The innervation of the frog's heart

I. An examination of the autonomic postganglionic nerve fibres and a comparison of autonomic and sensory ganglion cells

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[Plates 3 to 9]

The pattern of innervation in the frog's heart shown by silver staining has been compared with the results of histochemical methods. The muscle innervation is a series of dense networks of nerve bundles. These have been divided into four groups according to their size and distribution. The silver methods show fewer fibres than the histochemical methods but all the methods show the same distribution of fibres. The ganglion cells in the vagal, dorsal root and sympathetic ganglia, and in the vagosympathetic trunks and heart, have been examined with silver staining and histochemical methods. It has been concluded that the vagal ganglion cells are probably sensory; that the sympathetic ganglion cells are confined to the sympathetic chain; and that all the cells in the vagosympathetic trunks and heart are parasympathetic motor cells. Section of the vagosympathetic trunks causes loss of all fluorescent nerve fibres from the heart, while at the same time there are no changes in the distribution of acetylcholinesterase-containing fibres in the muscle. It is concluded that the fluorescence and acetylcholinesterase methods show respectively the sympathetic and parasympathetic postganglionic fibres. There is no evidence from this work to support the claims that some recently described and extensive plexuses of fibres on the muscle are in fact nervous in origin.

INTRODUCTION

Early work on the nerves in the frog's heart is fully reviewed by Jaques (1894) and Mollard (1908), and later work by Abrahám (1964) and Botar (1966). It is now generally accepted that the autonomic innervation of most organs in vertebrates consists of a terminal plexus of fibres of the type described by Hillarp (1946, 1959); however, Hirsch and his co-workers (see Cooper 1965) have described a much more extensive terminal innervation in the hearts of various species. They used a silver stain, and as these methods are notoriously liable to produce artefacts, the results of silver stains have been compared with histochemical methods, with which it was hoped to demonstrate specifically the sympathetic and parasympathetic nerve fibres.

The effects of cutting both vagosympathetic trunks on the nerve fibres in the frog's heart have been briefly reported (Woods 1967), and are to be more fully discussed in the following paper (Woods 1970). However, the locations of the

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sympathetic and parasympathetic ganglion cells, and the types of cells in the vagal ganglion are not clear from the anatomical descriptions (Gaupp 1899). Therefore the cells in the vagal ganglion have been compared with those in the vagosympathetic trunks and heart, the first cervical sympathetic ganglion, and the dorsal root ganglion of the second spinal nerve, in an attempt to identify the cells above and below the cuts through the vagosympathetic trunks.

**Methods**

**Animals**

Healthy specimens of *Rana temporaria* were kept in shallow running water between 15 and 20 °C for at least 1 week before use. All the animals were killed by pithing before the final removal of tissues.

For the operations to cut both vagosympathetic trunks, frogs were anaesthetized by immersion in 0.1 % M.S. 222 (tricaine methane sulphonate, Sandoz) for 5 to 10 min. The vagosympathetic trunks were located and cut about 3 mm below the vagal ganglion through an incision behind the ear and just in front of the blade of the scapula. When the animals were killed the success of the operation was verified.

Adrenaline or dopamine-hydrochloride in doses of 5 or 10 mg/kg were administered to some of the animals 1 h before they were killed. The drugs were dissolved in bicarbonate-buffered frog saline (Woods 1970) and injected into the peritoneal cavity. The adrenaline was brought into solution in a small quantity of 0.1 N hydrochloric acid before being diluted with saline.

**Tissue preparation**

The heart, vagosympathetic trunks and vagal ganglion, the dorsal root ganglion of the second spinal nerve, and the first cervical sympathetic ganglion were taken from the animals; without fixation for the histochemical methods, and after fixation in situ by perfusion or immersion, for the histological methods. Wherever possible whole mounts of the atria and atrial septum were used. For the silver methods, fixed tissue was mounted on gelatinized slides by the method described by Richardson (1960), and for histochemical methods fresh tissue was dried on to uncoated slides. However, sections of wax-embedded material, frozen sections of fixed material, and cryostat sections of fresh material had to be used to study the ventricle and ganglia.

**Histological methods**

The methods published by Richardson (1960) and Bodian (1936) were used without modification; batch N 4180 E of Winthrop Protargol was used in the latter. Schofield’s (1960) method was used with the following modifications: the tissues were fixed in Richardson’s (1960) fixative and the sections were pretreated in 5 % pyridine in 50 % alcohol overnight at room temperature, or for 1 h at 60 °C, before impregnation with silver nitrate. When supplies of Protargol that gave
good results were no longer available, an alternative method not using it, was
developed for staining wax sections. The method is based on the controlled reduc­
tion of silver by Peter’s (1955) p-hydroxyphenylglycine reducer, and it was carried
out as follows:

1) 5 to 10 μm Ester wax sections of tissue fixed in Bouin’s solution were
mounted on gelatinized slides and impregnated at 56 °C for 20 min to 1 h, in a
solution of 10 % silver nitrate and 2.5 % cupric nitrate.

2) After 5 min washing in distilled water the slides were reduced for 5 min
in a solution containing 5 g Johnson’s ‘Glycin’ (p-hydroxyphenylglycine), 10 g
anhydrous sodium sulphite, 5 g gelatine, 29.4 g sodium citrate, 3.5 g citric acid in
21 distilled water. To each 100 ml of this reducer 3 ml of 1 % silver nitrate were
added exactly 10 s before the slides were put into it.

3) After 5 min washing in distilled water the slides were toned for 5 min in
0.2 % yellow gold chloride, rinsed in distilled water, and intensified in 2 % oxalic
acid for 5 min. The slides were then fixed, washed, dehydrated and covered.

To provide a reference for the appearance of connective tissue fibres in the frog’s
heart, a series of 10 μm wax sections were stained with either Masson’s trichrome
stain, Gomori’s reticulin stain, or the orcein method for elastin fibres (Culling
1957).

Histochemical methods

(i) Catecholamines

Catecholamines were localized by the Falck & Hillarp fluorescence method
(Falck & Owman 1965; Hamberger 1967). Dried whole mounts and blocks of
freeze dried tissue were treated at 75 °C for 3 h with the gas from paraformalde­
hyde previously stored at a relative humidity of 50 %. 10 μm wax sections of the
blocks of tissue, and the whole mounts were mounted in liquid paraffin and
examined with a Leitz fluorescence microscope equipped with dark-field con­
denser, 3 to 5 mm BG 12 primary filter and a yellow barrier filter (Zeiss filter
no. 51).

(ii) Monoamine oxidase

Monoamine oxidase activity was demonstrated by the tetrazolium method of
Glenner, Burtner & Brown (1957). The incubations were carried out at a pH of
8.0 at room temperature. Fresh cryostat sections and whole mounts were used.
After incubation, and fixation in 10 % formalin, all the tissues were mounted on
slides and washed in 96 % alcohol, until all the red non-specific mono-formazan
had been washed away. Control slides that were incubated in the absence of sub­
strate, or in the presence of the inhibitor Marsilid (0.1 m) showed no reaction.

(iii) Esterases

Carboxylesterases were demonstrated with a naphthol AS-D acetate method
(Burstone 1962). The substrate was synthesized by acetylation of 3-hydroxy-2-
naphtho-O-toluidide (G. T. Gurr Ltd.). Fast Blue BR was used as the coupling
salt. The sections were mounted in aqueous mountant. As neither skeletal muscle motor end plates, nor vascular smooth muscle in the frog stained with this method, it does not demonstrate cholinesterase or acetylcholinesterase in the frog.

Cholinesterase and acetylcholinesterase were demonstrated by the Karnovsky & Roots (1964) method. All incubations were carried out at a pH of 6.5 and at room temperature. Butyrylthiocholine iodide was used as the substrate to show cholinesterase, which was completely inhibited by $10^{-4} \text{M}$ ethopropazine hydrochloride. Acetylthiocholine iodide was used to show acetylcholinesterase in the presence of $10^{-4} \text{M}$ ethopropazine hydrochloride, which did not inhibit it.

All three groups of esterases were also demonstrated successively on the same sections. It was found that replacing the copper sulphate in the Karnovsky & Roots medium with the same concentration of cobalt sulphate resulted in a green precipitate in the tissue instead of the brown one. A normal incubation to show cholinesterase followed by incubation in the cobalt medium to show acetylcholinesterase, was sometimes followed by incubation to show carboxylesterases. In the cobalt medium the substrate concentration was lowered to a quarter of normal. Ganglion cells containing various combinations of the esterases could be distinguished by their colours.

Photography

Photomicrographs were taken on 35 mm film using a Leitz Ortholux microscope. Agfa IFF film was used for normal subjects, Recordak Microfile film for low-contrast subjects, and Gevaert Scopix G High Definition film for fluorescent preparations.

Results

(a) The nerve supply to the heart

The ganglia in the neck region of the frog are shown in the diagram (figure 1). On leaving the vagal ganglion the vagosympathetic trunks separate from the other nerves and pass ventrally and caudally into the peritoneal cavity. A main cardiac branch runs medially to enter the heart at the sino-atrial junction, where an incomplete decussation takes place. The two nerves from this run directly on to the atrial septum and down it to the Bidder's ganglia against the atrioventricular valves, behind which they pass to enter the ventricle.

The nerve supplies to the different chambers of the heart are not discrete but are derived from a dense network of bundles running on the muscle of all the chambers. The sinus venosus is innervated by branches from the network on the precaval veins; the atria are largely supplied by branches of the septal nerves but also receive bundles of nerves from the outside of the sinus venosus; whilst the ventricle is supplied by the septal nerves and by bundles of nerves from the atrial wall also passing behind the atrioventricular valves. The conus arteriosus receives nerve fibres from the bundles lying in the loose epicardial connective tissue on the surface of the atria, and also from bundles in the ventricle that pass up into it in the connective tissue of the spiral valve.
Nerve fibres in the frog’s heart

(b) The innervation of the cardiac muscle, a nerve bundle classification

To facilitate the comparison of the nerve fibres shown by the different methods the nerve bundles in the heart have been divided into four artificial categories on the basis of their appearance with silver stains (figure 2).

Group I. The septal nerves and other large bundles of fibres giving rise to similar bundles only, and not connected with the plexuses on the muscle trabeculae. These bundles contain un-myelinated axons up to 2 μm in diameter and some myelinated fibres up to 5 μm in diameter. These latter fibres are never present in the ventricle.

Group II. Branches from group I bundles running in the connective tissue over and between the muscle trabeculae. The fibres in these bundles are mainly less than 1 μm in diameter, and are often displaced by the prominent Schwann cell nuclei. Some of the fibres have neurofibrillar rings or expansions along their course.

Group III. The fine plexus of nerve bundles running on the surface of the muscle trabeculae and in contact with them. These bundles contain a number of axons, but it is often difficult to distinguish them with silver stains, most have an irregular outline with expansions.
**Group IV.** The extremely fine bundles containing only a few or apparently single fibres, running on and within the muscle trabeculae. The swellings on these fibres are smaller and more regularly spaced than those on the group III fibres. With silver stains the group III and IV bundles are difficult to differentiate (figure 3, plate 3).

**Figure 2.** A diagram of the innervation of part of the atrial wall showing the nerve bundle classification. I, II, III, and IV are the bundle groups on the muscle.

**The fluorescence method**

The nerve bundles in the frog's heart show the typical green fluorescence indicating the presence of a catecholamine. The colour does not change along the fibres, but the intensity increases steadily from the dim cardiac branches of the vagosympathetic trunks to reach a peak usually in the group III bundles. The group IV fibres are less bright than the group III ones and are sometimes only visible as lines of varicosities. Fibres in all bundles except those in group I show regions of brighter fluorescence which become clearly defined as varicosities in group IV (figures 4 and 5, plates 3 and 4). The intensity of overall fluorescence varies between different animals, though they are prepared at the same time, and hence the number of fibres that is visible varies.

**The acetylcholinesterase method**

There is no cholinesterase activity in the heart. The nerve fibres contain only acetylcholinesterase. The depth of staining along the fibres is even, and appears similar in large and small bundles. The pattern of nerve fibres stained is the same.
Figure 3. A stretched preparation of the atrial septum showing many fine nerve fibres in groups III and IV amongst the muscle cells. (Bodian’s method, × 1000.)

Figure 4. The fluorescence of the nerve fibres in a stretched preparation of the atrial septum. The group IV fibres are just visible as lines of varicosities, and all the fibres have a varicose appearance including those in the group II bundle on the right. (× 245.)

(Facing p. 18)
Figure 5. The fluorescence of nerve bundles over a trabecula of muscle in a stretched preparation of the atrial septum. The thickest bundles running independently of the muscle are in group II; the smaller and brighter bundles on the muscle are in group III; and the finest varicose fibres, of which only a few are visible between these, are in group IV. (×245.)

Figure 6. A stretched preparation of the atrial septum incubated to show acetylcholinesterase. Bundles of fibres that are characteristic of groups II, III and IV run on and between the muscle trabeculae, which are just visible, though unstained. (×245.)
FIGURES 7 TO 9. 10 μm sections of the sympathetic, dorsal root and vagal ganglia respectively. Melanophores are present amongst the cells of the sympathetic and dorsal root ganglia. (The modified silver method, × 245.)
Figures 10 to 12. The fluorescence in 10 μm sections of the sympathetic ganglion, Bidder's ganglion and a group of chromaffin cells in the abdominal sympathetic chain, respectively. The swellings on the nerve fibres in Bidder's ganglion do not resemble the ganglion or chromaffin cells. (x 245.)
Figures 13 to 20. Ganglion cells in the heart, sympathetic ganglion, dorsal root ganglion and vagal ganglion, in each case; stained to show acetylcholinesterase (13 to 16) and carboxylesterase (17 to 20). (x 160.)
FIGURE 21. A ganglion cell and its spiral fibre stained to show acetylcholinesterase. A stretched preparation of the atrial septum. (x 1000.)

FIGURES 22, 23. 25 μm frozen section stained to show cholinesterase in the dorsal root and vagal ganglia respectively. (x 160.)
Figure 24. A fresh cryostat section incubated to show monoamine oxidase. The left part of the section is through a sympathetic ganglion, which has stained heavily; and the right part of the section is through a dorsal root ganglion that is almost unstained (×200.)

Figure 25. A fresh cryostat section of the heart incubated to show monoamine oxidase. Some of the nerve fibres and all the ganglion cells have stained. (×245.)
as that shown by both silver impregnation and the fluorescence method; The finest fibres form a continuous plexus amongst the muscle cells, supplied by a plexus of slightly larger bundles running mainly on the surface of the trabeculae (figure 6, plate 4). Varicosities are not conspicuous on the fibres, but can be found. The distribution of the fibres to the different chambers of the heart is the same as that of the fluorescent fibres. With both methods the density of innervation in the ventricle is less than that in the atria, which in turn have fewer fibres than the sinus venosus.

The effects of cutting both vagosympathetic trunks on the histochemical staining of the nerves in the heart

Five days after the vagosympathetic trunks have been cut there is almost no fluorescence of nerve fibres in the heart. Only a few fragmented fibres remain visible in the group I bundles and no finer bundles can be seen. No changes occur in the distribution of acetylcholinesterase within the nerve fibres to the cardiac muscle in this time, and the appearance of the nerve fibres is indistinguishable from that in normal hearts.

(c) Description and comparison of the ganglion cells

(i) Silver staining

The ganglion cells in the vagosympathetic trunks below the vagal ganglion, along the nerves in the heart, and in the first cervical sympathetic ganglion have the same appearance after silver staining. They are rounded unipolar cells 20 to 100 µm in diameter. They have no dendrites and taper through a large axon hillock region into an axon that may be up to 15 µm in diameter where it leaves the cell. A fine fibre or fibres approach each cell forming a spiral round the axon hillock. The number of turns in the spiral varies from 2 to 20 or more before the fibres pass onto the cell body and ramify over its surface. There is sometimes an appearance of boutons on the cell body near the axon hillock (figure 7, plate 5). Ganglion cells are absent from the ventricle.

The ganglion cells in the vagal ganglion and dorsal root ganglion are similar after silver staining. The cells are grouped at the peripheries of the ganglia. They are rounded and unipolar. They lack spiral fibres, endings and dendrites. Their axons curve towards the main fibre tracts in the centres of the ganglia where some of them divide symmetrically at T-shaped junctions.

(ii) The fluorescence method

Of the various ganglion cells only those in the first cervical sympathetic ganglion have a fluorescence typical of catecholamine containing cells, and only they take up adrenaline or dopamine injected into the animals before death. The axons from these cells can be traced into the vagal ganglion where they merge with the other fibre tracts.
Other fluorescent structures are found in the heart and sympathetic ganglia, which are apparently not ganglion cells. Groups of intensely fluorescent extra-adrenal chromaffin cells lie amongst the sympathetic ganglion cells; they appear to contain noradrenaline rather than adrenaline because their fluorescence develops after minimal treatment with formaldehyde. In Bidder's ganglia just above the atrioventricular valves there are irregular fluorescent objects 2 to 50 μm in diameter, amongst the non-fluorescent ganglion cells, and nerve fibres. They do not have nuclei and appear to be dilatations of the nerve fibres passing behind the valves. They disappear after the vagosympathetic trunks have been cut (figures 11 and 12, plate 6).

(iii) Staining for acetylcholinesterase

All the ganglion cells in the vagosympathetic trunks below the vagal ganglion, along the nerves in the heart, and in the first cervical sympathetic ganglion contain acetylcholinesterase. There is some variation in staining intensity of the cells in the sympathetic ganglion but not amongst the other cells. All the cells have a deeply stained spiral preganglionic fibre, but only the cells in the vagosympathetic trunks and heart have a strongly stained postganglionic fibre. The staining of the axons from the cells in the sympathetic ganglion is lost before they enter the vagal ganglion (figures 13 and 14, plate 7).

In the dorsal root and vagal ganglia many of the cells contain acetylcholinesterase, but there are wide variations in content and some of the cells remain uncoloured after prolonged incubation. There is no acetylcholinesterase in any of the glial cells or nerve fibres amongst the cells of these ganglia (figures 15 and 16, plate 7).

(iv) Staining for cholinesterase

There is no cholinesterase in any ganglion cells or nerve fibres in the heart, vagosympathetic trunks or cervical sympathetic ganglion. However, up to a quarter of the cells in the dorsal root and vagal ganglia contain cholinesterase; most of them also contain acetylcholinesterase as shown by the modified cobalt method carried out on the same sections. Cholinesterase is confined to the cytoplasm of the cells and never extends into their axons (figures 22 and 23, plate 8).

(v) Staining for carboxylesterase

Approximately half of the ganglion cells in all the ganglia contain carboxylesterase, but the activity in the cells varies widely. In the ganglia the staining is confined to the cytoplasm of the cells, but in the heart there is some staining of the nerve trunks and muscle (figures 17 to 20, plate 7).
Nerve fibres in the frog's heart

(vi) Staining for monoamine oxidase

All the ganglion cells in the vago-sympathetic trunks below the vagal ganglion, along the nerves in the heart, and in the first cervical sympathetic ganglion contain monoamine oxidase: myelinated fibres also stain in these situations. There is no staining of fibres or cells in the vagal or dorsal root ganglia (figures 24 and 25, plate 9).

(d) The differential distribution of the three esterases, and a summary of the properties of the ganglion cells

Approximately 2500 cells were examined after combinations of the esterase methods, and the results are shown in table 1. The distributions of the three enzymes overlap and many of the cells contain more than one, however each enzyme has a characteristic distribution. The results of the other methods are also summarized in the table. The sympathetic and parasympathetic motor cells have the same structure and all contain acetylcholinesterase and monoamine oxidase. The sensory cells in the dorsal root ganglion and the cells in the vagal ganglion do not have monoamine oxidase or spiral fibres, but do have similar structures and esterase contents.

Table 1. The percentage of cells in each ganglion that contain each combination of esterases, monoamine oxidase, catecholamines, or other distinguishing feature

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<th>dorsal</th>
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Discussion

The results of the tests on the ganglion cells show that the vagal ganglion cells are similar to those in the dorsal root ganglion, and are therefore probably sensory cells too. None of the cells in the vagal ganglion had characteristics of motor cells like those in the sympathetic ganglia or in the heart. It is concluded that parasympathetic ganglion cells are confined to the vago-sympathetic trunks and nerve
bundles in the heart, and sympathetic ganglion cells to the sympathetic chain. The only possible exceptions are the small fluorescent cells described in Bidder's ganglia by Falck, Häggendal & Owman (1963) and Falck (1964). However, the presence of cells in this position has not been confirmed. Massive dilatations of nerve fibres in the ganglia do occur, and they have a remarkable similarity to descriptions of nerves that have been partially constricted for a long time (Cajal 1928): it is possible that the nerves are mechanically constricted where they pass behind the valves and through the atroioventricular ring. Flourescent ganglion cells are only present in the sympathetic ganglia. The locations of the sympathetic and parasympathetic ganglion cells were further confirmed by the results of cutting the vagosympathetic trunks below the vagal ganglion.

A plexus of fine terminal fibres amongst the cardiac muscle cells, like that described by Hillarp in other tissues (1946, 1959), was shown by the silver stains and the histochemical methods. Fewer fibres were however impregnated by silver than were shown by either histochemical method. Silver probably stains mainly neurofibrils in the nerve fibres (Boycott, Gray & Guillery 1961), and these decrease in number in terminal fibres, whereas the histochemical methods rely on the presence of specific substances which would not be expected to diminish in the terminal fibres. Acetylcholinesterase is thought to be localized in or near to the membrane of parasympathetic axons (Robinson & Bell 1967), therefore though the fine fibres will be stained, any varicosities on them will not be especially conspicuous. On the other hand, adrenaline and other sympathetic transmitters are probably specifically concentrated in the varicosities, which explains their great prominence with the fluorescence method.

There were no differences in distribution or mode of termination of the sympathetic and parasympathetic fibres in the cardiac muscle of the frog, and the only differences in the innervation of the various regions of the heart were in the density of the terminal plexus. There was no evidence from any of the silver or histochemical preparations that the plexuses of fibres shown by Hirsch (Cooper 1965) were nervous in origin: the pattern of fibres illustrated was entirely different from that of the nerve fibres seen in this work but was, however, identical to the appearance of sections stained by Gomori's method for reticulin fibres.

Sympathetic neurons possibly contain acetylcholinesterase throughout the length of their axons, in addition to their cell bodies (Koelle 1965). Therefore the acetylcholinesterase method may be showing both the sympathetic and parasympathetic nerves in the heart. However, the axons from the frog’s cervical sympathetic ganglion no longer stain at the point where they join the vagal ganglion, and the disappearance of fluorescent fibres from the heart after section of the vagosympathetic trunks is not accompanied by changes in the distribution of acetylcholinesterase staining. It is concluded that the fluorescence and acetylcholinesterase methods separately demonstrate the sympathetic and parasympathetic postganglionic nerve fibres in the frog’s heart.
I would like to thank Dr M. Fillenz for instruction and guidance during this work, and Mr T. A. Marsland for tuition in histological techniques. I am also very grateful for the many discussions I have had with Sir Lindor Brown, F.R.S. and Dr A. G. H. Blakeley about this work. This work was carried out during the author’s tenure of a Medical Research Council Scholarship.

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