and solid contents of the tube were removed by cutting the tube. (iii) A saturated solution of ammonium sulfate was placed in a test tube, and the less dense azurin solution was added to the top of the ammonium sulfate solution forming a separate layer. Azurin crystals formed after several days. The crystals formed in these three processes were always blue-colored needles of square cross-section from 0.1 to 2.0 mm long, their width was about 1/20th of their length. Much smaller crystals were reported by Suzuki and Iwasaki (*4*).

X-ray diffraction photographs were taken with a precession camera with nickel-filtered CuKα radiation (wavelength, 1.542 Å). The photographs taken with the x-ray beam coincident with the direction of elongation of the crystal show *4mm* symmetry, and the photographs taken with the x-ray beam perpendicular to the elongation axis and the side of the cross section show *mm* symmetry. The Laue group is 4/*mmm*, the former photographs are of the *hk*0 reciprocal lattice plane, and the latter are of the 0*kl* or *h*0*l* reciprocal lattice plane (which are identical). The cell dimensions are $a = b = 53.2$ Å and $c = 101$ Å. The only extinctions observed are: 00*l* present only for $l = 4n$. This observation, together with the Laue group, defines the space group as 91 $P4_122$ (or its enantiomorph 95 $P4_322$). This space group is quite rare. This is also the first report of crystal data for a copper protein. There are eight equal asymmetric units in this space group, and therefore the volume of the asymmetric unit containing azurin and solvent of crystallization is about 36,000 Å^3. The most frequent ratio of volume to molecular weight is 2.2 Å^3/dalton (*5*), and this gives a molecular weight of 16,000 daltons, which is in agreement with the observed value of 16,300 (*4*).

GERALD STRAHS
Department of Biochemistry,
New York Medical College,
New York 10029

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9 April 1969; revised 23 May 1969

Biochemistry at 100°C: Explosive Secretory Discharge of Bombardier Beetles (Brachinus)

Abstract. *The defensive chemical spray of bombardier beetles is ejected at 100°C, with a heat content of about 0.2 calorie per milligram.*

More than 100 years ago, the entomologist J. O. Westwood, quoting a traveler returned from South America, wrote that certain large beetles of the genus *Brachinus* "on being seized . . . immediately . . . play off their artillery, burning . . . the flesh to such a degree, that only few specimens (can) be captured with the naked hand" (*1*). *Brachinus* is a widely distributed genus of Carabidae, whose members have long been known to naturalists as bombardier beetles. Their "artillery" is a defensive spray, ejected from a pair of glands that open at the tip of the abdomen. The spray is visible as a fine mist, and the discharge occurs with an audible detonation (*2*).

Each gland of *Brachinus* is a two-compartmented apparatus (Fig. 1). The inner compartment (reservoir) contains an aqueous solution of hydroquinones (hydroquinone and methylhydroquinone) and hydrogen peroxide, while the outer compartment (vestibule) contains a mixture of catalases and peroxidases (*3*). In order to effect a discharge, the beetle squeezes some reservoir fluid into the vestibule (*4*), thereby triggering what is essentially an instantaneous and explosive set of events: the catalases promote the decomposition of hydrogen peroxide, while the peroxidases force the oxidation of the hydroquinones to their respective quinones. Under pressure of the free oxygen, the mixture "pops" out. The active defensive principles of the secretion are the quinones generated in the discharge. They are strongly repellent to many predators (*2, 5*). Bombardiers can spray repeatedly and in quick succession; as many as 29 discharges have been elicited from a single beetle (*2*). Through rotation of the abdominal tip they can eject the spray in virtually any direction, and they always aim it toward the foe (*2*).

Given the known concentration of the reactants (hydrogen peroxide, 25 percent; both hydroquinones, 10 percent) (*6*), as well as the thermodynamic properties of the reaction they undergo, it is possible to predict within certain limits the heat content and temperature of the spray. The chemical reaction

$$\text{hydroquinone (aq)} + H_2O_2\text{ (aq)} \longrightarrow \text{quinone (aq)} + 2H_2O\text{ (l)} \qquad (a)$$

may be looked upon as the net result of the three steps (*7*):

$$\text{hydroquinone (aq)} \longrightarrow \text{quinone (aq)} + H_2\text{ (g)} \qquad (b)$$

$$H_2O_2\text{ (aq)} \longrightarrow H_2O\text{ (l)} + \tfrac{1}{2}O_2\text{ (g)} \qquad (c)$$

$$H_2\text{ (g)} + \tfrac{1}{2}O_2\text{ (g)} \longrightarrow H_2O\text{ (l)} \qquad (d)$$

The enthalpy changes for (c) and (d) are known (*8*): $\Delta H_c = -22.6$ kcal/mole and $\Delta H_d = -68.3$ kcal/mole, both at 25°C. That for (b) is calculable from $\Delta H_b = -2F\ d(E/T)/d(1/T)$, where E is the standard electromagnetic force of the quinhydrone electrode, T is the absolute temperature, and F is the Faraday. From the known (*9*) temperature dependence of E, we then find $\Delta H_b = +42.4$ kcal/mole at 25°C. Thus, $\Delta H_a = -22.6 - 68.3 + 42.4 = -48.5$ kcal/mole at 25°C; that is, 48.5 kcal of heat are evolved for each mole of hydroquinone that undergoes the reaction (a). It is not anticipated that this would depend very much on the temperature at which reaction occurs (between 25° and 100°C) or on whether the reactant is hydroquinone itself or methylhydroquinone. In a reservoir solution of 10 percent hydroquinone and 25 percent hydrogen peroxide, there are 0.91 μmole of hydroquinone and 7.40 μmole

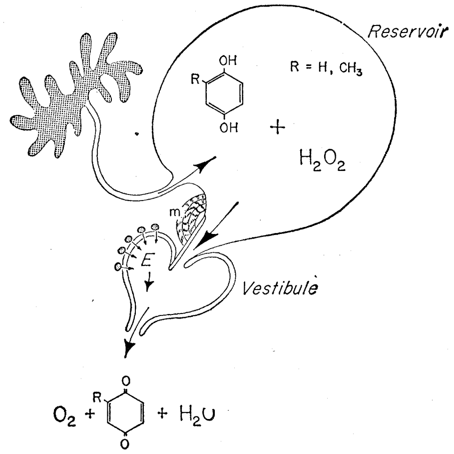

Fig. 1. Diagram of gland of bombardier beetle. The reservoir obtains its contents from a duct that drains an outlying cluster of secretory tissue. The enzymes (*E*) in the vestibule are secreted by cells on the wall of the vestibule itself. The muscle (*m*) controls the valve between the two compartments. Other details in text. [Based on Schildknecht *et al.* (*3*)]

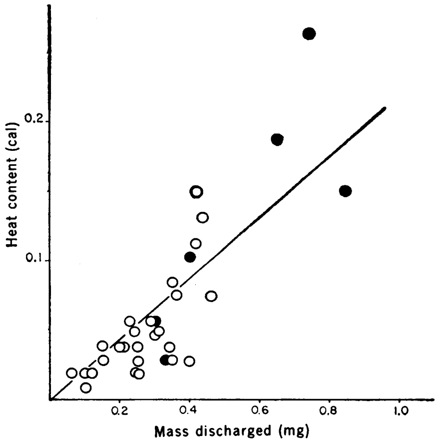

Fig. 2. Heat content of spray, plotted as a function of the mass of secretion discharged. (Open circles) Single discharges; (closed circles) double or triple discharges. The regression line was drawn through zero (its deviation from zero is statistically insignificant at the 95 percent confidence level).

of hydrogen peroxide per milligram of solution. The hydroquinone could react completely with an equivalent amount of hydrogen peroxide as in (a), the remaning hydrogen peroxide (6.49 μmole per milligram of solution) decomposing as in (c). Assuming that these reactions do indeed go to completion, we then conclude from the known ΔH_c and the estimated ΔH_a that they are accompanied by the evolution of 0.19 calorie of heat per milligram of solution. This quantity of heat is sufficient to heat the spray to the boiling point (~100°C) and to vaporize about one-fifth of it.

Measurements of the heat content of the spray were made with a calorimeter, consisting of a small cylinder of copper foil, encased in an insulating jacket of Styrofoam. A thermocouple on the cylinder wall, connected in conventional fashion by way of an amplifier to a storage oscilloscope, provided the means for recording cylinder temperature. One end of the cylinder was open and shaped so as to fit snugly around the beetle's posterior abdomen. The instrument was calibrated (*10*) and shown to have a heat capacity of 0.8 ± 0.01 calorie per degree Celsius.

Individual beetles (*11*), held by hooks cemented to the thorax and maneuvered with a micromanipulator, were slid rear-end-first into the open end of the calorimeter, until contact with a pointed shaft that had been preinstalled in the cylinder caused them to discharge into the instrument. Heat measurements were made on a total of 32 discharges, from three separate beetles. The amounts of secretion ejected were determined by weighing the beetles before and after each discharge. Heat content of the spray is a parameter of some variability (Fig. 2). The value given by the slope of the regression line, 0.22 calorie per milligram of secretion expelled, is in close accord with the theoretically predicted heat of formation of the spray.

Measurements of temperature of the spray were made with a thermocouple connected through an amplifier to a storage oscilloscope. The temperature-voltage relationship of the thermocouple, as well as its time-response characteristics, were ascertained by immersion of the sensing junction in water at various fixed temperatures between 25° to 100°C (*12*). Discharges were elicited from the beetles by poking them with the sensing junction itself, which was applied by its pointed end directly to the site of the gland openings. This insured that the sensing junction always received the full impact of the spray. Temperature measurements were made on several dozen discharges, from nine beetles. The value obtained was consistently 100°C, as predicted (*13*). Additional measurements made with a microthermistor circuit and storage oscilloscope confirmed this finding.

By causing bombardier beetles to discharge onto a thermocouple that acted as the trigger to an electronic flash unit,

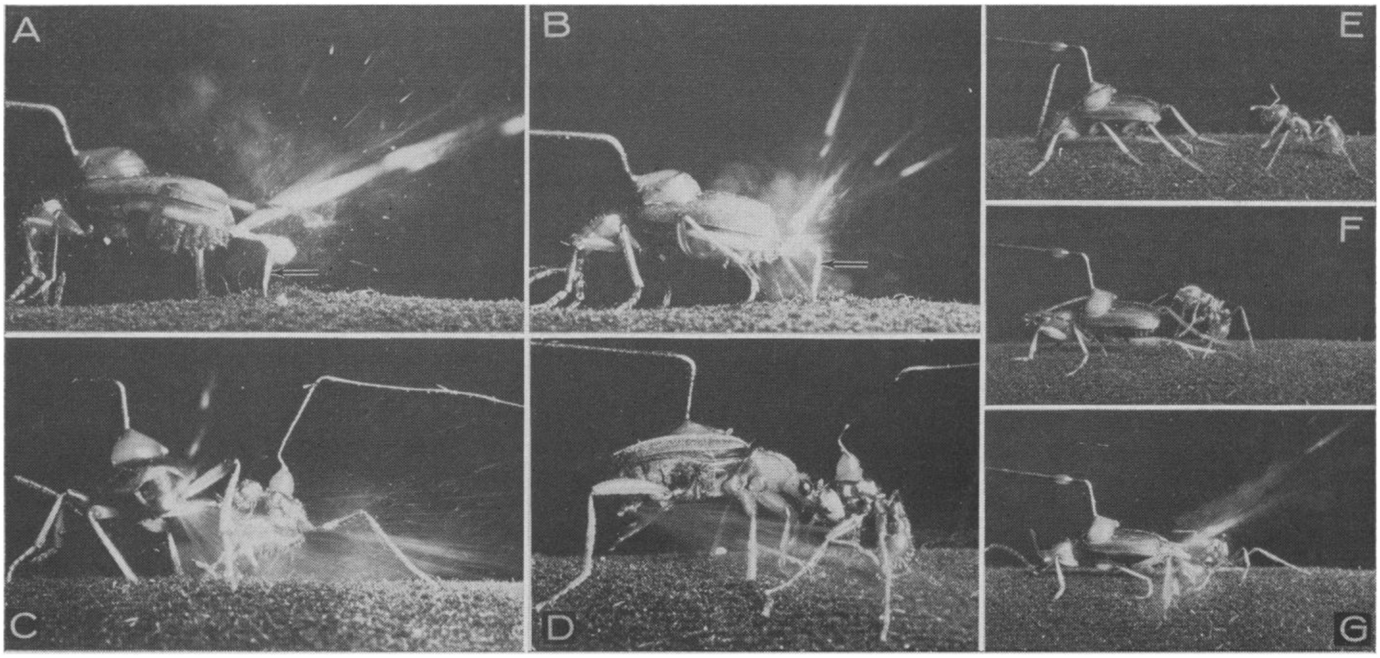

Fig. 3. (A and B) Bombardier beetles discharging. The beetles are fastened with wax to hooks on their back. The arrow points to the thermocouple used to prod the beetle and to trigger the electronic flash unit. The ejected droplets appear as tapering streaks, because the illuminating flash (about 1/4000 seconds) declines in intensity after an initial peak. (C and D) Bombardier beetles ejecting aimed discharges in response to "attack" by ants. The ants are fastened to hooks, and held in such a way as to prod the beetle's head (D) or to bite the beetle's right middle leg (C). (E to G) Consecutive stages in the attack of a bombardier beetle by an ant. After approaching (E), and poising itself for the attack (F), the ant bites the beetle's left rear leg and is accurately sprayed in return (G). (Bodies of beetles are 9 to 10 mm long.)

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*).

DANIEL J. ANESHANSLEY
Section of Neurobiology and Behavior and *Department of Electrical Engineering, Cornell University, Ithaca, New York 14850*

THOMAS EISNER
Section of Neurobiology and Behavior, Cornell University

JOANNE M. WIDOM, BENJAMIN WIDOM
Department of Chemistry, Cornell University

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6. Schildknecht and Holoubek (*3*), from whose paper these values are taken, do not indicate whether the percentage of hydroquinones is by volume or weight; in our calculations we have assumed it to be by weight.
7. It is not suggested that (b), (c), (d) constitute the mechanism for (a). Nevertheless, the enthalpy change associated with (a) is the sum of the enthalpy changes associated with (b), (c), and (d).
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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