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ATTRACTION OF THE NEMATODE *CAENORHABDITIS ELEGANS* TO PYRIDINE

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(*Received* 16 *August* 1975)

Abstract—1. The nematode *C. elegans* is attracted to pyridine.
2. The threshold is about 0·1 mM.
3. At concentrations above 1 mM the response weakens.
4. No indication of avoidance of high concentrations could be found.

INTRODUCTION

The nematode *Caenorhabditis elegans* has great promise for studies in neurogenetics as a result of its unusually simple nervous system and an uncommon sexual cycle (Brenner, 1973, 1974). As part of the effort to exploit the potential of this organism for studies in this area, the chemotactic abilities of *C. elegans* have been explored in some detail (Ward, 1973; Dusenbery, 1974, 1975). These studies identified a number of attractants and repellents. More recently a number of mutant strains that fail to respond to certain chemicals have been isolated (Dusenbery *et al.*, 1975). The value of chemotactic studies is further enhanced by the completion of very detailed studies of the sensory anatomy of *C. elegans* by Ward *et al.* (1975) and Ware *et al.* (1975). I report here experiments demonstrating that *C. elegans* is attracted to the chemical pyridine.

MATERIALS AND METHODS

The strain of *C. elegans* used and its methods of culture are those of Brenner (1974). The chemotaxis experiments were based on the method of countercurrent separation (Dusenbery, 1973). In this method a dense solution flows downward along the bottom side of an inclined tube, while a light solution, floating on the dense solution, flows upward along the top side. A response to an attractant or repellent carried in one of the solutions is determined by observing the proportion of animals which emerge from the tube with that solution.

When no chemotactic stimulus was present 99% or more of the recovered nematodes were found in the dense solution in this series of experiments. As a result attraction to a chemical was determined by simply measuring the proportion of recovered nematodes in the light solution when attractant was present in that solution.

In experiments where avoidance as well as attraction was possible, a pair of countercurrent tubes (a and b) was run in parallel using nematodes from the same population in both tubes but with the attractant distribution of the solutions reversed in the two tubes. An over-all measure, R, of the response to the attractant was then defined as $100\,(R'_a + R'_b - 1)$, where R'_a is the fraction of worms from tube a that were found in the solution with the higher concentration of attractant and similarly for R'_b. This measure defines a scale on which +100 corresponds to complete attraction, 0 corresponds to no response, and −100 corresponds to complete avoidance. This is the same measure as previously used (Dusenbery, 1974).

The nematodes were grown and tested at a temperature of 20°C. Chemotaxis experiments were performed in the presence of 0·5 mM KH_2HPO_4, plus 0·5 mM K_2HPO_4 which yielded a pH close to 7·0. The pyridine was from Mallinckrodt.

RESULTS AND DISCUSSION

Initial tests of chemotactic responses of *C. elegans* to pyridine indicated that there was a fairly strong attraction. Data indicating the strength of this attraction at various concentrations are presented in Fig. 1. As is usually observed in similar studies with other chemicals, responses of medium strength have a large degree of variability. Nonetheless it may be seen that above a threshold in the vicinity of 0·1 mM there is an attraction which is fairly strong around 1 mM but becomes weaker as concentration is increased to 10 mM.

The decline in response strength at high concentrations has several potential explanations. One likely possibility is that the higher concentrations of pyridine are toxic in some way and as a result interfere with chemotaxis generally. This hypothesis was tested by measuring the strength of attraction to NaCl in the presence of various concentrations of pyridine.

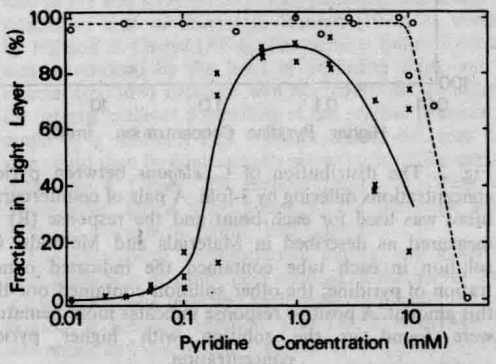

Fig. 1. The attraction of *C. elegans* to pyridine and to NaCl in the presence of pyridine. Crosses: no NaCl, pyridine of indicated concentration in light layer only; circles: 20 mM NaCl in light layer, pyridine of indicated concentration in both layers. Response measured as the fraction of nematodes in the light layer.

C.B.P. 53/1C—A

Previous experiments (Dusenbery, 1974) demonstrated that *C. elegans* has a strong attraction to NaCl with a threshold of about 0·1 mM. In each test discussed below pyridine was present in both solutions at the concentration indicated and NaCl was in the light solution at a concentration of 20 mM. These results are presented in Fig. 1. It is clear that above 10 mM pyridine does interfere with the response to NaCl. This decline in response strength, however, occurs at a higher concentration than does the decline in the response to pyridine. Such a situation could arise simply because the response to pyridine is weaker and thus more sensitive to disruption by toxic chemicals. I have observed that weak responses are generally more disrupted by various stresses than strong responses are.

A second possibility is that high concentrations of pyridine become repellent. Avoidance of high concentrations of an attractant is a fairly common observation and has, for instance, been observed in the response of paramecium to CO_2 or acetic acid (Jennings, 1906), of the cheese mite to skatol (Henschol. 1929) of ticks to butyric acid (Totze, 1933) and of mosquitoes to several organic acids (Muller, 1968).

The latter hypothesis may be tested by giving the nematodes a choice between two different concentrations of pyridine. If both concentrations are above that giving the maximum response, this hypothesis predicts that the nematodes will be found predominately in the lower concentration, producing negative response measurements. Such data for concentrations differing by 3-fold are given in Fig. 2. The responses are positive throughout the full range, indicating a complete lack of avoidance of pyridine. Thus the hypothesis that toxicity is responsible for the decline in response strengths is the more likely.

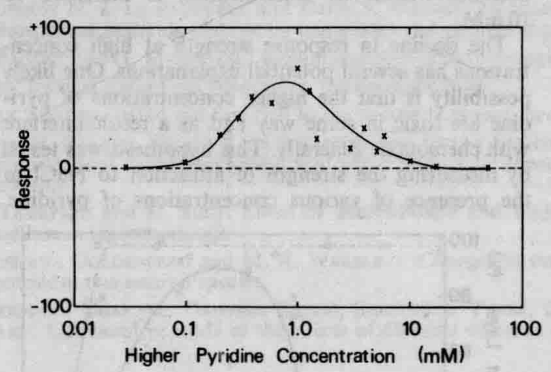

Fig. 2. The distribution of *C. elegans* between pyridine concentrations differing by 3-fold. A pair of countercurrent tubes was used for each point and the response (R) was measured as described in Materials and Methods. One solution in each tube contained the indicated concentration of pyridine; the other solution contained one-third this amount. A positive response indicates more nematodes were found in the solution with higher pyridine concentration.

The set of mutant strains previously isolated as defective in attraction to NaCl (Dusenbery *et al.*, 1975) has been tested for responsiveness to pyridine as well as other known stimuli (Dusenbery, in preparation). Several of these strains are also defective in their response to pyridine. The results demonstrate that responses to any of the set of stimuli—Na^+, Cl^-, OH^-, H^+, cAMP, and $NaHCO_3$ in phosphate buffer at pH 6·0—can be abolished without seriously affecting the response to pyridine or vice versa. This is good evidence that the receptor for pyridine is distinct from the receptors for these other chemicals.

The adaptive value of this response to the organism in its natural environment is far from obvious. In any case this response is a useful addition to the responses available for the characterization of behavioral mutants of *C. elegans*.

Acknowledgement—The author wishes to thank Ms. Georgann Hardin for carrying out the experiments. This research was supported by the National Science Foundation (Grant GB 43561).

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VAGO-SYMPATHETIC INNERVATION OF THE HEART OF THE PUFF ADDER, *BITIS ARIETANS*

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(*Received* 15 *August* 1975)

Abstract—1. The effects of drugs and vagal stimulation on the rate of the perfused heart *in situ* and on the heart rate and arterial blood pressure *in vivo* were studied in the puff adder. Fluorescent histochemistry of the catecholamines in the different parts of the heart was also performed.

2. The ventricle, atria and sinus venosus are all densely innervated by adrenergic fibres. Adrenergic fibres around coronary vessels were also observed by fluorescent histochemistry.

3. The adrenergic fibres reach the heart together with the vagi, and may join the vagi from the sympathetic chains. No direct sympathetic fibres to the heart were seen.

4. Acetylcholine causes bradycardia or cardiac arrest, and also arrythmia, while isoprenaline, adrenaline and noradrenaline increase the heart rate both *in situ* and *in vivo*. Vagal stimulation normally causes bradycardia or cardiac arrest, but in some cases a tachycardia was seen. Tachycardia during vagal stimulation was normally seen after pretreatment with atropine *in vivo*.

5. Reflex bradycardia in response to elevated blood pressure due to injection of noradrenaline or phenylephrine was seen only occasionally.

INTRODUCTION

In turtles and a lizard (*Uromastyx aegyptica*) sympathetic fibres join the vagus nerves and run with these to the heart (Gaskell & Gadow, 1884; Mills, 1885; Khalil & Malek, 1952) thus forming a vago-sympathetic trunk of the type also found in amphibians (Burnstock, 1969). The level of fusion between the vagi and sympathetic fibres varies from one species to the other. In the cervical part of the vagi no sympathetic fibres are present in the lizard (*Tiliqua rugosa*) or in crocodiles and fibres from the sympathetic ganglia run separately to the heart, or join the vagi close to the heart (Gaskell, 1884; Gaskell & Gadow, 1884; Berger, 1971).

By fluorescent histochemistry adrenergic fibres in the sinus venosus, auricles and ventricle have been demonstrated in turtles and lizards (Furness & Moore, 1970; Yamauchi & Chiba, 1973; Govyrin & Leontieva, 1965).

Like in mammals, the sympathetic fibres exert positive chrono- and inotropic effects on the reptilian heart, while parasympathetic cholinergic fibres of the vagi are inhibitory (Gaskell, 1884; Mills, 1885; Khalil & Malek, 1952; de la Lande *et al.*, 1962; Berger, 1971). Catecholamines increase the heart rate and blood pressure in lizards and a snake *in vivo* (Reite, 1970; Kirby & Burnstock, 1969), while acetylcholine has depressor effects and slows or stops the heart.

This study was made to give some basic information on the heart innervation and drug effects on the circulatory system in a representative from a group of reptiles which is less well studied in this respect, the snakes (*Ophidia*).

MATERIAL AND METHODS

Puff adder (*Bitis arietans*) of either sex with a body weight of 200–450 g were used in the experiments.

Experiments with the perfused heart in situ

The animals were anesthetized either with ether in a closed box for 45–120 min, depending on animal weight, or by intramuscular injection of fenemal (0·2 g/kg body wt). The depth of anesthesia was checked by touching the tongue or the tip of the tail, which are both normally rapidly withdrawn. Lack of this reflex was taken to indicate an anesthesia deep enough for surgery.

The animal was opened by a ventral incision and the heart exposed. Vena cava posterior was catheterized for the inflow of perfusion fluid at the level of the anterior part of the liver, and the left aortic arch was catheterized for the outflow of perfusion fluid at the level of the ventricle (Fig. 1). All other vessels to and from the heart were ligated.

Perfusion was performed with a fluid of the following composition: NaCl 7·62, KCl 0·36, $CaCl_2$ $2H_2O$ 0·52, $NaHCO_3$ 2·0, $MgSO_4$ $7H_2O$ 0·43, Na_2HPO_4 0·43 and glucose 1·0 g/l. The fluid was bubbled throughout with a mixture of O_2 and CO_2 (97·3%). The perfusion apparatus was similar to that of Davies & Rankin (1973) as modified by Nilsson & Grove (1974). The venous (inflow) pressure was determined by the level of perfusion fluid, and the arterial (outflow) pressure was controlled by constricting the arterial catheter. Recording of the arterial pressure was made by a Statham P23 pressure transducer, and heart rate could then be continuously recorded from the pressure signal by a Grass 7P4 tachograph on a Grass mod 79 recorder. Drugs were added to the perfusion stream in single doses by a syringe and catheter.

The anatomical distribution of nerves was studied by methylene blue or osmic acid staining of the nerves. Staining by methylene blue was performed by placing the preparation in Ringer's solution with 0·01% methylene blue overnight, and osmic acid staining by dripping 1% OsO_4 solution on the preparation during dissection. The preparations were studied under low power microscope, and photographs and drawings were obtained.

For further studies on the nerve function the vagi were dissected free of adjacent tissue, and electrodes made of two hooked silver wires 2–3 mm apart were placed in any of four points (1–4 in Figs. 1, 2, 3 and 4). Presence of

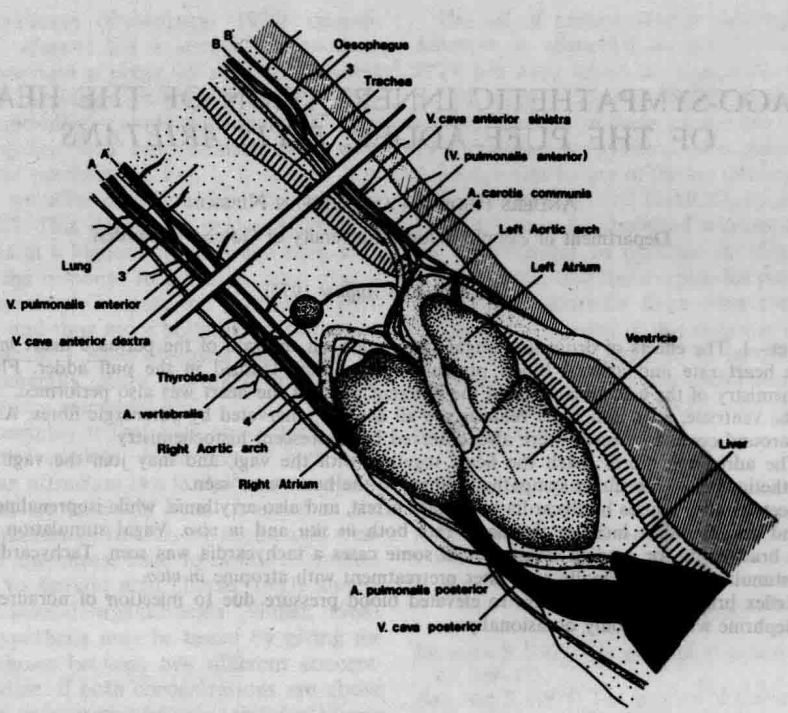

Fig. 1. Diagrammatic drawing of the lower neck region and heart region in the puff adder as seen from the ventral side. A and A', B and B' refer to branches of the vagi. Numbers (3 and 4) indicate application points for the electrodes during nerve stimulation.

antagonistic fibres within the vagi was shown by addition of atropine to the perfusion fluid.

Recordings of heart rate and blood pressure in vivo

The animals were anesthetized with mebumal sodium (50 mg/kg body wt) intramuscularly. A tracheal cannula was inserted to allow recording of the respiratory rate, and to make artificial respiration possible if necessary. Recording of blood pressure was made in the right aortic arch by a Statham P23 pressure transducer, and heart rate was continuously recorded from the blood pressure signal by a GRASS 7P4 tachograph. *Vena intestinalis* was catherized for the injection of drugs.

Fluorescent histochemistry

The heart was dissected out and the sinus venosus, parts of the atria and the ventricle were quick-frozen, freeze-dried and treated with formaldehyde vapour according to

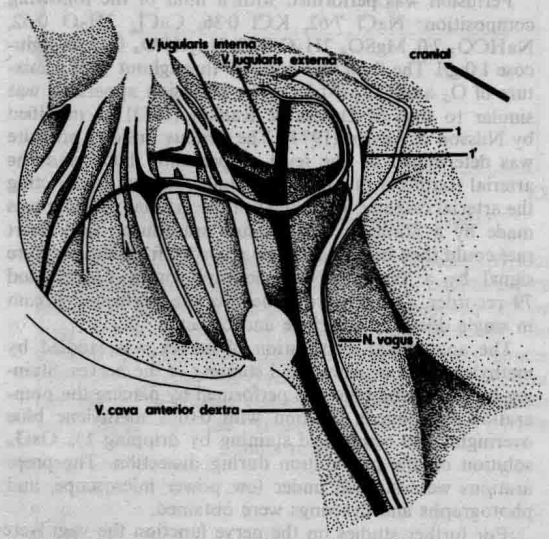

Fig. 2. Diagrammatic drawing of the neck region on the right side in the puff adder showing the exit of the right vagus from the skull. 1 and 1' indicate application points for the electrodes during nerve stimulation.

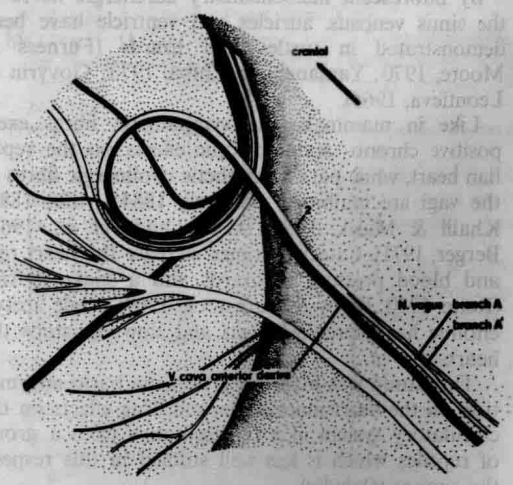

Fig. 3. Diagrammatic drawing of the upper neck region on the right side in the puff adder. The vagus nerve branches along its course forming two bundles, A and A'. 2 indicates application point for the electrodes during nerve stimulation.

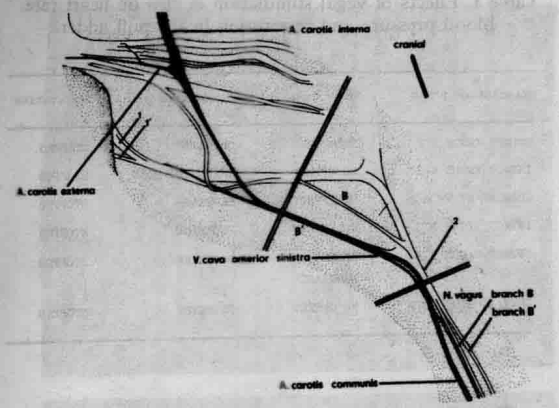

Fig. 4. Diagrammatic drawing of the upper and lower neck region on the left side in the puff adder. The branching of the left vagus nerve is shown. Numbers (1, 1' and 2) refer to application points for the electrodes during nerve stimulation.

the method of Falck & Owman (1965). Sections (10 μm) were mounted in Entellan (Merck) and viewed in a Leitz Ortholux microscope with top light illumination and a barrier filter at 460 nm. Photographs were taken by a Leitz Orthomat automatic camera on Kodak Tri-X film.

RESULTS

Drug effects on the perfused heart in situ

During perfusion with a venous pressure of 5–10 cm H_2O a heart rate of 46 ± 1 beats/min (mean ± S.E.M., $n = 17$) was recorded. Small amounts of isoprenaline, adrenaline and noradrenaline all caused dose-dependent tachycardia (Figs. 5 and 6). Acetylcholine $10^{-13} - 10^{-10}$ moles decreased the heart rate, but did also produce sustained arrythmia which made dose–response estimations impossible.

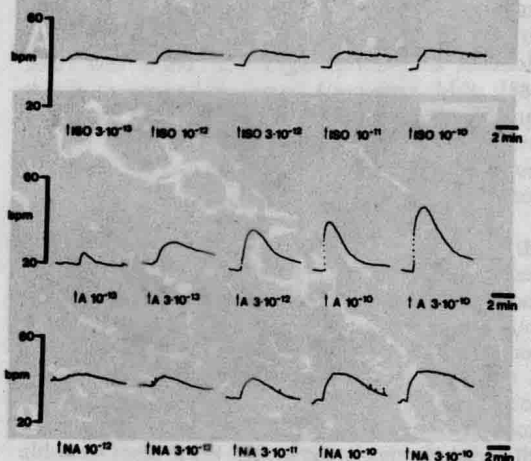

Fig. 5. Effects of increasing doses of isoprenaline (ISO), adrenaline (A) and noradrenaline (NA) on the heart rate in the *in situ* perfused heart of the puff adder. Doses are expressed in moles added to the inflowing perfusion fluid.

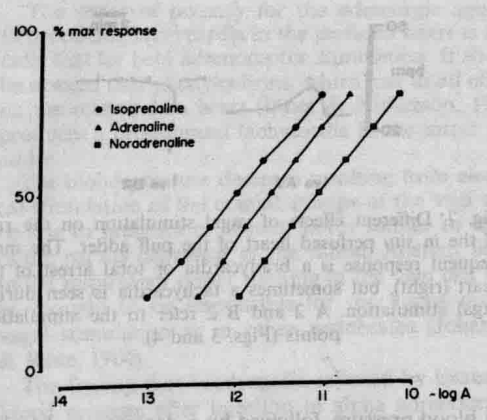

Fig. 6. Dose–response curves for the interval 20–80 per cent of the maximal response for isoprenaline, adrenaline and noradrenaline on the *in situ* perfused heart of the puff adder. The curves are mean values for 3–4 animals and show no differences in maximal effects (intrinsic activities) between the drugs. Single doses injected into the inflowing perfusion fluid are expressed in −log dose along the absciss.

Electrical stimulation of the vagi

The anatomical distribution of the autonomic nerves to the heart is summarized in Figs. 1, 2, 3 and 4. Both vagi leave the cranium at the level of the 4th ventral plate. On the right side, the nerve trunk runs along vena cava anterior, makes a loop and divides into two branches (A and A' in Fig. 3). These branches fuse again just anterior to the heart and run along the right vena cava anterior to sinus venosus. On the left side, the vagus follows the left vena cava anterior, crosses to arteria carotis communis and follows this to the heart branching and fusing along its course similar to the right vagus. The left vagus is divided a second time close to the heart sending one branch along the aorta to the stomach (Fig. 1 and 4).

It has not been possible to find any definite fusion point between the vagi and sympathetic fibres, neither was any direct sympathetic nerve to the heart seen.

Electrical stimulation with 20 Hz, 1 msec duration and 5–10 V for up to 1 min in any of the points 1–4 (Figs. 1, 2, 3 and 4) produced bradycardia or stopped the heart completely. In a few cases a significant tachycardia was seen instead, and after addition of atropine (10^{-5}M) to the perfusion fluid the result of vagal stimulation, if any, was always tachycardia (Fig. 7).

Recordings of heart rate and blood pressure in vivo

Mean values for the blood pressure and heart rate recorded *in vivo* in anesthetized animals were: heart rate 44 ± 2 beats/min, systolic blood pressure 33·5 ± 1·3 cm H_2O (24·6 ± 1·0 mmHg) and diastolic blood pressure 24·1 ± 1·6 cm H_2O (17·6 ± 1·1 mmHg) (Means ± S.E.M., $n = 16$). Injections of small doses of noradrenaline ($10^{-12} - 10^{-6}$ moles) or phenylephrine ($10^{-10} - 10^{-8}$ moles) produced tachycardia and elevated blood pressure (Fig. 8). Isoprenaline ($10^{-12} - 10^{-8}$ moles) caused a transient increase

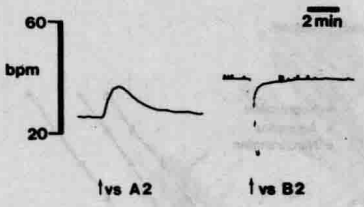

Fig. 7. Different effects of vagal stimulation on the rate of the *in situ* perfused heart of the puff adder. The most frequent response is a bradycardia or total arrest of the heart (right), but sometimes a tachycardia is seen during vagal stimulation. A 2 and B 2 refer to the stimulation points (Figs. 3 and 4).

Table 1. Effects of vagal stimulation *in vivo* on heart rate, blood pressure and respiration in the puff adder

Stimulation point	Heart rate	Blood pressure	Respiration
INTACT VAGUS A'2	DECREASE	DECREASE	STOPPED
INTACT VAGUS A 2	NO EFFECT	SMALL DECREASE	STOPPED
CRANIAL STUMP A 2	NO EFFECT	DECREASE	STOPPED
INTACT VAGUS B'2	NO EFFECT	DECREASE	STOPPED
INTACT VAGUS B 2	INCREASE OR DECREASE	DECREASE	STOPPED
CRANIAL STUMP B'2	NO EFFECT	DECREASE	STOPPED

in blood pressure, followed by a decrease, and tachycardia. Propranolol in high doses (up to 60 mg/kg body wt) did sometimes not completely block the chronotropic effects of the highest doses of the amines, although a decrease in the response could be seen. A sustained decrease in heart rate from 44 to 21 beats/min (average from six animals) was seen after injection of propranolol (10 mg/kg body wt).

The main effect of acetylcholine ($10^{-10} - 10^{-4}$ moles) was to produce cardiac arrythmia, and no dose-dependent chronotropic effects of this drug could therefore be recorded. Injection of atropine (up to 2 mg/kg body wt) caused an increase in heart rate from 44 to 56 beats/min (average of six animals).

The effects of nerve stimulation on heart rate, blood pressure and respiration are summarized in Fig. 8 and Table 1.

Reflex bradycardia caused by elevated blood pressure due to injections of noradrenaline or phenylephrine in presence of propranolol, was seen as a small decrease in heart rate in two cases out of six experiments.

Fluorescent histochemistry

A dense innervation by fluorescent fibres was seen in all parts of the heart (Figs. 9 and 10). Fluorescent fibres surrounding coronary arteries were also seen in the ventricle and atria (Fig. 9B).

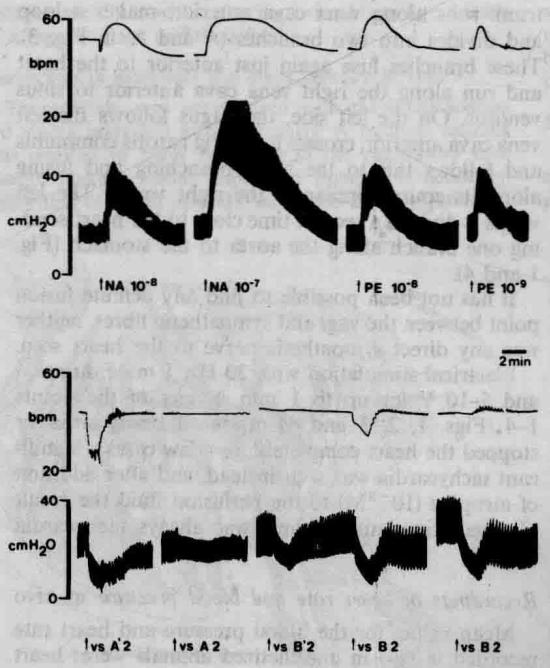

Fig. 8. Upper panel: Effects of noradrenaline (NA) and phenylephrine (PE) on the heart rate and arterial blood pressure in the puff adder. Doses are expressed in moles injected into the intestinal vein. Lower panel: Effects of electrical stimulation of the vagi on the heart rate and arterial blood pressure. Stimulation was carried out with 8V, 20 Hz and 1 msec duration for 1 min in each case. Numbers refer to the stimulation points (Figs. 3 and 4).

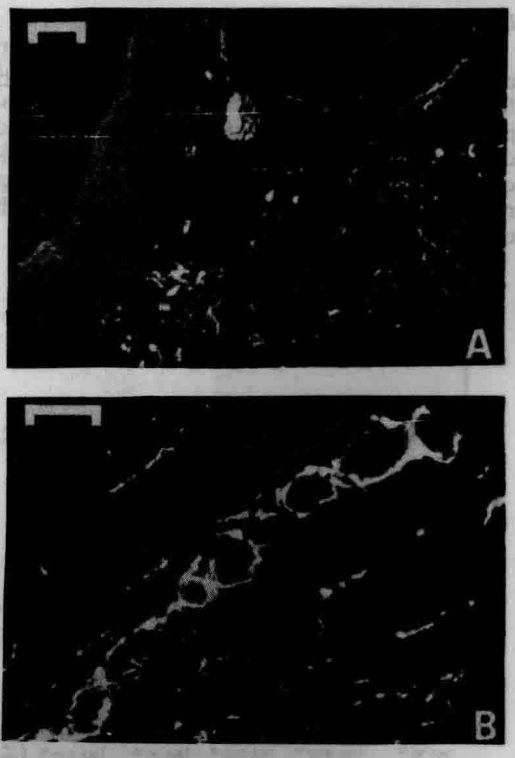

Fig. 9A. Specifically fluorescent fibres in the wall of the right atrium. Calibration = 100 μm. B. Specifically fluorescent fibres innervating the coronary arteries in the atrial wall. Calibration = 50 μm.

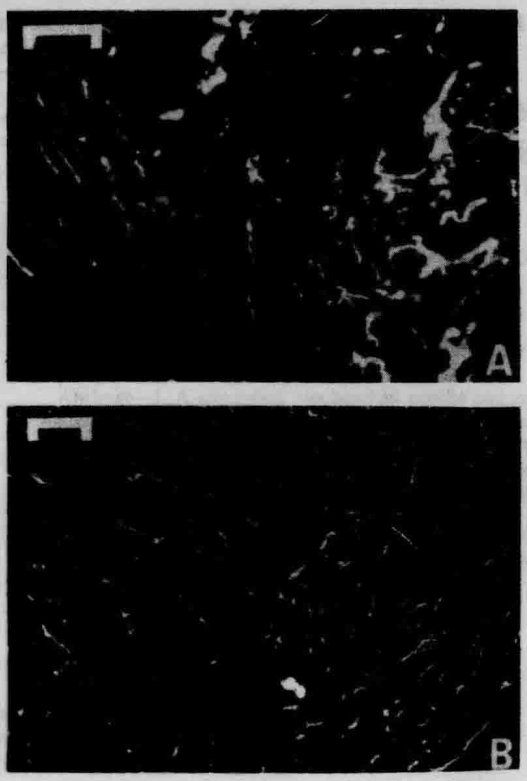

Fig. 10A. Specifically fluorescent fibres in the sinus venosus. Calibration = 100 μm. B. Dense innervation of specifically fluorescent fibres in the wall of the ventricle. Calibration = 50 μm.

DISCUSSION

The fluorescent histochemical study of the sinus venosus, the auricles and the ventricle shows a massive innervation by adrenergic fluorescent fibres in these parts. If these fibres are of sympathetic origin as concluded in other reptiles, they must enter the vagi and run with these as a vagosympathetic trunk to the heart since no direct sympathetic nerve to the heart was found. The organization thus resembles that in turtles and the lizard, *Uromastyx* (Mills, 1885; Malek & Khalil, 1952; Burnstock, 1969), although distinct point of fusion between sympathetic fibres and the vagi has not been seen. Sympathetic fibres may enter the vagi at several points along their courses.

The adrenergic fibres of the vagi accelerate the heart, as can be seen during vagal stimulation when the cholinergic inhibitory response has been abolished by atropine. The adrenergic fibres to the heart and/or presence of circulating catecholamines in the blood produce an adrenergic tonus on the heart, which is shown by the decrease in heart rate after injection of propranolol or sotalol in the *in vivo* preparation, although these drugs seldom completely abolished the effects of injected amines even in very high concentrations. A similar, although smaller, cholinergic inhibitory tonus is shown by the increase in heart rate after injection of atropine *in vivo*.

The order of potency for the adrenergic agonists in producing tachycardia in the perfused heart is typically that for beta adrenoceptor stimulation. It should be noticed that phenylephrine, which has small effects on the mammalian heart (Innes & Nickerson, 1970), produces a pronounced tachycardia in the intact puff adder.

The blood pressure decrease resulting from electrical stimulation of the cranial stumps of the vagi may be due to presence of afferent fibres similar to those of the nervus depressor of the rabbit. Afferent fibres to the respiratory center running in the vagi can also be thought to be responsible for the apnea during vagal stimulation as in other vertebrates (Johansen & Reite, 1964).

The finding that bradycardia induced by increased blood pressure after injection of alpha adrenoceptor agonists was very small, if at all present, indicates that such vaso-motor reflexes are poorly developed in this species.

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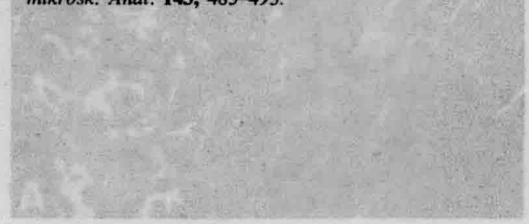

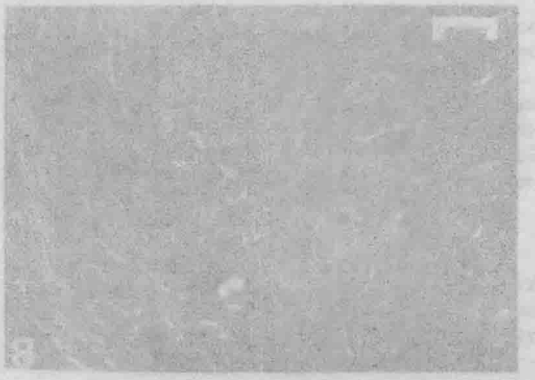

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DEVELOPMENT OF A BIOCHEMICAL PROFILE FOR MASS-REARED BOLL WEEVILS* (COLEOPTERA:CURCULIONIDAE)

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Abstract—1. A simplified biochemical profile consisting of whole body levels of cholesterol, lipid, uric acid, and soluble protein was obtained on mass-reared *Anthonomus grandis* Boheman of three adult ages for 8 successive weeks representing four generations.

2. All parameters showed significant variation with adult age, sex, and chemosterilant treatment.

3. Levels of cholesterol, uric acid, and soluble protein were significantly depressed in weevils treated with busulfan.

4. The feasibility of obtaining biochemical data on large numbers of insects as a means of quality control is verified, but the utility of this approach requires the long-term accumulation of such information prior to its application.

INTRODUCTION

The importance of a quality control program for mass rearing of insects for inundative releases was reviewed by Boller (1972). The objectives of such a program are to monitor those parameters of the reared insect on which its success in the field is dependent as well as those parameters which reflect its overall vigor and fecundity, and to perform this in a short period of time at a minimum cost.

As a part of the pilot boll weevil eradication experiment, one million boll weevils, *Anthonomus grandis* Boheman, were reared each week for an 8-week period, beginning July 1, 1972, sterilized, and released in a cotton growing area in south Mississippi. As an integral part of this experiment, the development of a quality control program was initiated. In addition to key behavioral, pathological, and nutritionally-related parameters being monitored, the present study was undertaken to obtain direct biochemical information pertaining to the physiological state of both the mass reared and chemosterilized weevils. The objectives were to determine the feasibility of obtaining a simplified biochemical profile on large numbers of insects each week, to establish baseline profiles for both normal and chemosterilized weevils, and to determine if significant variation in the profile parameters existed among successive generations.

MATERIALS AND METHODS

Adult boll weevils were obtained routinely from the Robert T. Gast Rearing Laboratory at the rate of 2400 weevils/week. These insects were sexed and divided into equal numbers and fed on either standard adult diet (Gast, 1966) or diet containing 0·1% busulfan (1–4, butanediol dimethane sulfonate) (Klassen & Earle, 1970). All insects were held in constant light at 27°C. Weevils of adult ages 5, 6 or 7 days were collected in replications of ten weevils each, weighed to the nearest mg, and homogenized in an all glass homogenizer for the following determinations. Weevils were homogenized with chloroform–methanol (2:1) to a final tissue concentration of 10 mg/ml. The homogenate was quantitatively transferred to Whatman No. 1 filter paper. The filtrate was collected and 4 ml used for duplicate determinations of cholesterol by the method of Caraway (1960). The remaining filtrate was used for the gravimetric determination of lipid. The tissue brei and filter paper were then mixed with 0·6% lithium carbonate to reconstitute to the original sample concentration of 10 mg/ml (Bhattacharya & Waldbauer, 1969). The tubes were stirred and placed in a boiling water bath for 30 min to insure solution of the uric acid. The mixture was filtered through glass wool and 5 ml of the filtrate was centrifuged for 3 min at 3500 rev/min. One ml of supernatant was adjusted to a final volume of 10 ml by the addition of glycine buffer (0·1 M, pH 9·4). Three ml aliquots were removed and 50 μl of uricase (Sigma 292 UV) were injected. After 30 min incubation at 25°C, absorbance was read at 292 nm on a Beckman Model DB-GT dual beam spectrophotometer.

Separate homogenates of 50 weevils each were centrifuged for 10 min at 4000 rev/min and the supernatant, at a concentration of 50 mg/ml was used for protein analysis by the method of Lowery *et al.* (1951).

RESULTS AND DISCUSSION

Table 1 shows a comparison of the biochemical profiles of males and females. The mean values represent data obtained on three successive days of adult age, and for 8 weeks representing four different generations of mass-reared weevils. All profile parameters except lipid were significantly lower in busulfan sterilized weevils.

The depression of protein in busulfan treated weevils is apparent for both sexes although there is more variation in females. Mitlin & Wiygul (1971) reported a depression of nucleic acid and protein synthesis in

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Table 1. Biochemical profile of mass-reared boll weevils fed normal and busulfan diet

Insect - Treatment[1]		Cholesterol[2]	Lipid	Protein	Uric acid
Male	N5	2.08[3]	73.74 d	52.25	2.11
Male	N6	1.90 a	78.74 c	52.90	1.71 ab
Male	N7	1.91 a	90.27	50.59	1.86 a
Male	B5	1.35	73.00 de	49.67 a	1.83 ab
Male	B6	1.43	75.32	47.32 bc	1.64 b
Male	B7	1.69	79.44 bc	45.76	1.16 d
Female	N5	1.80	71.76 e	54.03	1.69 ab
Female	N6	1.89 a	85.04 a	49.58 a	1.23 cd
Female	N7	1.94	80.62 b	46.76 cd	1.36 c
Female	B5	1.27	67.09	47.50 b	1.41 c
Female	B6	1.40	84.28 a	46.64 d	1.66 b
Female	B7	1.58	87.78	48.39	0.94

[1] N and B represent normal and busulfan-treated diets, 5, 6, and 7 are adult age in days.

[2] All values expressed as mg/g wet weight.

[3] Numbers in each column not followed by a common letter are significantly different at the 0·05 level by Duncan's New Multiple Range Test.

the boll weevil by busulfan treatment. [U-^{14}C]-Lysine incorporation into protein was reduced from 66·5 to 48·7% and [^{14}C]-thymidine incorporation into nucleic acid was reduced from 33·6 to 11·7%. The greater variability in the depression of protein in female weevils is to be expected since there is such a difference between males and females for both protein composition (Chen, 1958, 1963) and protein synthetic demands during reproductive phases (Chen, 1971). The general decline in protein content of males of ages 5–7 days agrees with earlier studies. Mitlin *et al.* (1968) showed a decline in free amino acids in the hemolymph of adult boll weevils from ages 0–5 days. A lower, but parallel trend exists for females. The decrease in labeled lysine and tryptophan with the concomitant increase in protein synthesis within 24 hr of injection indicates the high degree of protein synthesis in early adult ages of boll weevils (Mitlin *et al.*, 1968; Mitlin & Wiygul, 1969). This decline in soluble protein content of adult weevils agrees with the pattern in crickets (Nowasielski & Patton, 1965) and may in fact be the general trend among insects for a reduced turn-over rate with increased adult age (Chen, 1971).

Although there was a significant increase in lipid levels of weevils from 5 to 7 days adult age (Table 1), there was such a wide variation on lipid content from week to week that interpretation is difficult. Table 1 also indicates that busulfan treatment did not affect lipid levels consistently in either sex as was noted for protein. A depression in lipid content of 5-day weevils occurred at week 3, the same time as an increase in infection by several types of bacteria of the mass-reared colony (P. P. Sikorowski, 1973, personal communication). The effect of these pathogens on lipid levels of boll weevils is unknown although infection by the protozoan *Matesia grandis* McLaughlin is known to depress the lipid content of infected weevils (R. L. McLaughlin, 1973, personal communication).

The depression of cholesterol in both sexes parallels that of protein, and is quite consistent for all ages over the entire 8-week period. Cholesterol was chosen as a quality control parameter, since the adult boll weevil is unique in requiring a constant dietary supply of cholesterol for sustained egg production and normal longevity (Earle *et al.*, 1965; Vanderzant & Richardson, 1964). The cholesterol of adult weevils has a rapid turnover rate as shown by radiotracer studies of Earle *et al.* (1965) in contrast to the low turnover rates of house flies (Kaplanis *et al.*, 1960), and two species of cockroaches (Robbins *et al.*, 1961; Ishii *et al.*, 1963; Vroman *et al.*, 1964). Although an increase in lipids storage is accompanied by an increase in total sterols in adult weevils (Earle *et al.*, 1965; Lambremont *et al.*, 1964) no such correlation was apparent in a comparison of the lipid and cholesterol profiles over the 8 weeks. It appears that in addition to the previously reported depression of protein and DNA synthesis by busulfan, the decreased levels of cholesterol indicate that busulfan is affecting a wide variety of metabolic pathways in the weevil as suggested by Mitlin & Wiygul (1971).

Although the mean level of uric acid was statistically lower in busulfan fed weevils, there was extremely wide variation over the 8 weeks. Uric acid was chosen as a profile parameter to monitor nitrogen metabolism. In the boll weevil, Mitlin (1965) determined that uric acid is in a dynamic state in the tissues, and appears to be involved in amino acid synthesis. Mitlin & Wiygul (1972) reported that busulfan inhibited metabolism of pyrimidines and therefore DNA biosynthesis. Although there is a more consistent depression of uric acid in 7-day adults than at the other ages, by busulfan, we are unable to correlate any environmental or nutritional fluctuations with the numerous changes in uric acid content.

Since the profile parameters showed significant variation with sex, adult age, and chemosterilant treatment, data were separated by these factors for analysis of the differences among generations. Table 2 gives the mean values of 7-day-old males and females fed normal diet over the 8-week period. This represents four generations of mass-reared weevils. These data indicate that even though these values were obtained from large numbers of adults of constant age, diet, and laboratory rearing conditions, there are significant differences among nearly all weekly values for each parameter. Cholesterol levels were the same for males in weeks 1 and 7 and 4 and 7, but different for all other weeks. Cholesterol levels in females were the same in weeks 3 and 5, but different in all other weeks. Lipid, uric acid, and protein values likewise contain much heterogeneity, with no distinct pattern among weeks or generations. Furthermore, the data from 5-, and 6-day-old weevils showed even more hererogeneity than the data in Table 2. We conclude from these data that even though we used large samples of insects that were mass-reared under controlled laboratory conditions, there is such a large degree of variability within a generation that no meaningful interpretations of differences among generations are possible.

Thus, it was possible to obtain a simplified biochemical profile on large numbers of mass-reared insects. The data presented here were obtained by one professional entomologist and one technician during a 40-hour week. The information content of this profile was somewhat limited in spite of the vast amount

Table 2. Biochemical profile of four generations of mass-reared boll weevils

Week	Generation	Males C	Males L	Males UA	Males Pro.	Females C	Females L	Females UA	Females Pro.
1	I	2.44 b	95.15	1.55	40.50	1.27	88.25 a	1.08	40.97
2	II	1.45	92.80	1.76 b	50.60 ab	1.78	76.75 c	1.31 a	46.12 a
3		2.04	88.40 a	1.91 a	57.25	1.98 a	80.50 b	1.39 a	53.60
4	III	2.49 a	88.40 a	1.81 b	51.90	2.25	80.50 b	2.32	50.50
5		2.28	125.00	1.43	53.30	2.01 a	89.00 a	0.96	45.90 a
6		1.78	85.00	2.50	50.85 a	1.72	71.00	1.60	46.80
7	IV	2.47 ab	59.00	2.04	49.70 b	2.62	78.50 bc	0.85	43.60
8		1.71	88.40 a	1.86 ab	50.59 ab	1.87	80.50 b	1.36 a	46.60 a

C, L, UA, and Pro refer to cholesterol, lipid, uric acid, and protein, respectively. All values expressed as mg/g wet weight. Values not followed by a common letter are significantly different at the 0·05% level as determined by Duncan's New Multiple Range Test. Values obtained from 7-day-old adults fed normal diet.

of basic information that has been accumulated on this insect (Dunn, 1960; Mitlin & Mitlin, 1968). The tissue levels of cholesterol, uric acid, and protein did reflect chemosterilant treatment and possibly could be used as an indicator of sterility; however, these undoubtedly reflect secondary effects of the chemosterilant and are less desirable than the values of either metabolites or enzymes at the testicular tissue level. Even under the controlled laboratory conditions of mass-rearing, there are such a large number of factors that could be contributing to the observed variability that meaningful interpretation is not now possible. Accumulation of this kind of information over a considerable time period, together with stepwise modifications of the rearing environment, could result in clinically useful values that could reflect disease levels, diet nutrient fluctuations, and genetic changes resulting from inadvertent selection pressures in mass-rearing techniques. The variability in the quality of mass-reared insects is presently recognized, but we are as yet unable to quantitate it to the degree that will be required for future success inundative release programs.

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ROLE OF CYCLIC NUCLEOTIDES IN THE EFFECT OF TRANSMITTERS ON THE HEART OF *HELIX POMATIA* L.

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Abstract—Studying the membrane effect of the substances relating to the second messenger system on the *Helix* heart it was found:

1. At the beginning of their application cAMP, DBcAMP, theophylline and imidazole increase diastolic depolarization, the duration of the plateau phase and the frequency of the spontaneous action potentials. At the second period of their action, the amplitude of the action potentials decreased then the generator potential ceased.
2. The above substances depolarize or hyperpolarize the membrane only slightly, synchronously lowering membrane resistance.
3. The changes in the different phases of the action potentials are attributed to the decrease in K-permeability and increase in Ca-permeability. During the application of cyclic nucleotides the alterations in permeability were only secondary appearing as a consequence of phosphorylation of the membrane components.
4. After pretreatment with cyclic nucleotides or theophylline the 5HT and ACh failed to affect the membrane of *Helix* heart. The simultaneous application of 5HT with cyclic nucleotides decreased, while with theophylline increased the resistance of the surface membrane, but in the presence of ACh both the cyclic nucleotides and theophylline caused a decrease in membrane resistance.
5. The changes in membrane effects of the above transmitters can be explained by prolonged alteration of permeability caused by cyclic nucleotides. The effect of DA was not blocked after treatment of the heart with cyclic nucleotides or theophylline. On the contrary, the potentiation of DA effect and the decrease in membrane resistance was observed.

INTRODUCTION

An increasing amount of evidence emphasizes the participation of cyclic nucleotides in the effect of transmitters on vertebrates and invertebrates (Jost & Rickenberg, 1971). Our earlier experiments showed the positive inotrope effect of cyclic nucleotides as well as their modulatory influences on transmitter actions on molluscan and insect hearts (S.-Rózsa, 1968; S.-Rózsa, 1974). Meanwhile the presence of adenylate cyclase and its activation by 5HT and DA was shown in *Helix* and *Anodonta* hearts (Wollemann & S.-Rózsa, 1975). The participation of the cyclic nucleotides in transmitter processes was shown on other species of Molluscs (Higgins, 1974; Higgins & Greenberg, 1974). Nevertheless there is limited information about the membrane effect of cyclic nucleotides especially as regards their sites of actions. The present account was undertaken to study the membrane effect of cyclic nucleotides and their influences on the transmitter actions.

MATERIAL AND METHODS

The experiments were carried out on spontaneously beating or driven by electrical stimuli ventricle of *Helix pomatia* L. The experimental conditions were described elsewhere in detail (S.-Rózsa, 1974; Kiss & S.-Rózsa, 1975). The following substances were used: cAMP–adenosine-3′, 5′–cyclic phosphate (Calbiochem); DBcAMP–N^6, O^2 dibutyryl adenosine-3′, 5′–cyclic phosphate (Calbiochem); theophylline (Reanal); imidazole (Sandoz); 5HT–5-hydroxytryptamine creatinine sulphate (Reanal); Ach–acetylcholine chloride (Sigma); DA–dopamine hydrochloride (Sigma).

For the measurement of the membrane (MP) and action potentials (AP) glass microelectrodes filled with 3M KCl and having 5–15 MΩ resistance were used with conventional equipment. The recording of the response was made 5–10 min after the application of substances with the exception of transmitters, when the response was registered 1 min after their application.

RESULTS

1. *The membrane effects of cAMP, DBcAMP, theophylline and imidazole*

The heart membrane of *Helix* was sensitive to the substances related to the second messenger system. No dose-dependency was found between the range of 10^{-6}–10^{3} M concentrations. cAMP, DBcAMP and theophylline were found to be effective only in high doses (10^{-3}–10^{-4}M) on *Helix* heart. The maximum effect of cyclic nucleotides was observed 5–10 min following their applications.

cAMP at the beginning of its action increased the frequency of the spontaneous action potentials, the diastolic depolarization and prolonged the phase of repolarization. In the second phase of its action cAMP decreased the amplitude of the action potentials, then the spontaneous beating of the heart cells ceased. The evoked action potentials were affected in a similar way by cAMP.