References and Notes

1. R. Damadian, *Science* **171**, 1151 (1971).
2. H. E. Frey, R. R. Knispel, J. Kruuv, A. R. Sharp, R. T. Thompson, M. M. Pintar, *J. Natl. Cancer Inst.* **49**, 903 (1972); G. P. Raaphorst, J. Kruuv, H. Frey, in *The Nuclear Resonance Effect in Cancer*, R. Damadian, Ed. (Pacific Press, New York, in press); C. F. Hazlewood, D. C. Chang, D. Medina, G. Cleveland, B. L. Nichols, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1478 (1972); G. L. Cottam, A. Vasek, D. Lusted, *Res. Commun. Chem. Pathol. Pharmacol.* **4**, 495 (1972); R. G. Parrish, R. J. Kurland, W. W. Janese, L. Bakey, *ibid.* **183**, 438 (1973); D. P. Hollis, L. A. Saryan, H. P. Morris, *Johns Hopkins Med. J.* **131**, 441 (1972); D. P. Hollis, J. S. Economou, L. C. Parks, J. C. Eggleston, L. A. Saryan, J. L. Czeisler, *Cancer Res.* **33**, 2156 (1973); R. A. Floyd, J. S. Leigh, B. Chance, M. Miko, *ibid.* **34**, 89 (1974); I. C. Kiricuta, D. Demco, V. Simplaceanu, *Arch. Geschwulstforsch.* **42**, 226 (1973); R. E. Block, *FEBS Lett.* **34**, 109 (1973); W. R. Inch, J. A. McCredie, R. R. Knispel, R. T. Thompson, M. M. Pintar, *J. Natl. Cancer Inst.* **52**, 353 (1974); W. Bovee, P. Huisman, J. Smidt, *ibid.*, p. 595; L. A. Saryan, D. P. Hollis, J. S. Economou, J. C. Eggleston, *ibid.*, p. 599; C. F. Hazlewood, G. Cleveland, D. Medina, *ibid.*, p. 1849; R. E. Block and G. P. Maxwell, *J. Magn. Reson.* **14**, 329 (1974); B. M. Fung, *Biochim. Biophys. Acta* **362**, 209 (1974); S. Ratkovic and C. Rusov, *Period. Biol.* **76**, 19 (1974); S. S. Ranade, S. S. Shah, K. S. Korgaonkar, C. V. Talwalkar, S. R. Kasturi, paper presented at the Fifth International Symposium on Magnetic Resonance, Bombay, India, 14 to 18 January 1974; C. F. Hazlewood, in *The Nuclear Resonance Effect in Cancer*, R. Damadian, Ed. (Pacific Press, New York, in press); I. D. Weisman, L. H. Bennett, L. R. Maxwell, in *ibid.*; W. Bovee and J. Smidt, in *ibid.*; N. Iijima, S. Saitoo, Y. Yoshida, N. Fujii, T. Koike, K. Osanai, K. Hirose, in *ibid.*; M. M. Pintar, in *ibid.*; S. Ratkovic and C. Rusov, in *ibid.*; S. S. Ranade, in *ibid.*; R. E. Gordon, J. R. Singer, L. Crooks, in *ibid.*; Z. Abe and K. Tanaka, in *ibid.*
3. I. D. Weisman, L. H. Bennett, L. R. Maxwell, M. W. Woods, D. Burk, *Science* **178**, 1288 (1972).
4. R. Damadian, U.S. Patent 3,789,832, filed 17 March 1972.
5. A. Kumar, D. Welti, R. R. Ernst, *Naturwissenschaften* **62**, 34 (1975).
6. P. M. Morse and H. Feshbach, in *Methods of Theoretical Physics II*, (McGraw-Hill, New York, 1953), p. 1265.
7. R. Damadian, in *The Nuclear Resonance Effect in Cancer*, R. Damadian, Ed. (Pacific Press, New York, in press).
8. R. Freeman and H. D. Hill, *J. Chem. Phys.* **54**, 3367 (1971).
9. Supported in part by grant R01-CA 14988-06 from the National Institute of Health.

23 July 1976; revised 24 September 1976

Hormonal Release of Programmed Behavior in Silk Moths: Probable Mediation by Cyclic AMP

Abstract. *The eclosion hormone triggers a stereotyped preprogrammed pattern of behavior in silk moths. The effects of the hormone were duplicated by the injection of dibutyryl adenosine 3',5'-monophosphate, adenosine 3',5'-monophosphate (cyclic AMP), or guanosine 3',5'-monophosphate (cyclic GMP) into theophylline-treated pharate moths. Treatment with theophylline reduced the latency of the response to a low dose of hormone, presumably by blocking phosphodiesterase. Endogenous levels of cyclic AMP, but not cyclic GMP, increased significantly in the central nervous system within 10 minutes after hormone injection. We conclude that an early step leading to the release of the eclosion motor program is an increase in cyclic AMP in target neurons of the central nervous system.*

In many animals, hormones can act rapidly to alter behavior (*1*). The exact mechanisms by which these behavioral changes come about are unknown, but it is likely that the initial responses in the nervous system are similar to those shown by nonneural tissues. In the case of peptide and amine hormones, these agents typically act at the surface of their target cells to alter the level of adenosine 3',5'-monophosphate (cyclic AMP) (*2*). Recently, guanosine 3',5'-monophosphate (cyclic GMP), has also been implicated in the action of certain hormones (*3*). We report here that cyclic nucleotides play a central role in the hormonal mediation of complex behavioral changes in a silk moth.

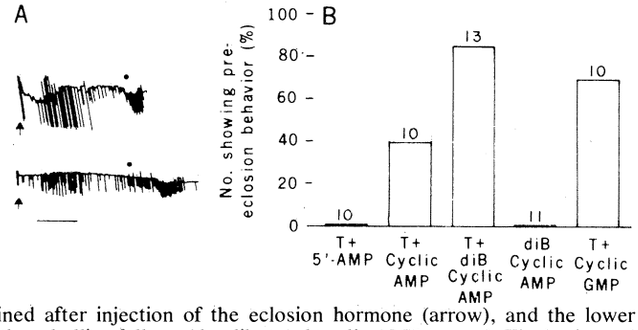

Fig. 1. The ability of cyclic nucleotides to stimulate preeclosion behavior when injected into isolated abdomens of pharate *H. cecropia* moths. (A) Tracings of movement were recorded by attaching the tip of the abdomen to a lever that wrote on a revolving drum (5); the upper tracing was obtained after injection of the eclosion hormone (arrow), and the lower tracing after injection of theophylline followed by dibutyryl cyclic AMP (arrow). The horizontal line equals 0.5 hour; the dot identifies the onset of the peristaltic movements. (B) The percentage of abdomens showing the preeclosion behavior after injection of 1 mg of a nucleotide. Theophylline (50 μg) was injected at *T*, 5 minutes before nucleotide injection; *AMP*, adenosine monophosphate; *diB-cyclic AMP*, dibutyryl cyclic AMP. The number gives the size of each group.

In the pharate (*4*) stage of the moth *Hyalophora cecropia*, a brain-derived hormone, the eclosion hormone, triggers a species-specific sequence of motor acts that culminates in the moth's escape from the pupal cuticle (eclosion) and the activation of its repertoire of adult behavior (*5*). The first portion of the emergence sequence, the preeclosion behavior (Fig. 1A) begins about 10 to 15 minutes after hormone application. It consists of three phases: an initial 0.5-hour period of frequent abdominal rotations; a period of quiescence of about the same duration; and, finally, a second hyperactive period during which strong peristaltic contractions move anteriorly along the abdomen to cause eclosion. Experiments on deafferented abdominal nervous systems and on isolated abdominal nervous systems indicate that the information for the pattern of preeclosion behavior is preprogrammed in the abdominal ganglia and that this behavioral program is triggered by the direct action of the hormone on the abdominal central nervous system (CNS) (*6*).

Since the eclosion hormone appears to be proteinaceous (*7*), we sought to determine whether its effects are exerted by way of changes in cyclic nucelotide levels. To guard against our treatments causing a release of endogenous hormone from the brain, abdomens isolated from pharate adults were routinely utilized (*8*). The behavior of each abdomen was continuously monitored by attaching the tip of the abdomen to a lever that wrote on a revolving drum; preparations were also inspected at 10- to 15-minute intervals. The preeclosion behavior was recognized by the characteristic temporal pattern of activity coupled with the appearance of the distinctive peristaltic movements during the final phase.

The effects of injecting one of four purine nucleotides (*9*) into isolated abdomens of *H. cecropia* are summarized in Fig. 1. Before the nucleotides were injected, theophylline [Sigma; 50 μg in 10 μl of Ringer (*10*) solution] was injected to inhibit the high cyclic nucleotide phosphodiesterase activity in moth nervous tissue (*11*). Dibutyryl cyclic AMP [which may enter cells more readily, or have greater resistance to phosphodiesterase than cyclic AMP (*2*)] released the preeclosion behavior in 11 of 13 abdomens. Cyclic AMP was somewhat less active whereas 5'-adenosine monophosphate was inert. Injections of cyclic

GMP proved to be nearly as effective as dibutyryl cyclic AMP. However, the preeclosion behavior shown by some of the abdomens injected with cyclic GMP was atypical in that the quiescent phase was abnormally prolonged.

To examine these findings in further detail, abdomens isolated from pharate male moths were injected with a standard dose of an extract containing the eclosion hormone (*12*), and the concentrations of cyclic AMP and cyclic GMP in the abdominal nervous system were measured at various times thereafter (*13*). Cyclic AMP was assayed in extracts prepared from individual chains of ganglia. The lower endogenous levels of cyclic GMP required it to be measured in extracts pooled from up to five abdominal nervous systems.

The results, summarized in Fig. 2, show that in the unstimulated abdomen the nerve cord contained about 1.8 pmole of cyclic AMP [7.9 pmole per milligram of protein (*14*)]. This is within the range reported for the CNS of other insects (*15*). Injection of the eclosion hormone was followed by an approximately twofold increase in cyclic AMP that peaked after 10 to 15 minutes and then declined to basal level by 30 minutes after injection. The initial level of cyclic GMP was approximately one-fifth that of cyclic AMP; it seemed to be little affected by the injection of either saline or hormone.

Because of the low basal levels of cyclic GMP in the CNS and because pooled samples had to be used for these determinations, we cannot exclude the involvement of cyclic GMP in the response to the eclosion hormone. However, the marked rise in cyclic AMP after hormone injection strongly argues that it is the cyclic nucleotide of physiological significance. Since exogenous cyclic GMP has been shown to produce cyclic AMP–like effects in several systems (*3*), the effectiveness of cyclic GMP in stimulating the preeclosion behavior (Fig. 1) may be due to its ability to mimic or protect endogenous cyclic AMP (*16*).

As described elsewhere (*17*), the latency between hormone injection and eclosion decreases as one increases the amount of hormone injected. In an experiment performed on pharate male *Antheraea pernyi* moths, we examined whether prior treatment with theophylline (75 μg per animal) could reduce the latency of response to a standard low dose of the eclosion hormone (*18*). The inhibition of cyclic nucleotide destruction by theophylline would presumably allow a more rapid buildup of these agents after hormonal stimulation and thereby produce a faster response. The results, summarized in Table 1, show that the latency was markedly reduced by the prior injection of theophylline. The same result was also obtained in parallel experiments performed on 30 females. These results are consistent with the hypothesis that the action of the eclosion hormone is mediated through an increase in cyclic AMP in its target cells.

Table 1. Effect of theophylline (75 μg per animal) on the latency of response to the eclosion hormone (0.25 corpora cardiaca equivalent per gram of live weight) by pharate *A. pernyi* moths.

Material injected	Number	Number eclosing	Latency*
Theophylline	10	1	3.33
Ringer solution plus eclosion hormone	10	10	2.17 ± 0.25
Theophylline plus eclosion hormone	11	11	1.27 ± 0.05

*Time between hormone injection and eclosion (hours ± standard error of the mean).

Because cyclic nucleotides are known to mediate certain classes of chemical synapses (*19*) in the nervous system, it is difficult to interpret the data. But the three lines of evidence presented above strongly argue that an increase in cyclic AMP is an integral step in the hormonal triggering of a complex motor behavior. Consequently, cyclic nucleotides appear to be involved not only in relatively short-term phenomena, such as synaptic transmission, but also in longer term, hormonally induced alterations in neuronal function. Although we assume that the increase in cyclic AMP occurs within central neurons, increases within glial elements cannot be ruled out. Through the use of immunofluorescent techniques for localizing cyclic AMP (*20*), it is feasible to establish the cellular origin of the increase. It should thereby be possible to identify target neurons—that is, the cells responsible for initiating the preeclosion behavior.

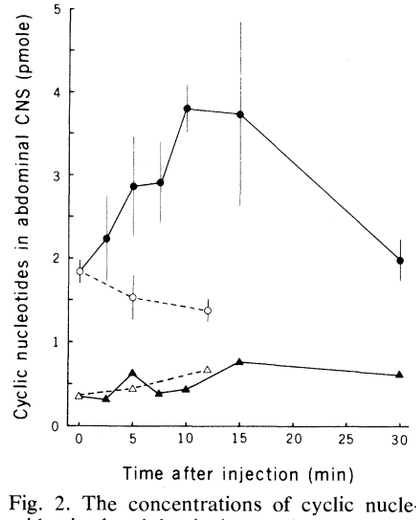

Fig. 2. The concentrations of cyclic nucleotides in the abdominal central nervous system (CNS) of *H. cecropia* after eclosion hormone was injected into isolated, pharate abdomens. Symbols: Circles are for cyclic AMP, triangles for cyclic GMP. Open symbols, abdomens injected with Ringer solution; solid symbols, abdomens injected with eclosion hormone (0.7 corpora cardiacia equivalents per gram of live weight). Each point represents determinations on approximately five nervous systems. Cyclic AMP was measured in individual nerve cords and cyclic GMP in pooled extracts. Error bars represent the standard error of the mean.

Note added in proof: Cyclic AMP has recently been implicated in the hormonal alteration of bursting patterns of certain large neurons in the CNS of *Aplysia* and *Helix* (*21*).

JAMES W. TRUMAN
Department of Zoology, University of Washington, Seattle 98195
ANN MARIE FALLON
G. R. WYATT
Department of Biology, Queen's University, Kingston, Ontario K7L 3N6

References and Notes

1. F. L. Strand, *BioScience* **25**, 568 (1975); J. W. Truman and L. M. Riddiford, *Adv. Insect Physiol.* **10**, 297 (1974).
2. R. W. Butcher, G. A. Robinson, E. W. Sutherland, in *Biochemical Action of Hormones*, G. Litwack, Ed. (Academic Press, New York, 1972), vol. 2, p. 21; P. Cuatrecases, *Adv. Cyclic Nucleotide Res.* **5**, 79 (1975).
3. N. D. Goldberg, R. F. O'Dea, M. K. Haddox, *Adv. Cyclic Nucleotide Res.* **3**, 154 (1973); N. D. Goldberg, M. K. Haddox, S. E. Nicol, D. B. Glass, C. H. Sanford, F. A. Kuehl, Jr., R. Estensen, *ibid.* **5**, 307 (1975).
4. The term "pharate" moth refers to a moth prior to eclosion, that is, a moth which is still encased in the pupal cuticle [H. E. Hinton, *Nature (London)* **157**, 552 (1946)].
5. J. W. Truman, *J. Exp. Biol.* **58**, 805 (1971); *Am. Sci.* **61**, 700 (1973).
6. ——— and P. G. Sokolove, *Science* **175**, 1491 (1972); J. W. Truman, unpublished data.
7. S. E. Reynolds and J. W. Truman, unpublished data.
8. It was important to have preparations that had not already been exposed to the eclosion hormone. In the photoperiod (LD 17 : 7) to which the moths were exposed, the brain does not release the eclosion hormone until after the lights are turned on. Therefore, prior to lights-on, moths that would emerge on a given day (as evidenced by the full resorption of the molting fluid) were clamped with a hemostat at the juncture of the thorax and abdomen and the anterior portion cut away. The abdomens were routinely used about 1 hour after isolation.
9. All nucleotides were obtained as the sodium salts from Sigma.
10. B. Ephrussi and G. W. Beadle, *Am. Nat.* **70**, 218 (1936).
11. E. E. Albin, S. J. Davison, R. W. Newburgh, *Biochim. Biophys. Acta* **377**, 364 (1975).
12. The paired brain neurohemal organs (corpora cardiaca) were dissected from 60 pharate *Manduca sexta* moths. The glands were homogenized in 1.2 ml of Ringer solution (*10*) and the

homogenate centrifuged at 1000*g* for 15 minutes. The supernatant with an effective concentration of 0.1 corpora cardiaca equivalent per microliter was divided into 200-μl portions and frozen at −20°C until needed. Abdomens were injected near the anterior midline with 0.7 corpora cardiaca equivalent per gram of live weight.

13. At appropriate times, the abdomens were opened along the dorsal midline, the gut removed, and the remainder pinned out under ice-cold Ringer solution [R. G. Weevers, *J. Exp. Biol.* **44**, 163 (1966)]. The nerve cords were rapidly dissected free of contaminating fat body tissue and frozen on Dry Ice. The dissection time ranged from 1.5 to 2 minutes. Extracts were prepared with internal standards of ^{3}H-labeled cyclic nucleotides to monitor recovery. Cyclic AMP and cyclic GMP were separated chromatographically and measured by binding protein assays as previously described [A. M. Fallon and G. R. Wyatt, *Anal. Biochem.* **63**, 614 (1975); B. L. Brown, R. P. Ekins, J. D. M. Albano, *Adv. Cyclic Nucleotide Res.* **2**, 25 (1972)]. The average recovery of cyclic AMP and cyclic GMP was 89 percent and 70 percent, respectively. Tritiated cyclic AMP (22.1 c/mmole) was from New England Nuclear, and the tritiated cyclic GMP (21 c/mmole) was from Amersham/Searle.
14. Protein was determined according to the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall [*J. Biol. Chem.* **193**, 265 (1951)] with bovine serum albumin being used as standard.
15. J. A. Nathanson and P. Greengard, *Science* **180**, 308 (1973).
16. The ability of exogenous cyclic GMP to activate cyclic AMP–dependent systems has been attributed to the ability of cyclic GMP to penetrate cells more readily than does cyclic AMP [J. H. Exton, J. G. Hardman, T. F. Williams, E. W. Sutherland, C. R. Park. *J. Biol. Chem.* **246**, 2658 (1971)] and, at high levels, to activate cyclic AMP–dependent protein kinases [J. F. Kuo and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 1349 (1969)]. In addition, cyclic GMP has been shown to inhibit hydrolysis of cyclic AMP in intact and broken cell systems [E. N. Goren and O. M. Rosen, *Arch. Biochem. Biophys.* **142**, 720 (1971); F. Murad, V. Manganiello, M. Vaughan, *J. Biol. Chem.* **245**, 3352 (1970)].
17. J. W. Truman, *Biol. Bull. (Woods Hole, Mass.)* **144**, 200 (1973).
18. The hormone was prepared from 132 pairs of corpora cardiaca as described in (*12*) except that the homogenate was exposed to 100°C for 5 minutes before centrifugation. This treatment removed much of the large protein from the homogenate but had little effect on the eclosion hormone activity.
19. B. J. Hoffer, G. R. Siggins, A. P. Oliver, F. E. Bloom, *Adv. Cyclic Nucleotide Res.* **1**, 411 (1972); P. Greengard and J. W. Kebabian, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **33**, 1059 (1974).
20. H. J. Wedner, B. J. Hoffer, E. Battenberg, A. L. Steiner, C. W. Parker, F. E. Bloom, *Histochem. Cytochem.* **20**, 293 (1972).
21. S. N. Treistman and I. B. Levitan, *Nature (London)* **261**, 62 (1976).
22. We thank L. M. Riddiford and R. Logan for helpful discussions. Supported by NSF grant BMS 75-02272 to J.W.T. and NIH grant HD-07951 and a grant from the National Research Council of Canada to G.R.W.

24 March 1976; revised 13 July 1976

Infidelity of DNA Synthesis in vitro: Screening for Potential Metal Mutagens or Carcinogens

Abstract. *Thirty-one metal salts have been tested for their ability to affect the accuracy of DNA synthesis in vitro. All ten salts of metal carcinogens decreased the fidelity of DNA synthesis. Of the three metals which beforehand were considered to be possible mutagens or carcinogens, only one decreased fidelity. In contrast, 17 noncarcinogenic metal salts did not affect fidelity even when present at concentrations that were clearly inhibitory.*

The role of environmental agents in causing human malignancy (*1*) suggests the possibility that their identification and subsequent elimination from the environment could reduce the incidence of cancer in the general population. In the past, animal studies have generally been used to identify environmental carcinogens. Unfortunately, these studies require several years to complete and may require inordinate capital expenditures to test the plethora of new chemicals introduced annually into the environment. Thus, attention has focused on the development of assays in vitro to screen initially for potential carcinogens. These tests offer the possibility of rapidly and inexpensively examining a multitude of chemicals to select those to be evaluated further in animal systems. As there is a close correlation between carcinogenicity and mutagenicity, most assays in vitro examine the ability of exogenous agents to act as mutagens (*2*). For example, Ames and his colleagues (*2*) have developed tester strains of *S. typhimurium* in which suspected agents are evaluated by their ability to revert previously induced mutations. Over 300 compounds have been studied, and the correlation of mutagenicity in their system with reported carcinogenicity is about 90 percent. However, this test has not been applied for screening metal compounds for mutagenicity, although it and other systems may be potentially adaptable (*3*). Because many metals have been identified as carcinogens (*4*), through occupational exposure as well as in the laboratory, it may be of importance to develop a test system to screen for these carcinogens. We now report an assay in vitro which may be used to screen for potential mutagenic or carcinogenic metal compounds. All of the known metal mutagens or carcinogens (*5*) which have been tested could be identified through this analysis. In addition, three metals were analyzed that beforehand were assigned as possible mutagens (*6*) or carcinogens (*7*); one was found to be positive in this assay. None of the noncarcinogenic metals tested were identified as potential mutagens or carcinogens.

For this analysis we have measured perturbations in the fidelity of DNA synthesis in vitro caused by soluble metal salts. Metals which increased base substitution in this assay in vitro were designated as suspected mutagens or carcinogens in vivo. Each reaction mixture contained a DNA polymerase, a template-primer of restricted base composition, Mg^{2+}, and complementary and noncomplementary deoxynucleoside triphosphates each labeled with different radioactive isotopes. One of the complementary deoxynucleotides was labeled with α-^{32}P of low specific activity (approximately 5 to 20 dpm/pmole), while the noncomplementary deoxynucleotide was labeled with ^{3}H of high specific activity (approximately 10,000 to 50,000 dpm/pmole). From the ratio of radioactive substrates incorporated, we calculated the error frequency, that is, the ratio of noncomplementary to complementary deoxynucleotide incorporation. For these initial studies, we chose to use the DNA polymerase (E.C. 2.7.7.7) from avian myeloblastosis virus (AMV), since this polymerase is well characterized (*8*), incorporates noncomplementary deoxynucleotides as single base substitutions (*9*) and, in common with all purified eukaryotic DNA polymerases, lacks any associated exodeoxynuclease activity which might excise noncomplementary bases after incorporation (*10*). The propensity of this DNA polymerase to make mistakes increased the sensitivity of the assay and emphasized further the inability of noncarcinogenic metal compounds to affect fidelity.

The effect of each metal compound on the fidelity of DNA synthesis was determined at multiple concentrations between 20 μM and 150 mM as limited by solubility and by the effect of the metal salt on DNA synthesis. Compounds which increased the error frequency by greater than 30 percent at two or more concentrations were scored as positive. Each experiment contained reaction mixtures without additional metal salts as well as reaction mixtures with $CoCl_2$ (4 mM) as a positive control. This cobalt concentration increases the error frequency by at least 50 percent (*11*).

In this study, 31 compounds were tested (Table 1); 22 compounds were tested in triple-blind experiments. The assays, computations, and the designation of the unknown compounds with respect to fidelity were carried out by three separate individuals, each unaware of the compounds to be analyzed. All metal salts assayed in the triple-blind experiments were designated as carcinogens, possible carcinogens, or noncarcinogens prior to the analysis. Of the total metal com-