Biochemical Pharmacology, Vol. 26, pp. 2325–2330. Pergamon Press, 1977. Printed in Great Britain.

PHOSPHODIESTERASE IN THE LIVER FLUKE, *FASCIOLA HEPATICA*

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(*Received* 14 *December* 1976; *accepted* 25 *February* 1977)

Abstract—Phosphodiesterase was found in homogenates of the liver fluke, *Fasciola hepatica*, and was distributed between a supernatant and particulate fraction after centrifugation at 2000 *g*. Mg^{2+} was necessary for enzyme activity; Ca^{2+} in the presence of Mg^{2+} did not affect enzyme activity. Enzyme kinetics followed the Michaelis–Menten model with a K_m of 8 μM for cAMP and 300 μM for cGMP as the substrate. The most potent inhibitor tested was 1-ethyl-4-(isopropylidenehydrazino)-1 *H*-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, HCl (SQ 20009) which had a K_i of 26 μM. The K_i for isobutyl methyl xanthine (IBMX) was 45 μM; for 6,7 dimethyl-4 ethylquinazoline (Quazodine) 75 μM; papaverine, 100 μM; theophylline, 550 μM; and for caffeine or D-lysergic acid diethylamide (LSD), 800 μM. The effects on fluke motility of these phosphodiesterase inhibitors were tested. All phosphodiesterase inhibitors except caffeine stimulated the rhythmical movement of the flukes. None of the inhibitors tested significantly increased the endogenous cAMP concentrations of fluke heads. IBMX potentiated the rise in endogenous cAMP caused by 5-hydroxytryptamine (5-HT) but SQ 20009, LSD, and papaverine prevented it. The latter results could not be explained on the basis of phosphodiesterase inhibition, but might be attributed to interference with the stimulation of adenylate cyclase by 5-HT.

Cyclic 3',5'-nucleotide phosphodiesterase (EC 3.1.4.17) is the only enzyme known to hydrolyze the cyclic nucleotides to the 5'-nucleotide monophosphates. Adenylate cyclase and phosphodiesterase regulate the concentration of cyclic nucleotides in the cell and in extracellular fluids.

For many years this laboratory has been studying the biochemistry of the liver fluke, *Fasciola hepatica*, frequently using it as a model for the effects of 5-hydroxytryptamine (5-HT)* and related psychoactive agents. 5-HT appears to be one of the regulators of carbohydrate metabolism and neuromuscular activity in the liver fluke. 5-HT stimulates glycolysis and glycogenolysis [1, 2], activates glycogen phosphorylase and phosphofructokinase [3–5] and stimulates the motility of this organism [6]. It increases the endogenous concentration of cAMP by activating adenylate cyclase [7] and it activates protein kinase [8]. LSD antagonizes these last two effects of 5-HT, but stimulates motility [7, 8]. Indolamine analogs of 5-HT stimulate fluke motility and activate adenylate cyclase in direct relation to their structural similarity to 5-HT [7]. Externally added 5-HT has the same effects as that synthesized internally from its metabolic precursor, 5-hydroxytryptophan [9]. For these reasons we postulated that 5-HT or a related indolamine may have a hormonal function in the flukes similar to that of epinephrine in higher organisms, and that cAMP acts as a second messenger for 5-HT.

Since we have reported the presence of phosphodiesterase in the liver fluke [3] the present investigation was undertaken to study the properties of the enzyme and to test the effects of compounds which were known to inhibit phosphodiesterases from other sources. Specifically, we wanted to know whether these inhibitors would affect the levels of endogenous cAMP and whether they could potentiate the action of 5-HT in raising the endogenous levels of cAMP in the flukes. We also wanted to compare the motility of flukes treated with 5-HT or phosphodiesterase inhibitors.

MATERIALS AND METHODS

Crotalus atrox venom, cAMP, cGMP and 5-HT were purchased from Sigma; LSD from Sandoz; [8-^{14}C] cAMP and [8-^{14}C] cGMP from ICN; and IBMX from Aldrich. Quazodine was a gift of Mead-Johnson (Dr. G. R. McKinney) and SQ 20009 was given by E. R. Squibb and Sons, Inc. by the courtesy of Dr. S. M. Hess. Instagel was purchased from Packard and anion exchange resin, AG 1-X8, 200–400 mesh from Bio-Rad and prepared according to the directions of R. Ho (personal communication). It was washed with water until pH of effluent was 5–6, washed with 0.2 M NaOH until Cl-free ($AgNO_3$ test), washed with water until pH was 6–7, washed with 0.2 N formic acid until pH was 2.5 and washed with water until pH of effluent was approximately 4. The resin was then suspended in 2 vol. of 0.1 M ammonium formate and stored at 4°. Before use the resin was shaken thoroughly and 1 ml was pipeted into each 0.7 × 4 cm polypropylene column and washed with 3 ml of 20 mM ammonium formate (pH 7.4).

Assay procedure. The reaction sequence for phosphodiesterase assay was similar to the one originally

* Abbreviations used: cAMP (cyclic 3',5'-adenosine monophosphate), cGMP (cyclic 3',5'-guanosine monophosphate), 5-HT (5-hydroxytryptamine), LSD(D-lysergic acid diethylamide), IBMX (iso-butyl-methyl xanthine), Quazodine (6,7 dimethyl-4 ethylquinazoline), SQ20009 (1-ethyl-4-(isopropylidenehydrazino)-1H-pyrazolo-(3,4-b)-pyridine-5-carboxylic acid, ethyl ester, hydrochloride).

described by Butcher and Sutherland [10] except that a ^{14}C-iabeled substrate was used.

[^{14}C] nucleoside-3′,5′-phosphate *Phosphodiesterase* → [^{14}C] nucleoside 5′-phosphate *Crotalus atrox venom* → [^{14}C]nucleoside + P_i.

The unreacted nucleoside 3′,5′-phosphate was separated from the nucleoside by anion exchange chromatography which was essentially that of Wells *et al.* [11].

The reaction medium contained 6 mM magnesium acetate, 25 mM Tris–HCl (pH 7.5), 25 mM imidazole (pH 7.5), enough [^{14}C] cAMP to give 10,000 to 12,500 cpm, non-radioactive cAMP which varied from 1 μM to 0.5 mM and water to make a total volume of 0.45 ml. The reaction was started by adding 0.05 ml enzyme diluted with 0.25 M sucrose so that 5–30% of the substrate was hydrolyzed in 10 min in a shaking water bath at 37°. The reaction was stopped by adding 0.05 ml of 0.1 M EDTA. Immediately 0.1 ml *Crotalus atrox* venom (5 mg/ml) was added and the tube returned to the shaking bath for an additional 10 min. Blanks were obtained by adding enzyme preparations after EDTA and snake venom. After the incubation with snake venom 0.35 ml of 0.1 mM adenosine was added to each tube and an aliquot (0.8 ml) was removed and placed on an anion exchange column. (See Materials) Scintillation vials were placed under the columns and after the aliquot had run through the adenosine was eluted with 4 ml of 20 mM ammonium formate (pH 7.4). 5 ml Instagel was added to each vial and they were counted for 10 min in a liquid scintillation spectrometer. When cGMP was the substrate the procedure was varied by diluting the reaction mixture with guanosine after the incubation with snake venom. Six ml of 20 mM ammonium formate was used to elute guanosine from the columns and 7 ml Instagel was added to the scintillation vials. Results were calculated as nmoles of cyclic nucleotide hydrolysed/min/0.1 ml extract or per mg protein. Protein was determined by the method of Lowry *et al.* [12].

Preparation of phosphodiesterase. Whole flukes, heads or tails were homogenized in 0.33 M sucrose (6 ml/g wet weight) in a motor-driven glass homogenizer. Supernate and particulate fractions were prepared from homogenate by centrifuging for 20 min at 2000 *g*. For kinetic experiments particulate fractions were suspended in the original volume with 0.25 M sucrose, centrifuged again, and resuspended in the same volume of 0.25 M sucrose. Before assay supernate was usually diluted 1:10 and particulate enzyme 1:5 in 0.25 M sucrose. Fluke heads were prepared by cutting off the anterior end of the fluke just behind the suckers. The posterior 80 per cent of the fluke is referred to as the tail. All operations except cutting off heads were carried out at 4°.

Determination of endogenous cAMP. cAMP was determined by the Gilman binding assay [13]. Flukes were prepared for the assay as described previously [7]. Test compounds were added to the saline medium to a final concentration of 1 mM (except LSD—1 μM). Fluke heads were immersed in the medium for 10 min at 37° before 5-HT was added. Motility was estimated as reported previously [7] and aliquots of fluke heads were frozen on Wollenberger clamps after various time intervals.

Table 1. Distribution of phosphodiesterase activity between supernate and unwashed particles prepared from whole-fluke homogenates by centrifugation at 2000 *g* for 20 min

	nmoles/g wet weight*/min				
Expt. No.	Homogenate	Supernate	% of Hom.	Particles	% of Hom.
1	59	39	67	14	25
2	93	55	59	20	21
3	86	41	48	16	22
4	59	46	78	20	34
Mean	74	45.3		17.5	

* One gram wet weight is 10–12 flukes.

RESULTS

Properties of phosphodiesterase in flukes. Table 1 shows how activity (after centrifugation at 2000 *g*) was distributed between the supernatant and the particulate fractions. These results are expressed in terms of g wet weight so that the actual distribution of enzyme activity within the flukes can be understood and also so phosphodiesterase activity could be measured in the same fractions that had previously been used to measure adenylate cyclase activity [7]. It is interesting to note that 21–34 per cent of phosphodiesterase activity was present in the particulate fraction that was reported to contain the major adenylate cyclase activity [3].

The specific activity (at 10 μM cAMP) of homogenates of whole flukes was 0.3 nmoles cAMP/mg protein/min at 37°. In addition to whole-fluke homogenates we determined the specific activity of supernates from whole flukes, heads, and tails. The specific activity of supernate prepared from fluke heads was 1.7 nmoles/mg protein/min, almost twice as high as that of supernate from tails (1.1) or from whole flukes (0.95).

Phosphodiesterase activity in freshly prepared fractions was stable at 4° for 8 hr. The activity was reduced 10–50 per cent by freezing either at −20° or in liquid nitrogen, but subsequently remained stable for several months. Consequently, fractions were frozen routinely whenever there was an excess of fresh flukes. Although frozen fractions were used for many experiments the results were always checked using freshly prepared material. No significant differences were noted between fresh or frozen extracts.

A buffer system composed of 25 mM Tris–HCl and 25 mM imidazole at pH 7.5 was used routinely because enzyme activity was maximal in the presence of this buffer. Activity was only 38 per cent of the maximum at pH 8.6 and only 75 per cent of the maximum at pH 6.6. Imidazole was essential for maximal activity. Mg^{2+} was necessary for phosphodiesterase activity. Elimination of Mg^{2+} by using EDTA in the reaction mixture resulted in complete loss of enzyme activity. The presence of 6 mM Magnesium acetate in the reaction mixture was optimal. If Mn^{2+} was substituted for Mg^{2+} the activity was not significantly affected.

Ca^{2+} has frequently been reported to be either an activator or an inhibitor of phosphodiesterase. Addition of EGTA to the phosphodiesterase reaction mixture to eliminate Ca^{2+} caused no change in phospho-

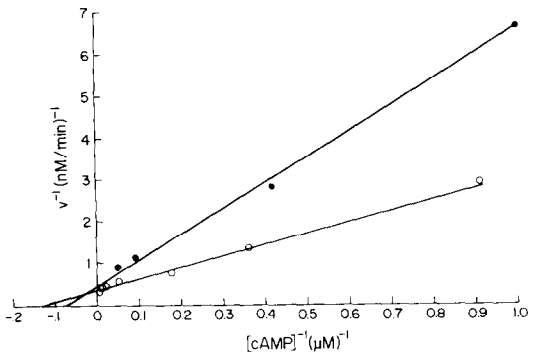


Fig. 1. Lineweaver–Burke plot of cAMP hydrolysis by supernate ○ and particulate ● fractions from *F. hepatica*. The concentration of cAMP varied from 1.1 to 500 μM for the supernate and from 1.0 to 20.4 μM for the particulate enzyme. The protein concentration of the supernate in the assay was 0.8 mg/ml, and for the particulate it was 1.6 mg/ml. All lines in Lineweaver–Burke plots were drawn by linear regression.

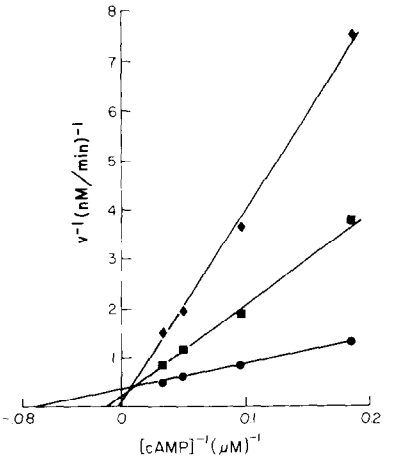


Fig. 3. Lineweaver–Burke plot of cAMP hydrolysis by particulate phosphodiesterase ● and inhibition by 100 μM ■ and 400 μM ◆ IBMX. cAMP concentration varied from 3.4 to 20.4 μM.

diesterase activity. Enzyme activity also remained unchanged whether or not 25 or 50 μM $CaCl_2$ was added to the reaction. Therefore, we concluded that Ca^{2+} neither stimulates nor inhibits the phosphodiesterase from *F. hepatica*.

Enzyme kinetics. Experiments were done to determine the kinetics of the enzyme. Figure 1 shows a Lineweaver–Burke plot for supernatant and particulate fractions using cAMP as substrate. The K_m for the enzyme in the supernate was 8 μM and for the particulate enzyme was 12 μM. The kinetics appear to follow the Michaelis–Menten model. When cGMP was the substrate for supernate the kinetics appeared similar to those when cAMP was used, but the K_m was 300 μM.

Several of the more commonly used phosphodiesterase inhibitors were tested to determine their relative inhibition of the fluke enzyme and whether the inhibition was competitive or non-competitive. Figure 2 shows a Lineweaver–Burke plot of the inhibition of enzyme activity in the supernate by IBMX. Figure 3 is a comparable plot for the particulate enzyme. Both figures illustrate an apparent competitive inhibition. The K_i for supernate of 55 μM and the K_i for particulate enzyme of 45 μM were calculated from multiple experiments using either Dixon or Hunter–Downs plots as described in Dixon and Webb [14]. Figure 4 is the plot for SQ 20009 inhibition of the supernatant enzyme. The inhibition appears to be competitive and the K_i is calculated to be 26 μM.

Since it was previously reported that LSD antagonizes the 5-HT elevation of endogenous cAMP levels in the liver fluke [7] it was thought desirable to test its effect on phosphodiesterase. Figure 5 is the plot for inhibition of supernate phosphodiesterase by LSD. The inhibition is apparently competitive.

Other compounds which inhibited fluke phosphodiesterase were caffeine, theophylline, papaverine and Quazodine. K_i's were determined for compounds by Dixon plots or Hunter–Downs plots (not shown) [14] and are listed in Table 2. Neither 5-HT nor guanosine inhibited phosphodiesterase from the liver fluke.

The effect of phosphodiesterase inhibitors on motility and endogenous cAMP concentrations in fluke heads. Whole flukes or fluke heads in saline medium undu-

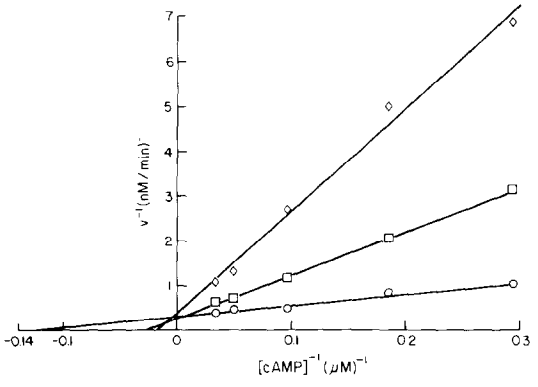


Fig. 2. Lineweaver–Burke plot of cAMP hydrolysis by supernate ○ and inhibition by 100 μM □ and 400 μM ◇ IBMX. cAMP concentration varied from 3.4 to 30.4 μM.

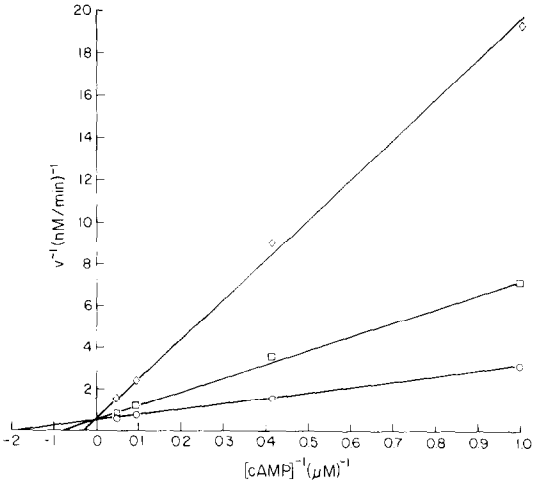


Fig. 4. Lineweaver–Burke plot of cAMP hydrolysis by supernate ○ and inhibition by 20 μM □ and 100 μM ◇ SQ 20009. cAMP concentration varied from 1 to 20.4 μM.

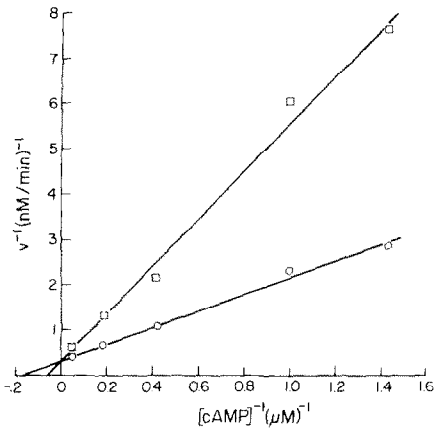
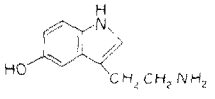
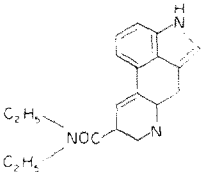
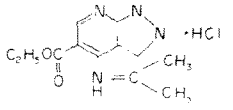
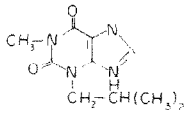
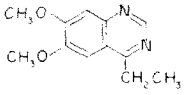
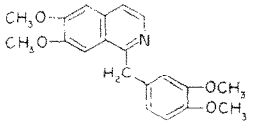
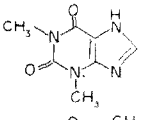
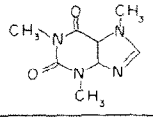


Fig. 5. Lineweaver-Burke plot of cAMP hydrolysis of supernate ○ and inhibition by 0.8 mM LSD □. cAMP concentration varied from 0.7 to 20.4 μM.

late slowly. When 5-HT (1 mM) is added the undulations increase in frequency and amplitude [6]. The resting state is rated 0 and the exitation caused by 1 mM 5-HT is rated +4. The effect on motility can be observed less than 1 min after adding the test compound to the flukes in saline medium. Of the compounds reported in this paper, all except SQ 20009 caused the same kind of undulation that 5-HT produced. The movements caused by SQ 20009 were not undulations, but resembled spastic contractions with a thickening body and decreased perimeter of the whole fluke or fluke head. Among the compounds tested (with the exception of LSD) the stimulation of motility was inversely related to the K_i for phosphodiesterase inhibition. (Table 2).

It has previously been reported that 5-HT and related indolamines cause a marked increase in endogenous cAMP [7]. This effect is more apparent in fluke heads probably because they are relatively

Table 2. The structures and K_i's of phosphodiesterase inhibitors and their effects on mobility, concentration of endogenous cAMP, and 5-HT mediated elevation of endogenous cAMP

Compound*	Structure	Motility	K_i (μM)	pmoles cAMP per mg protien	+ 1mM 5-HT
No additions		0	—	13.7±7.7 (12)	432±120
5-HT		+4	—	432±120 (12)	—
LSD		+4	790	14†	64†
SQ 20009		+4‡	26	16.4±5.8 (3)	134±32 (3)
IBMX		+4	55	22.7±9.8 (9)	600 1112 mean=856
Quazodine		+3	75	Not tested	339 (1)
Papaverine		+2	100	4.5 14 mean=9.2	27.5 97.6 mean=62.6
Theophylline		+1	550	Not tested	399 (1)
Caffeine		0	800	32 (1)	327 (1)

Numbers in parentheses indicate the number of experiments done. * All concentrations are 1 mM except LSD which was 1 μM. † Data from Abrahams *et al.* [7]. ‡ See Results.

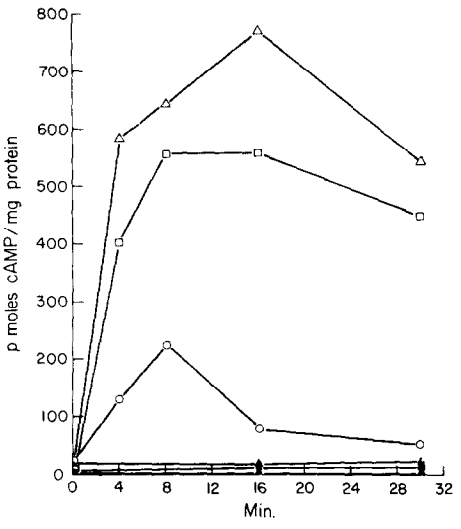


Fig. 6. Time course for formation of endogenous cAMP in heads of flukes. Fluke heads were incubated for 10 min in saline medium ■, containing 1 mM IBMX ▲ or 1 mM SQ 20009 ●. At 0 time 1 mM 5-HT was added to fluke heads in saline medium □, and to fluke heads in medium containing 1 mM IBMX △, or 1 mM SQ 20009 ○. Aliquots of 20 heads were removed at the indicated times and immediately frozen on Wollenberger tongs. cAMP was determined as described in Methods.

richer in ganglionic and neuromuscular structures. Experiments were done to determine if phosphodiesterase inhibitors would raise the concentration of endogenous cAMP in fluke heads, and whether phosphodiesterase inhibitors would potentiate the 5-HT mediated rise in endogenous cAMP. The two most potent inhibitors, SQ 20009 and IBMX, were tested first. Representative results using 20 heads per experimental condition are shown in Fig 6. IBMX amplified and slightly prolonged the increase in cAMP caused by 5-HT, but had no significant effect on the endogenous cAMP concentration by itself. SQ 20009, however, antagonized the increase in cAMP caused by 5-HT, although it, too, had no significant effect on the control cAMP concentration.

Other phosphodiesterase inhibitors were also tested for effects on motility and on endogenous cAMP concentrations in fluke heads with and without 5-HT. Results summarized in Table 2 show that none of the phosphodiesterase inhibitors tested caused a significant rise in the concentration of endogenous cAMP in fluke heads. IBMX was the only inhibitor which had the expected effect of increasing the elevation of endogenous cAMP caused by 5-HT. LSD, SQ20009, and papaverine effectively prevented the rise in endogenous cAMP mediated by 5-HT and incubation with Quazodine, theophylline, or caffeine had little effect on the elevation of cAMP by 5-HT.

DISCUSSION

Phosphodiesterases have been known to be present in multiple forms. Based on their affinities to the substrate, cAMP, low (about 10^{-6} M) and high (10^{-4} M) affinity phosphodiesterases were characterized [15]. From the results reported above it is apparent that the liver fluke phosphodiesterase is a low K_m enzyme. This was shown both for the enzyme from the soluble fraction of the organism as well as for the enzyme from the particulate fraction. The results do not rule out the possibility that there may be more than one enzyme present. However the kinetic data do not support the existence of a phosphodiesterase with a K_m in the millimolar range. Kinetic analysis of the enzyme shows no evidence of cooperative kinetics similar to what has been reported with a variety of tissues [16]. The enzyme was shown to hydrolyse cGMP with a K_m 30 times higher than that for cAMP. Therefore, it appears that the enzyme is more specific for the degradation of cAMP. The specific activity of phosphodiesterase in the supernate from fluke heads is comparable to that reported for a similar supernate from rat brain [17]. However, a preparation from beef heart had a specific activity 50 times higher [10] and from dog heart 100 times higher [18]. As is the case with many other phosphodiesterases [11, 18] the fluke enzyme requires Mg^{2+}, a cation that can be replaced by Mn^{2+}.

The inhibitors that were tested in this investigation can be considered as structural analogs of the phosphodiesterase substrate, cAMP. This includes LSD which can, by comparing space filling molecular models, be seen to be a structural analog of cAMP. It is, therefore, not surprising that all these analogs show typical competitive inhibition of phosphodiesterase. Of the inhibitors tested, SQ 20009 was the most potent.

In order to better understand the role of phosphodiesterase in the regulation of levels of cAMP in the fluke we investigated the effect of these inhibitors on steady-state levels of the cyclic nucleotide. The results with two of the most potent inhibitors, SQ 20009 and IBMX, at concentrations which should be adequate to inhibit the enzyme, revealed no significant change in the levels of cAMP in the fluke heads. This is particularly evident when compared with the marked increase in the levels induced following incubation with 5-HT, an agent which increases the synthesis of cAMP through activation of adenylate cyclase. This is consistent with similar experiments carried out with several tissues treated with similar phosphodiesterase inhibitors [19]. One expected effect of the phosphodiesterase inhibitors is to potentiate the elevation of endogenous cAMP caused by 5-HT. This was only observed following treatment with IBMX. Such an effect of IBMX can easily be interpreted as inhibition of the degradation of cAMP synthesized. The effect of SQ 20009 and papaverine in preventing the rise in endogenous cAMP mediated by 5-HT was contrary to our expectations. Our results, therefore, suggest the interesting possibility that these agents antagonized a component of the stimulation of adenylate cyclase by 5-HT. This may be similar to the effect of LSD on adenylate cyclase stimulation by 5-HT [7, 20]. It has been suggested by Rodbell [21] that GTP or one of its analogs may be involved in hormone activation of liver adenylate cyclase. Unpublished observations from our laboratory (Northup and Mansour) indicate that GTP also activates fluke adenylate cyclase. It remains to be seen whether these phosphodiesterase inhibitors antagonize the 5-HT stimulated increase in cAMP levels through competi-

tion with GTP or a related activator at the same sites.

Although the liver fluke is phylogenetically distant from mammals it is interesting to note that many of the phosphodiesterase inhibitors demonstrate neuromuscular stimulatory effects which may be interpreted as analogous to some of their effects in mammals. The data reported above shows that the higher the phosphodiesterase inhibitory potency the greater the neuromuscular stimulatory effect. The only exception to this finding is LSD which was one of the poorest phosphodiesterase inhibitors, but caused neuromuscular stimulation at very low concentrations. LSD was shown previously to act as an activator of adenylate cyclase at low concentrations [3, 7]. The stimulatory effect on neuromuscular activity by SQ 20009 appears to be distinctly different from that of the other phosphodiesterase inhibitors. The physiological characteristics of this effect is beyond the purpose of this phase of our investigation. The relationship between physiological effects and endogenous cAMP concentration is not seen in these results since these compounds did not, by themselves, raise the steady-state levels of cAMP in intact organisms or in fluke heads. The possibility still exists that the effect of the phosphodiesterase inhibitors on cAMP levels is restricted to a specific set of synpases that represent only a minor part of the cAMP pool in the fluke. A more localized determination of cAMP in the synapses is necessary to ascertain such a possibility.

Acknowledgement—This investigation has been carried out under grant MH 23464 from the National Institute of Mental Health.

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