

Cyclic Adenosine Monophosphate in the Nervous System of *Aplysia californica*

II. *Effect of serotonin and dopamine*

HOWARD CEDAR and JAMES H. SCHWARTZ

From the Departments of Pathology and Microbiology, New York University Medical Center and the Public Health Research Institute of the City of New York, New York 10016

ABSTRACT Serotonin and dopamine, both likely transmitter substances in *Aplysia*, stimulated formation of adenosine-3',5' monophosphate (cAMP) in ganglia, connectives, and identified nerve cell bodies. This widespread distribution suggests that receptors for the response are localized throughout the nervous system, as is adenylyl cyclase. Both synthesis of cAMP-³H from precursor previously labeled in incubations with adenine-³H and total content of cAMP were stimulated up to 15-fold. The acetylcholine analogue carbachol, glutamate, norepinephrine, and histamine were inactive. Full stimulation occurred within 2–4 min of applying serotonin; the extent of the effect was half maximal at 6 μ M serotonin. Even in the continued presence of serotonin, the increased cAMP diminished with time. When serotonin was removed, tissue remained refractory for 15–20 min; sensitivity returned after 25 min. Serotonin stimulated cAMP after removal of extracellular Na, K, or Cl and in isotonic sucrose, with all extracellular ions removed. Elevating Mg, which blocked the stimulation of cAMP caused by synaptic activity, did not affect the response to serotonin. Thus the response appeared to be independent of transmitter release and of changes in synaptic potentials and current flow. The role of cAMP in neuronal functioning remains to be determined. Conditions which markedly increased cAMP in neurons, however, did not affect the rate of RNA synthesis, nor did they alter the distribution of phosphorylated adenine or uridine nucleotides.

INTRODUCTION

Electrical stimulation of nerves or connectives at physiological rates and for short periods of time increased the synthesis of adenosine 3',5' monophosphate (cAMP) in the abdominal ganglion of *Aplysia* (Cedar, Kandel, and Schwartz, 1972). This increase appeared to result from synaptic activation; it occurred only under conditions required for the release of a transmitter substance. We therefore tested a variety of substances considered to be neurotransmitters in *Aplysia* in order to examine their effects on the formation of cAMP.

MATERIALS AND METHODS

Care and maintenance of *Aplysia* are described in the accompanying paper (Cedar et al., 1972). After isolation, nervous tissue was washed and kept at room temperature for about an hour in artificial seawater (Instant Ocean, Aquarium Systems, Inc., Eastlake, Ohio), buffered with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.6). Connectives were cut from the abdominal ganglion immediately after removal from the animal. In order to label cellular pools of adenine nucleotides (Shimizu, Creveling, and Daly, 1970), tissue was incubated for another hour in 80 μ M adenine-2- 3 H (New England Nuclear Corp., Boston, Mass., 6–9 Ci/mmol) as already described (Cedar et al., 1972); the content of ATP was unaffected by incubation in 80 μ M adenine. The total amount of radioactivity and its distribution among the various phosphorylated adenine nucleotides remained essentially unchanged for at least several hours after the initial 1 hr period of labeling. We also used adenosine-2- 3 H but the amount of material taken up and converted to phosphorylated derivatives was found to be considerably less. In order to obtain amounts of cAMP- 3 H sufficient to detect in cell bodies of individual neurons it was necessary to use 0.8 mM adenine- 3 H and incubate 3–4 hr.

Testing of Various Transmitter Substances and Pharmacological Agents

Serotonin creatinine sulfate was obtained from Schwarz/Mann, Orangeburg, N. Y.; lysergic acid diethylamide (LSD) from the Center for Studies of Narcotics and Drug Abuse (Food and Drug Administration—National Institute of Mental Health), and methysergide maleate was the gift of Sandoz Pharmaceuticals (East Hanover, N. J.). Other materials were obtained commercially. Usually substances were applied to nervous tissue in 10 ml of freshly prepared solutions in buffered artificial seawater at room temperature.

Assays for nucleotides

We purified cAMP- 3 H from tissue by thin-layer chromatography on polyethyleneimine cellulose-coated plates (Brinkmann Instruments, Inc., Westbury, N. Y.) followed by treatment with ZnSO_4 and $\text{Ba}(\text{OH})_2$ using procedures modified from Shimizu et al. (1970). Total content of cAMP was determined by isotopic displacement (Gilman, 1970), which in our hands detected 0.5 pmoles reliably. cAMP-8- 3 H (14.2 Ci/mmol) was obtained from Schwarz/Mann.

Content of adenosine triphosphate (ATP) was assayed with firefly luciferase (Strehler, 1965) obtained from Worthington Biochemical Corp., Freehold, N. J.; distribution of labeled adenine derivatives, by high voltage paper electrophoresis at pH 4.4 (Cedar et al., 1972).

Determination of Adenyl Cyclase Activity

Freshly isolated nervous tissue was washed at room temperature first with artificial seawater and then three times with an isotonic solution containing 0.2 M sucrose, 0.3 M NaCl, and 10 mM Tris-HCl (pH 7.6). Tissue was blotted, chilled on ice, and homogenized in a ground glass homogenizer with 50 mM Tris-HCl (pH 7.6) con-

taining 12.5 mM MgSO_4 and 0.1 % bovine serum albumin (TM buffer) estimated to be 10 times the volume of the tissue. The homogenate was centrifuged at 1000 *g* for 10 min, and the resulting pellet was homogenized again in TM buffer. (In some experiments the original 1000 *g* supernatant was saved to be added back to the particulate material during the incubation for assaying adenyl cyclase.) After a second low speed centrifugation, the particulate material was resuspended in TM buffer at a concentration of 0.5–1 mg protein/ml. For activation, 10 mM NaF was added and the suspension kept at 15°C for 15 min. In other experiments 0.2 mM serotonin was added to all solutions used for preparing the enzyme. Enzyme protein was estimated nephelometrically by comparing the A_{600} of the washed particulate fraction with those of similar preparations, made in the absence of bovine albumin, which had been assayed for protein colorimetrically (Lowry, Rosebrough, Farr, and Randall, 1951).

Incubations with uridine- ^3H

Ganglia or severed connectives were incubated at room temperature each under a 40 μl droplet of buffered artificial seawater containing 18 μM uridine-5- ^3H (28 Ci/mmole, New England Nuclear Corp.). After the incubation they were washed for 3 min in seawater. The cell body of R2 was isolated by dissection at room temperature. Tissues or cell bodies were homogenized in 0.1 ml of iced 10 % TCA, and centrifuged.

DETERMINATION OF URIDINE INCORPORATED INTO RNA Acid-precipitable material was homogenized again in 10 % TCA (containing 200 μg serum albumin added as carrier to the precipitated material from individual cell bodies). Incorporated radioactivity was measured by scintillation directly on Whatman GF/C glass fiber pads (Schwartz, Castellucci, and Kandel, 1971).

ANALYSIS OF ACID-SOLUBLE URIDINE COMPOUNDS Duplicate samples (5 or 10 μl) of the resulting supernatant, containing the soluble derivatives of uridine, were applied to strips (0.5 \times 2.5 cm) of polyethyleneimine-coated plastic already containing 60 nmoles of carrier uridine triphosphate (UTP), and air-dried. TCA was neutralized by placing individual strips in 7 ml of 0.12 % Tris base in methanol for 10 min. Free uridine was removed completely by washing the strips batchwise in 100 ml of methanol with gentle agitation for an additional 15 min, leaving phosphorylated derivatives to be counted by scintillation directly on the strips.

In some experiments the distribution of nucleotides was determined by electrophoresing 5- μl samples of the TCA supernatant at pH 4.4. Uridine diphospho sugar (UDPS) was characterized by its mobility on electrophoresis, which corresponded to that of uridine diphosphoglucose (UDPG) (Sigma Chemical Co., St. Louis, Mo.). The mobility of radioactivity which migrated with UDPG was unaffected after incubation at 30°C with 0.7 U of highly purified alkaline phosphomonoesterase of *Escherichia coli* (Schwartz and Lipmann, 1961); it was characterized further by two-dimensional thin-layer chromatography in two different sets of solvents (Randerath, 1965; 1966). Measurements of uridine incorporation were done during the months of November and December; all other experiments, from September to May.

Mean values for the various measurements are presented in the tables \pm SEM for (n) determinations.

RESULTS

Stimulation of cAMP Formation

We tested several putative transmitter substances for their effects on the production of cAMP in *Aplysia* nervous tissue (Table I). Of the substances tested, only serotonin and dopamine stimulated formation of both cAMP-³H and total cAMP. Since serotonin was administered as the creatinine sulfate, we tested creatinine sulfate itself at 0.2 mM (two experiments), and found no effect. Glutamate, histamine, norepinephrine, and the acetylcholine analogue carbachol, each at a concentration of 0.2 mM, were without effect. Presumably application of these substances might have induced release of biogenic amines

TABLE I
EFFECT OF NEUROTRANSMITTER SUBSTANCES ON FORMATION OF cAMP

Substance	cAMP- ³ H	Total cAMP
	% of total radioactivity	pmoles/ganglion
None	0.07±0.01 (27)	7.0±0.7 (27)
Serotonin	0.20±0.01 (16)	60.1±8.1 (16)
Dopamine	0.21±0.03 (4)	42.8±5 (2)
Carbachol	0.06±0.01 (5)	6.4±0.7 (5)
Glutamate	n.d.	6.8±0.8 (2)
Histamine	0.07±0.02 (2)	5.8±0.7 (2)
Norepinephrine	0.07±0.01 (2)	n.d.

Effect of various putative neurotransmitter substances on formation of cAMP-³H and on the total content of cAMP. cAMP was assayed in either abdominal or in left pleural-pedal ganglia after application of the various substances for 5 min at concentrations of 0.2 mM. Ganglia for cAMP-³H determinations were from animals weighing an average of 65 g; for total cAMP, from animals weighing an average of 101 g.

n.d., not done.

through actions on interneurons; the amounts of serotonin and dopamine released, however, were evidently insufficient to produce detectable changes in cAMP. There is some question whether norepinephrine occurs in molluscan nervous tissue (Carpenter, Breese, Schanberg, and Kopin, 1971; Jurorio, 1971); nevertheless, we tested this substance and histamine because both have been found to stimulate markedly the synthesis of cAMP in vertebrate brain slices (Kakiuchi and Rall, 1968 *a, b*; Kakiuchi, Rall, and McIlwain, 1969). We studied the effect of serotonin in greatest detail since the stimulation brought about by dopamine was variable. Solutions of dopamine in seawater were unstable, possibly because of spontaneous oxidation catalyzed by high concentrations of metal ions.

Animal weight has previously been shown to provide an indication of postembryonic development of the abdominal ganglion (Coggeshall, 1967;

Frazier, Kandel, Kupfermann, and Coggeshall, 1967; Giller and Schwartz, 1971 *a, b*). Content of cAMP in the ganglion also varied with animal weight. The amounts of cAMP synthesized from adenine- ^3H in isolated ganglia exposed to serotonin and in control ganglia isolated from *Aplysia* weighing between 40 and 360 g are shown in Fig. 1. Ganglia from heavier animals contained more cAMP. Moreover, the extent of stimulation by serotonin was also greater in ganglia from heavier animals. The total content of ATP as well as the uptake of adenine- ^3H into ganglia also increased with animal weight. These increases in amounts of radioactive precursors were similar in extent to the increase in amount of cAMP in control ganglia from *Aplysia* in the

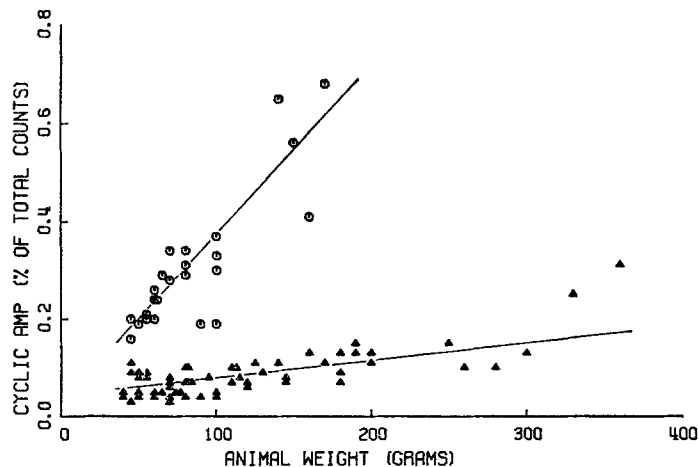


FIGURE 1. Formation of cAMP in abdominal ganglia during postembryonic growth. The conversion of radioactive adenine nucleotides to cAMP was determined after application of 0.2 mM serotonin for 5 min to: (a) ganglia isolated from *Aplysia* of various weights (○); (b) control ganglia in the absence of serotonin (▲). Curves were drawn by a computer using a least-squares fitting program.

weight range tested. The fate of the labeled precursor did not vary, however. Thus the proportion of radioactivity in the form of ATP did not change with animal weight, remaining at about 50% of the total, as determined previously (Cedar et al., 1972). Therefore, the increased amounts of radioactivity in cAMP measured in ganglia from heavier animals could not have resulted from an increased specific radioactivity of its precursor, ATP. A further indication that this is so was provided by measurements of total cAMP. Although we do not have data over as wide a range of animal weights for total cAMP, the data we do have indicate a similar weight dependence not only of cAMP in control ganglia but also in the extent of stimulation of total cAMP synthesis by serotonin. Since the experiments were done using *Aplysia* within a small weight range, the data to be presented have not been normalized by animal weight.

All regions of the nervous system responded to serotonin by increasing both cAMP-³H and total cAMP. Stimulation in the pleural and pedal ganglia was similar in extent to that found in the abdominal ganglion. The extent of the effect in clumps of cell bodies and neuropil removed from the abdominal ganglion was similar to that obtained in the intact ganglion (three experiments, data not shown). It was not possible to isolate cleanly the connective tissue sheath of the ganglion since it was always contaminated with residual neuropil and nerve stumps. Stimulation in the pleuro-abdominal connectives was consistently greater than in ganglia (Table II). In all of these tissues the newly synthesized cAMP-³H constituted about 5% of the total cAMP. In order to obtain amounts of cAMP-³H in individual cell bodies sufficient for analysis we were forced to use a 10-fold greater concentration of adenine-³H and a longer labeling period. Under these conditions, 10 times the amount of radioactivity was taken up by ganglia, but only 25% was in the form of ATP-³H; 20% remained as unconverted adenine. The lower values of conversion to cAMP-³H obtained in the cells (Table II) probably reflected this

TABLE II
FORMATION OF cAMP IN VARIOUS COMPONENTS OF *APLYSIA* NERVOUS
SYSTEM AFTER APPLICATION OF 0.2 mM SEROTONIN FOR 5 MIN

Neural component	cAMP	
	Control	Serotonin
	<i>pmoles/component</i>	
A. Abdominal ganglion	6.7±0.7 (14)	41.4±7.2 (14)
Left pleural-pedal ganglion	8.0±0.8 (14)	69.2±5.1 (11)
Connectives	2.8±0.3 (15)	36.5±2.8 (18)
Cell bodies: R2	0.6±0.1 (5)	1.4±0.3 (5)
Left pleural giant	0.6±0.1 (7)	1.2±0.1 (7)
	<i>% of total radioactivity</i>	
B. Abdominal ganglion	0.09±0.01 (7)	0.51±0.18 (7)
Left pleural-pedal ganglion	0.08±0.01 (4)	0.54±0.09 (5)
Connectives	0.06±0.02 (9)	1.2±0.3 (9)
C. Abdominal ganglion	0.04±0.01 (7)	0.12±0.03 (7)
Left pleural-pedal ganglion	0.05±0.01 (2)	0.12±0.02 (2)
Cell bodies: R2	0.06±0.01 (7)	0.21±0.04 (7)
R15	0.05±0.01 (3)	0.15±0.02 (2)
Left pleural giant	0.05±0.01 (2)	0.12±0.02 (2)

Cell bodies of R2 and R15 were dissected free-hand from the abdominal ganglion, and the left pleural giant cell from the left pleural ganglion, in about 3-4 min. A. Total content of cAMP was determined in neural components from animals weighing an average of 100 g. B. Formation of cAMP-³H in ganglia and nerves previously incubated for 1 hr in 80 μM adenine-³H, and obtained from animals weighing an average of 120 g. C. Formation of cAMP-³H in cell bodies. Nervous tissue, from animals weighing an average of 70 g, was previously incubated in 800 μM adenine-³H. Nerve cell bodies were dissected just after exposure to serotonin.

change in the proportion of radioactivity in ATP. The smaller extent of stimulation in the presence of serotonin found in the cells, however, remains unexplained. The contents of cAMP found in the cell bodies of the identified neurons were approximately what might be expected from their volumes: cAMP in R2 amounted to 5% of the total in the abdominal ganglion as a whole; R15 contained 2%.

Dependence on Serotonin Concentration and Time-Course of Stimulation

The concentration of serotonin required to stimulate half maximally was approximately 6×10^{-6} M (Fig. 2). This concentration is possibly an overestima-

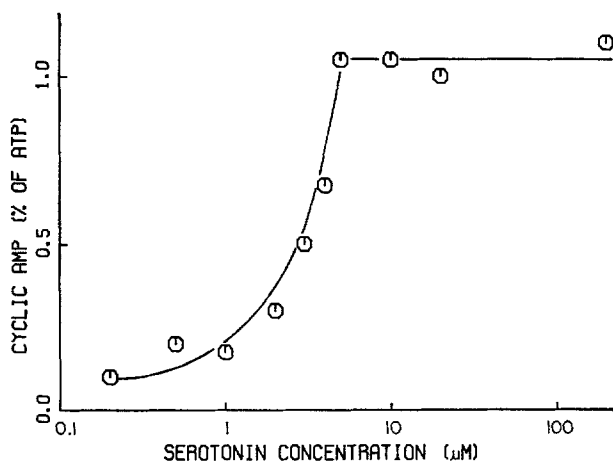


FIGURE 2. Effect of serotonin concentration on the content of cAMP in *Aplysia* nervous tissue. Serotonin at the various concentrations was applied for 4 min to connectives isolated from *Aplysia* weighing an average of 120 g. Contents both of cAMP and of ATP were assayed, and the results expressed as pmoles cAMP/pmole ATP $\times 100$. Each point is the average of duplicate or triplicate determinations. The content of ATP in a connective was $5.3 \text{ pmoles} \pm 0.9$ (15). A similar dependence on serotonin concentration was observed with other parts of the *Aplysia* nervous system, and when we measured formation of cAMP- ^3H rather than the total content of cAMP.

tion. The values presented in Fig. 2 were obtained after a 5 min exposure of the nerves to serotonin, and therefore may not represent the initial rate of stimulation at all concentrations. Especially at the lower concentrations, penetration of serotonin through the connective tissue sheath might be rate-limiting. Similar results were obtained with ganglia.

Maximal stimulation at saturating concentrations of serotonin occurred within 2 min of application (Fig. 3). In part, this latency may reflect the time required for serotonin to reach receptors in an effective concentration. Transmitter substances and drugs of low molecular weight appear to penetrate to neural components of ganglia quite rapidly, since their effects can be detected

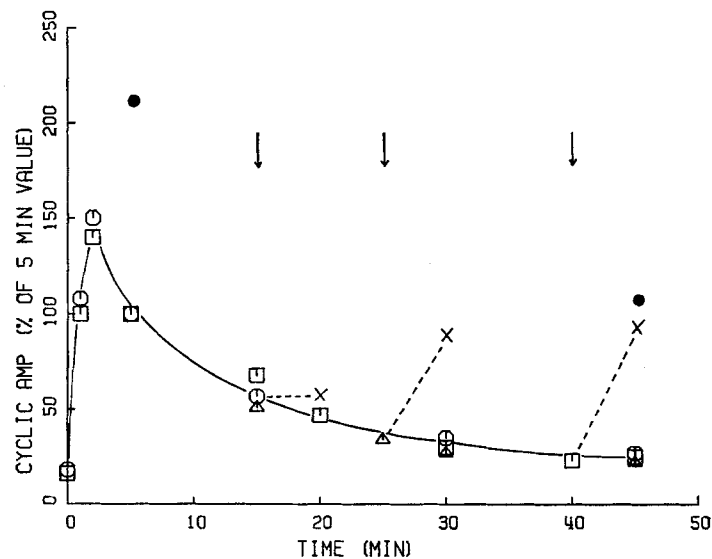


FIGURE 3. Time-course of the appearance of cAMP after the application of 0.2 mM serotonin. *cAMP in the continued presence of serotonin.* Serotonin was added to connectives, abdominal or left pleural-pedal ganglia from *Aplysia* weighing an average of 130 g. cAMP (both the total content \square and formation of the radioactive nucleotide from adenine- ^3H in the absence \circ and in the presence \bullet of 1 mM theophylline) was determined at the times indicated, and values from two or more determinations are presented as a per cent of the cAMP in the tissue after 5 min in the presence of serotonin, in order to normalize the different contents of cAMP characteristic of each ganglion or nerve (see Table II for these values). *cAMP after brief application of serotonin.* After 5 min, serotonin was removed by washing with 10 ml of artificial seawater three times, each time for 2 min. The tissue was kept in artificial seawater, and then analyzed for formation of cAMP- ^3H Δ . *Return of sensitivity to serotonin.* With nervous tissue washed after the initial application of serotonin, formation of cAMP- ^3H (\times) was tested after a second 5 min exposure to 0.2 mM serotonin at the times indicated by arrows.

electrophysiologically within 1 min of application in the bath (see, for example, Gerschenfeld and Tauc, 1961; Gerschenfeld, Ascher, and Tauc, 1967). Since this period of latency extended from 2–4 min in different experiments, we chose an incubation period of 5 min for all our other experiments, even though the response to serotonin at that time might have been somewhat diminished.

Whether or not serotonin was constantly present, the amount of cAMP in the nervous tissue diminished with time (Fig. 3). Presumably cAMP was hydrolyzed enzymatically since the addition of 1 mM theophylline together with serotonin resulted in a twofold greater maximal stimulation both of cAMP- ^3H and total cAMP, and also prevented its rapid disappearance (Fig. 3). Incubation with theophylline for as long as 1 hr in the absence of serotonin, however, had no effect on the control amount of cAMP (Cedar et al., 1972) or on the

increased synthesis during electrical stimulation. We have no simple explanation for these differences in the effects of theophylline.

The elevated content of cAMP diminished even in the presence of serotonin, and this suggested that the tissue rapidly lost its capacity to respond. When serotonin was removed after an incubation period of 5 min and the tissue maintained for an additional period of 20 min in its absence, however, and then fresh serotonin added, stimulation was again seen to an extent similar to that initially observed. The tissue was refractory to serotonin for a period of at least 10 min after its removal (Fig. 3). We have not tested the effect of theophylline on the refractory period. This transient loss of sensitivity might have resulted from desensitization of receptors for serotonin; alternatively, it might have been caused by formation of an antagonist which blocks synthesis of cAMP. Indications of the existence of an inhibitor of this kind have been reported in rat adipocytes after incubation with epinephrine and with other lipolytic hormones (Manganiello, Murad, and Vaughn, 1971; Ho and Sutherland, 1971).

Effect of Drugs and Ionic Composition of Medium on Stimulation of cAMP by Serotonin

Several studies with various transmitter substances have demonstrated the existence of four types of ionic mechanisms associated with receptors to chemical transmitters which alter membrane permeability of neurons in *Aplysia* (Ascher, 1968; Blankenship, Wachtel, and Kandel, 1971; Kehoe, 1969) and in snails (Chiarandini and Gerschenfeld, 1967; Chiarandini, Stefani, and Gerschenfeld, 1967; Gerschenfeld, 1971). In *Helix*, Gerschenfeld (1971) has found that three of these four ionic mechanisms are associated with receptors for serotonin. These mechanisms can be probed by substituting an impermeant ion for the critical ionic species controlled by a given receptor. We removed each ion individually, substituting various impermeant ions, and also collectively, substituting sucrose. We presume that ion-trapping did not occur in these experiments in which nervous tissue was washed at least three times in 10 ml of the bathing solution to be tested before applying serotonin. In a defined salts solution with Na^+ , Cl^- , or K^+ omitted, stimulation of cAMP by serotonin was unaffected (Table III). This suggests that the stimulation did not involve a potential change due to movement of these ions, nor did it result from current-flow associated with either Cl^- or Na^+ moving down its normal concentration gradient. Moreover, removal of external K^+ did not enhance responsiveness to serotonin, suggesting that a K^+ conductance mechanism may also not be directly involved. Indeed, when all ions were replaced by 0.8 M sucrose (adjusted to pH 7.5 with a negligible amount of Tris base), stimulation of cAMP by serotonin was still observed to the full extent (Table III). More-

TABLE III
THE EFFECT OF IONS ON INCREASED FORMATION OF cAMP-³H

Solution	cAMP- ³ H	
	Control	Serotonin
	% of total radioactivity	
A. Complete salts	0.07±0.01 (6)	0.22±0.03 (6)
Na ⁺ -free	0.07±0.01 (6)	0.21±0.02 (6)
K ⁺ -free	0.07±0.01 (4)	0.19±0.02 (4)
Cl ⁻ -free	0.07±0.01 (6)	0.19±0.02 (6)
B. Artificial seawater (Instant Ocean)	0.09±0.01 (11)	0.53±0.15 (12)
Salt-free (0.8 M sucrose)	0.08±0.01 (3)	0.61±0.14 (3)
C. Complete salts	0.09±0.01 (3)	0.72±0.2 (3)
Complete salts + high Mg ²⁺	0.09±0.01 (3)	0.91±0.2 (3)

Abdominal or left pleural-pedal ganglia, isolated from *Aplysia* and incubated for 1 hr in adenine-³H in buffered artificial seawater (Instant Ocean) were washed with the experimental solution for 5 min, incubated with 0.2 mM serotonin for an additional 5 min in the same solution, and then assayed for cAMP-³H. A. Complete salt solution contained (millimoles/liter): NaCl, 425; KCl, 10; CaCl₂, 10.23; MgCl₂, 22.03; NaHCO₃, 2.8; MgSO₄, 26.22; and Tris-HCl (pH 7.6), 50. To make the solution Na-free, Na was replaced by Tris. K was replaced by Na. To prepare Cl-free solution, Cl was replaced by acetate. Ganglia were from *Aplysia* weighing an average of 70 g. B. Isotonic (0.8 M) sucrose was made pH 7.6 by the addition of a negligible volume of 2 M Tris base. C. Left pleural-pedal ganglia were incubated in the complete salts solution in the presence and in the absence of 152 mM additional MgCl₂. Ganglia were from *Aplysia* weighing an average of 130 g.

over, serotonin increased cAMP in nervous tissue already depolarized by ouabain (data not shown).

Increasing Mg²⁺ in seawater to a concentration which blocked synaptic transmission did not alter the effect of serotonin on cAMP-³H (Table III) nor total cAMP (data not shown); it also did not change the amount of cAMP in the absence of serotonin. Thus serotonin can act when the natural release of transmitter substances is blocked.

In addition to their specific ionic mechanisms, the three types of serotonin receptors described in *Helix* were also distinguished by their sensitivities to various drugs (Gerschenfeld, 1971). LSD blocked them all. Neostigmine (Hoffmann-LaRoche, Inc., Nutley, N. J.) at a concentration of 1 μM or curare at 10 μM blocked one receptor, and neostigmine together with curare blocked another; the third was unaffected by these drugs. We found that LSD had no effect (Table IV). Methysergide maleate, which has been shown to block transmission by giant serotonergic neurons in *Helix* (Cottrell, 1970) also had no effect on the stimulation of cAMP (Table IV). At the concentrations of the inhibitors used by Gerschenfeld (1971), who applied serotonin to nerve cell bodies iontophoretically, we found no inhibition of stimulation of cAMP brought about by serotonin administered in the bath at 20 μM. This concen-

TABLE IV
EFFECT OF DRUGS WHICH BLOCK SEROTONIN RECEPTORS ON
THE FORMATION OF cAMP

Drug	cAMP	Per cent inhibition
	% of ATP	
None	0.7±0.1 (15)	—
Curare (14 μ M)	1.3±0.3 (3)	None
Curare (140 μ M)	0.34±0.06 (6)	50
Neostigmine (30 μ M)	0.71±0.1 (2)	None
Neostigmine (300 μ M)	0.41±0.05 (3)	41
LSD (500 μ M)	0.62±0.05 (8)	14
Methysergide (10 ⁻⁴ g/ml)	0.71±0.1 (4)	None

After 10 min in the presence of the drug, nervous tissue was exposed to 20 μ M serotonin together with the drug for 5 min, and then assayed for both total cAMP and ATP. In order to normalize data from the various parts of the nervous system, values from isolated right and left connectives and left pleural-pedal ganglia are presented as (picomoles cAMP/picomoles ATP) \times 100. (The actual contents of cAMP in these components have been presented in Table II.) With the range of concentrations used, none of the drugs, in the absence of serotonin, had any effect on the proportion of cAMP, which was $0.07 \pm 0.01\%$ (15).

tration of serotonin was saturating (Fig. 2). Although lower than our standard dose, it was chosen with the aim of detecting more sensitively the action of the drugs, which we presume to be competitive. Indeed, higher concentrations of curare and neostigmine appeared to inhibit to some extent (Table IV). These effects were slight, however, and may not be specific.

Distribution of Adenyl Cyclase

We measured synthesis of cAMP in extracts of various parts of the nervous system of *Aplysia*. In preliminary experiments with ATP-³H we detected slight synthesis; it was apparent, however, that cAMP was rapidly being destroyed even in the presence of theophylline, caffeine, or papavarine. Because of the presence of phosphodiesterase in the washed particulate material, we assayed adenyl cyclase using the isotope displacement method, which was sensitive enough to detect accumulation of cAMP in reaction mixtures at concentrations (0.5–5 μ M) which were below the affinity of the diesterase activity. Even so, caffeine or theophylline enhanced the appearance of cAMP (Table V). The specific activity of adenyl cyclase in washed particulate material from the abdominal ganglion was 0.24 ± 0.02 (3) pmoles/min per μ g protein, and from the right and left connectives, 0.11 ± 0.02 (6). Activity in the entire nervous system was about twice that in the abdominal ganglion (Table V). These relatively small differences in specific activity of the various parts of the nervous system possibly reflect amounts of connective tissue associated with the neural components rather than a true difference in the neuronal content

TABLE V
REQUIREMENTS FOR ASSAYING ADENYL CYCLASE IN EXTRACTS
OF *APLYSIA* NERVOUS TISSUE

Condition	Activity	
	pmoles/6 min	%
Complete	19	100
– Caffeine	10	53
– Caffeine + theophylline (2 mM)	22	116
Complete + 0.5 mM serotonin	22	116
– NaF	0	0
– NaF + 0.5 mM serotonin	0	0

The complete system contained, in a volume of 100 μ l, 1 mM ATP, 8 mM MgSO_4 , 10 mM caffeine, 0.1% bovine serum albumin, 30 mM Tris-HCl (pH 7.6), and 33 μ g of the washed particulate material prepared from the five major ganglia of the *Aplysia* central nervous system. Samples of 20 μ l were removed at 6, 14, and 30 min at 25°C, pipetted into 50 μ l water, and boiled for 3 min. 25- μ l portions were assayed for cAMP (Gilman, 1970). Appearance of cAMP was linear for 14 min. A zero time blank of 1.4 pmoles has been subtracted from the data, which is presented as activity in 20- μ l samples.

of the enzyme. We failed to detect formation of cAMP in extracts of single cell bodies of identified neurons (R2 and R15). With the methods used, however, we could not have measured reliably less than 5–10% of the cyclase activity present in the entire abdominal ganglion.

Adenyl cyclase from *Aplysia* was similar to the enzyme from other sources (Robison, Butcher, and Sutherland, 1971). All of the enzyme was particulate, and sedimented at 1000 *g* in 10 min. Adding back soluble material did not enhance the activity. Formation of cAMP was maximal when ATP was present at a concentration between 1 and 2 mM, and when Mg^{2+} was between 6 and 12 mM. Addition of bovine serum albumin (0.1%), dithiothreitol (0.01 M), and 0.2 M NaCl were without effect. The effect of 0.1 to 0.25% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) could not be tested, since the detergent interfered with the subsequent isotope displacement assay for cAMP.

We detected no activity in the absence of NaF (Table V). Addition of 0.5 mM serotonin during assay of the enzyme had no effect either in the presence or in the absence of NaF (Table V). Two other tests failed to show that serotonin activates the enzyme *in vitro*. First, in the absence of NaF, there was no activity in cyclase prepared in buffers containing 0.5 mM serotonin from ganglia which had been incubated for 5 min in the presence of 0.1 mM serotonin before homogenization. Second, it could be shown that the activating effect of NaF was reversible: suspensions of particulate material incubated in the presence of NaF lost their activity when washed, even if the washing were carried out in the presence of serotonin. Our assay was not sufficiently sensitive, however, to detect a small degree of activation.

Serotonin Had No Effect on RNA Synthesis

cAMP has been shown to mediate increased RNA and protein synthesis in several tissues in response to hormones (Robison et al., 1971). In order to test whether cAMP might be involved in stimulations of RNA synthesis reported in *Aplysia* (Berry, 1969; Peterson and Kernell, 1970; Kernell and Peterson, 1970) we measured incorporation of uridine-³H into RNA ganglia connectives and in the cell body of R2 (Table VI) under conditions which we have already

TABLE VI
INCORPORATION OF URIDINE-³H INTO RNA IN THE PRESENCE AND
THE ABSENCE OF SEROTONIN

Neural component	No.	Condition	Incorporation into RNA	RNA/total nucleotides	RNA/UTP
<i>pmoles/2 hr per component</i>					
Abdominal	3	+	7.9±2.9	0.073±0.004	0.16±0.007
ganglion	3	—	13.2±4.0	0.081±0.007	0.15±0.01
R2 cell body	7	+	0.53±0.13	0.18±0.02	—
	7	—	0.48±0.12	0.13±0.02	—
Left pleural-pedal	3	+	12.3±2.2	0.056±0.002	0.11±0.01
ganglion	3	—	13.3±2.3	0.074±0.010	0.14±0.02
Connectives	5	+	7.4±1.4	0.064±0.007	0.11±0.01
	5	—	8.4±1.8	0.064±0.008	0.11±0.015

Nervous tissue from *Aplysia* weighing 40–60 g was exposed to 20 μ M serotonin and 1 mM theophylline for 4 min, washed twice each time for 90 sec, and then incubated with uridine-³H for 1 hr; exposure to serotonin was repeated again, and the tissue incubated with uridine-³H for a second hour. Solutions for both control (—) and experimental (+) tissue were changed in parallel, and both contained 1 mM theophylline throughout the period of incubation. Incorporation into RNA was measured on glass fiber pads (1 pmoles of uridine-³H was 15,000 cpm). Total uridine nucleotides were determined on strips of polyethyleneimine with about a third the efficiency of counting. UTP was measured on Whatman 3 MM paper after separation by high voltage electrophoresis, with about 10% the efficiency of counting on glass fiber pads.

shown resulted in greatly increased amounts of cAMP in neurons (cell bodies and axons). We exposed nervous tissue to serotonin for 4 min and, after washing, incubated the tissue in uridine-³H. This was repeated a second time. Exposure to serotonin produced no significant differences ($P < 0.05$, Mann-Whitney U test) in the amounts of uridine-³H incorporated either in the presence or in the absence of theophylline. The continued presence of theophylline, which by itself did not alter the amount of cAMP in nervous tissue or in cell bodies, was also without effect on the incorporation. This protocol, involving brief exposure to serotonin, then its removal by washing, followed by a longer period during which sensitivity of the tissue to serotonin returns, was modeled on experiments shown in Fig. 3. Since measurements of uridine incorporation were quite variable (Schwartz et al., 1971), we also determined

the amounts of phosphorylated uridine derivatives within tissues and cells at the same time as we measured incorporation into RNA, in an attempt to normalize the incorporation data. About half the acid-soluble radioactivity was UTP; a substantial proportion was in the form of UDPS, however ($28.8 \pm 1.0\%$ [6] in the abdominal ganglion, 65% of which was identified as UDPG by two-dimensional thin-layer chromatography). As with adenine nucleotides, we found no differences in the distribution of uridine compounds after treatment of the tissue with serotonin.

DISCUSSION

In the vertebrate central nervous system, the greatest increases in cAMP were evoked by histamine and norepinephrine (Kakiuchi and Rall, 1968 *a, b*; Kakiuchi, Rall, and McIlwain, 1969; Krishna, Forn, Voight, Paul, and Gessa, 1970; Shimizu et al., 1970). Serotonin and dopamine produced only small or insignificant effects (Chasin, Rivkin, Mamrak, Samaniego, and Hess, 1971; Kakiuchi and Rall, 1968 *b*; Krishna et al., 1970; Shimizu et al., 1970). Dopamine, however, has recently been shown to stimulate formation of cAMP in the bovine sympathetic ganglion (Kebabian and Greengard, 1971) and retina (Brown and Makman, 1972). We now show that in *Aplysia*, serotonin and dopamine stimulate formation of cAMP markedly both in central ganglia and in peripheral nerves. Moreover, we found that serotonin can act in isotonic sucrose in the absence of external ions, indicating that its action on the synthesis of cAMP is probably independent of synaptic potential changes or current flow.

Even though we have observed stimulation by serotonin in bodies of identified neurons isolated by microdissection, we cannot be certain that the increased cAMP was entirely neuronal, and we have not excluded the possibility that some of these changes occurred in glial cells. As with other invertebrates, nerve cell bodies and axons of *Aplysia* are covered with glial cells (Coggeshall, 1967) and the glial contaminants cannot be completely removed by microdissection (Giller and Schwartz, 1971 *a*). Moreover, Gilman and Nirenberg (1971) and Shultz, Hamprecht, and Daly (1972) found that cAMP in cell lines derived from several glial cell tumors of rats was greatly increased after exposure to catecholamines.

Mechanism and Localization of Serotonin Action

Two conditions, electrical stimulation of nerves and connectives (Cedar et al., 1972) and the application of serotonin or dopamine to the bathing solution, initiated increased synthesis of cAMP in nervous tissue of *Aplysia*. Although these two effects were not identical, they may result from a similar mechanism. The differences can be explained if we assume that the effect of electrical stimulation is mediated by the synaptic release of a chemical transmitter sub-

stance (perhaps serotonin or dopamine) and is restricted specifically to synaptic regions. On the other hand, serotonin applied in the bathing solution may affect both junctional and extrajunctional receptors which are distributed throughout the nervous system, as is the biosynthetic enzyme adenylyl cyclase. This assumption would explain why the increase in cAMP in response to serotonin in the bath is much larger and more widespread than the restricted response to electrical stimulation. This assumption would also account for the different effects of the high Mg^{2+} concentration, since only electrical stimulation would require release of a transmitter substance.

Serotonin and Dopamine as Possible Natural Transmitter Substances in Aplysia

We have as yet no direct evidence that the natural transmitter substance which mediates increased synthesis of cAMP during electrical stimulation is either serotonin or dopamine. These substances do, however, simulate the actions of natural transmitters at certain molluscan synapses including those in *Aplysia* (Ascher, 1968; Gerschenfeld, 1971; Gerschenfeld and Stefani, 1968). Carpenter et al. (1971) found both serotonin and dopamine in the abdominal ganglion; they also described a specific uptake mechanism for serotonin in the heart, which is innervated by neurons located in the ganglion (Mayeri, Kupfermann, Koester, and Kandel, 1971). In addition, James Goldman (unpublished) has found that serotonin is synthesized from tryptophan- 3H in the ganglion. Furthermore, serotonin depolarized R15 (Gerschenfeld, Ascher, and Tauc, 1967) and R2 (Kandel and Schwartz, unpublished experiments), two cells which responded to serotonin with increased cAMP. Thus, serotonin might be an excitatory transmitter for both R15 and R2. In addition, Ascher (1968) reported that dopamine hyperpolarized some neurons in the ganglion (including R15) and excited others.

Robison, Butcher, and Sutherland (1967) have proposed that hormones activate adenylyl cyclase in a variety of tissues by acting on specific receptors. We do not have direct evidence that receptors for serotonin activate adenylyl cyclase, but this possibility is suggested because the isolated enzyme failed to respond to serotonin in vitro and because the response desensitized (see Fig. 3). Gerschenfeld and Stefani (1968) have found that the cell bodies of *Aplysia* and other molluscan neurons, which are free of synapses, are none the less electrically sensitive to the iontophoretic application of serotonin. Indeed, it is probable that molluscan neurons have receptors to some transmitter substances along their entire surface. If receptors activate adenylyl cyclase in *Aplysia* it would be attractive to suppose that they are similar or identical to junctional and extrajunctional receptors that are associated with the ionic mechanisms controlling conductance changes produced by serotonin.

A possible difficulty with this idea is that LSD, which has been shown to

block ionic mechanisms associated with receptors for serotonin in molluscan neurons (Gerschenfeld, 1971), does not block increased formation of cAMP brought about by serotonin. The effects of LSD, however, are complex. For example, in the liver fluke, *Fasciola hepatica*, serotonin increased motility; concomitantly it also brought about an accumulation of cAMP together with several characteristic changes in the activity of enzymes involved in the utilization of glucose (Stone and Mansour, 1967). LSD acted like serotonin on motility, and did not block the biochemical actions of serotonin, although it was itself biochemically inactive (Mansour, 1957; 1959). LSD has also been shown to cause the release of serotonin in the heart of *Aplysia*, thereby stimulating contraction paradoxically (Carpenter et al., 1971).

Role of cAMP in Neuronal Functioning

We have presented evidence that elevated amounts of cAMP within neurons did not result in increased rates of RNA synthesis, nor did they alter the distribution of phosphorylated uridine or adenine nucleotides. Thus, it would appear that an elevated content of cAMP is not a sufficient cause for the increased incorporation of uridine into the RNA of R2 reported by Berry (1969), Peterson and Kernell (1970), and Kernell and Peterson (1970) to result from synaptic stimulation. Biochemical alteration of the postsynaptic neuron as a consequence of synaptic activity presumably is essential for plastic interactions between neurons. cAMP is known to be important in the regulation of metabolism and function in some cells (see Robison, Butcher, and Sutherland, 1971). It remains to be determined what role it might play in neuronal functioning in *Aplysia*.

We thank Doctors Irving Kupfermann and W. Alden Spencer for reading the manuscript critically. This research was supported by U. S. Public Health Service Career Award GM-28,125 to Dr. J. H. Schwartz, as well as by research grants from the National Science Foundation (GB-25046) and from the National Institutes of Health (NS 08159).

Received for publication 19 April 1972.

REFERENCES

- ASCHER, P. 1968. Electrophoretic injections of dopamine on *Aplysia* neurones. *J. Physiol. (Lond.)* **198**:48P.
- BERRY, R. W. 1969. Ribonucleic acid metabolism of a single neuron: correlation with electrical activity. *Science (Wash. D. C.)* **166**:1021.
- BLANKENSHIP, J. E., H. WACHTEL, and E. R. KANDEL. 1971. Ionic mechanisms of excitatory, inhibitory and dual synaptic actions mediated by an identified interneuron in abdominal ganglion of *Aplysia*. *J. Neurophysiol.* **34**:76.
- BROWN, J. H., and M. H. MAKMAN. 1972. Stimulation by dopamine of adenylate cyclase in retinal homogenates and of adenosine-3',5'-cyclic monophosphate formation in intact retina. *Proc. Natl. Acad. Sci. (U.S.A.)* **69**:539.

- CARPENTER, D., G. BREESE, S. SCHANBERG, and I. KOPIN. 1971. Serotonin and dopamine: distribution and accumulation in *Aplysia* nervous and non-nervous tissues. *Int. J. Neurosci.* **2**:49.
- CEDAR, H., E. R. KANDEL, and J. H. SCHWARTZ. 1972. Cyclic adenosine monophosphate in the nervous system of *Aplysia californica*. I. Increased synthesis in response to synaptic stimulation. *J. Gen. Physiol.* **60**:558.
- CHASIN, M., I. RIVKIN, F. MAMRAK, S. G. SAMANIEGO, and S. M. HESS. 1971. α - and β -adrenergic receptors as mediators of accumulation of cyclic adenosine 3',5'-monophosphate in specific areas of guinea pig brain. *J. Biol. Chem.* **246**:3037.
- CHIARANDINI, D. J., and H. M. GERSCHENFELD. 1967. Ionic mechanisms of cholinergic inhibition on molluscan neurons. *Science (Wash. D. C.)*. **156**:1595.
- CHIARANDINI, D. J., E. STEFANI, and H. M. GERSCHENFELD. 1967. Ionic mechanism of cholinergic excitation in molluscan neurons. *Science (Wash. D. C.)*. **156**:1597.
- COGGESHALL, R. E. 1967. A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**:1263.
- COTTRELL, G. A. 1970. Direct post-synaptic responses to stimulation of serotonin-containing neurones. *Nature (Lond.)*. **225**:1060.
- FRAZIER, W. T., E. R. KANDEL, I. KUPFERMANN, and R. E. COGGESHALL. 1967. Morphological and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**:1288.
- GERSCHENFELD, H. M. 1971. Serotonin: two different inhibitory actions on snail neurons. *Science (Wash. D. C.)*. **171**:1252.
- GERSCHENFELD, H. M., and L. TAUC. 1961. Pharmacological specificities of neurones in an elementary nervous system. *Nature (Lond.)*. **189**:924.
- GERSCHENFELD, H. M., P. ASCHER, and L. TAUC. 1967. Two different excitatory transmitters acting on a single molluscan neurone. *Nature (Lond.)*. **213**:358.
- GERSCHENFELD, H. M., and E. STEFANI. 1968. Evidence for an excitatory transmitter role of serotonin in molluscan central synapses. *Advan. Pharmacol.* **6B**:369.
- GILLER, E., and J. H. SCHWARTZ. 1971 *a*. Choline acetyltransferase in identified neurons of abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **34**:93.
- GILLER, E., and J. H. SCHWARTZ. 1971 *b*. Acetylcholinesterase in identified neurons of abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **34**:108.
- GILMAN, A. G. 1970. A protein binding assay for adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. (U.S.A.)*. **67**:305.
- GILMAN, A. G., and M. NIRENBERG. 1971. Effect of catecholamines on the adenosine 3':5'-cyclic monophosphate concentrations of clonal satellite cells of neurons. *Proc. Natl. Acad. Sci. (U.S.A.)*. **68**:2165.
- HO, R. -J., and E. W. SUTHERLAND. 1971. Formation and release of a hormone antagonist by rat adipocytes. *J. Biol. Chem.* **246**:6822.
- JURORIO, A. V. 1971. Catecholamines and 5-hydroxy-tryptamine in nervous tissue of cephalopods. *J. Physiol. (Lond.)*. **216**:213.
- KAKIUCHI, S., and T. W. RALL. 1968 *a*. The influence of chemical agents on the accumulation of adenosine 3',5'-phosphate in slices of rabbit cerebellum. *Mol. Pharmacol.* **4**:367.
- KAKIUCHI, S., and T. W. RALL. 1968 *b*. Studies on adenosine 3',5'-phosphate in rabbit cerebral cortex. *Mol. Pharmacol.* **4**:379.
- KAKIUCHI, S., T. W. RALL, and H. MCILWAIN. 1969. The effect of electrical stimulation upon the accumulation of adenosine 3',5'-phosphate in isolated cerebral tissue. *J. Neurochem.* **16**:485.
- KEBABIAN, J. W., and P. GREENGARD. 1971. Dopamine-sensitive adenylyl cyclase—possible role in synaptic transmission. *Science (Wash. D. C.)*. **174**:1346.
- KEHOE, J. S. 1969. Single pre-synaptic neurone mediates a two component post-synaptic inhibition. *Nature (Lond.)*. **221**:866.
- KERNELL, D., and R. P. PETERSON. 1970. Effects of spike activity versus synaptic activation on metabolism of ribonucleic acid in a molluscan giant neurone. *J. Neurochem.* **17**:1087.

- KRISHNA, G., J. FORN, K. VOIGHT, M. PAUL, and G. L. GESSA. 1970. Dynamic aspects of neurohormone control of cyclic 3',5'-AMP synthesis in brain. In *Role of Cyclic AMP in Cell Function*. P. Greengard, and E. Costa editors. Raven Press, New York. 155.
- LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. L. RANDALL, JR. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193**:265.
- MANGANIELLO, V. C., F. MURAD, and M. VAUGHN. 1971. Effects of lipolytic and antilipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells. *J. Biol. Chem.* **246**:2195.
- MANSOUR, T. E. 1957. The effect of LSD, 5-HT and related compounds on the liver fluke, *Fasciola hepatica*. *Br. J. Pharmacol.* **12**:406.
- MANSOUR, T. E. 1959. The effect of serotonin and related compounds on the carbohydrate metabolism of the liver fluke, *Fasciola hepatica*. *J. Pharmacol. Exp. Ther.* **126**:212.
- MAYERI, E., I. KUPFERMANN, J. KOESTER, and E. R. KANDEL. 1971. Neural coordination of heart rate and gill contraction in *Aplysia*. *Am. Zool.* **11**:667.
- PETERSON, R. P., and D. KERNELL. 1970. Effects of nerve stimulation on metabolism of ribonucleic acid in a molluscan giant neurone. *J. Neurochem.* **17**:1075.
- RANDERATH, K. 1965. Ion-exchange thin-layer chromatography. XIV. Separation of nucleotide sugars and nucleoside monophosphates on PEI-cellulose. *Anal. Biochem.* **13**:575.
- RANDERATH, K. 1966. Thin-Layer Chromatography. Academic Press, Inc. New York. 2nd edition. 230.
- ROBISON, G. A., R. W. BUTCHER, and E. W. SUTHERLAND. 1967. Adenyl cyclase as an adrenergic receptor. *Ann. N. Y. Acad. Sci.* **139**:703.
- ROBISON, G. A., R. W. BUTCHER, and E. W. SUTHERLAND. 1971. Cyclic AMP. Academic Press, Inc. New York.
- SHULTZ, J., B. HAMPRECHT, and J. W. DALY. 1972. Accumulation of adenosine 3':5'-cyclic monophosphate in clonal glial cells: labeling of intracellular adenine nucleotides with radioactive adenine. *Proc. Natl. Acad. Sci. (U.S.A.)*. **69**:1266.
- SCHWARTZ, J. H., V. F. CASTELLUCCI, and E. R. KANDEL. 1971. Functioning of identified neurons and synapses in abdominal ganglion of *Aplysia* in absence of protein synthesis. *J. Neurophysiol.* **34**:939.
- SCHWARTZ, J. H., and F. LIPMANN. 1961. Phosphate incorporation into alkaline phosphatase of *E. coli*. *Proc. Natl. Acad. Sci. (U.S.A.)*. **47**:1996.
- SHIMIZU, H., C. R. CREVELING, and J. DALY. 1970. Stimulated formation of adenosine 3',5'-cyclic phosphate in cerebral cortex: synergism between electrical activity and biogenic amines. *Proc. Natl. Acad. Sci. (U.S.A.)* **65**:1033.
- STONE, D. B., and T. E. MANSOUR. 1967. Phosphofructokinase from the liver fluke, *Fasciola hepatica*. I. Activation by adenosine 3',5'-phosphate and by serotonin. *Mol. Pharmacol.* **3**:161.
- STREHLER, B. L. 1965. Adenosine-5'-triphosphate and creatine phosphate. Determination with luciferase. In *Methods of Enzymatic Analysis*. H.-V. Bergmeyer, editor. Academic Press, Inc. New York. 559.