Cardioaccelerator Release in Periplaneta americana (L)

Abstract. *Focal electrical stimulation of the brain of* Periplaneta americana *evokes the release of a factor with high cardioaccelerator activity. Results of extirpation of components of the retrocerebral complex from an isolated head preparation imply that the corpus cardiacum is the critical component of this system. The cardioaccelerator is a heat-stable proteinaceous compound.*

Studies of the structure and function of the corpora cardiaca in insects have established that these bodies are neuroendocrine organs. The corpora cardiaca are characterized by axon profiles which contain several different types of neurosecretory granules (*1*). The complexity suggested by the ultrastructure is emphasized by the variety of pharmacological activities which can be evoked by extracts of the corpora cardiaca (*2*–*6*). Using a modification of classical perfusion techniques, I have demonstrated the release of a factor from the corpora cardiaca which accelerates cardiac frequency.

It is generally accepted that a cardioaccelerator substance is secreted from the corpora cardiaca (*2*, *4*). However, Cameron (*3*) and Davy (*4*) used extraction procedures which left undetermined the nature of the cardioaccelerator substance actually released. The corpora cardiaca undoubtedly contain several substances which can accelerate the cardiac frequency. "Adrenergic substances" (*5*), 5-hydroxytryptamine (*7*), and two compounds which appear to be proteins (*8*) are all present in corpora cardiaca extracts, and, in appropriate concentrations, will accelerate the cardiac frequency. The nature of the substance actually released from the corpora cardiaca as a functional cardioaccelerator is unclear.

The assay procedure used by Ralph (*6*) was chosen and modified to facilitate interpretation of the results. A mechanical transducer was placed on the heart and coupled to a chart recorder so that continuous records of cardiac frequency could be obtained. The transducer stylus was placed adjacent to the heart in the third abdominal segment of the isolated preparation. The effect of replacing the normal saline on the test heart with 50 μl of perfusion solution was expressed as the percentage of change relative to the basal heart rate (BHR). The BHR of a test heart was determined by calculating the mean number of beats per minute, over the 2-hour period which preceded the assay.

The isolated head preparation (Fig. 1) was used as the source of the cardioaccelerator substance. Adult male *Periplaneta americana,* 3 to 12 days after metamorphosis, were used for these experiments. An isolated head, with the retrocerebral complex exposed and the gut ligated, was mounted on a wax block. No anesthetic was used for the operation. The preparation was perfused with 75 μl of Pringle's insect Ringer (*9*). This solution drained across the brain, corpora cardiaca, and corpora allata, and was then collected in a small glass vessel as it ran off the ligated gut. The solution was withdrawn from the vessel and again perfused over the preparation approximately once per minute for the duration of the 15-minute test period. At the end of this period the solution was removed from the glass vessel. This procedure was repeated for eight successive 15-minute periods. All eight samples were then individually assayed on an isolated heart.

The dark portion of the histogram in Fig. 2 shows the results of a typical experiment. While the first seven perfusion samples were being obtained electrodes were in position, but no electrical stimulation was applied. Concomitant with the eighth perfusion period, a 15-volt, 0.3-msec pulse was applied at a frequency of once per second. The brain was stimulated by a silver–silver chloride electrode (20 μ in diameter) on the anterior dorsomedial surface of each brain lobe. A pin through the labium served as the anode. The position of the anode had no influence upon the results.

The basic pattern displayed by the black portion of the histogram (Fig. 2) was observed in all 14 cases performed in this series. Perfusion samples 1 and 2 characteristically contained relatively high cardioaccelerator activity. This may be due to postoperative trauma since perfusions were always started within 5 minutes after the initiation of operative procedures. Subsequent perfusion samples, in the absence of electrical stimulation, contained considerably less activity. This appears to represent a steady-state level of release, which begins with the fourth perfusion. Even when 26 consecutive perfusion samples were obtained in the absence of electrical stimulation, the activity of individual samples (after the first two) was within 10 percent of that in the fourth sample. When electrical stimulation of the brain accompanied a perfusion, as in sample 8, there was always a marked increase in cardioaccelerator activity.

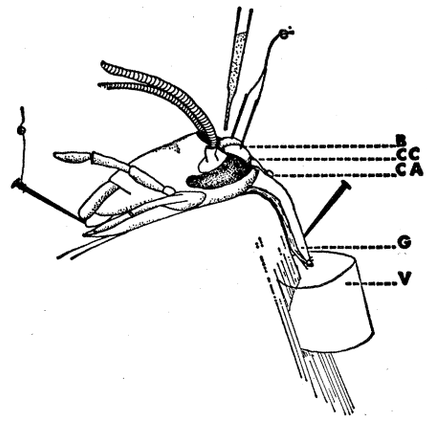

Fig. 1. The isolated roach head used in the stimulation and perfusion of the retrocerebral complex. *B*, Brain; *CC*, corpora cardiaca; *CA*, corpora allata; *G*, gut; *V*, glass collecting vessel.

In seven additional preparations, the corpora allata were removed before the first perfusion sample was collected. This operation did not alter the ability of brain stimulation to evoke cardioaccelerator release into the perfusion medium. Perfusion sample 8 (during stimulation), in the allotectomized preparations, contained between two and a half to four times the activity of the seventh sample. The overall pattern for allotectomized preparations was that of preparations with the retrocerebral complex intact. When the corpora cardiaca were removed, before the first perfusion period, brain stimulation failed to evoke increased cardioaccelerator activity in

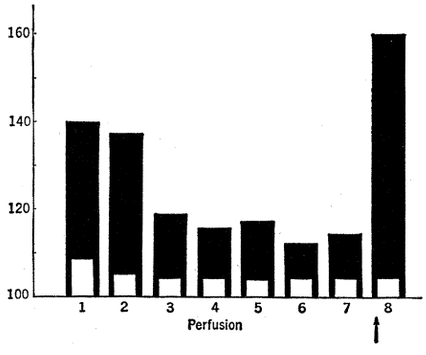

Fig. 2. The cardioaccelerator activity, expressed as the percentage increase in the basal heart rate (BHR) of the assay heart for individual 15-minute perfusion samples. The dark histogram shows the results with an intact retrocerebral complex; the white histogram shows the results from a preparation with the corpora cardiaca removed. The arrow indicates electrical stimulation to the brain for both cases.

the perfusion solution (Fig. 2). In addition, the cardioaccelerator activity was decreased in all perfusion samples. These results were observed in all nine experiments of this type.

When the corpora cardiaca, rather than the brain, were stimulated, release of cardioaccelerator was increased. This raised a question concerning the mode of release when the brain was stimulated. Electrotonic spread of a stimulus applied to the brain might affect the corpora cardiaca directly. As a control, the brain was stimulated in the usual fashion, but all of the nerves to both sides of the corpus allatum–corpus cardiacum complex were cut (Fig. 3). In all of the seven cases of this series, brain stimulation failed to evoke cardioaccelerator release. The amount of cardioaccelerator released was significantly different from that released by intact preparations ($P < .01$). This finding implies that neural connections are necessary for brain stimulation to evoke cardioaccelerator release. It also tends to discount the possibility that electrotonic spread of the stimulus affected the corpora cardiaca directly. Direct stimulation of the bulbous area of the corpora cardiaca in denervated preparations resulted in the release of three to six times the cardioaccelerator activity contained in perfusion samples obtained in the absence of stimulation. Thus, denervation did not alter the ability of the corpora cardiaca to release the cardioaccelerator.

To determine whether a specific neural pathway between the brain and corpora cardiaca mediates cardioaccelerator release, the isolated head preparation was again employed, but in each a specific nerve (*10*) to each side of the corpus cardiacum–corpus allatum complex was severed (Fig. 3). The brain was then stimulated in the usual manner. The effect of cutting a particular nerve was determined by the ability of brain stimulation to evoke increased cardioaccelerator activity in the perfusion solution. Separate roaches were used for each experiment, and the results of at least five experiments were pooled for calculation of the mean values. Severing only one of the eight nerves (*NCC 1*) consistently resulted in cardioaccelerator activity significantly lower than that of intact preparations ($P < .01$).

My evidence demonstrates that a cardioaccelerator substance is released from the corpora cardiaca and suggests that a neural pathway between the brain and corpora cardiaca can mediate this release. Additional observations have been made on some characteristics of the substance released. The isolated heart preparation displayed a characteristic response to the cardioaccelerator. Within 5 seconds after the application of a perfusion solution cardiac frequency increased. Visual examination indicated a concomitant increase in the amplitude of the heart beat, but the transducer used in these assays did not accurately measure this parameter. The cardiac frequency reached a peak after about 6 minutes and then decreased slightly during the next 5 minutes. Depending upon the length of the stimulation period used to evoke cardioaccelerator release, a given perfusion sample gave a maximum cardiac frequency which was between 40 and 300 percent BHR. The cardiac frequency then leveled out at approximately 85 percent of the maximum value and maintained this rate for 5 to 8 hours. The plateau phase was followed by a slow decline, over about 2 hours, to the BHR. The effect of the cardioaccelerator is readily reversible at any stage of its action. Washing the test heart with fresh Ringer solution reestablishes the BHR within 5 minutes.

The instability of the cardioaccelerator substance as collected in perfusion solutions has hindered attempts at its characterization. Within 24 hours after release from the corpora cardiaca, the cardioaccelerator activity in a perfusion solution has decreased by about 80 percent. This loss is not retarded by freezing. Boiling the perfusion solution for 10 minutes does not affect the cardioaccelerator activity. When a perfusion sample which initially had a high cardioaccelerator activity is incubated for 1 hour with Pronase, trypsin, or chymotrypsin, it loses all assayable activity. These results demonstrate the presence of at least two peptide bonds in the cardioaccelerator molecule. The cardioaccelerator released under the conditions of these experiments may be one or both of the peptides extracted from the corpora cardiaca of *Periplaneta* (*8*).

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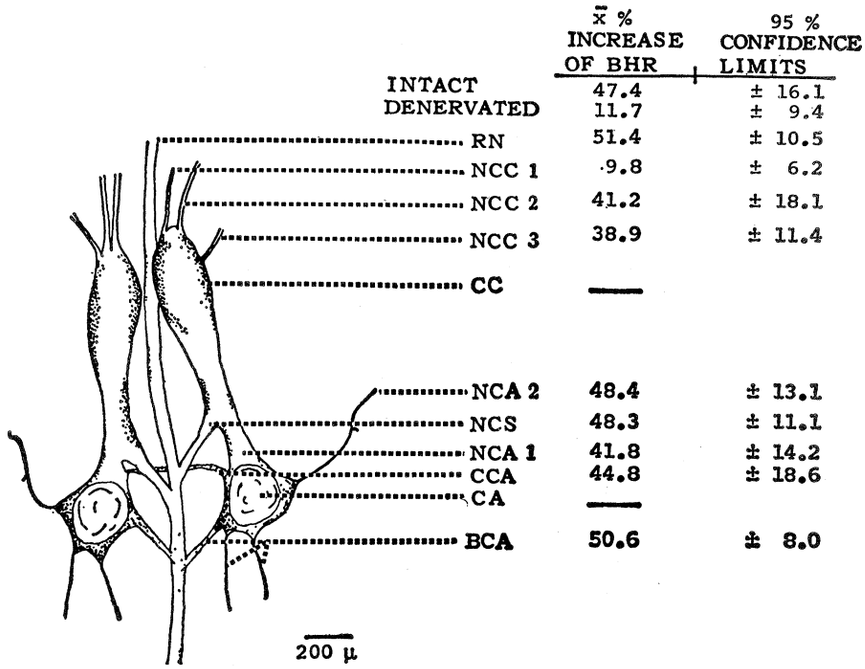

Fig. 3. The effects of cutting nerves on the ability of brain stimulation to evoke cardioaccelerator release. Cardioacclerator release is expressed as the mean percentage increase in BHR evoked by brain-stimulated perfusion samples from intact preparations, completely denervated preparations, and preparations in which specific nerves have been cut bilaterally. *RN*, Nervus recurrens; *NCC 1, 2, 3*, nervus corporus cardiaci; *NCA 1, 2*, nervus corporis allati; *NCS*, nervus cardiostomatogastricus; *CCA*, commissurus corporis allati; *BCA*, three nerves branching off the corpus allatum (*CA*) which are treated as a single nerve; *CC*, corpus cardiacum. Nerve notations after Willey (*10*).

References and Notes

1. B. Bowers and B. Johnson, *Gen. Comp. Endocrinol.* **6**, 213 (1966); B. Scharrer, *Z. Zellforsch.* **60**, 761 (1963); U. Smith and D. S. Smith, *J. Cell Sci.* **1**, 59 (1966); R. B. Willey and G. Chapman, *J. Ultrastruct. Res.* **4**, 1 (1960).
2. F. S. W. Knowles, D. B. Carlisle, M. Dupont-Raabe, *J. Marine Biol. Ass. U.K.* **34**, 611 (1955); N. S. Milburn, E. Weiant, K. D.

Roeder, *Biol. Bull.* **118**, 111 (1960); N. S. Milburn and K. D. Roeder, *Gen. Comp. Endocrinol.* **2**, 70 (1962); S. Ozbas and E. S. Hodgson, *Proc. Nat. Acad. Sci. U.S.* **44**, 825 (1958); J. E. Steele, *Nature* **192**, 680 (1961).
3. M. L. Cameron, *Nature* **172**, 349 (1953).
4. K. G. Davey, *Gen. Comp. Endocrinol*, **1**, 24 (1961); *Nature* **192**, 284 (1961); *J. Insect Physiol.* **9**, 375 (1963).
5. L. Barton-Browne, L. F. Dodson, E. S. Hodgson, J. K. Kiraly, *Gen. Comp. Endocrinol.* **1**, 232 (1961).
6. C. L. Ralph, *J. Insect Physiol.* **8**, 431 (1962).
7. E. H. Colhoun, *Experientia* **19**, 9 (1963); M. Gersch, F. Fischer, H. Unger, W. Kabitzer, *Z. Naturforsch.* **166**, 351 (1961).
8. B. E. Brown, *Gen. Comp. Endocrinol.* **5**, 387 (1965).
9. J. W. S. Pringle, *J. Exp. Biol.* **15**, 101 (1938).
10. R. B. Willey, *J. Morphol.* **108**, 219 (1961).
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Tyramine-H^3: Deaminated Metabolites in Neuroblastoma Tumors and in Continuous Cell Line of a Neuroblastoma

Abstract. *Neuroblastoma tumors, as well as cultured cells of neuroblastoma, contain high monoamine oxidase activity. The major deaminated metabolite of tyramine-H^3 in the incubation mixtures with the tumors or with the cultured cells is* p-*hydroxyphenylacetaldehyde. Upon addition of reduced nicotinamide-adenine dinucleotide phosphate, the aldehyde was further metabolized by the reductive pathway to* p-*hydroxyphenylethanol, whereas upon addition of nicotinamide-adenine dinucleotide phosphate the aldehyde was only metabolized to a minor extent by the oxidative pathway to* p-*hydroxyphenylacetic acid. Aldehyde dehydrogenase activity is very low in the neuroblastoma tumors and in the cultured neuroblastoma cells. The generation of aldehydes and alcohols by the action of monoamine oxidase suggests that the deaminated metabolites of biogenic amines might exhibit some toxic effects in neuroblastoma patients.*

In view of the common histogenetic origin of the chromaffin cells and of the sympathetic nerve cells it was not unreasonable to assume that the tumors arising from the latter might also be metabolically active. However, only in the last decade has the increased excretion of catecholamines and their metabolites been described in patients with neuroblastomas (*1*). The sympathetic manifestations, such as hypertension, frequently observed in pheochromocytoma patients are rather uncommon in patients with neuroblastoma. These clinical observations, as well as the excretion spectrum of catecholamine metabolites in neuroblastoma patients, suggest that the tumor mainly secretes the breakdown products of the corresponding catecholamine (*2*). Therefore, we undertook the determination and identification of the metabolic breakdown products of biogenic amines in these tumors. We have investigated the deamination of tyramine by monoamine oxidase and have identified the deaminated products which were formed in a continuous cell line of neuroblastoma and in fresh neuroblastoma tumors.

The neuroblastoma cells were in a continuous culture for 2 years. The line was started from a lymph node obtained from a 2-year-old child immediately after its death. The fresh neuroblastoma tissues were obtained immediately after surgery and they were kept at −10°C.

Tissue culture cells of neuroblastomas were suspended in 0.5*M* phosphate buffer (*p*H 7.4) and incubated with tyramine-H^3 at 37°C for 2 hours. Neuroblastoma tissue tumors were homogenized in 0.5*M* phosphate buffer (*p*H 7.4) and incubated under the same experimental conditions as the tumor culture cells. At the end of the incubation period, the reaction mixtures were stopped by addition of 0.1 ml of concentrated HCl. Upon deproteinization the supernatant was adjusted to *p*H 2, and the deaminated products were extracted into ethylacetate. Samples of the ethylacetate fraction were counted in a liquid scintillation spectrometer. The deaminated tyramine-H^3 metabolites were analyzed and identified by the following procedures. Samples of the ethylacetate fraction were subjected to paper chromatography in a mixture of isopropyl alcohol, ammonia, and water (8:1:1). In this solvent system *p*-hydroxyphenylacetic acid was separated from *p*-hydroxyphenylethanol and from *p*-hydroxyphenylacetaldehyde. The radioactive peak which contained *p*-hydroxyphenylethanol and *p*-hydroxyphenylacetaldehyde was eluted with methanol and the radioactive material was acetylated (*3*). Upon acetylation, the radioactive material was submitted to paper chromatography in the "C" solvent system of Bush (*4*). This system separates the acetylated alcohol from the acetylated aldehyde. Other samples of the ethylacetate fraction were chromatographed in toluene, methanol, ethylacetate, and water (1:1:1:1). In this system *p*-hydroxyphenylacetaldehyde separates from *p*-hydroxyphenylethanol and from *p*-hydroxyphenylacetic acid. The R_F values of the deaminated tyramine metabolites in these different solvent systems are presented in Table 1.

Table 1. The R_F values of deaminated tyramine metabolites in different solvent systems for paper chromatography. (A) Isopropyl alcohol, ammonia, and water (8:1:1); (B) toluene; ethylacetate, methanol, and water (1:1:1:1); (C) toluene, ethylacetate, methanol, and water (1:0.1:0..5:0.5) [Buch "C" solvent system (*4*)]. The R_F values in the "C" solvent system are listed for the acetylated metabolites.

Metabolite	R_F in different solvents		
	A	B	C
p-Hydroxyphenylacetic acid	0.36	0.60	
p-Hydroxyphenylethanol	0.90	0.64	0.75
p-Hydroxyphenylacetaldehyde	0.90	0.90	0.95

A further identification of *p*-hydroxyphenylacetaldehyde was achieved by the addition of aldehyde dehydrogenase into some of the incubation mixtures. The enzyme aldehyde dehydrogenase which catalyzes the conversion of the aldehydes into the corresponding acids was isolated and purified from guinea pig kidney as previously described (*5*).

Neuroblastoma tissue, as well as cells of neuroblastoma tissue cultures, contain monoamine oxidase activity (Table 2). The enzyme activity is inhibited by such known inhibitors of monoamine oxidase as pheniprazine or iproniazid, as well as by sulfhydryl blocking agents and by disulfiram.

In incubation mixtures with neuroblastoma tumors as well as with cells of neuroblastoma tissue cultures, the only deaminated metabolite which was formed from tyramine-H^3 was *p*-hydroxyphenylacetaldehyde-H^3 (Table 2). Upon addition of reduced nicotinamide-adenine dinucleotide phosphate (NADP) to the incubation mixtures, two radioactive deaminated metabolites of tyramine-H^3 were formed. The major one was *p*-hydroxyphenylethanol-H^3 and the minor was *p*-hydroxyphenylacetaldehyde-H^3. Upon addition of NADP also two radioactive deaminated metabolites of tyramine-H^3 were