it was possible to obtain photographs of the spray during the moment of its emergence. The pictures (Fig. 3, A and B) generally conform to prediction. Part of the secretion is forcibly ejected as discrete droplets, while the remainder is emitted in what appears to be a turbulent cloud of vapor.

Photographic proof was also obtained of the unerring marksmanship of the beetles. Attacks were staged in which individual live ants (*Pogonomyrmex badius*) were induced to bite appendages of tethered bombardiers. The moment an ant clamped down with its mandibles, the beetle responded by spraying, and the discharge invariably hit the assailant "full blast" (Fig. 3, C through G).

Although the quinones are intrinsically repellent even when cold, the thermal properties of the spray undoubtedly contribute to the overall defensive potential of the secretion. Even a relatively small specimen of *Brachinus* can impart a distinct burning sensation when it is held in the fingers, and particularly if it should discharge in rapid succession, as it often does. At a more sensitive site, such as on the human lip, even a single discharge may be felt as "hot" (as we determined by actual trial). The burning sensation is truly attributable to a thermal effect, since the general integument is not immediately sensitive to the irritant properties of benzoquinones (only the eyes, other mucous surfaces, and sites of injured skin are instantly irritated). Thus, a bombardier beetle can make itself felt thermally, even where the chemical "message" cannot get through. Nothing is known about the beetle's own ability to withstand the effects of the discharge. Whether tolerance is attributable to heat insensitivity or to the possession of a thermally insulated integument (and possibly vestibule wall) remains to be determined.

The defense mechanism of *Brachinus*, although anomalous, is not unique. We have studied two other carabid beetles, a species of *Metrius*, and a Panamanian member of the tribe Ozaenini, which also discharge hot quinones, but which apparently have evolved this capability independently from *Brachinus*. Even man has developed a device which, although nondefensive, resembles the ejection mechanism of the bombardier. Some of his latest shaving-cream dispensers are designed to administer a lather that is pleasantly hot rather than cold. In some of these dispensers, the generation of heat relies on a catalytic process (activated when the lather is squirted from the dispenser), in which hydrogen peroxide acts as the oxidant (*14*).

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10. Calibration was achieved by means of a resistor that had been installed as part of the cylinder wall, and which could be subjected to quantitatively controlled electrical heating.
11. Identification to species in *Brachinus* is difficult or impossible. The specimens used in this study were a mixed lot from Ithaca, New York, and Lake Placid, Florida.
12. Water was used, because the secretion is largely (65 percent) aqueous (*3*).
13. Actually, the registered temperatures of the spray were always somewhat lower than 100°C, usually 90° to 95°C. This was due to a limitation of the thermocouple, whose response time (50 msec for 99.3 percent of total equilibration in water) was longer than the duration of even the longest discharges (30 msec). Thus, the thermocouple could never reach equilibration with the temperature of the spray. The value of 100°C was obtained when the oscilloscope records were extrapolated to equilibration, by mathematical inference from the known response curves of the thermocouple to heated water.
14. United States Patent 3,341,418; 12 September 1967.
15. Study supported by NIH grant AI-02908. We thank W. S. Gale (Gillette Toiletries Co.) and D. W. Whyte (S. C. Johnson & Son) for suggestions, our colleagues N. Bryant, J. Meinwald, B. Nichols, and D. S. Robson for advice and loan of equipment, and R. Archbold for help and hospitality during our stay at the Archbold Biological Station, Lake Placid, Florida, where some of this work was done. Contribution No. 24 in the series *Defense Mechanisms of Arthropods.*

14 January 1969

Adenosine 3′,5′-Monophosphate–Dependent Protein Kinase from Brain

Abstract. *Adenosine 3′,5′-monophosphate at a concentration of 5×10^{-7} mole per liter causes a 400 percent increase in the rate of phosphorylation of histone catalyzed by a partially purified enzyme preparation from rabbit brain. The data provide the first direct evidence of a biochemical action of adenosine 3′,5′-monophosphate in the brain.*

Sutherland and his associates (*1*) have provided strong evidence that adenosine 3′,5′-monophosphate (cyclic AMP) mediates the actions of many hormones. There has not been any demonstration of a biochemical or physiological effect of cyclic AMP in the brain; nor has it been possible to assign a definite role to cyclic AMP in brain function. However, experimental evidence does suggest a possible linkage between the metabolism of cyclic AMP and brain function: (i) of all mammalian tissues the brain has the highest activity of adenyl cyclase, the enzyme responsible for the synthesis of cyclic AMP (*2*); (ii) the brain also has the highest activity of cyclic 3′,5′-nucleotide phosphodiesterase, which destroys cyclic AMP (*3*); (iii) subcellular distribution studies with brain tissue suggest that adenyl cyclase may be localized in the synaptic membranes (*4*); (iv) the methyl xanthine class of stimulants of the central nervous system (caffeine and theophylline) inhibit phosphodiesterase (*3*); (v) neurohumors can stimulate or inhibit the activity of adenyl cyclase in cell-free preparations (*5*); (vi) certain neurohumors, including norepinephrine, histamine, and serotonin, produce large increases in the

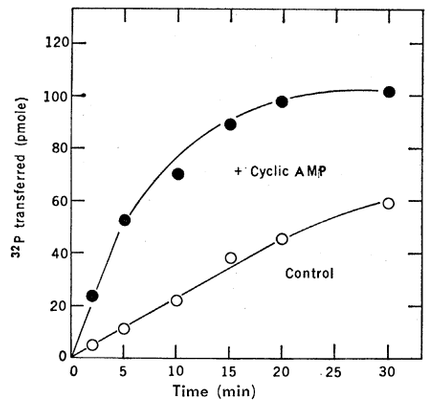

Fig. 1. Effect of cyclic AMP on the activity of protein kinase, as a function of incubation time. Incubation conditions were as described in the text, except for the variation in incubation time. The amount of purified protein kinase used was 45 μg.

concentrations of cyclic AMP of slices of rabbit brain, and these increases are potentiated by theophylline (*6*); and (vii) electrical stimulation of brain slices leads to large increases in cyclic AMP levels (*7*).

The studies of Krebs and his associates (*8–10*) have greatly clarified the mechanism by which cyclic AMP mediates the metabolic effects of epinephrine in skeletal muscle. Recently, Walsh, Perkins, and Krebs (*10*) reported the occurrence and partial purification of a protein kinase from rabbit skeletal muscle with a complete dependence on

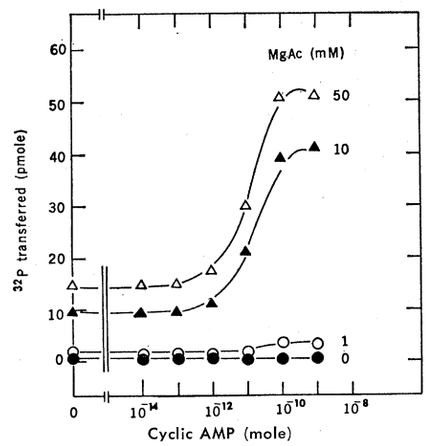

Fig. 2. Effect of concentration of cyclic AMP on the activity of protein kinase in the presence of various amounts of magnesium acetate. Incubation conditions were as described in the text, except for the variation in amount of cyclic AMP and magnesium acetate as indicated. The amount of purified protein kinase used was 45 μg.

cyclic AMP. This enzyme catalyzed the phosphorylation of three different proteins, namely, casein, protamine, and phosphorylase kinase. The reaction between the protein kinase and phosphorylase kinase presumably is a link in the sequence of reactions by which epinephrine causes increased glycogenolysis in muscle. The occurrence in skeletal muscle of a protein kinase dependent on cyclic AMP for activity led us to examine brain tissue for such an enzyme. More recently, Langan (*11*) reported the occurrence of such a protein kinase from liver that phosphorylates histones, lending further impetus to the search for a similar enzyme in brain tissue.

A protein kinase dependent on cyclic AMP for activity has now been found in rabbit brain, and the enzyme has been partially purified by techniques similar to those used by Walsh, Perkins, and Krebs (*10*). Frozen whole brain (57 g) from four rabbits was thawed and homogenized with 170 ml of 4 m*M* ethylenediaminetetraacetic acid (EDTA) (*p*H 7.0) in a Waring blender. The homogenate was centrifuged for 30 minutes at 27,000*g*, and the *p*H of the supernatant was adjusted to 5.7 with 1*N* acetic acid. The precipitate formed was removed by centrifugation. The *p*H of the clear supernatant solution was readjusted to 6.9 with 1*M* potassium phosphate buffer (*p*H 7.2), and solid ammonium sulfate (0.33 g/ml) was slowly added to the enzyme solution. The protein kinase in the precipitate was collected by centrifugation, dissolved in 20 ml of 5 m*M* potassium phosphate buffer (*p*H 7.0) containing 2 m*M* EDTA, and dialyzed against 100 volumes of the same buffer with two changes of buffer. All buffers used in the succeeding steps of the purification contained 2 m*M* EDTA. The enzyme was adsorbed on a diethylaminoethyl cellulose (Sigma) column (2 by 15 cm). The column was washed with 0.1*M* potassium phosphate buffer (*p*H 7.0), and the enzyme was eluted from the column with 0.5*M* potassium phosphate buffer. The active fractions were pooled and dialyzed against 100 volumes of 5 m*M* tris-Cl buffer (*p*H 7.5) with two changes of buffer; the enzyme was adsorbed on a DE-52 (Whatman) column (2 by 8 cm). The column was washed with 0.1*M* tris-Cl (*p*H 7.5) and the activity was eluted with 0.25*M* tris-Cl (*p*H 7.5). Active fractions were pooled and dialyzed against 5 m*M* tris-Cl (*p*H 7.5) as above. To the dialyzed enzyme

Table 1. Substrate specificity of purified protein kinase. Incubation conditions were as described in the text, except for the replacement of 1.2 mg of histone by 1.2 mg of the indicated substrates. The amount of ^{32}P transferred was corrected for that occurring in the absence of added substrate; the correction amounted to 1.99 and 2.13 pmole, in the absence and in the presence of cyclic AMP, respectively.

Substrate	^{32}P transferred (pmole)		Increase (%)
	Control	+ Cyclic AMP	
Histone	6.11	31.63	418
Protamine	1.73	4.01	132
Phosvitin	1.23	2.07	68

was added 15 ml (one-fifth volume) of calcium phosphate gel containing 300 mg (dry weight) of calcium phosphate. The gel was collected by centrifugation and washed with 0.03*M* potassium phosphate buffer (*p*H 7.0). The kinase was eluted from the gel with 0.3*M* potassium phosphate buffer and dialyzed against 10 m*M* tris-Cl buffer (*p*H 7.5) with two changes of buffer; the purified enzyme was stored at −20°C.

The activity of the cyclic AMP–dependent protein kinase was assayed in an incubation volume of 0.2 ml containing: sodium glycerol phosphate buffer (*p*H 6.0), 10 μmole; histone (*12*), 1.2 mg; γ^{32}P-ATP, 3.2×10^{-10} mole, containing about 350,000 count/min; magnesium acetate, 2 μmole; sodium fluoride, 2 μmole; theophylline, 0.4 μmole; ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid, 0.06 μmole; with or without 10^{-10} mole of cyclic AMP. Incubations were carried out for 5 minutes at 30°C in a shaking water bath. The reaction was terminated, and the protein-bound ^{32}P was determined as described for the enzyme from skeletal muscle (*9*, *10*).

By use of the purification procedure described above, the specific activity of the partially purified enzyme was increased about 150-fold over that of the crude extract. For the purpose of calculating the activity and extent of purification of the cyclic AMP-dependent protein kinase, the difference between the rate of phosphorylation in the presence and absence of cyclic AMP was used. It was not possible to determine total recovery of the enzyme with accuracy, since highly inhibitory material was removed during the course of the purification; the final preparation contained 101 percent of the starting units of enzyme activity.

The effect of cyclic AMP on the activity of the purified protein kinase as a function of incubation time is shown in Fig. 1. Cyclic AMP increased the initial rate of phosphorylation of histone. The rate in the presence of cyclic AMP was linear for about 5 minutes. The activity of the protein kinase was proportional to enzyme up to 45 μg, the highest amount tested.

The effect of the concentration of cyclic AMP on the activity of the protein kinase is shown in Fig. 2. Near maximal stimulation was obtained with about $5 \times 10^{-7}M$ cyclic AMP. It is clear that Mg^{2+} ions are required for the kinase activity dependent on cyclic AMP. (The presence in the purified preparation of protein kinase activity not dependent on cyclic AMP is also indicated by the data in Fig. 2.) In other experiments, designed to determine the apparent K_m, half-maximum stimulation of histone phosphorylation was achieved at a concentration of cyclic AMP of $8 \times 10^{-8}M$. This value is similar to the values for the cyclic AMP–dependent protein kinase from muscle (*10*) as well as that from liver (*11*). Adenosine 5′-monophosphate, in concentrations from 5×10^{-8} to $5 \times 10^{-6}M$, could not replace cyclic AMP for the activation of the protein kinase of rabbit brain.

A comparison of the ability of various proteins to serve as substrate for the purified protein kinase preparation is shown in Table 1. Protamine was able to serve as a phosphate acceptor, but was much less effective than histone, and phosvitin was still less effective. Langan (*11*) found protamine to have little or no ability to serve as phosphate acceptor for the cyclic AMP-dependent histone kinase from liver. The protein kinase from skeletal muscle, prepared according to the procedure of Walsh, Perkins, and Krebs (*10*), also phosphorylates histone much more readily than protamine (*13*). Thus, the cyclic AMP-dependent protein kinases found in liver, muscle, and brain all use histone preferentially to protamine as substrate.

The demonstration of a cyclic AMP–dependent protein kinase in brain tissue would appear to be the first evidence of a biochemical or physiological effect of cyclic AMP in nervous tissue and, taken together with data (*5–7*) indicating changes in brain cyclic AMP levels in response to various neurohumors or electrical stimulation, provides rather strong evidence for a role of cyclic AMP in brain function. The nature of the interaction between cyclic AMP and protein kinase must await further purification of the enzyme. However, the recent demonstration that cyclic AMP is a high-energy compound, with a free energy of hydrolysis substantially greater than that of ATP, and that it is capable of adenylylating pyrophosphate, makes it feasible to consider the possibility that cyclic AMP interacts with the protein kinase by adenylylating it (*14*). Another interesting problem, raised by the finding of a protein kinase in brain tissue, is whether this protein kinase is the only "receptor" with which cyclic AMP interacts in the brain. Finally, further investigation is required to determine the nature of the naturally occurring substrate for protein kinase in brain tissue.

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15 April 1969

Aspirin: Its Effect on Platelet Glycolysis and Release of Adenosine Diphosphate

Abstract: *Incubation of human platelets with aspirin inhibited glycolysis and produced a fall in the concentration of adenosine triphosphate. When platelets were exposed to collagen there was an increase in glycolysis and release of adenosine diphosphate. Prior incubation of the platelets with aspirin for 5 minutes did not totally suppress the increase in glycolysis after exposure to collagen but completely inhibited the collagen-induced reaction of the release of adenosine diphosphate. It is suggested that aspirin acts on human platelets by inhibiting both release of adenosine diphosphate and the transport of glucose across the platelet membrane.*

The fundamental contribution of platelets to hemostasis and thrombosis is well recognized. When a vessel is injured, platelets adhere to the exposed subendothelial collagen, release adenosine diphosphate (ADP), and then aggregate (*1*). The importance of this reaction has recently been emphasized by the demonstration that aspirin and other drugs that impair the release, by collagen, of platelet ADP in vitro inhibit thrombus formation and hemostatic plug formation in vivo (*2, 3*).

The effect of salicylates on platelet metabolism is unknown; however, salicylates have been reported to interfere with carbohydrate metabolism in several other tissues by uncoupling oxidative phosphorylation (*4*), inhibiting erythrocyte glycolysis (*5*), and increasing liver and muscle glycogenolysis (*6*).

The possibility exists that aspirin also interferes with platelet carbohydrate metabolism and that through this effect inhibits release of ADP. Alternatively, aspirin may specifically inhibit the release of platelet ADP without interfering with other metabolic changes caused by the interaction of platelets with collagen.

These questions were examined by first investigating the influence of aspirin on platelet carbohydrate metabolism and then by comparing its effect on release of platelet ADP with its