4 shows the antagonism of 5-HT stimulated adenylate cyclase by various indoleamines. The antagonism by all these compounds saturates as a single class of sites as determined by linear inverse plots (data not shown). The K_i values determined from Woolf plots are given in Table 1. As expected, these compounds do not all decrease velocity to the basal level, rather the partial agonists N,N-dimethyltryptamine and 5-methoxy-N,N-dimethyltryptamine inhibit to a level of activity comparable to that expected were they tested as activators of the enzyme. A second indication that all of these activators utilize a common class of activating sites is obtained when the data of Fig. 3 are treated according to Barlow (22). These inverse plots are all linear (data not shown) and yield K_B values consistent with the K_A values when corrected for relative efficacy. Data for the interaction of a variety of indole derivatives and other drugs is presented in Table 2. All compounds that were found to activate the adenylate cyclase also antagonized the activation by 5-HT.

Fig. 5 shows the activation of adenylate cyclase by derivatives of lysergic acid. Lysergic acid amides are poorer agonists than 5-HT but they possess much higher affinity. Lysergic acid diethylamide (D-LSD) was the most potent derivative, activating max-

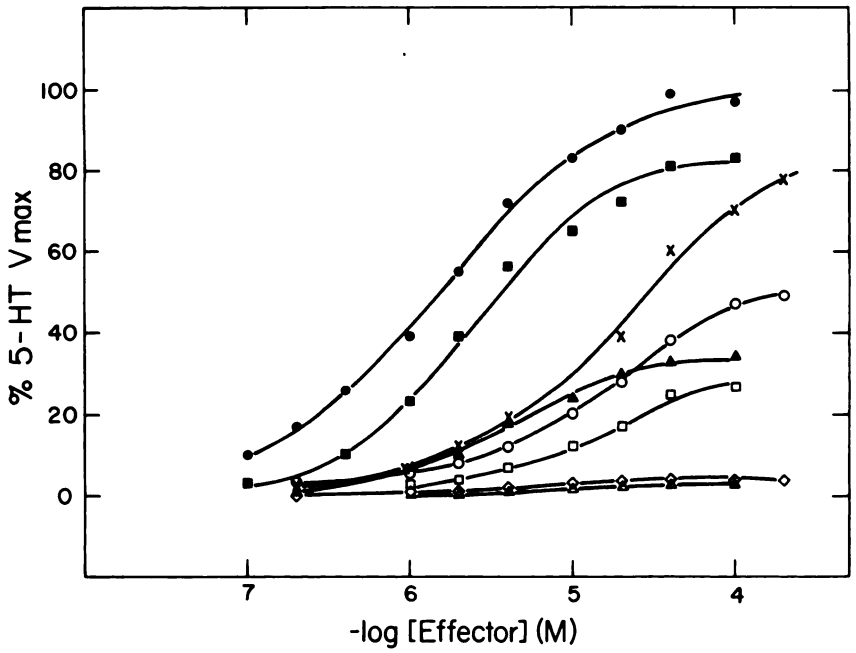

FIG. 3. *Activation of adenylate cyclase by 5-HT analogs*

Activation by analogs of 5-HT was determined by addition of the compounds at indicated concentrations to reaction mixtures containing 100 μM GTP, 1 mM ATP, and 5 mM $MgCl_2$. Adenylate cyclase was assayed as described in METHODS. Basal velocity was subtracted from all values, and V was normalized as the fraction of Vm for 5-HT determined in the same experiment. All points are the average of values from duplicate incubations in three separate experiments except for tryptamine and 5-methoxytryptamine which are from two experiments. Symbols are as follows: 5-hydroxytryptamine (●), 5-hydroxy-N-methyl tryptamine (□), 5-methoxytryptamine (×), tryptamine (○), N-methyl tryptamine (■), 5-hydroxy-N,N-dimethyl tryptamine (▲), 5-methoxy-N,N-dimethyl tryptamine (◇), and N,N-dimethyl tryptamine (△). Maximal activation by 5-HT averaged 1.25 nmoles/min·mg protein.

imally about 25% of 5-HT activity. Half-maximal activation by D-LSD occurred at 46 ± 2 nM (mean ± SD for six experiments). Any substitution other than the diethyl on the amide nitrogen decreased the potency of the derivative. The half-maximal constants and relative efficacies of these derivatives are given in Table 1. Both the monoethyl and dimethyl derivatives have apparent affinity roughly similar to D-LSD with K_A values of 53 and 43 nM, respectively. The relative efficacies of these compounds are somewhat reduced from that of D-LSD (0.17) and of 5-HT (0.18). The butanolamide substitution reduces efficacy and affinity greatly to 0.12 and 500 nM. Substitution on the indole nitrogen in both the diethyl and butanolamide derivatives substantially decreases affinity. Finally, the 2-bromo substitution produces a high affinity antagonist of 5-HT which is discussed more fully below. These data not only show the effects of substitutions on the amide, but also show that activity is confined solely to the D-isomer of LSD. The levo-rotatory isomer, L-LSD, is totally devoid of activity when tested up to a concentration of 10^{-3} M (Fig. 5; see also Fig. 7 below).

The detailed kinetics of activation by D-LSD show that it saturates a single class of sites. Fig. 6 shows a direct plot as well as a Woolf inverse plot for D-LSD activation of adenylate cyclase. Linearity of the inverse plot indicates a single class of sites with K_A of about 40 nM. This site is the same as that for 5-HT, as seen by antagonism studies. Fig. 7 shows the effect of D- or L-LSD alone and the antagonism by D- or L-LSD or 5-HT activation. Woolf inverse plots indicate a single class of sites with a K_i of 40

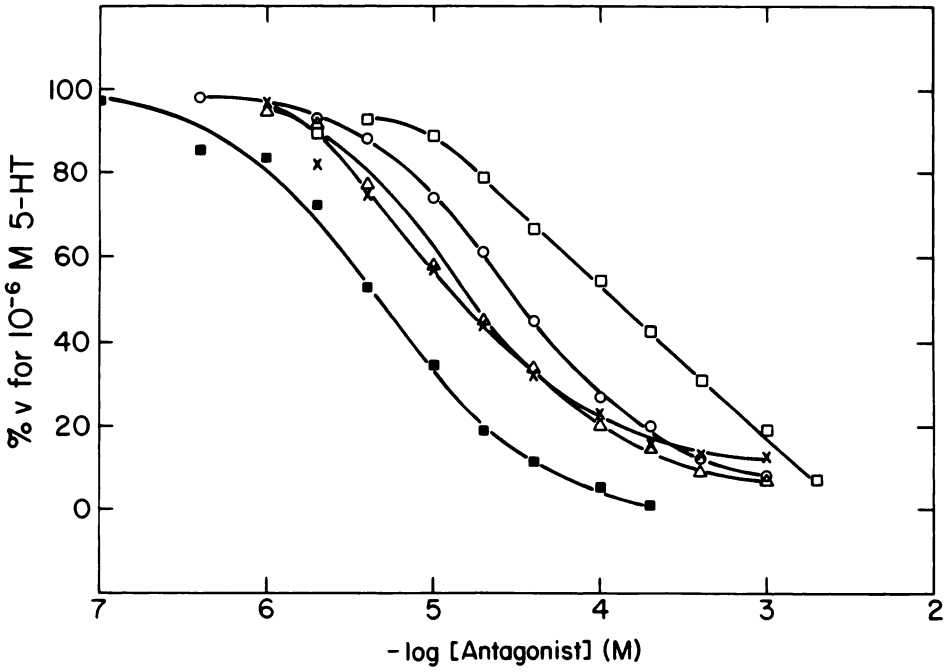

FIG. 4. *Antagonism of 5-HT by indoleamine and harmala derivatives*

Compounds were tested as antagonists of the 5-HT receptor by incubation at the indicated concentrations in reaction mixtures containing 1 μM 5-HT, 100 μM GTP, 1 mM ATP, and 5 mM $MgCl_2$. Adenylate cyclase activity was determined as described in METHODS. Basal velocity was subtracted from all values and the activity normalized as the fraction of activity due to 1 μM 5-HT alone. The points shown are the average values obtained for duplicate incubations in two separate experiments. Symbols are as follows: harmaline (■), gramine (○), N,N-dimethyl tryptamine (△), 5-methoxy-N,N-dimethyl tryptamine (×), and 12-hydroxy tetrahydroharman (□). Activation by 1 μM 5-HT averaged 0.44 nmoles/min·mg protein.

nM for D-LSD. Fig. 7 also shows that the stereoisomer, L-LSD, has considerably decreased affinity as an antagonist of 5-HT, with a K_i of about 2.5 μM. Both the activation of adenylate cyclase and antagonism of 5-HT activation, then, are stereo-specific for the hallucinogenic D-isomer.

Inhibition by 2-bromo LSD of basal as well as 5-HT-stimulated activity. The 2-bromo derivative of D-LSD (BOL) which is devoid of psychoactive properties in man, does not activate fluke adenylate cyclase. Rather, BOL inhibits basal (non-activated) enzyme activity and competes for the 5-HT receptor, as seen by antagonism of 5-HT stimulation (Fig. 8). The antagonism of serotonin can be seen to occur at nanomolar concentrations of BOL, and the inhibition of basal adenylate cyclase activity also occurred at the nanomolar range. The possibility that BOL might inhibit adenylate cyclase through an interaction with the serotonin receptor prompted us to explore its actions in considerable detail. Fig. 8A shows inhibition by BOL of adenylate cyclase stimulated with 1 μM 5-HT. A replot of these data as change in enzyme velocity versus BOL concentration in Fig. 8B shows hyperbolic saturation by BOL. The Woolf inverse plot, shown as an inset of Fig. 8B, is linear. This data are consistent with a single class of sites. This plot yields a K_i of 28 nM for BOL antagonism of 5-HT and a ΔV_{max} of 293 pmoles/mg·min. This ΔV_{max} is 12 pmole/mg·min below basal activity (no additions to the reaction mixture). In four experiments the average K_i was 30 ± 10 nM (mean ± SD) and the average ΔV_{max} was 10 ± 5 pmole/mg·min below basal activity.

We wished to determine the kinetics of the inhibitory action of BOL on basal activity. Since the inhibitory effect of BOL on

TABLE 1

Activation of adenylate cyclase by different serotonin and LSD analogs

Half-maximal constants and V_{max} were determined from the data in Figs. 3 to 5 by linear regression of Woolf inverse plots. For all activators, these were determined as an increase over basal, and for antagonists as a decrease from 5-HT stimulated activity. For compounds exhibiting apparent negative cooperativity (5-HT,5-methoxytryptamine, tryptamine and 5-hydroxy-N-Methyl tryptamine) only points on the high $K_{1/2}$ lines were used for the regression. All lines fit with $r^2 > 0.95$.

Effector	Relative efficacy V_m as fraction of 5-HT V_m	Apparent affinity		
		K_A[a]	K_B[b]	K_i[c]
			μM	
5-Hydroxytryptamine	1.00	2.00	—	—
5-Hydroxy-N-methyltryptamine	0.84	2.5	16	—
5-Hydroxy-N,N-dimethyltryptamine	0.38	5.0	9	—
5-Methoxytryptamine	0.84	20	—	—
Tryptamine	0.54	15	41	—
N-Methyltryptamine	0.28	13	13	—
N,N-Dimethyl-5-methoxytryptamine	0.04	5	—	8
N,N-Dimethyltryptamine	0.04	10	10	8
Harmaline	0.00	—	—	3
12-Hydroxy tetrahydroharman	0.00	—	—	80
Gramine	0.00	—	—	20
D-Lysergic acid diethylamide	0.25	0.046	0.055	0.030
D-Lysergic acid ethylamide	0.17	0.053	—	—
D-Lysergic acid dimethylamide	0.18	0.043	—	—
1-Methyl-D-lysergic acid diethylamide	0.18	0.125	—	—
D-Lysergic acid butanolamide	0.12	0.50	—	—
1-Methyl-D-lysergic acid butanolamide	0.13	2.0	—	—
2-Bromo-D-lysergic acid diethylamide	−0.02	—	0.008	0.030

[a] K_A values are simply the half-maximal activation constants calculated as the x-intercept of the Woolf inverse plot.

[b] K_B values for agonists were calculated by treating the data according to Barlow (22). Only points of [5-HT] > 0.4 μM were used. These data yielded linear plots ($r^2 > 0.99$), and K_B values were calculated from the y-intercept. For comparison, K_B is related to K_A as follows: $K_B = K_A(1 - e)$ where e is the relative efficacy of the compound compared to 5-HT.

[c] Half-maximally inhibitory concentrations were used to calculate K_i values on the basis of the following formula: $K_i = K_{1/2}\left(\frac{K_{A(5\text{-}HT)}}{1 + [5\text{-}HT]}\right)$.

basal activity was small, we carried out quadruplicate assays for each concentration in order to analyze the statistical significance. Fig. 9 shows the effect of increasing concentrations of BOL on basal adenylate cyclase activity. A decrease in enzyme velocity with increasing BOL can be seen. This inhibition also saturates hyperbolically and the inverse plot is linear as shown by the Woolf plot of this data in the inset. Half-maximal inhibition of the basal enzyme velocity occurred at 33 ± 15 nM BOL, and the ΔV_{max} was 6.8 pmole/mg·min below basal. In five independent experiments in which saturating concentrations of BOL were tested on basal adenylate cyclase, velocity in the presence of saturating levels of BOL was consistently inhibited, averaging 72.8 ± 12.6% (mean ± SD) of basal. Basal velocities ranged from 30 to 78 pmole/mg·min. Hence, the effect of BOL represents a statistically significant change of about 8–16 pmole/mg·min. In those experiments in which BOL was tested as an antagonist of 5-HT, there was good agreement between the maximal direct inhibition of basal velocity and the ΔV_{max} from 5-HT stimulated velocities. In the presence of 1 μM 5-HT the average ΔV_{max} at infinite BOL concentration of 10 ± 5 pmole/mg·min below basal velocity agrees well with direct inhibition of adenylate cyclase of 8–16 pmole/mg·min below basal activity.

TABLE 2

Activation and inhibition of adenylate cyclase activity by different agents

Compounds were added at a concentration of 100 μM to adenylate cyclase reaction mixtures with 100 μM GTP, 0.1 mM ATP, 2 mM $MgCl_2$ and with or without 1 μM 5-HT. Adenylate cyclase was assayed as described in METHODS. Each value is the average of values obtained from duplicate incubations in two separate experiments. Since these were data obtained in several experiments, the change of velocity from control levels is expressed relative to that for 100 μM 5-HT. Increase over basal velocity for 100 μM 5-HT averaged 1.6 nmoles/min.mg protein.

Compound	Activation % of activity for 10^{-4}M 5-HT	Inhibition % of activity for 10^{-6}M 5-HT
5-Hydroxy indole acetic acid	−0.1	4
1-Methyl-N,N-dimethyltryptophol	0.2	22
5,6-Dihydroxytryptamine	26	−18
N-Acetyl-5-hydroxytryptamine	−0.3	51
N-Acetyl-5-methoxytryptamine	0.2	47
4-Phosphoryl-N,N-dimethyltryptamine	0.7	12
Harmine	0.9	92
Harmaline	−0.3	97
Tetrahydroharman	0.1	73
12-Hydroxy tetrahydroharman	1.1	63
Histamine	−0.5	20
Epinephrine	0.5	22
Octopamine	0.7	13
Carbachol	−0.2	37
Dopamine	−0.1	—

The nature of the antagonism by BOL was further studied by Schild analysis (23). For a competitive antagonist, the Schild analysis should yield a line with a slope of 1.0. Our studies indicate a competitive interaction between BOL and 5-HT. Fig. 10 is a representative antagonism experiment. This plot is linear with a slope not significantly different from 1.0. Apparent negative cooperativity of 5-HT activation was evident in reciprocal plots of these data (not shown). Only the data points corresponding to the higher K_A lines on the reciprocal plots were used in this analysis. These results confirm the findings of negative cooperativity in 5-HT activation and are consistent with competitive antagonism by BOL. The Schild analysis yields an apparent affinity, K_B, of 8 nM for BOL interaction with the 5-HT site.

In addition to the compounds already discussed, a number of compounds were tested at only one or two concentrations to determine if they interacted with 5-HT-activated adenylate cyclase. These results are summarized in Table 2. It can be seen that only indoleamines and lysergic acid derivatives activated adenylate cyclase. Among the indoleamine compounds are a number of antagonists of 5-HT activation. These compounds have not, however, been tested sufficiently to state the nature of the antagonism or their K_i values. No compounds other than 5-HT or LSD derivatives have been found to have significant activity in the fluke system. Various other biogenic amines and drugs including histamine, epinephrine, dopamine, octopamine, norepinephrine, and carbachol were found to be without significant effect at 100 μM concentration. Several of these were found, however, partially to antagonize the activation of 1 μM 5-HT as shown in Table 2.

DISCUSSION

The present studies on adenylate cyclase from the liver fluke support our long held view that receptors regulating its activity are indoleamine receptors (24). This is supported by the finding that activators of the enzyme are indoleamines and lysergic acid derivatives which possess an indole nucleus as part of their structures. The data also show that both classes of activator compete for the serotonin receptors. Agents that are known to activate mammalian adenylate cyclase such as epinephrine, histamine, octopamine and dopamine were found to have no effect on the liver fluke enzyme. While we cannot disregard some other undiscovered ligands for an additional class of receptors, our evidence suggests nonetheless that the fluke adenylate cyclase contains only a single class of receptors regulating a single population of adenylate cyclase. Since the presence of serotonin or a related indoleamine and its biosynthesis have been indicated in the liver fluke (25) and since 5-HT

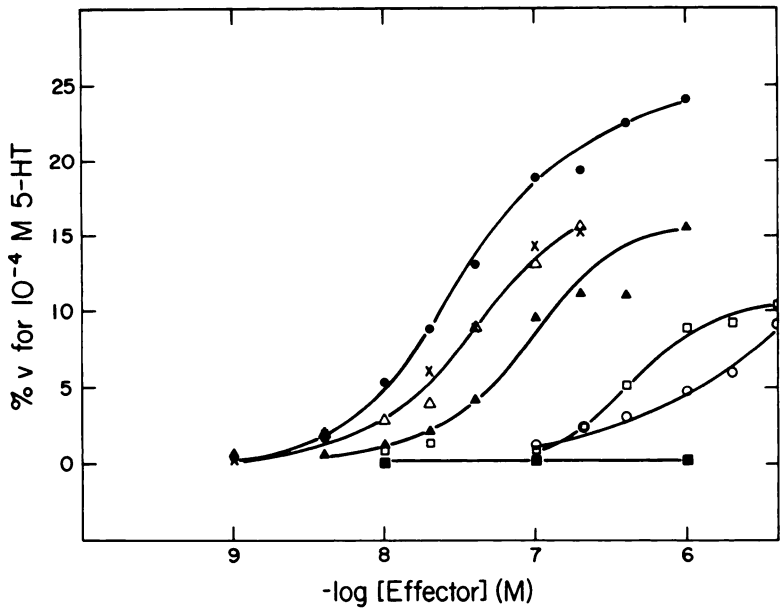

FIG. 5. *Activation of adenylate cyclase by lysergic acid derivatives*

Activation by derivatives of lysergic acid was determined by addition of the compounds at the indicated concentrations to reaction mixtures containing 100 μM GTP, 1 mM ATP, and 5 mM $MgCl_2$ and assaying adenylate cyclase as described in METHODS. Basal velocity was subtracted from all values and activity normalized as the fraction of activity due to 100 μM 5-HT determined in the same experiments. Symbols are as follows: D-lysergic acid diethylamide (●), D-lysergic acid ethylamide (△), D-lysergic acid dimethylamide (×), 1-methyl-D-lysergic acid diethylamide (▲), ergonovine (□), 1-methyl-D-lysergic acid butanolamide (○), and L-lysergic acid diethylamide (■). 100 μM 5-HT activation averaged 0.70 nmoles/min·mg protein.

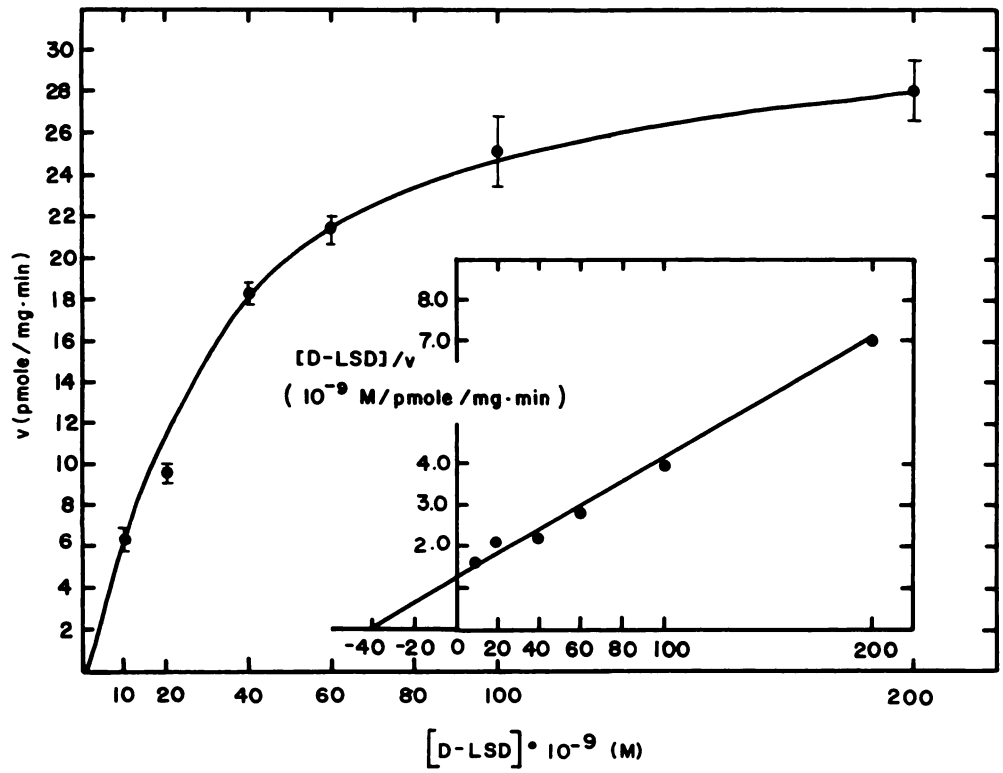

FIG. 6. *Kinetics of activation by D-LSD*

The saturation of activation by D-LSD was determined by adding the indicated concentrations of D-LSD to reaction mixtures and assaying adenylate cyclase in the presence of 1 mM ATP, 5 mM $MgCl_2$ and 100 μM GTP as described in METHODS. This figure shows the direct plot of change in velocity over basal (V) as a function of [D-LSD]. The inset is the Woolf inverse plot of these data determined by linear regression ($r^2 > 0.99$).

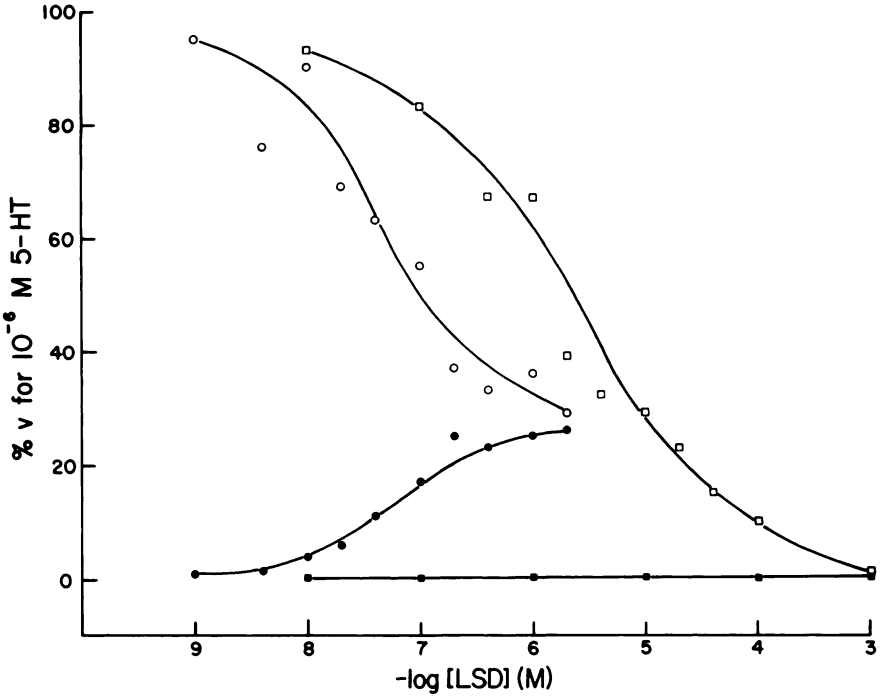

FIG. 7. *Antagonism of 5-HT activation by D- and L-LSD*

In these experiments the indicated concentrations of D- (○, ●) or L-LSD (□, ■) were added to reaction mixtures containing 1 mM ATP, 5 mM $MgCl_2$ and 100 μM GTP with (○, □) or without (●, ■) 1 μM 5-HT. Adenylate cyclase was assayed as described in METHODS. Basal velocity has been subtracted from all values and the activities have been normalized as the fraction of activity due to 1 μM 5-HT.

is the most potent of the known indoleamines in activating adenylate cyclase, it is likely that 5-HT or a related indoleamine is the natural regulator of this enzyme in the fluke.

Evidence for all of these activators utilizing a common class of receptor sites comes from studies of their interactions. Competition for the 5-HT receptor by inactive indoleamines was demonstrated with antagonism studies. The dimethyl indoleamines (dimethyltryptamine, 5-methoxydimethyltryptamine, and gramine) and Harmala alkaloids (harmaline and 12-hydroxytetrahydroharman) all appeared to be competitive antagonists of 5-HT. Furthermore, the partial agonist compounds (dimethyltryptamine and 5-methoxydimethyl tryptamine) were shown to be partial antagonists of 5-HT activation. Thus, these derivatives activate adenylate cyclase through a common receptor site. These results were expected as all of the indoleamines tested in detail were structurally related to serotonin. Lysergic acid derivatives also competed for the 5-HT site. LSD, which activated the adenylate cyclase half-maximally at 46 nM, was previously reported to antagonize serotonin-activated accumulation of endogenous cAMP in the fluke (6). The earlier findings are consistent with the results reported above that LSD is a partial agonist with efficacy of 25% that of 5-HT. The competitive antagonism of 5-HT activation of adenylate cyclase by BOL determined by Schild analysis showed that this compound, too, interacted with the serotonin site. Furthermore, the agreement between K_i and V_{max} for both inhibition of basal adenylate cyclase and antagonism of 5-HT activation suggests that the direct inhibition by BOL of basal activity also is mediated by the serotonin site. This interpretation means that BOL has a negative efficacy for the serotonin receptor. Our data, then, are consistent with a single class

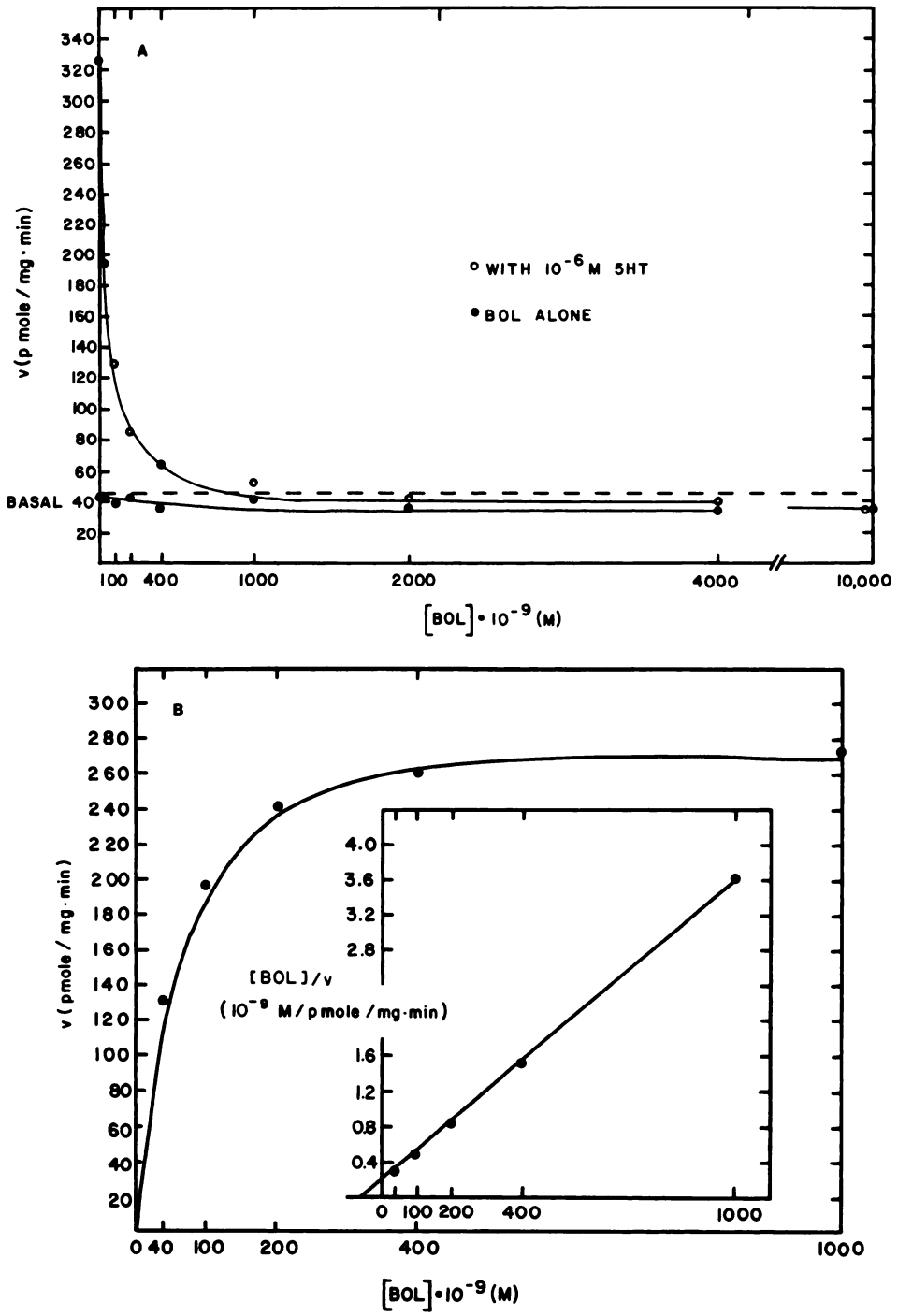

FIG. 8. *Inhibition of basal and 5-HT stimulated adenylate cyclase by BOL*

(a) The effect of BOL on basal (●) and 1 μM 5-HT activated (○) adenylate cyclase velocities was determined by incubation of the indicated concentrations of BOL with 100 μM GTP, 1 mM ATP, 5 mM $MgCl_2$ and with or without 1 μM 5-HT in adenylate cyclase reaction mixtures. Adenylate cyclase was assayed as described in METHODS. Basal velocity is indicated by the dashed line.

(b) The data of BOL antagonism of 1 μM 5-HT activated adenylate cyclase from Fig. 8(a) are replotted here. ΔV was computed as the decrease in velocity from that of 1 μM 5-HT alone. The inset shows a Woolf inverse plot of this data. The line was computed by linear regression ($r^2 > 0.99$).

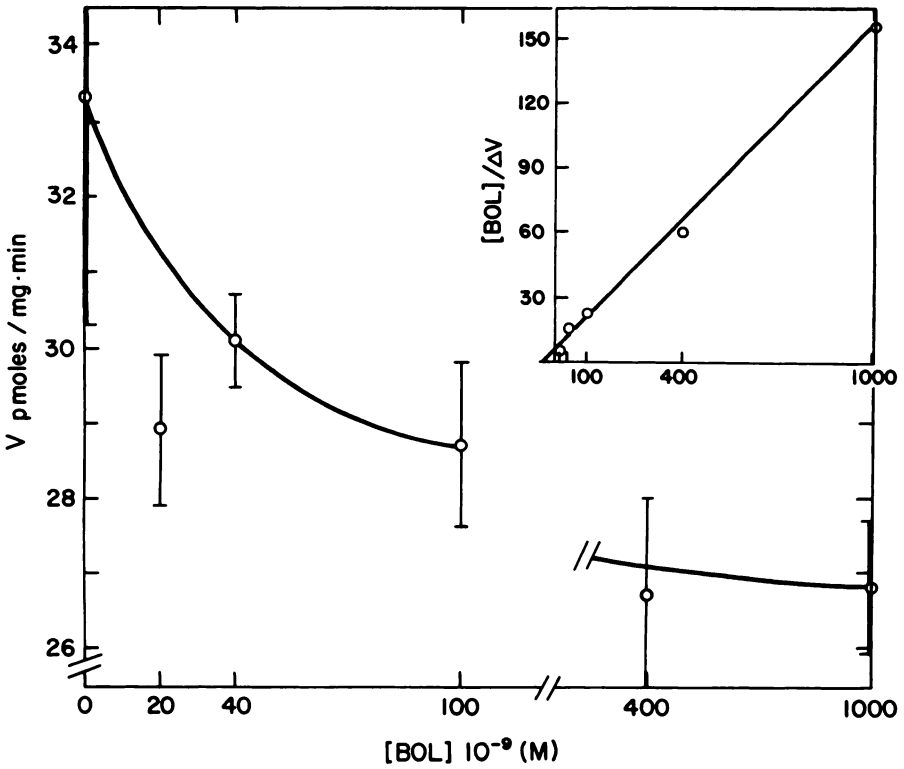

FIG. 9. *BOL inhibition of basal adenylate cyclase*

The concentration dependence of BOL inhibition of basal adenylate cyclase was determined by quadruplicate incubations of the indicated concentrations of BOL in adenylate cyclase reaction mixtures. Adenylate cyclase was determined as described in METHODS in the presence of 1 mM ATP and 5 mM $MgCl_2$. The values plotted are the means of the quadruplicate incubations, and error bars indicate standard deviations for each mean value. The adenylate cyclase velocities for 400 nM and 1 μM BOL were significantly different from control (basal) activity ($p < 0.01$). These data are replotted in the inset as a Woolf plot of the decrease in adenylate cyclase activity from basal (ΔV). The line drawn is the least-squares best fit ($r^2 > 0.99$). Linearity indicates a single class of sites for BOL. Half-maximal decrease in velocity was calculated to occur at 33 ± 15 nM (95% confidence). ΔV_{max} with BOL was −7 pmol/min·mg protein.

of serotonin sites interacting with ligands of positive, zero, and negative efficacy.

Because of the unique class of activating ligands, this adenylate cyclase provides an uncomplicated model system of serotonin receptors. The structure activity studies for indoleamines and lysergic acid derivates show the importance of the 5-hydroxyl and free ethyl amine for agonists of this receptor. The high apparent affinity of D-lysergic acid amides and reduced affinity of dimethylated indoleamines suggest that the preferred conformation for binding has the ethylamine coplanar with the indole ring as in LSD. The decreased apparent affinity of L-LSD and of all other amide substitutions of D-LSD suggests an additional interaction of the amide region of the LSD molecule which cannot occur for indoleamines. Equilibrium dissociation constants determined by ligand binding methods will strengthen these observations, but the data reported here for activation provide important information of pharmacologic activity for these compounds which cannot be obtained by ligand binding. It is of interest that all of the hallucinogenic substances tested proved to be partial agonists of the receptor, a finding in keeping with the studies of the raphé nucleus, where the compounds were found to be serotonin agonists (26).

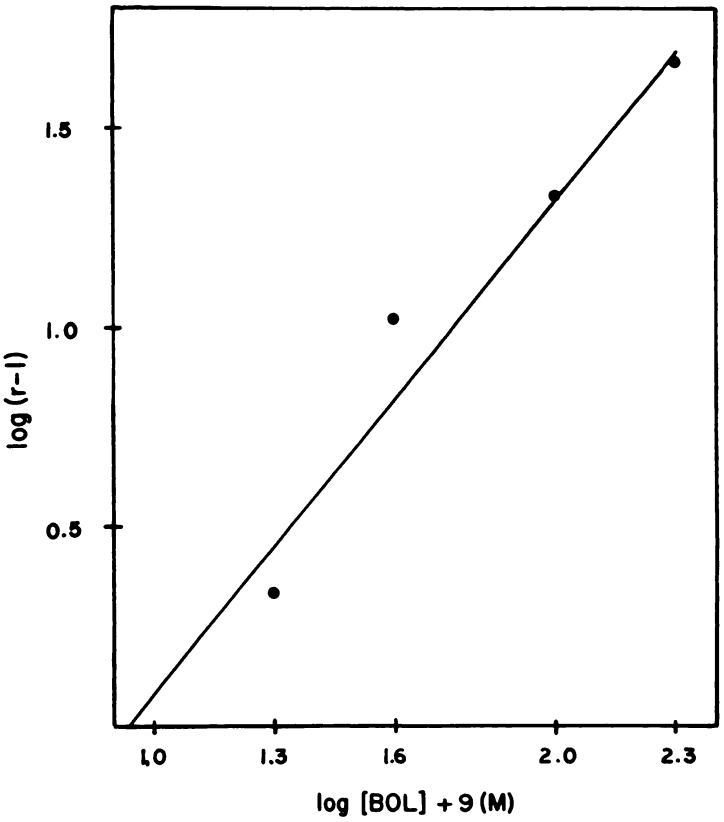

FIG. 10. *Antagonism of 5-HT activation by BOL*

A Schild analysis of the interaction of 5-HT and BOL was performed on data obtained for 5-HT activation in the presence of fixed concentrations of BOL. Seven concentrations of 5-HT (1–100 μM) were added to tubes containing each of the indicated concentrations of BOL, and adenylate cyclase was determined in the presence of 1 mM ATP, 5 mM $MgCl_2$ and 100 μM GTP as described in METHODS. Linear regression of inverse plots of increase in velocity above BOL alone was used to determine $K_{1/2}$ and V_{max}. The concentration of 5-HT required for a V of .300 nmoles/min·mg (×300) was computed for each concentration of BOL. The value of r was calculated as the ratio of ×300 in the presence and absence of BOL. The Schild plot was fitted by linear regression (r^2 = 0.94, a_1 = 1.25 ± 0.22, 95% confidence). Apparent affinity for BOL (K_B = 8 ± 4 nM, 95% confidence), was determined as the concentration of BOL at which log (r − 1) = 0.

The apparent negative cooperativity of 5-HT activation and the existence of a ligand with negative efficacy (27, 28) both argue for two states of the serotonin receptor associated with this adenylate cyclase. Our enzyme activation data suggest two states of the serotonin receptor with different affinities for 5-HT (K_A 0.14 and 2.6 μM). Similarly, our results indicated two apparent affinities for BOL—the Schild analysis yielded an apparent affinity (K_B = 8 nM) while the K_i value was 30 nM. The latter discrepancy in fact is predicted by a two-state model for receptor activation (27, 28).

Ligand binding studies in membranes from the mammalian central nervous system have suggested that receptors for several neurotransmitters and for opiates exist in two conformations with preferential affinity for agonist or antagonist drugs (29). Also, models involving two states of the receptor have been proposed to describe hormonal activation of adenylate cyclase (30). Ligand binding studies of presumptive serotonin receptors in the mammalian central nervous system (16, 17) indicated that these receptors also exist in two binding conformations. Based upon the difference

between K_d values determined for displacing labelled LSD or 5-HT, the latter studies suggested that BOL should be a potent antagonist and D-LSD a partial agonist of 5-HT receptors. Our results which are based on enzyme activity directly confirm these predictions. In fact, the inhibitory action of BOL on basal adenylate cyclase is in agreement with the interpretation of higher affinity for the inactive or antagonist form of the receptor (17).

The inhibitory action of BOL need not signify two states of the 5-HT receptor, but may rather be explained by contamination of the particle preparation by an endogenous activator. This seems unlikely because variations in basal activity among preparations of fluke particles generally correlate well with variations in V_{max} for 5-HT stimulation (about 25–30 fold), which would not be expected if basal activity were due to contaminating ligands. The lack of activation by GTP in the absence of added 5-HT reported in the accompanying paper (21) also is inconsistent with contamination by an activating ligand. The interpretation of apparent negative cooperativity for the kinetics of 5-HT activation also cannot be unqualified. While there is no other evidence for heterogeneity of adenylate cyclase, the existence of several classes of 5-HT regulated adenylate cyclase cannot be excluded, and this may account for the apparent cooperativity. This suggestion, and that of ligand contaminations of the particles, cannot be tested directly at this time and must be considered as alternative explanations to the suggested two-state interpretation.

The present experiments demonstrate a preparation of 5-HT receptors regulating adenylate cyclase in *F. hepatica* from which detailed pharmacological data have been obtained. The motility response of intact liver flukes to LSD derivatives was previously reported to give a good rank-order potency correlation with hallucinogenic potency in humans (11). The present data expand on this with the additional result that all hallucinogens tested were partial agonists of the 5-HT receptor. Since adenylate cyclase can be studied in cell-free membrane systems amenable to ligand binding methods, detailed molecular studies of this receptor should be possible. It will be of interest to compare these results to those now obtainable with the recently demonstrated serotonin-sensitive adenylate cyclase of mammalian brain (31, 32).

REFERENCES

1. Lefkowitz, R. J. (1976) *Life Sci.*, **18**, 461–472.
2. Mansour, T. E. (1959) *J. Pharmacol. Exp. Ther.*, **126**, 212–216.
3. Mansour, T. E. (1962) *J. Pharmacol. Exp. Ther.*, **135**, 94–101.
4. Mansour, T. E., Sutherland, E. W., Rall, T. W. & Bueding, E. (1960) *J. Biol. Chem.*, **235**, 466–470.
5. Stone, D. B. & Mansour, T. E. (1967) *Mol. Pharmacol.*, **3**, 161–176.
6. Abrahams, S. L., Northup, J. K. & Mansour, T. E. (1976) *Mol. Pharmacol.*, **12**, 49–58.
7. Nathanson, J. A. & Greengard, P. (1974) *Proc. Nat. Acad. Sci., U. S. A.*, **71**, 797–801.
8. Berridge, M. J. & Prince, W. T. (1973) *Nature New Biol.*, **243**, 283–284.
9. Berridge, M. J. & Prince, W. T. (1974) *Br. J. Pharmacol.*, **51**, 269–278.
10. Mansour, T. E., Lago, A. D. & Hawkins, J. L. (1957) *Fed. Proc.*, **16**, 319.
11. Beernick, K. D., Nelson, S. D. & Mansour, T. E. (1963) *Int. J. Neuropharm.*, **2**, 105–112.
12. Shaw, E. & Wooley, D. W. (1956) *Science*, **124**, 121–125.
13. Erspamer, V. (1966) in *Handbook of Experimental Pharmacology* XIX, N.Y.
14. Gyermek, L. (1966) in *Handbook of Experimental Pharmacology* XIX, N.Y.
15. Bennett, J. P. & Aghajanian, G. K. (1975) *Life Sci.*, **15**, 1935–1944.
16. Bennett, J. P. & Snyder, S. H. (1975) *Brain Res.*, **94**, 523–544.
17. Bennett, J. P. & Snyder, S. H. (1976) *Mol. Pharmacol.*, **12**, 373–389.
18. Lovell, R. A. & Freedman, D. X. (1976) *Mol. Pharmacol.*, **12**, 620–630.
19. Salomon, Y., Londos, C. & Rodbell, M. (1975) *Anal. Biochem.*, **58**, 541–548.
20. Mansour, T. E. & Mansour, J. (1977) *Biochem. Pharmacol.*, **26**, 2325–2330.
21. Northup, J. K. & Mansour, T. E. (1978) *Mol. Pharmacol.*, **14**, 820–833.
22. Barlow, R. B., Scott, N. C. & Stephenson, R. P. (1967) *Br. J. Pharmac.*, **31**, 188–196.
23. Schild, H. O. (1949) *Br. J. Pharmac.*, **4**, 227–232.
24. Mansour, T. E. (1970) in Blum, J. J. (ed.) *Biogenic Amines as Physiological Regulators.* Prentice Hall, Englewood Cliffs.
25. Mansour, T. E. & Stone, D. B. (1970) *Biochem. Pharmac.*, **19**, 1137–1146.

26. Aghajanian, G. K. & Haigler, H. G. (1974) *Adv. Biochem. Psychopharmacol.*, **10**, 167–177.
27. Thron, C. D. (1973) *Mol. Pharmacol.*, **9**, 1–9.
28. Colquhoun, D. (1973) in *Drug Receptors* (Rang, ed.), University Park Press, Baltimore.
29. Synder, S. H. (1975) *Biochem. Pharmacol.*, **24**, 1371–1374.
30. Hammes, G. & Rodbell, M. (1976) *Proc. Nat. Acad. Sci. U.S.A.*, **73**, 1189–1192.
31. Enjalbert, A., Bourgoin, S., Harmon, M., Adrien, J., & Bockaert (1978) *Mol. Pharmacol.*, **14**, 2–10.
32. Enjalbert, A., Hamon, M., Bourgoin, S., and Bockaert (1978) *Mol. Pharmacol.*, **14**, 11–23.