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DO THE MONOAMINES IN CRAB PERICARDIAL ORGANS PLAY A ROLE IN PEPTIDE NEUROSECRETION?

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INTRODUCTION

The experiments reported in this paper seek to examine the possible involvement of monoamines in the release, by electrical stimulation, of a cardio-excitor peptide from crab pericardial organs. The preceding paper (Cooke & Goldstone, 1970) describes systems of axons and terminals containing dopamine (DA) and 5-hydroxytryptamine (5-HT). These are intermingled with the neurosecretory axons and terminals in this neurohaemal structure. This intermingling of secretory and 'ordinary' nervous elements is a morphological feature of neurohaemal organs in many groups of animals. Monoamine-containing elements are present in the vertebrate neurohypophysis (e.g. Enemar & Falck, 1965; Odake, 1967; Björklund, 1968). Electronmicroscopical observations show synaptic-like structures between processes containing 'elementary neurosecretory granules' and other elements in the vertebrate median eminence (Oota & Kobayashi, 1963; Kobayashi, Hirano & Oota, 1965; Knowles & Vollrath, 1966) and in insect corpora cardiaca (Scharrer, 1963; Normann, 1965). These observations provide a morphological basis for suggesting that interactions between such elements may play a role in neurosecretory release. However, there is physiological evidence supporting the hypothesis that neurosecretory cells release their products when action potentials depolarize the neurosecretory terminal (for references see Cooke & Goldstone, 1970). There appear to have been few studies which directly correlate propagated electrical activity and neurosecretory release (e.g. Cooke, 1964; Kater, 1968; Dyball & Koizumi, 1969). Such studies all suffer from uncertainty about the role which non-neurosecretory elements known to be present might play.

The crab pericardial organ is a neurohaemal structure which can be removed to a chamber for electrical stimulation and recording. The bathing fluid, of normal or of altered composition, can be withdrawn and assayed on the isolated crab heart for cardio-excitor effects. The release of cardio-excitor has been correlated with the presence of a slowly conducted component of the compound action potential elicited by stimulation (Cooke, 1964). In the following paper evidence is presented that the cardio-excitor released by such stimulation is a peptide. Its release is not accompanied by detectable amounts of protein or enough monoamine to affect the heart assay (Berlind & Cooke, 1970*a*).

This study attempts to demonstrate in three ways a role of the monoamine-contain-

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ing axons and terminals in peptide neurosecretion. (1) The effect of DA and 5-HT on release of cardio-excitor hormone is examined; (2) the effect of a drug which blocks 5-HT receptors is tested; and (3) release from neurohaemal organs depleted of histochemically demonstrable monoamines is investigated.

The results allow the conclusion that the release of peptide neurosecretory material is controlled by the electrical activity of the neurosecretory cells. It is not detectably influenced by the dopamine-containing and 5-HT-containing systems of axons and terminals with which they are intermingled.

METHODS

Pericardial organs of the spider crab *Libinia emarginata* or *L. dubia* were prepared for electrical stimulation and recording of electrical activity by methods described previously (Cooke, 1964; Berlind & Cooke, 1968).

The fluid bathing the pericardial organs was drawn off at intervals and assayed for cardio-excitor content by perfusion through an isolated heart of the same species. The response of the heart to the peptide hormone is an increase in frequency and amplitude of beat.

All drug solutions applied to isolated preparations were made up in crab saline. Experiments with 5-HT and DA are complicated by the significant increases in frequency and amplitude of heart-beat caused by the substances. However, Cooke (1962, 1966) has shown that though both the hormone and 5-HT have their effect via the cardiac ganglion, their site of action is different.

A heart was made tachyphylactic by continuous perfusion with 10^{-7} M 5-HT (as creatinine sulphate, Regis Chemical Co.). At the start of perfusion with this concentration the frequency of heartbeat increases sharply, but after 15–20 min of perfusion the heart slows to approximately its original rate (20–30 beats/min). At this time it will no longer respond to a test dose of 10^{-6} M 5-HT, but exhibits a frequency increase in response to the peptide from the pericardial organ. The effect of three sets of test conditions on the pericardial organ were analysed: (1) the pericardial organ was bathed in 0·5 ml of crab saline for 15 min without stimulation; (2) the pericardial organ was bathed for 15 min in saline with 10^{-6} M 5-HT added; (3) the pericardial organ was stimulated for 8 out of 15 min in saline. At the end of each period each sample (0·5 ml, the entire volume bathing the preparation) was passed through the isolated heart for assay of cardio-excitor activity.

In experiments using 10^{-5} M dopamine (as HCl, Mann Research Labs., Inc.), assays were performed on hearts perfused continuously with the same concentration of that substance. Unlike the hearts perfused with 5-HT, hearts perfused with dopamine do not return to their original frequency. They continue beating for several hours at frequencies of 60 to 80 contractions per minute. Test doses of 10^{-5} M dopamine have no effect on these hearts. Pericardial organ extracts, or saline in which the pericardial organ has been stimulated electrically, cause a marked increase in amplitude of beat. The effect of four sets of test conditions on the pericardial organ were analysed: (1) the pericardial organ was bathed for 2 min in normal saline without stimulation; (2) the preparation was stimulated for 2 min in normal saline; (3) the preparation was bathed for 2 min without stimulation in saline with 10^{-5} M dopamine added; (4) the preparation was stimulated for 2 min in saline with 10^{-5} M dopamine added.

UML (1-methyl-D-lysergic acid butanolamide bimaleate, Sandoz Pharmaceuticals), a blocker of 5-HT action in various vertebrate and invertebrate preparations (Gyermek, 1966), was tested on the pericardial organ at a concentration of 10^{-5} M. This drug has no effect on the crab heart when a small dose is passed through the heart rapidly. The effects of four sets of test conditions on the pericardial organ were analysed. The preparation was tested for spontaneous (1) and electrically evoked (2) release in normal saline. After 30 min in UML the pericardial organ was tested for spontaneous release in UML (3) and for electrically evoked release in UML (4). All test periods were of 2 min duration.

Reserpine phosphate (CIBA) dissolved in distilled water (with the addition of a drop of glacial acetic acid to increase solubility) was injected into spider crabs according to the following procedure. A total of approximately 20 mg/kg body weight was injected in two doses at an interval of 24 h. A control animal was injected with two doses of the solvent. Both animals were kept in aerated sea water at 18 °C. Twenty-four hours after the second injection one pericardial organ from each animal was removed for physiological study. The other pericardial organ was freeze-dried and analysed by the fluorescence histochemical method (e.g. Falck & Owman, 1965) for the presence of catecholamines and 5-HT as described in Cooke & Goldstone (1970).

The heart used for bioassay of fluids bathing the pericardial organs of reserpinized and control-injected animals was taken from a third animal which was kept under the same conditions but was not injected.

RESULTS

Six experiments were performed to test the effect of 10^{-6} M 5-hydroxytryptamine on the release of heart-exciting peptide from an isolated pericardial organ. In each experiment no more hormone was released during a 15 min period in 5-HT than during the same length of time in normal saline. Immediately following exposure to the 5-HT, electrical stimulation of the neurohaemal structure in normal saline resulted in the release of cardio-excitor activity at a rate significantly greater than in periods without stimulation and at a rate not obviously different from those observed in preparations not previously treated with drugs. Bioassay results from a single experiment of this type are shown in Fig. 1, and the results of all six experiments are summarized in Fig. 2.

Six experiments were performed to determine the effect of 10^{-5} M dopamine on the release of hormone from isolated pericardial organs. In these six experiments spontaneous release in 10^{-5} M dopamine was not significantly different from spontaneous release in normal saline. Electrically elicited release in dopamine was not significantly different from that in normal saline ($P < 0.05$). These experiments are summarized in Fig. 3.

Three experiments were performed in which the pericardial organ was soaked for 30 minutes in 10^{-5} M UML. If 5-HT acts within the neurohaemal organ, treatment with UML would be expected to block its action and result in altered rates of release of hormone in response to stimulation. In each of the three experiments the same amount of hormone was released by stimulation in UML as was released before exposure to the drug (Fig. 4). UML does not affect spontaneous release of hormone.

Three animals were treated with reserpine to deplete the stored 5-hydroxytryptamine

and catecholamine of the pericardial organs. In the three pericardial organs from reserpinized animals hormone was released in response to a 1 min test period with stimulation at normal rates, and release without stimulation was undetectable or barely detectable (Fig. 5). In two of the experiments the amount of hormone released during a 1 min period of stimulation from the reserpinized pericardial organ was approximately the same as that released from the pericardial organ of the control-injected

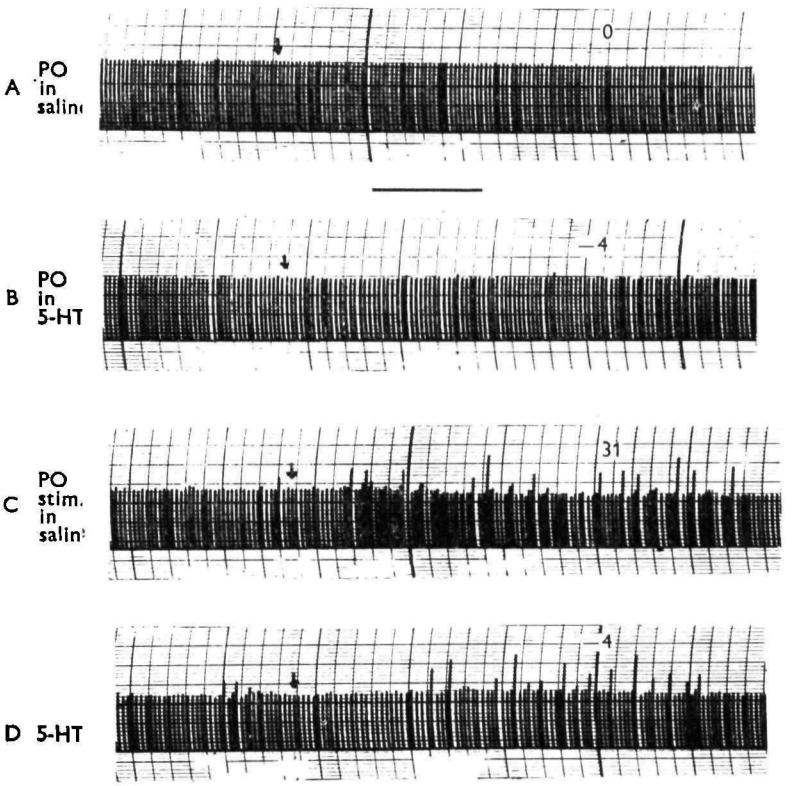

Fig. 1. Effects of 5-HT on release of cardio-excitor from the pericardial organ. Assays of solutions bathing the pericardial organ (A, B, and C), and of 10^{-6} M 5-HT (D) were performed on a heart being perfused continuously with 10^{-7} M 5-HT. A, Pericardial organ bathed for 15 min in saline without stimulation. B, Pericardial organ bathed for 15 min in saline with 10^{-6} M 5-HT, but without electrical stimulation. C, Pericardial organ stimulated electrically for 8 out of 15 min in saline. Numbers at the right give the percentage increase in frequency for each assay. The arrow marks the switching of the perfusion system to the channel with the test dose. Time marker, 1 min.

animal stimulated for the same time. In the third experiment, such a comparison was not possible, since the control preparation released hormone at a high rate without being stimulated. (This was probably the result of injury during dissection.)

In each of the three experiments fluorescence microscopy of freeze-dried pericardial organs from the reserpinized animals indicated an almost complete loss of the bright terminal 'blebs' characteristic of nerve endings with a high content of catecholamines or 5-HT (Pl. 1, and Pl. 7 of Cooke & Goldstone, 1970).

In all of the experiments electrical activity was monitored from the dorsal trunk of the isolated pericardial organ with an extracellular electrode. Neither the frequency of

the occasional potentials recorded during periods without stimulation nor the amplitude and shape of the compound action potential recorded in response to electrical stimulation were affected in any consistent or noticeable way by any of the procedures used in these experiments.

DISCUSSION

These studies provide strong evidence against a participation of the monoamine-containing neurones in the acute control of peptide hormone release from crab pericardial organs. 5-HT (10^{-6} M) and DA (10^{-5} M) do not evoke release of cardio-

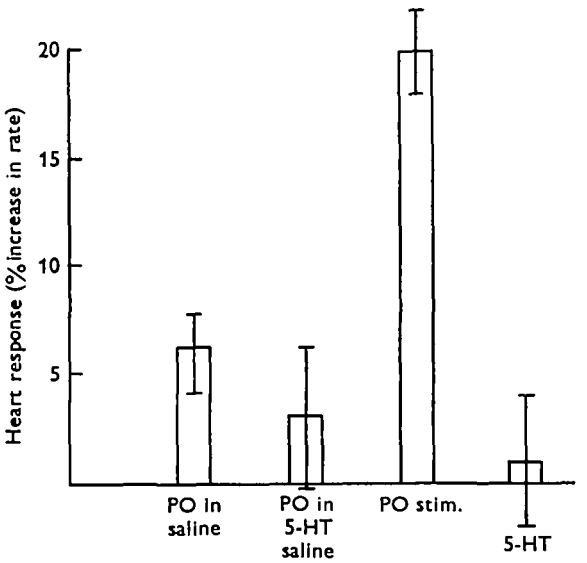

Fig. 2. Summary of 5-HT effects. Percentage increase in frequency of heart-beat in response to the following test solutions (left to right): pericardial organ (PO) bathed in saline for 15 min without stimulation; pericardial organ bathed in saline with 10^{-6} M 5-HT, without electrical stimulation; pericardial organ stimulated electrically for 8 out of 15 min in saline; 10^{-6} M 5-HT. Each bar is the mean of 6 experiments. Bathing the pericardial organ in saline with 5-HT added does not increase release of hormone.

excitor when directly applied to the isolated crab pericardial organ. DA, and probably 5-HT, do not alter the release of hormone in response to electrical stimulation. The effect of 5-HT on electrically elicited release was not examined, but an effect is almost certainly ruled out by the failure of UML (10^{-5} M) to alter release in response to stimulation.

The device of making hearts tachyphylactic could not be extended to higher concentrations of 5-HT and DA since hearts being perfused continuously with 5-HT concentrations above 10^{-7} M, or with DA concentrations greater than 10^{-5} M, become erratic and unsuitable for assay. However, if concentrations greater than 10^{-6} M 5-HT or 10^{-5} M DA did alter release of pericardial organ hormone the observation would be of doubtful physiological significance.

There seems to be no reason to doubt that 5-HT, DA or UML penetrate the neurilemma which separates the terminals of the neurohaemal organ from the surrounding fluid. We have never been able to detect any delay in the appearance of the peptide

hormone in the bath after stimulation; tetrodotoxin (MW 319) blocks conduction within seconds, and small ions have rapid effects on electrical activity and release (Berlind & Cooke, 1968, 1970*b*).

Pericardial organs depleted of histochemically demonstrable monoamines by reserpinization of the animals release cardio-excitor hormone in a manner indistinguishable from that of a control-injected animal. Experiments with reserpine cannot provide conclusive evidence as to whether monoamines inhibit release since the

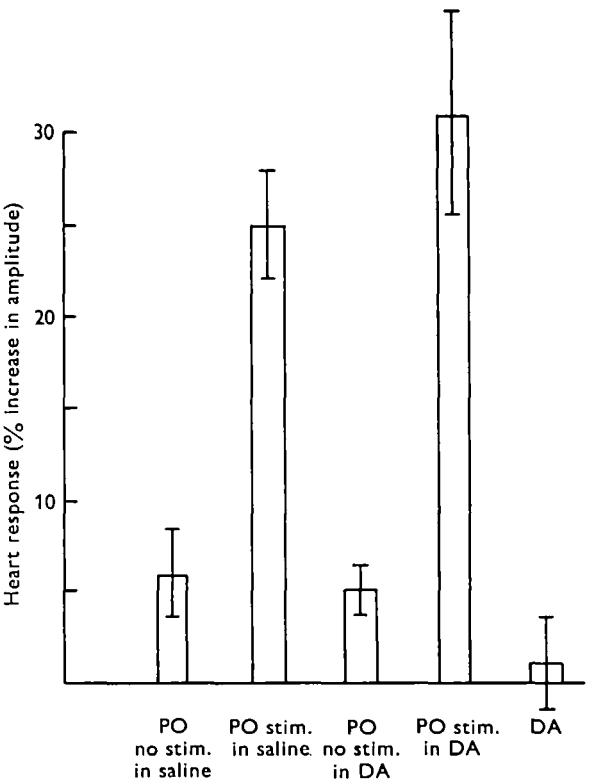

Fig. 3. Summary of dopamine effects. Percentage increase in amplitude in response to solutions bathing the pericardial organ (first four columns). Assays were performed on a heart being perfused with 10^{-5} M dopamine. The fifth column shows the effect of passing through the heart a test dose of 10^{-5} M dopamine. Each bar is the mean value of six experiments. Dopamine does not increase spontaneous release of hormone from the pericardial organ, and does not significantly alter release in response to stimulation.

experiments involve a comparison of pericardial organs from different animals. Any differences observed in these experiments between control-injected and reserpinized preparations were well within the limits of differences observed in pericardial organs from different normal animals.

Fluorescent histochemical studies of sympathetic ganglia have shown that only in terminals which have been depleted of more than 90 to 95% of their content of catecholamines can absence of fluorescent 'blebs' be expected (Fuxe & Sedvall, 1964; Andén *et al.* 1966). Drastic reduction in the efficiency of synaptic transmission following reserpinization has been demonstrated at various vertebrate peripheral adrenergic

junctions (e.g. Burnstock & Holman, 1962; Andén & Henning, 1966). Pericardial organs which have no specific fluorescence remaining can therefore be considered unlikely to have functional monoamine-containing terminals. Thus the normal release of hormone from pericardial organs of reserpinized crabs adds further evidence in support of the conclusion that the monoamine-containing axons and terminals in this

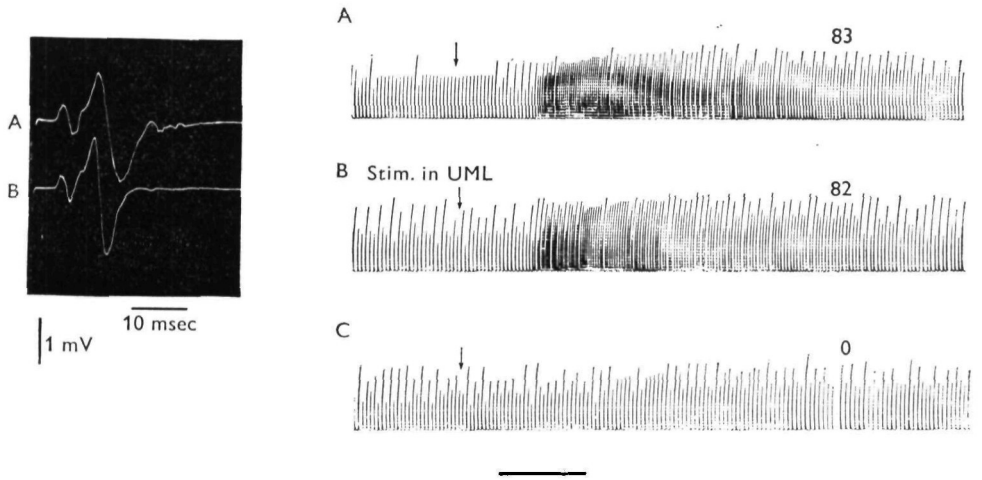

Fig. 4. Effects of UML on release of hormone from the pericardial organ. (A) and (B) Electrical activity recorded from the dorsal trunk of the pericardial organ before (A) and after (B) 30 min in UML, and corresponding heart assays (right) for released material. (C) Assay for spontaneous release in UML taken just *before* test B. Numbers at the right give percentage increase in frequency for each assay. UML does not effect release in response to stimulation or spontaneous release.

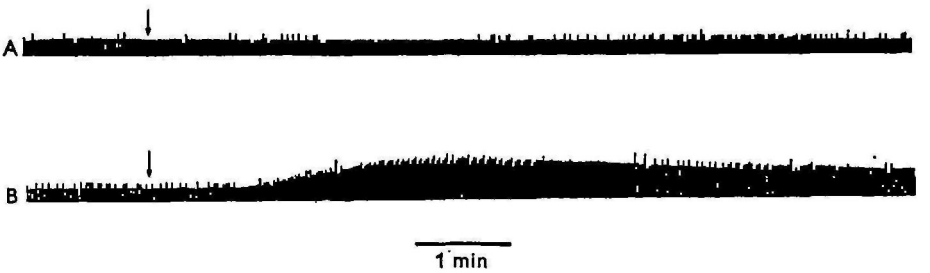

Fig. 5. Release of hormone from the pericardial organ of a reserpinized crab. (A) Assay of solution bathing the pericardial organ for 1 min without electrical stimulation. (B) Assay of fluid from the same pericardial organ as in (A), stimulated for 1 min in saline, 5 min after (A). Cardio-excitor release was indistinguishable from that of controls.

neurohaemal structure do not participate in the process of peptide hormone release Our experiments do not rule out the possibility of interactions between these elements over a relatively long time span.

These experiments remove most of the uncertainty which qualified the conclusion that propagated action potentials in the neurosecretory axons of crab pericardial organs control the release of the neurosecretory product (Cooke, 1964). Evidence is provided that a conspicuous population of non-neurosecretory elements does not

participate in neurosecretion. However, we cannot be certain that we have accounted for the activity of the entire non-neurosecretory population of axons.

It seems reasonable to suppose that the release of substances from the 5-HT-containing and DA-containing terminals is under independent central nervous control. The role of 5-HT and DA in the physiology of the animal is unknown.

The general hypothesis that neurosecretory hormone release results from depolarization of the terminals by action potentials conducted in the axons of the neurosecretory cells remains unchallenged. We know of no physiological evidence for direct activation of the terminals by other means. It will be of interest to learn to what extent the conclusions reached from observations on the pericardial organ can be generalized to other neurohaemal structures in which monoaminergic elements are present.

SUMMARY

1. Pericardial organs of *Libinia emarginata* and *L. dubia* were isolated, the nerves from the thoracic ganglion were stimulated electrically, and propagated electrical activity was recorded. Fluid which bathed the neurohaemal organ was assayed for cardio-excitor effects on the isolated crab heart, when necessary, made tachyphylactic to drugs.

2. Neither 10^{-6} M 5-hydroxytryptamine (5-HT), 10^{-5} M dopamine (DA), nor 10^{-5} M UML applied directly to the pericardial organ evoke release, nor do DA or UML alter release in response to stimulation.

3. The rate of cardio-excitor release is normal in pericardial organs taken from reserpinized animals. Histochemical examination of the pericardial organs by the formaldehyde-induced fluorescence method confirmed depletion of the monoamines.

4. These experiments, taken together, provide strong evidence against a role of monoamines in the release of cardio-excitor hormone from this neurosecretory structure.

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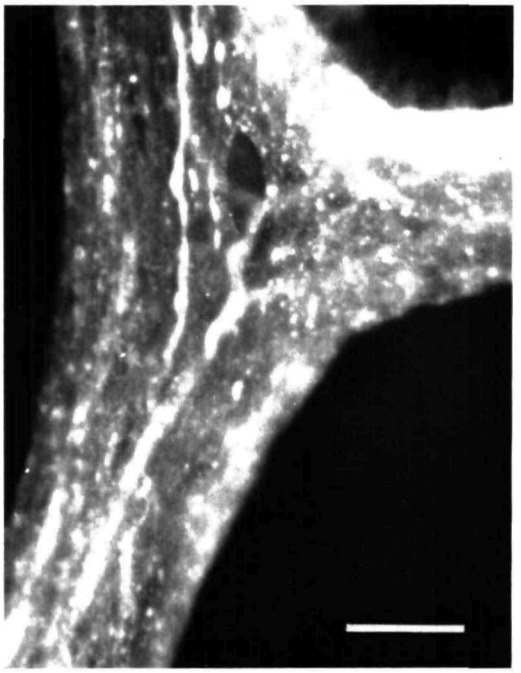

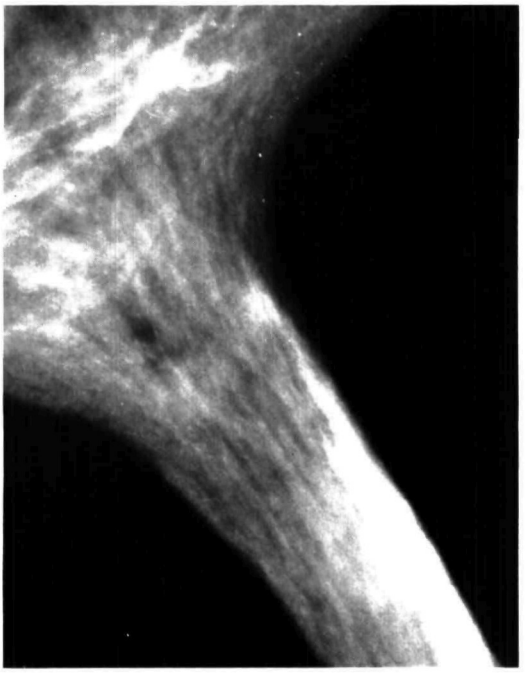

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EXPLANATION OF PLATE

Examination of pericardial organs by the fluorescence histochemical technique for monoamines. (A) Pericardial organ from a reserpinized animal used for the physiological records shown in Fig. 5. (B) Pericardial organ from a control-injected animal. The negative for (A) was exposed for twice as long as the negative for (B), because the overall intensity of fluorescence was much lower. Note the loss of fluorescent 'blebs' (which are indicative of a high content of monoamines), in photograph (B) as compared to photograph (A). Whole mounts, scale 100 μ.