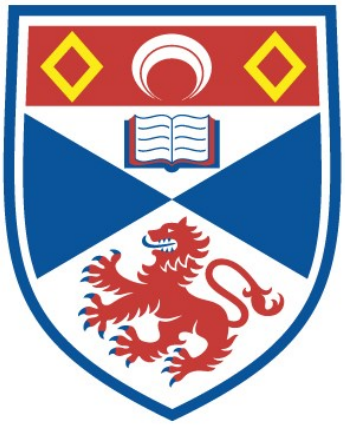
ULTRASTRUCTURAL, HISTOCHEMICAL AND PHYSIOLOGICAL STUDIES ON CARDIAC STRUCTURE AND FUNCTION IN THE TELEOST, PLEURONECTES PLATESSA L.

Robert M. Santer

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Ultrastructural, histochemical and physiological studies on cardiac structure and function in the teleost, Pleuronectes platessa L.

by

Robert M. Santer

A thesis presented for the degree of Doctor of Philosophy of the University of St. Andrews.

Gatty Marine Laboratory,
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ABSTRACT

Ultrastructural, histochemical and physiological studies on cardiac structure and function in the teleost Pleuronectes platessa L.

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A general study has been made on cardiac function in the plaice (*Pleuronectes platessa*) involving ultrastructural histochemical and physiological studies on the adult and developing fish.

Myocardial cells have an average diameter of 3.2μ. They lack a T-system and nexus contacts and have a very sparse sarcoplasmic reticulum. The sino-atrial region is heavily innervated but no pacemaker cells have been identified. Cells resembling Purkinje cells of higher vertebrate myocardia are seen in the plaice myocardium but they do not run in tracts. There is no coronary blood supply which is a consequence of the absence of an outer cortical layer of myocardium.

The development of the heart resembles that process as seen in the chick, but there are minor sequential differences. By Day 24 the "early larval" heart has formed which is a tri-laminar structure - a myocardial layer bounded externally by epicardium and internally by endocardium. This condition lasts until the 4a (Ryland) stage with the onset of endocardial invagination. This is the criterion distinguishing the "late larval" heart which persists until two months post-metamorphosis. Thus cardiogenesis is independent of hatching and metamorphosis.

The heart is innervated only parasympathetically through the cardiac branch of the vagus. The cardiac ganglion

situated at the sino-atrial region. The atrium is sparsely innervated and the ventricle is aneural. The absence of a sympathetic innervation is concluded from the following a) No specific catecholamine fluorescence is seen with the Falck technique. b) 6-hydroxydopamine does not degenerate any axons. c) Intraaxonal granular vesicles are not depleted by reserpine and AChE was localised around axons containing such vesicles. d) No adrenergic-type axons were seen by electron microscopy.

Differential vagal stimulation of 3Hz and 7Hz causes excitation and inhibition respectively, both effects being blocked by 10^{-6} g/ml atropine and are thus cholinergically mediated. On cessation of prolonged inhibitory stimulation there is marked increase in heartrate, and in quiescent hearts one or two beats are initiated after stimulation. Vagal stimulation causes a hyperpolarisation in atrial cells. It is proposed that all the excitatory effects of vagal stimulation are due to rebound excitation from an inhibitory hyperpolarisation. At high frequencies the hyperpolarisations summate to give total inhibition. At lower frequencies of stimulation the heartbeat is increased to rates dependent upon the time course of the hyperpolarisation and the refractory period of the heart. The rebound excitation persists in the presence of atropine and bretylium (both at 10^{-6} g/ml) and is therefore probably a response of the muscle cell membrane and is not nerve-mediated.

SUPERVISOR'S CERTIFICATE

I certify that Robert Baxter has fulfilled the conditions laid down under Ordinance General No 12 of the University Court of St. Andrews and is accordingly qualified to submit this thesis for the degree of Doctor of Philosophy.

DECLARATION

I declare that the work reported in this thesis is my own and has not previously been submitted for any other degree.

VITAE

I was educated at Marlborough College, Wiltshire, and the University of St. Andrews where I graduated in zoology in June 1970. The work described in this thesis was carried out between October 1970 and December 1972.

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase.
A-V	Atrio-ventricular
BA	Bulbus arteriosus
BuChE	Butyrylcholinesterase
5-HT	5-hydroxytryptamine
ID	Intercalated disc.
6-OHDA	6-hydroxydopamine
SR	Sarcoplasmic reticulum
S-A	Sinoatrial.
SV	sinus venosus

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I. INTRODUCTION

The heart of Osteichthyes together with that of cyclostomes is the most primitive vertebrate heart. It demonstrates few of the features associated with the hearts of higher vertebrates, and its functions are to circulate the blood and to supply the gills with venous blood. The development of a double circulation in the vertebrates is first noted in the Dipnoan heart where oxygenated and deoxygenated blood are separated from each other on entering the heart.

During ontogeny the fish heart develops in the ventral embryonic midline as a cylindrical tube. This tube subsequently differentiates into four chambers separated by constrictions: the sinus venosus (SV), atrium, ventricle and bulbus cordis. The sinus venosus is a thin-walled chamber which receives venous blood from the hepatic veins and the ducts of Cuvier. This chamber consists mainly of connective tissue but also contains a small amount of cardiac muscle. Only in the eel (Anguilla anguilla) is the SV actively contractile (Grodzinsky, 1954) assisting blood flow to the atrium. The atrium is a highly distensible muscular chamber in which venous blood collects. Contraction of the atrial trabeculae draws the roof of the atrium downwards in order to force blood into the ventricle. The ventricle is the main propulsive chamber of the heart and is much more massive than the atrium. The forceful contraction of this chamber drives blood through the bulbus cordis into the ventral aorta. The structure of the bulbus cordis varies greatly among fish. In cyclostomes it is present only as a slight post-ventricular swelling whilst in elasmobranchs it is a muscular, actively contractile chamber called the conus arteriosus. In teleosts it is not

contractile, but passively distensible and called the bulbus arteriosus.

The fish heart is a gill-heart, being situated almost immediately behind the gills. As a result, the ventral aorta is very short and all of the output of the heart passes directly to the gills. Therefore the heart pumps only venous blood. The pericardium, in which the heart is situated, varies greatly in its structural rigidity which is sometimes reinforced by association with adjacent skeletal muscles. The heart lies free within the pericardium, surrounded by lymph, but in some teleosts, as in eels, thin connective tissue strands unite the ventricle to the dorsal wall of the pericardium.

In 1849, Stannius studied the anatomy of the autonomic nervous system of a number of teleosts. He was the first worker to describe the cardiac branches of the vagus. Moreover, he stated that this was the only nerve to the heart and that the sympathetic nervous system provided no cardiac nerves. Thus the teleosts were considered to be unique amongst vertebrates in having only a vagal innervation of the heart. Besides ligature studies on the pacemakers of teleost hearts, Stannius described a small connection between the anterior sympathetic chain and the vagus nerve. It is not, however, a consistent feature of teleosts since Cole & Johnstone (1901) found no such ramus in the plaice and likewise Mandrick (1901) in Argyropelecus and Lampanyctus.

With the advent of wax histology as a routine laboratory technique in the latter decades of the nineteenth century (Klebs, 1869), the teleost heart did not escape the attentions of the morphologists of the time. However, virtually all the anatomical studies on the adult heart were undertaken with a

view to understanding the pacemaker system and the innervation of the heart. Very few morphological studies on the teleost heart were undertaken before the development of the electron microscope.

The use of methylene blue and osmic acid for staining nerves was a great advance in the study of the innervation of visceral organs. Kazem-Beck & Dogiel (1882) made a study on the innervation of the heart of the pike (Esox lucius) and the sturgeon (Acipenser ruthenus) using methylene blue staining followed by ammonium molybdate fixation and wax histology. They concluded that there were two possibilities for pathways by which nerves might reach the heart. First was the two cardiac branches of the vagus running to the sino-atrial junction and second was the coronary blood supply to the outer layer of ventricular myocardium. These blood vessels have a perivascular plexus of nerves which may ramify into the myocardium. Kazem-Beck and Dogiel also described the cardiac ganglion, the innervation of the atrium and of the atrio-ventricular (A-V) junction and showed these to have a vagal origin.

Before 1900, two very important facts were discovered regarding the effect of the vagus nerve upon the teleost heart. First, weak stimulation (usually mechanical) of the cardiac nerves caused the heart rate to slow, total inhibition occurring after strong stimulation. Second, these effects were blocked by atropine. These results were reported for a variety of teleost species by McWilliam (1885), Mills (1886), Chevral (1887) and Thesen (1896).

The discovery of inhibitory vagal control of the teleost heart was subsequently confirmed by many workers (Young, 1930; von Skramlik, 1935; Jullien & Ripplinger, 1957; Kulaev, 1957;

Laurent, 1962; Randall, 1966), but found to be restricted to the SV and atrium, the ventricle being insensitive to vagal stimulation and to applied acetylcholine (ACh). This is not limited to teleosts but also applies to the elasmobranch heart (Chevral, 1887; Lutz, 1930; Young, 1933). An exception to this rule occurs in lampreys (cyclostomata) where the action of the vagus is excitatory, as it is in skeletal muscle (see Fange, 1972, for review).

Subsequent investigations on teleost cardiac physiology followed two lines of approach until the present time. The first was to find the pacemaker regions and the second to investigate the mechanism of autonomic control of the heart.

Many anatomical studies on the hearts of higher vertebrates show the presence of well-defined pacemaker regions or nodal tissue at the S-A and A-V junctions. Keith and Flack (1907) and Keith and Mackenzie (1910) described the circular bands of muscle at these junctions in teleosts as consisting of cells with a larger diameter, having fewer myofibrils, less dense cytoplasm and bigger nuclei than ordinary myocardial cells. They concluded that this was definately nodal tissue and interpreted the pacemaker system accordingly. Laurent (1962) contested these proposals by saying that the annular muscles were not anatomically distinct from the rest of the myocardium and they probably acted to reinforce the valves. Also he pointed out that the special pacemaker cells were not confined to these bands of muscle but occurred in islets throughout the myocardium. Whilst agreeing that the dense innervation of the S-A and A-V regions was doubtless of physiological significance he concluded that the idea that this was true nodal tissue was not tenable.

von Skramlik (1935), using ligatures, nominated the ventral S-A junction and the A-V junction as pacemaker regions in all the teleosts that he studied except in the eel where such regions are more extensive including the whole of the SV and also the ventriculo-bulbar junction. However Wardle (1962) found that the only region showing pacemaker activity in the flounder (Platichthys flesus) was the median dorsal region of the S-A junction. This region is densely innervated, whilst no nerves were seen at the A-V junction. In a study of the electrocardiogram of the pike, Oets (1950) showed that the V-wave originated from the S-A junction. Until recently, there had been no electrophysiological demonstration of pacemaker activity in the teleost heart. Saito (1973) has recorded potentials showing the characteristic slow diastolic depolarisation of pacemaker potentials from the base of the S-A valves in the carp (Cyprinus carpio). The exact localisation of the pacemaker cells in the carp is as yet obscure, but it is evident that in all teleost hearts, there is a pacemaker region at the S-A junction.

By assuming that the teleost heart received only a vagal innervation two problems arose with regard to the autonomic control of the heart. First, what is the mechanism of the vagal inhibition and second, in the absence of any observable excitatory nerve, how is direct excitation possible?

In higher vertebrates which possess both a parasympathetic and sympathetic cardiac innervation, the heart is controlled by the direct effects of the nerves upon the pacemaker cells (Hutter and Trautwein, 1956). Vagal stimulation causes a hyperpolarisation of the pacemaker cell membrane with a resultant cardio-inhibition; this effect can be mimicked by acetylcholine.

Sympathetic stimulation causes an increase in the rate of diastolic depolarisation with a resultant cardio-acceleration; this effect is mimicked by noradrenaline. No such effects have been described in teleost hearts until very recently (Saito, 1973; see below).

Jullien and Ripplinger (1957) suggested that the cardiac ganglion cells in the tench (Tinca tinca) were tonically active, acting in an inhibitory fashion upon presumed pacemaker cells and that the effect of the vagus nerve was to modulate the degree of inhibition by an inhibitory effect on these ganglion cells. In this way, an increase in vagal impulse frequency would inhibit the ganglion cells and cardio-acceleration would result. Cardio-inhibition would be due to the lessening of vagal impulses and a concomitant increase in the tonic activity of the ganglion cells. However this is unlikely since section of the vagus in tench causes cardio-acceleration (Randall, 1966), indicating that vagal activity is tonic in nature, holding the heart under an inhibitory drive. If this is the case then an increase in vagal frequency will cause cardio-inhibition and a decrease (as a result of section) will result in cardio-acceleration. Furthermore, intracellular recording from pacemaker cells in the carp heart of the effects of vagal stimulation upon them indicate that, as the stimulating frequency increases, there is an increase in the amplitude of the hyperpolarisation of the pacemaker cell, and a concomitantly increased inhibitory effect.

A reflex cardio-inhibition can be elicited by many external stimuli (see Randall, 1968) and by stimulation of visceral afferent fibres (Kulaev, 1957b; Rodinov, 1959). These

last two authors deny that there is any vagal inhibitory tonus and propose that the heartrate is affected by direct vagal influences. Both Kulaev and Rodinov note different effects on the heart in response to differential vagal stimulation: high frequency stimulation produces inhibition and low frequency stimulation produces excitation. Also Mills (1886) elicited a reflex cardio-acceleration in the toadfish (Opsanus tau) at a lower stimulus strength than was required to achieve reflex cardio-inhibition. Thus, most of the evidence suggests that control of the teleost heart is due to vagal effects acting directly on pacemaker cells as in higher vertebrates.

As described above, excitatory effects have been noted in response to vagal stimulation. However, the possibility of an excitatory sympathetic innervation has been considered by certain workers despite the absence of a demonstrable sympathetic branch to the heart in teleosts. Izquierdo (1930) described very slight cardio-acceleration during vagal stimulation of the atropinised heart of the elasmobranch Scyllium and in this respect, the recent discovery of a few adrenergic fibres in the SV of the shark (Heterodontus portusjacksoni) by Gannon et al. (1972) using the fluorescence histochemical technique may be significant. Gannon & Burnstock (1969) have described an atropine-resistant cardio-acceleration in the trout. They have also described an extensive adrenergic innervation of the SV, atrium, ventricle and coronary blood vessels. Subsequent pharmacological work by Gannon (1971) supports the contention that there is an excitatory adrenergic innervation of the trout heart. He also notes that there is a connection between the vagus nerve and the sympathetic chain

and suggests that the cardiac vagus is therefore a vago-sympathetic trunk. On Gannon's evidence, it is no longer possible to state that the heart of all teleosts receives only a cholinergic vagal innervation.

The role of aneural cardio-regulation in fish must also be considered. Catecholamines are known to be present in fish blood and have an excitatory effect upon the heart. The plaice heart has β-receptors (Falck et al. 1966) as does the trout heart (Gannon, 1971) and it is possible that cardio-regulation could be affected by the endogenous release of catecholamines into the blood, perhaps from distant chromaffin cells. Such a system is proposed by Gannon et al. (1972) for elasmobranchs. Chromaffin cells abound in the cyclostome heart (Bloom et al. 1961; Hoffmeister et al. 1961) and may well act in controlling the heart by releasing catecholamines. Catecholamines are also known to be present in teleost hearts (Ostlund, 1954; Govyrin & Leonteva, 1965; Gannon & Burnstock, 1969).

Laurent's (1961, 1962) study on the anatomy and physiology of teleost cardiac function will be discussed in detail later. Most of his study concerned the catfish (Ictalurus nebulosus) but he compared his results with the carp, eel and tench. He used silver and osmium staining in combination with degeneration studies and extracellular electrophysiology to study the innervation of the hearts. At this point it is relevant to note that he was unable to find any correlation between efferent vagal activity in the cardiac nerve and changes in heart rate. Thus the suggestion of Mills (1886), Kulaev (1957) and Rodinov (1959) that a reduction in vagal impulse frequency results in cardio-acceleration has not yet received experimental confirmation. Laurent (1962) also made the very important

observation that there is a considerable variation in the pattern of cardiac innervation within teleosts and that this is likely to affect the physiology of the heart.

Studies on the morphology of the teleost myocardium have been undertaken with the electron microscope. All the investigators agree that the ultrastructural features of this tissue are fundamentally the same as in higher vertebrates (Kisch & Philpott, 1963; Kilarsky, 1964, 1967; Yamamoto, 1968; Yamauchi & Burnstock, 1969). One investigation (Ostadal & Scheibler, 1971) involving light and electron microscopy concludes that the presence of an outer layer of ventricular myocardium is present only in fish whose heart weight: body weight ratio is high. Consequently in these fish a more powerful stroke is required from the ventricle. There is also a coronary blood supply associated with this outer layer of muscle. This feature is common to elasmobranchs and to teleosts and probably reflects the work required of the heart by the fish.

The hearts of embryonic and larval fish have been used in several experimental investigations, but only one histological study on the developing heart has been made (Senior, 1909). Most of the experimental studies have concerned either the effects of temperature upon heartrate. (Grodzinski, 1952; Huggel, 1959), or the effect of applied drugs (Armstrong 1931; 1935; Brinley, 1933). Brinley's (1933) work involved the injection of adrenaline into the embryos of Fundulus heteroclitus and noting its excitatory effects on the heart. His conclusion that the adrenaline was stimulating sympathetic nerves to the heart must be questioned since the observed effects could be due to the stimulation of adrenergic receptors on the cardiac tissue itself.

It is evident that there is comparatively little available information on the structure and physiology of the fish heart and there are some important gaps to be filled. Also, the great interspecific variation between teleosts makes any generalisations difficult and unreliable. The fluorescence microscopical technique has shown adrenergic nerves in the trout heart (Gannon & Burnstock, 1969) but there is no evidence to show that this is a standard feature of all teleost hearts.

The present study was undertaken on the heart and its innervation of a single species of teleost to answer some of the following specific questions:-

1. Does the ultrastructure of the adult and developing heart provide morphological correlations with the physiology of the heart, and in particular with the pacemaker system?
2. How is the innervation of the heart related to its function, with special reference to the possibility of an excitatory sympathetic innervation and the control of heartrate?
3. Does the development of the heart reflect the state of the developing fish?

The species chosen for this study was the plaice (Pleuronectes platessa L.). This fish is not only an important commercial flatfish, but is one that has been chosen for various studies including breeding in captivity with a view to fish-farming (The Whitefish Authority) and swimming behaviour and prolonged excercise physiology in relation to trawling (Ministry of Agriculture, Fisheries and Food). The whole animal experiments on the physiology of excercise and swimming

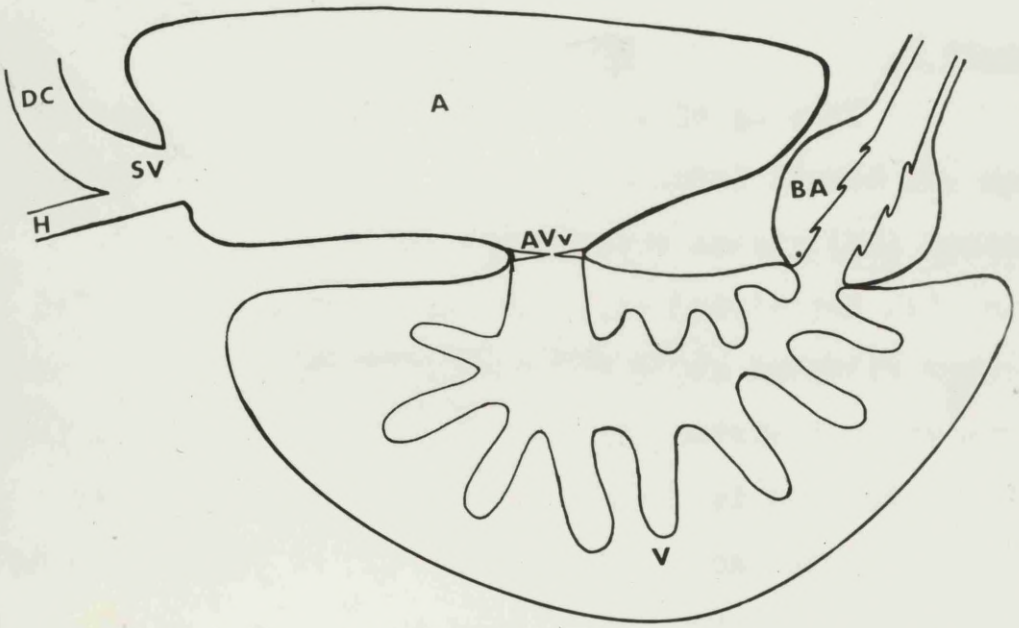
behaviour use changes in heartrate for monitoring the activity of the fish. Thus information on the structural and physiological basis of cardiac function in the adult and developing fish may be applicable to the above types of investigation, particularly in those fish subjected to stress.

Figure 1.

Drawing of the isolated plaice heart viewed from the dorsal (ocular) side of the fish. The sinus venosus (SV) passes through the pericardium (P) to join with the atrium (A). The atrium is a flat-roofed chamber situated above the ventricle (V) which has the shape of an inverted pyramid. The bulbus arterious (BA) leaves the ventricle close to the atrio-ventricular junction. Note the cardiac branch of the vagus passing over the SV And breaking up into a plexus.

Figure 2.

Diagram of a longitudinal section through the plaice heart to show the morphological arrangement of the chambers. DC, duct of Cuvier; H, hepatic vein; SV, sinus venosus; A, atrium; AVv, atrio-ventricular valves; V, ventricle; BA bulbus arteriosus.



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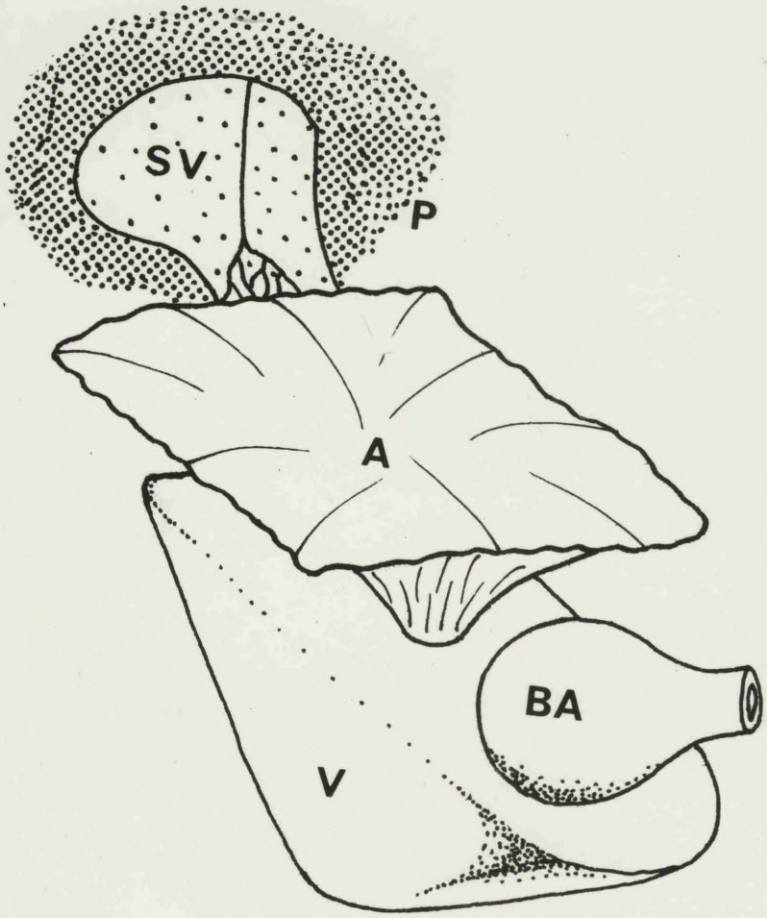

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II. MATERIALS AND METHODS

1. ANIMALS AND THEIR DISSECTION

i) Animals

Approximately 700 fish were used in this study. Fish were trawled in St. Andrews Bay and after landing were kept in an aquarium with constantly circulating seawater. They were not fed and whenever possible were used within three days of catching. Unless otherwise stated, all fish used were from the size range 6-14 inches and were younger than seven years old.

All fish were killed by decerebration with a fine needle, this procedure taking no more than five seconds to complete. When pithed, the animal could remain in a satisfactory physiological state for up to three hours if adequately supplied with well-oxygenated Ringer, provided that the cerebral vasculature was not too severely damaged during the process of decerebration.

ii) Dissection

Dissection to expose the heart was usually carried out on the ocular side of the fish although dissection from the eyeless ventral side is equally possible. Following decerebration, the outer edge of the operculum was removed. In order to remove the overlying pectoral girdle, an incision was first made at the base of the pectoral fin and continued in an anterior direction along the line of the pectoral girdle and posterior to it for approximately 1.5 cms. A second incision, starting at the initial point of the first incision, (just posterior to the pectoral girdle) was made and continued peripherally, cutting through the superficial musculature as far as the lateral edge of the fish. The pectoral girdle was

then cut about 1 cm. above the pectoral fin. A third incision, anterior to the pectoral girdle, was made along its contour, posterior to the gills as far as the lateral edge of the fish. By severing any attached muscles and connective tissue and cutting the bone peripherally the necessary lateral part of the girdle (the pectoral fin, coracoid and cleithrum) could be removed. This dissection revealed the duct of Cuvier, pericardial membrane and cardiac nerve. The heart can then be exposed by slitting the pericardial membrane and removing a large part of it. In this way the heart is prepared for intra- and extracellular recording in situ, for easy removal or for intracardiac injection of vital stains etc.

2. STRETCH PREPARATIONS AND LIGHT MICROSCOPY

i) Stretch preparations

In order to display the sinus venosus and sino-atrial regions of the heart, it was necessary to slit the sinus venosus and atrium longitudinally to form a flat sheet of tissue. Such a preparation could then be used more conviently for examination of these regions than was possible in the intact heart. The whole heart was excised and cut at the atrio-ventricular junction. The tube formed by the sinus venosus and the atrium was then slit longitudinally with fine scissors along the ventral midline. The result of this operation was a flat piece of tissue which could then be pinned out on a square of dental wax with either side of the heart uppermost, ready for use. The thicker walled chamber of the two is the atrium whose maximum thickness is approximately 700μ and therefore, to ensure that fixatives etc. were able to penetrate the tissue from both sides, it was slightly raised off the dental wax whilst still being firmly held by the pins.

A 'stretch' preparation as described above could be produced within five minutes of pithing the fish.

Such preparations were used for fixation in routine electron microscopy, light and electron microscope histochemistry, vital staining, fluorescence histochemistry and for some intracellular electrical recording.

ii) Vital staining

For an examination of the cardiac innervation 1 ml. of 1.0% methylene blue (Gurr) in Ringer was injected into the duct of Cuvier of decerebrate fish. Since the general circulation is not unduly impaired by the decerebration, the fish was left in well oxygenated Ringer (see later) for two hours before the heart was removed. Stretch preparations were made and fixed in 10% ammonium molybdate for 24 hours at 4°C with several changes thereof. After washing in aq. dist for 2 hours, the preparation was dehydrated and mounted in D.P.X. (BDH).

iii) Light microscopy

Whole hearts, or pieces thereof, were fixed in either Bouin's fixative or in methyl-formol-acetic acid (80% Abs. Methyl alcohol, 10% formalin and 10% glacial acetic acid) for 24 hours and then routinely dehydrated and embedded in wax. Sections, 10μ thick, were stained in Mallory Heidenhein's Azan stain or in the Rapid One-step Cazon (1950) modification. For general morphological purposes, the standard Azan stain produced consistently better results.

For the demonstration of connective tissue, a modification of the Azan stain was devised with success. By omitting the first stain (Azocarmine) and lengthening to 4 hours the mordanting time, it is possible to demonstrate the connective tissue as dark blue against a virtually colourless background.

The second stain is composed of Aniline blue and Orange G. which stains the cardiac muscle very lightly and makes the visualisation of the processes of connective tissue cells readily photographable with Ilford Pan F film.

In order to demonstrate the extrinsic innervation of the heart and especially the cardiac ganglion at the light microscopical level, stretch preparations were made and treated in one of two ways:

a) Immediately fixed in 1% OsO_4 in veronal acetate buffer at pH 7.2 at 4°C for 1 hour. Acetone dehydration was followed by embedding in Araldite for examination of the whole mount or in paraffin wax for sectioning. This method was consistently reliable for the demonstration of myelinated nerves.

b) Champy-Maillet stain

Following Bennett and Cobb (1969a) the stretch preparations were placed in a freshly prepared mixture of 2% OsO_4 buffered with veronal acetate to pH 7.2 (1 part) and ZnI solution (3 parts). The ZnI was prepared by adding 15g of Zn dust and 5g of Iodine crystals to 200 mls. of Aqua. dist. and filtering the product. The ZnI is unstable and should be freshly prepared prior to use. The tissue was fixed in this solution for about 16 hours in the dark before being dehydrated, cleared and embedded in paraffin wax. The sections were mounted in liquid paraffin and viewed on a stage warmed with a conventional hair dryer. This method seemed to produce results of better quality than those achieved by dewaxing of the sections and the preparation of permanent mounts.

iv) Ringer solutions

Throughout the experiments described herein the Ringer solution used was one devised specifically for the plaice and

which is ionically and osmotically equivalent to the serum of this fish. It has been experimentally shown to be suitable both in electrophysiological experiments and in the preparative procedures for electron microscopy (Cobb et al. 1973).

The composition of the plaice Ringer solution was:

NaCl		8.22	gm/litre
KCl		0.387	"
$CaCl_2$	$2\ H_2O$	0.72	"
$MgCl_2$	$6\ H_2O$	0.229	"
$NaHCO_3$		0.2	"
NaH_2PO_4	$2\ H_2O$	0.28	"
Glucose		1.0	"

In making up the Ringer it is important to add the calcium chloride last to prevent precipitation of calcium phosphate, and to store it below $10^{o}C$.

3. ELECTRON MICROSCOPY

Stretch preparations of the sinus venosus and atrium or small pieces of tissue from the ventricle and bulbus arteriosus were used for electron microscopy. In no cases were any of the pieces of greater thickness than 1 mm.

Fixation Tissue pieces were either taken from the heart immediately after its removal from the fish and placed in the fixative or the fixative was injected into the duct of Cuvier with the heart *in situ*. In the latter case, tissue pieces from the excised heart were subsequently placed in fresh cool fixative. All fixatives were kept at $4^{o}C$ and fixation was carried out at this temperature.

Of the methods of fixation attempted, two proved to be the most consistently reliable and were therefore routinely used:

a) 1% OsO_4 in 0.1M Sorensen's PO_4 buffer at pH 7.2 for 1 hour.

b) 3% gluteraldehyde in 0.1M Sorensen's PO_4 buffer at pH 7.2 for 2 hours.

Veronal acetate (Palade 1952) was used as a buffer with osmium fixation but the quality of the results was not as satisfactory as with the phosphate buffer. Gluteraldehyde proved a better fixative than osmium alone and it was also found that fixation in phosphate buffered gluteraldehyde for up to 36 hours at 4°C produced equally good results as a shorter duration of fixation. s-Collidine and sodium cacodylate were also used as buffers but with little notable success. The triple fixative of Imaizumi and Hama (1969) (Osmium-gluteraldehyde-osmium) did not fix the tissue satisfactorally, not obviously as a result of osmotic stress, but was therefore not used at all subsequently. Gluteraldehyde-fixed tissue was briefly rinsed in the buffer for 5 mins and then post-fixed in 1% OsO_4 in 0.1M PO_4 buffer for 1 or 2 hours. Some material was stained <u>en bloc</u> in 2% uranyl acetate after osmium treatment and prior to dehydration. The tissue was then dehydrated either in a graded acetone series over a period of 30 minutes or in a graded ethanol series followed by two changes of propylene oxide. After placing in a 50:50 mixture of either absolute acetone or propylene oxide and epoxy resin for a period of at least 4 hours and not more than 16 hours, the fixed tissue was embedded in epoxy resin using a vacuum embedding technique. All material was embedded in Araldite (Fluka, Buchs) which was polymerised in a 60°C oven for 36 hours.

For orientation of the tissue prior to thin-sectioning, 1μ sections were cut on a Porter-Blum microtome, placed on a glass microscope slide and stained for 1 minute with 1% toluidine

blue. This was particularly necessary in the study of the developing heart.

Light gold-silver sections were cut on a Huxley Ultramicrotome and mounted on unfilmed copper grids. The sections were then stained with lead citrate (Reynolds, 1963) for 5 minutes followed by 2% uranyl acetate for 10 minutes.

All sections were viewed and photographed with an AEI EM6B electron microscope at 60 and 80 KV.

4. HISTOCHEMICAL DEMONSTRATION OF ACETYLCHOLINESTERASE (AChE)

i) Light microscopy

Following the technique of Gomori (1952), in which the substrate, a thiocholine ester is hydrolysed by cholinesterase to release thiocholine which is then reacted with Cu^{++} ions, stretch preparations of the sinus venosus and atrium were washed in ice-cold phosphate buffer for 3 minutes before incubating at 37°C for one hour in 10 mls. of incubation medium. The incubation medium (see below) contained 15 mg of the substrate acetylthiocholine (BDH).

Incubation medium

Na_2SO_4 (95% saturated)	170 mls.
$CuSO_4 \cdot 5H_2O$	0.3 g.
Glycine	0.375g.
$MgCl_2 \cdot 6H_2O$	1.0g.
Maleic acid	1.75g.
4% NaOH	30 mls.

Control stretch preparations were also incubated in the above medium as follows:

a) lacking the substrate acetylthiocholine.

b) with substrate and 10.0 mg. of eserine (physostigmine. BDH).

c) with substrate and 1.0 mg. of iso-OMPA, a specific butyrylcholinesterase inhibitor.

After incubation, the tissue was rinsed in three changes of sodium sulphate to remove excess copper ions, and then placed in 5% ammonium sulphide for five minutes in order to precipitate brown insoluble copper sulphide at the reaction site. The tissue was then mounted on a microscope slide in glycerine for examination under the light microscope. The length of incubation time was determined by trial and error as that which resulted in a dense localised deposit of copper sulphide without any undue signs of diffusion.

ii) Electron microscopy

Electron microscope localisation of acetylcholinesterase was undertaken following the technique of Karnowsky and Roots (1964) for mammalian cardiac muscle. Whilst the fixative of Karnowsky was used, the mode of operation and preparation of the fixative followed P.M. Robinson (personal communication).

In this method the principle followed is that thiocholine reduces ferricyanide to ferrocyanide preferentially. The ferrocyanide then combines with the Cu^{++} ions to form insoluble copper ferrocyanide at the reaction site, directly as the enzymatic activity produces thiocholine. Thus, unlike the light microscope method, there is no time-lapse before the visualization of enzymatic activity by the production of a visible end product with ammonium sulphide.

a) Fixation: The fixative was 4% formaldehyde in 0.1M phosphate buffer. Stretch preparations were fixed for 2-3 hours at $4^{o}C$.

The fixative was prepared as follows:

1. 100 mls. of distilled water was heated in a conical flask to 60°C and 8 drops of N NaOH were added.

2. 8 gms of Paraformaldehyde (Merck) were added and the flask was kept at 60°C until the precipitate dissolved (within 45 seconds).

3. The resultant 8% solution was rapidly cooled under running tapwater and mixed 50:50 with 0.2M PO_4 buffer. The end-product was therefore 4% HCHO in 0.1M PO_4 buffer.

This fixative preserved the morphology satisfactorally. The gluteraldehyde-formaldehyde fixative of Karnowsky (1965) was also used, with similar results. Due to the thinness of the sinus venosus and of the atrial trabeculae, it was not necessary to cut tissue slices for this procedure.

b) Washing: the fixed tissue transferred from the fixative to 0.1M PO_4 buffer, in 10% steps over a period of 30 minutes at 4°C.

c) Preincubation. In the demonstration of AChE, the tissue was preincubated in 10^{-5} iso-OMPA (Tetraisopropylpyrophosphoramide, Koch-Light) for the specific inhibition of pseudocholinesterases. Controls were also preincubated in BW284C51 dibromide for the specific inhibition of AChE. All inhibitors were made up in phosphate buffer and preincubation was for 30 minutes at 4°C.

d) Incubation: The tissue was incubated in the Karnowsky medium for 30, 45, 60, 75 or 90 minutes with 15 mg of acetylthiocholine present as substrate, at 4°C. The incubation medium was as follows:

1. 15 mg of acetylthiocholine in 6.5 mls of 0.065M sodium hydrogen maleate buffer.

2. 0.5 mls. of 100 mM sodium citrate.

3. 1.0 ml. of 5 mM copper Sulphate.

4. 1.0 ml. of distilled water.

5. 1.0 ml. of 0.5 mM potassium ferricyanide.

e) The tissue was then washed for 5 minutes in ice-cold buffer and transferred to 1% OsO_4 in 0.1M PO_4 buffer. Subsequent electron microscopical preparative procedures were as described above.

5. FLUORESCENCE HISTOCHEMISTRY

The fluorescence technique for the demonstration of biogenic monoamines developed by Falck & Owman (1965) was used to demonstrate the cellular distribution of these substances in both the cardiac nerve and the heart itself.

In order to view the sinus venosus, stretch preparations were air-dried for three or four hours over phosphorous pentoxide. Whole hearts or pieces thereof were frozen in liquid propane (cooled in liquid nitrogen) and then freeze-dried for 2-3 days in a Millitorr freeze-dryer at -40^{o}C and 10^{-5} torr.

The dry tissue was then incubated at 80^{o}C with paraformaldehyde that had been stabilized for 5 days over sulphuric acid giving an air humidity of about 80%, for either 1 or 3 hours, the times necessary for noradrenaline and, adrenaline respectively to produce their fluorophores. Stretch preparations were mounted in liquid paraffin on a glass slide underneath a coverslip, whereas whole hearts were vacuum-embedded in paraffin wax, sectioned and then mounted on a glass slide as above.

Specimens were viewed with a Zeiss microscope equipped for fluorescence microscopy with a BG12 exciter filter

and an HB0200 mercury vapour lamp.

The level of tissue catecholamines in fish tissues is, in general, approximately 10% of that in analogous mammalian tissues (D.J. Grove, personal communication). Therefore it is especially important to attempt to preserve all the catecholamine in such a tissue. For this purpose, tissue was incubated for 2 hours prior to drying, in Ringer containing 100µg/ml of nialamide, an inhibitor of monoamine oxidase. Also, in other experiments, sometimes following nialamide treatment, the tissue was incubated in the false transmitter α-methyl-noradrenaline (10µg/ml Ringer) which is known to increase the intensity of any weakly fluorescing structures (Read & Burnstock, 1969).

6. PHARMACOLOGY & ELECTROPHYSIOLOGY

i) Pharmacology: In order to test the inotropic and chronotropic responses of the isolated heart to applied drugs, it was considered necessary to apply the drug to the internal surfaces of the heart rather than to the external surfaces. Initial experiments had shown that applying drugs to the isolated heart in an organ-bath demonstrated a) irregular diffusion of the drug to the tissue, b) efficiency of the valves of the heart in preventing the access of the drug to the chambers and c) the relative insensitivity of the functionally less important external surfaces of the heart.

A cannula was inserted into the S-A region of the heart which was secured to it by a ligature just above the S-A junction. The placing of the ligature was important in order that the heartbeat remained unimpaired. The cannula was connected to a perfusion apparatus which supplied Ringer

(at 10°C) to the heart at a constant pressure, the level of which was determined visually as being suitable. The apex of the ventricle was attached to a small hook which in turn was attached to a flexibly mounted lever. Contractions were recorded on a Kymograph. Drugs were applied via a short side-tube close to the heart. A period of at least five minutes was allowed to elapse between the last noticeable effect of one drug and application of the next. Blocking drugs were applied about 30 seconds before their associated agonists. Twelve hearts were used in this study.

ii) Electrophysiology: Approximately ninety plaice were used in this study. The hearts was either used in situ or as an isolated preparation, whole animal baths and organ baths being held in ambient local sea-water termperatures using a water jacket connected to a Churchill chiller-theromo-circulator. The specific plaice Ringer solution (see above) was used throughout.

Extracellular recording was carried out using conventional suction electrodes of finely drawn polythene tubing of 0.1mm tip diameter and a differential amplifier.

Intracellular recordings were made using flexibly mounted glass microelectrodes (Woodbury & Brady, 1956). Successful impalements were made of atrial cells using microelectrodes made from glass tubing of a small diameter (0.9mm-1.2mm external diameter). The microelectrodes were filled with 2M KCl, suspended by silver wire approximately 2 cms long from a micromanipulator, and had a resistance of the order of 25MΩ. A Bak unity gain pre-amplifier with high input impedance was used. All recordings were displayed on a Tektronix 502A oscilloscope.

The stimulators were Tektronix 161 pulse and 162 waveform generators connected by an optically coupled gallium arsenide isolation unit providing constant voltage (Roommele, in preparation). A suction electrode was used to apply the stimulus to the tissue. The stimulating electrode resistance was approximately 20MΩ and the shocks delivered were just supra-threshold and above. The experimental arrangement for individual experiments is described in the text.

iii) Drugs used in pharmacological and electrophysiological experiments: All doses of drugs are given as salts. In all cases the drugs were made up in plaice Ringer for administration.

Pronethalol (Alderlin)	I.C.I.
Adrenaline	B.D.H.
Atropine sulphate	B.D.H.
Bretylium tosylate	Burroughs Wellcome
Noradrenaline bitartrate monohydrate	Sigma
5-Hydroxytryptamine	Koch-Light.
Acetylcholine hydrochloride	B.D.H.
Physostigmine (eserine)	B.D.H.
Tubocurarine	Burroughs Wellcome
Tyramine	Koch-Light
Carbachol	B.D.H.
Hexamethonium bromide	Koch-Light
Nicotine	Koch-Light

iv) Other drugs: 6-hydroxydopamine (Sigma) in doses of 100 mg/Kg body weight (dissolved in 0.5 mls of Ringer containing 0.2μg/ml of ascorbic acid) was injected intravenously into decerebrate fish. Fish were sacrificed 2 hours later. Reserpine (Serpasil. CIBA) in doses of 5 mg/Kg was also used. Upon

sacrifice, the heart was prepared for electron microscopy as described above.

7. DEVELOPMENTAL STUDIES

The development of plaice larvae from hatching to metamorphosis in aquaria was studied by Ryland (1966) who described in detail the developmental stages through which the larvae pass, the duration of these stages and the effect of food supply and temperature upon their rate of development. Ryland's description have been used throughout this developmental study in order to identify and hence determine the age of the experimental material used. The temperature at which the experimental animals used in this study were reared is higher than that used by Ryland who followed ambient local seawater temperatures. Consequently there was a reduction in the length of the larval life to approximately 54 days. Ryland's post-hatching larval stage numbers are therefore quoted in lieu of ages in days in the present experiments.

The technique of rearing plaice larvae to metamorphosis and beyond follows that of Ryland (1966) and that adopted by the Whitefish Authority of Great Britain. The technique was developed since it has long been considered that a considerable proportion of the high mortality of marine teleosts occurs during the stages prior to metamorphosis and that any attempt to breed such fish on a commercial scale could not succeed without first overcoming this problem.

Artificially fertilized plaice eggs were obtained from the Whitefish Authority Marine Fish Cultivation Unit at Hunterston, Ayrshire, and transported to St. Andrews in a vacuum flask of well-oxygenated seawater. The eggs were trans-

ferred to a seawater tank at 8°C, the temperature at which they had been maintained since fertilization. Bacteriocides (Streptomycin SO_4, 64 mg/l seawater) and Penicillin-G (Solupen, 30 mg/l seawater) were present in the tank because bacteria are a serious cause of egg mortality due to the damage they inflict on the egg membrane. However, bacteria do not affect larvae to such an extent. Since the eggs and larvae were not reared in circulating seawater, the seawater in the tank was changed every 3 days and any detritus removed. Throughout the experimental period, the seawater was aerated and the tank left undisturbed as far as possible. Bounded by a tough egg membrane, the eggs are relatively hardy, but the larvae, particularly in their early stages, are very frail and it is important that they be left undisturbed. The larvae are sensitive to all contaminants and especially to transitional metal ions. Thus tank cleanliness is an important factor for successful rearing.

The temperature of 8°C was maintained until hatching was about 50% complete after which it was raised to 10°C over a period of 24 hours since larvae will not feed satisfactorily below 10°C. The temperature was then slowly raised to 15°C over 5 days and was then maintained at this level for the duration of the experiment by a chiller-thermocirculator.

Before hatching and during the first three weeks of larval life, the ambient light was kept low, since, in the early stages, the retinal pigment is relatively unstable and is quickly broken down by excess light (Shelbourne et al. 1963). Plaice larvae are predatory and rely solely on vision to catch their prey. Thus any visual deficiency will result in death from starvation. Older larvae were never exposed to

excessively bright light.

Nauplii of Artemia salina up to 36 hours old were used to feed the developing larvae. They were first introduced into the rearing tank three days after hatching at an approximate concentration of 10 nauplii/larva. This concentration was gradually increased until a level of approximately 30 nauplii/larva/day was reached. No difficulty was experienced in weaning the plaice larva onto this food and they survived well and completed metamorphosis in about 54 days.

In order to examine the development of the heart, embryos removed from the egg and young larvae were placed whole in the fixative. Larger larvae were dissected and only the anterior end was fixed, but after metamorphosis it was possible to excise the whole heart. Location of cardiac tissue within fixed, whole specimens was achieved by cutting 1μ sections and staining with toluidine blue. Ultrathin sections were then cut from the same block. The larvae were fixed in gluteraldehyde and processed for electron microscopy as previously described.

III. STRUCTURE OF THE ADULT HEART.

1. INTRODUCTION

There have been few reports of electron microscopical investigations of the teleost heart. Kisch (1966), Yamamoto (1966) and Kilarsky (1964, 1967) have studied the myocardial cells and the general structure of the atrium and ventricle of the goldfish (*Carassius auratus*) was studied by Kisch & Phillpott (1963). Kilarsky (1964, 1967) found the teleost myocardial cell to be simpler but essentially similar in structure to that of higher vertebrates but smaller in diameter. He also noted a very poorly developed sarcoplasmic reticulum and the absence of a T-system in *Gobius minutus* and *Perca fluviatilis*. Kisch and Phillpott (1963) paid particular attention to the epicardial and endocardial tissues as well as to the proposed "lymph" system and to the capillary bed of the heart of *Carassius auratus*.

Other specific electron microscopic investigations of the teleost heart are by Yamauchi and Burnstock (1968) on the relative density of innervation of the heart chambers of the trout (*Salmo trutta*), and by Howse et al. (1970) on the surface coatings of cardiac cells of *Galeichthys felis*. There have been however, two electron microscope studies on the hearts of cyclostomes (Bloom 1962, Leak 1969). Ostadal & Scheibler (1971) included the elasmobranch heart in their general study of the terminal blood bed of the heart of fishes in which they note that those with a small body weight (*Lampetra planeri*, *Hippocampus hippocampus* and *Labrus mixtus*) have no outer cortical layer of myocardium, whilst those with a large body weight (*Scyllium canalicula*, *Torpedo marmorata* and *Cyprinus carpio*) do.

2. RESULTS

1) Gross morphology of the plaice heart.

The plaice heart lay in the pericardium which was bounded by a flexible pericardial membrane. The pericardial membrane gained some rigidity from the adjacent muscles to which it was attached.

The sinus venosus (SV) was a thin-walled chamber which extended from just outside the posterior dorsal surface of the pericardium at the entry of the two ducts of Cuvier, to the sino-atrial (S-A) junction (Fig. 5). The SV also received the two hepatic veins. In the plaice the SV was not contractile unlike that of the eel (Grodzinski, 1954). There was a single, large atrium which was positioned above the posterior two-thirds of the ventricle. The atrium had a thin outer wall of cardiac muscle but the bulk of the atrial tissue was composed of trabeculae of cardiac muscle (Fig. 3). The "roof" of the atrium was approximately square, but underneath this "roof", the chamber narrowed considerably to form a circular junction with the ventricle at the atrio-ventricular (A-V) junction (Fig. 6). This junction was reinforced by a considerable amount of connective tissue which appeared *in vivo* as a white ring around this junction. There was a single ventricle which had the shape of an inverted pyramid with an oblong base. On the base of this pyramid (the dorsal surface), the apertures of the A-V junction and the ventricular-bulbar junction lay close together, separated by a region of connective tissue. The ventricular volume was not as great as that of the atrium but the ventricle was a more massive structure with very thick muscular walls (Fig. 4). The cavity of the ventricle was highly subdivided by the profusion of trabeculae. There was no

outer, cortical layer of cardiac muscle in the plaice heart as is present in many teleosts. This is a highly significant feature when the ventricle is considered since, in those teleosts that do possess an outer cortical layer, there is a coronary blood supply to this layer of muscle arising from efferent epi-branchial vessels which run to the heart along the ventral aorta. In the plaice, concomitant with the absence of this muscle layer was the absence of a coronary blood supply. It has been suggested that the coronary blood vessels could provide a pathway for nerves innervating the heart (Gannon & Burnstock, 1969). The bulbus arteriosus (BA) was an onion-shaped chamber consisting solely of connective tissue and was continuous with the ventral aorta. The luminal surface was lined by a series of small ridges which prevented backflow of blood. At the S-A junction, there was a small but effective S-A valve and the A-V junction, a pair of semi-lunar valves which isolated the chambers from one another. The valve consisted of connective tissue only.

Fine Structure of the Plaice Heart

For this study, material from hearts of about thirty-five adult plaice were used. In this case, the term "adult" includes animals between one and six years old in an attempt to describe the "normal" adult heart. That is to say, heart-tissue that is fully mature but as yet showing no specific age-associated changes.

ii) Sinus venosus

The sinus venosus of the plaice heart was a thin-walled chamber between 60μ -90μ thick which extended from the junction of the ducts of Cuvier and the hepatic vein sphincters to the sino-atrial junction. The matrix of the sinus venosus was a collagenous tissue, bounded internally by a layer of endocardium, one cell thick, and externally by a similar epicardium. Striated collagen fibres, actively produced by the many irregularly arranged fibrocytes, formed a thick meshwork of fibres, either in bundles surrounded by cellular fibrocyte extensions or simply in undivided sheets. This collagen supported the other components of the sinus venosus, namely localised bundles of myocardial cells and also the plexus of the parasympathetic cardiac ganglion. Chromatophores commonly occurred under the epicardial layer (Fig.7).

Myocardial cells occurred in small bundles of 4-6 cells in cross section, running longitudinally along the sinus venosus (Fig. 8). They did not form a continuous layer of cells and were not confined to this region. These muscle bundles were rarely found more than 1 mm away from the S-A junction. Nerve bundles and individual profiles were closely apposed to some of the muscle cell bundles and presumably innervate them, although no end-plates have been observed. Satchell (1971) has

suggested that the action of these muscles is in controlling the flow of blood into the atrium. How effective this mechanism is, must be questioned since there was a relatively small amount of myocardial tissue in the plaice sinus compared with that of the eel (Anguilla anguilla) which has a strongly contractile sinus and which is apparently also active as a pacemaker region (von Skramlik, 1935). The eel is the only fish in which the sinus venosus has been ascribed a contractile function, and probably has an active functional role in the passage of blood through the heart. These cardiac muscle cells of the SV were similar in structure to those of the atrium and ventricle, but showed a more random distribution of myofibrils. The bundles of muscle cells were bounded by a thin epimysium. The cardiac branch of the vagus passed over the sinus venosus to a point about 2 mm. from the junction with the atrium where it broke up into an extensive plexus which contained the somata of the paracympathetic innervation of the heart. Although the cardiac nerve passed over the sinus venosus it penetrated the epicardial layer and entered the connective tissue matrix. The myelinated axons of the plexus differed from those in other parts of the heart in that they were individually enclosed by a collagenous sheath, as well as a whole nerve bundle being likewise enclosed. There were a considerable amount of glial cells amongst the ganglion cells in the plexus. The cardiac plexus and innervation will be described in detail (see later).

The extent of the sinus venosus was limited anteriorly by the atrium and posteriorly by its junction with the ducts of Cuvier and by the hepatic vein sphincters which have been reported in elasmobranchs by Johnson & Hanson (1967)

and these also occur in teleosts. The sphincters consisted of a thin band of smooth muscle 3-5 cells thick directly continuous with the collageneous matrix of the sinus venosus.

iii) Sino-atrial junction

The atrial end of the sinus venosus was a circular band of connective tissue which also gave rise to the S-A valve. This valve was very much reduced in the plaice, projecting only 250 (approx.) into the S-A orifice. Similarly, on the sinus end of the atrium, the muscle cells formed a thin band which encircled the heart. The combined action of the S-A valve and this circular band of muscle was to isolate the sinus venosus from the atrium and to prevent any backwards flow of blood. Whilst the sinus venosus and the atrium are only joined by connective tissue and a small amount of muscle which projects backwards from the atrium there were many large tracts of both myelinated and unmyelinated nerves between the two chambers. These were derived from the cardiac plexus many of whose branches split up and encircled the S-A junction before passing into the atrium to innervate the myocardium. Ganglion cells and their processes were also found in this region.

This region has hitherto been designated a pacemaker region in the trout by Yamauchi & Burnstock (1968) and in the flounder by Wardle (1962) but whilst the density of innervation of the myocardium was definitely greater here, there was no basic difference in the structure of the myocardial cells themselves, from the rest of the myocardium. The sino-atrial cells were slightly smaller in diameter, averaging 1.76μ, than those of the rest of the myocardium (see later). This is similar to that seen in the trout (Yamauchi & Burnstock, 1969).

The concept that pacemaker cells are recognisable by the relatively sparse density of myofibrils and by their random distribution within the cells (Trautwein & Uchizono, 1963) cannot as yet be applied to the S-A tissue of the plaice. The muscle cells in this region had an obvious external lamina (Fig. 9) completely surrounding each one unlike the pacemaker tissue of the S-A node of the rabbit which consist of muscle cells juxtapposed to within 200Å in bundles, each bundle of cells being surrounded by an external lamina. Trautwein & Uchizono suggested that this arrangement could well be the functional unit of pacemaker activity. Recently, Saito (1973) has recorded pacemaker potentials from the S-A region and valve of the carp (Cyprinus carpio) heart.

iv) Myocardial tissue

a) General structure

The arrangement of the component cell types of the atrium and ventricle (Fig. 10) have the same basic plan. Kisch & Phillpott (1963) described the histological arrangement of these chambers in the goldfish and this arrangement applied to the plaice. The myocardial cells are sandwiched by an external epicardium and sub-epicardial space and by an internal endocardium. The trabeculae are lined with endocardium, but there is no well-defined sub-endocardial space. It should be noted that this pattern is that generally seen in the hearts of all vertebrates.

The epicardial layer which covers the outside of the atrium and ventricle was a monolayer being formed of large, epithelial cells with lightly staining cytoplasm containing a simple endoplasmic reticulum and, in some cells, lipid droplets of 0.3μ -0.4μ diameter. The epithelial cells of the

epicardium and endocardium had desmosomal (macula adhaerentes) junctions between them as well as intermediate junctions consisting of very localised points of apposition of neighbouring cell membranes with small amounts of filamentous material in the adjacent cytoplasm. No contacts of the nexus (fascia occludens) type were seen between epithelial cells. The sub-epicardial space was 0.25μ -0.8μ in thickness and was filled with loosely packed collagen fibres and a small number of fibrocytes. The trabeculae and thus the lumen of the atrium and ventricle were lined by similar epithelial cells forming the endocardium which invaginated to divide up the trabecular myocardium irregularly into groups of myocardial cells numbering approximately twenty-five cells in transverse section. There was collagenous tissue accompanying these invaginations and occasionally, very electron-dense fibrous tissue. Apart from the trabeculae the thin outer layer of myocardium constituted the wall of these chambers but it was not invaginated by endocardium. Thus the heart was a heterogeneous tissue, consisting of muscle cells, connective tissue and an innervation.

b) <u>The Teleost myocardial Cell</u>.

The myocardial cell of the plaice conformed to the model described for teleosts by Kilarsky (1967) for the goby and the perch and by Kisch & Phillpott (1963) for the goldfish, but there were definite differences in the plaice.

The myofibrils of the myocardial cells were arranged peripherally (Fig. 11) sometimes forming a complete tube around the mitochondria. These myofibrils showed the typical array of six thin actin filaments surrounding each thick myosin filament (Fig. 12). The sarcomere length in relaxed tissue was shorter (1.4μ) than that previously reported for

teleosts by Kilarsky (1967), of 2.0μ, even in preparations which were fixed in a stretched position. A, I and Z-bands were prominent but the H-band is less distinct and in most preparations the M-line, or the central thickening of myosin filaments was not observed. In comparison with the myocardium of most higher vertebrates, the number of myofibrils per myocardial cell was lower in teleosts, even accounting for the smaller diameter of the teleost myocardial cell. The diameter of plaice myocardial cells in the atrium ranged from 1.3μ- 6.0μ with an average of 3.2μ. There was no significant difference in diameter between cells of the atrium and those of the ventricle. At the nuclear region, the measured diameter was slightly greater, averaging 4.3μ. The cell length was variable and difficult to measure. These values were smaller than those recorded in teleosts by Kilarsky (1967) and those of the hagfish (Jensen, 1965) but of a similar size to those of the amphibian Necturus (Hirakow, 1971).

The single nucleus of each cell was oval and centrally placed, and was associated with closely packed mitochondria at the nuclear poles. The mitochondria had many cristae and were round in cross section, but were elongate, usually being less than 1.5μ in length. The 'tube' of myofibrils was thus filled with mitochondria and, in the middle of the cell, by the nucleus. The sarcoplasmic reticulum (SR) was, as in other teleosts (Kilarsky, 1967), very sparsely distributed. Sub-sarcolemmal cisternae were fairly frequently observed (Figs. 14,16) but other components of the SR were scarce. Occasionally short lengths of tubule were observed near, or in contact with, the myofibrils but never in apparent contact with Z-lines or intercalated discs. It was not possible to determine whether

the sarcoplasmic reticular system was continuous from cell to cell. The sparseness of this system was reminiscent of that observed in the amphibian Necturus by Hirakow (1971).

In common with other cardiac cells of small diameter there was no T-system. The cells were bounded by a sarcolemma which was generally convoluted over much of its length (Fig. 13) (and this does not appear to be a fixation artifact) and by a 250Å external lamina which was most obvious in the sino-atrial region. Elsewhere, in the myocardium, cells were often grouped tightly together in bundles limited by an external lamina. However, between individual cells the intercellular space was narrow (about 300Å) and no obvious external lamina was observed. This is a similar arrangement to that encountered in frogs (Sommer & Johnson, 1969) and in birds (Forbes & Sperelakis 1971) but very different to the situation in most mammals where the working fibres are loosely assembled into bundles in which the intercellular space may be 0.5μ. There were desmosomal regions along the sarcolemma, presumably associated with cell-to-cell attachment. The long axis of the myocardial cells was delineated by intercalated discs formed mainly of intermediate junctions (fasciae adherentes) but also containing localised desmosomal regions. The intercalated discs varied in length and were only moderately convoluted. They generally spanned the width of a single myocardial cell. There was often an extensive filamentous mat associated with the intercalated discs, stretching at least 500Å on either side of the disc into the cytoplasm. Nexuses were not observed in the plaice but they have been reported in the goldfish (Martinez-Palomo & Mendez, 1971) as "gap junctions". Golgi complexes are sparsely distributed. There were considerable

amounts of glycogen, often densely accumulated around the mitochondria and myofibrils, as very electron-dense granules. The distribution of glycogen granules within the myocardial cells varied considerably from dense "pools" of granules to an evenly spread distribution throughout the cytoplasm. Though glycogen was abundant within the cytoplasm of the adult heart, it was not as densely distribution as in the myocardium of newly-metamorphosed fish. (see later).

In the cytoplasm of atrial cells, there were large membrane-limited granular vesicles of 1200-1500Å diameter which occurred in considerable numbers. (Fig. 13). Approximately 10% of myocardial cell profiles contained large groups of these vesicles and many other profiles had them in smaller numbers. These granules were comparable in size to those in cyclostome cardiac tissue which are known to be the intracytoplasmic storage sites of biogenic amines (Bloom, 1962). These amines were present in teleost cardiac tissue in low concentrations (0.5µg of noradrenaline and 0.64 µg of adrenaline per gm of tissue in Gadus callerias) (Ostlund, 1954). von Euler (1952) and von Euler and Fange (1961) have also reported intracellular catecholamines in teleost heart cells.

c) Differing cell populations in the myocardium.

The myocardial cells of the atrium and ventricle were not all identical. In the myocardium of higher vertebrates, and mammals in particular, there were two types of myocardial cell namely the contractile "working" myocardial cell and the "conducting" or Purkinje cell. In the plaice two types of cell could be recognised. The bulk of the myocardium consisted of

the ordinary myocardial cells as described above. There were certain differences between those of the atrium and those of the ventricle which will be described in detail in the next section, but it is important to note that the ventricular myocardial cells contained a higher proportion of myofibrils and mitochondria than do the atrial cells and as a result had less free cytoplasm. The second cell type resembled the Purkinje fibre of the mammalian and avian myocardium in having a very large amount of free cytoplasm, a comparative lack of glycogen and a random distribution of only a small number of myofibrils and mitochondria. All these features gave the cells a "light" appearance (i.e. electron lucent), in contrast to the ordinary myocardial cells (Fig. 15) and were identical to those seen in the Necturus myocardium by Hirakow (1971) who was the first author to call these cells "light" cells. The "light" cells occurred in both atrium, and ventricle and were easily distinguishable from the ordinary "working" cells of these chambers. However, unlike Purkinje cells (Hirakow 1966, 1970, Scott, 1971) they did not run in tracts and their distribution was impossible to follow in thin section, and appeared, as in Necturus (Hirakow, 1971) to be totally random. The diameter of the "light" cells of the same order of magnitude as the "working" cells, as compared to the large diameter Purkinje cells of the bird and mammal.

3) Comparison of atrial and ventricular myocardium

The ventricle was a more massive structure than the atrium having a thicker outer layer of muscle and more trabeculae. The outer layer of the plaice ventricle was not the same as "compact" or "cortical" layer reported by Oestadal & Schiebler

(1971) to occur in fish of large body weight, which is richly supplied with capillaries. Intracellular channels were scarce in the plaice ventricle and absent in the atrium, the thickness of the trabeculae apparently not being too great to prevent adequate diffusion of oxygen from the blood in the heart. Where such channels did occur, they were lined by single epithelial cells and were mostly confined to the outer layer of ventricular myocardium and closely resembled those seen in cyclostome hearts (Leak, 1969).

There were certain differences of the myocardial cells between atrium and ventricle. Atrial cells contained more large (1200-1500Å) granular vesicles within them than the ventricle and cells containing them were more widespread. Membrane-limited intracytoplasmic structures with a homogeneous, light contents occurred randomly throughout the ventricular myocardium, of 0.1μ-0.3μ diameter, being distributed throughout the cell space. They resembled the lipid droplets of the mammalian myocardium in size, shape and position within the cell, but did not have the slight opacity of their lipid contents. This may be a result of the fixation and embedding of the tissue or perhaps due to the more soluble nature of teleost lipid as compared with the mammalian counterpart (Travis & Travis, 1972).

The diameters of atrial and ventricular cells showed no significant difference, ranging from 1.3-6.0μ but the ventricular cells contained more myofibrils and mitochondria than did the atrial cells (Figs. 16,17).

v) <u>The Atrio-ventricular junction</u>

The point at which the atrium and the ventricle meet

comprised the connective tissue involved in joining the two chambers, a pair of semi-lunar valves and a connecting strip of myocardium. There was a concentration of fibrocytes and connective tissue in the region of the atrio-ventricular junction, which occurred in the sub-epicardial space. This widened to accomodate the extra tissue up to 500 μ away from the junction (Fig. 18). The area proximal to the junction showed fibrocytes lined up in great numbers on both the atrial and ventricular sides and a greatly increased thickness of collagen fibres in the sub-epicardial space which widened to 6 μ-7μ . The atrial and ventricular myocardia communicated across the A-V junction by means of a thin strand of muscle cells running along the inside of the sub-epicardial connective tissue layer. This strand of muscle was about 15-20μ in width (4 or 5 cells thick) (Fig. 6) and extended completely around the junction. The presence of this muscle strand provided a morphological correlate to transmission of the heartbeat from the atrium to the ventricular myocardium.

The length of the muscle strand between the two chambers was about 20μ. The myocardia showed no specialised sphincters but there were a number of muscle fibres running in a circular band on the ventricular side of the junction, though this was unlikely to have any significant effect on the passage of blood flow from one chamber to the other. There was also a considerable amount of connective tissue amongst the muscle cells of the two chambers for about 20μ from both sides of the junction which might strengthen the join. There was no aggregation of nervous tissue into a node at this junction.

Two semi-lunar atrio-ventricular consisting solely

of connective tissue bounded on the outside by a thin endothelial layer arose from the junction. There was a row of regularly arranged fibrocytes along the edge which produced thick bundles of closely packed collagen fibres (Fig. 18), which were held together by processes from the fibrocytes, the effect of which would be to add rigidity to the structure. There were no sites of attachment of muscles to the base of these valves which makes it unlikely that there is any muscular control of the action of the valves. No structures which could be interpreted as mechanoreceptors were seen at the valve bases, nor was any innervation apparent.

vi) Bulbus arterious

The bulbus arterious was the most anteriorly situated chamber of the heart. This onion-shaped chamber was the only one of the four that was totally elastic, having no muscular elements at all. Satchell (1971) claims that the bulbus arterious consists of layers of smooth muscle and elastic tissue but this was not confirmed by electron microscopy of the plaice bulbus arterious, and it would therefore appear that this chamber is not actively contractile. There may however be interspecific differences since Womersley (1973) notes smooth muscle cells in the bulbus arterious of Cottus scorpius. The outside of the bulbus arterious was covered by a single-celled epicardial layer and the lumen lined by large flat endocardial cells which contained oblong nuclei and many lipid granules of varying electron density and of 1000Å-3000Å diameter. Between these two cell layers was the main tissue component of the bulbus arterious which was a granular mass of material resembling yellow elastic tissue packed with collagen fibres and many

fibrocytes (Figs. 19,20).

These fibrocytes, not being closely confined by other cells, were large and irregular in size and outline, though their shape was basically tubular. Fibrocyte cytoplasm contained large numbers of small, round mitochondria and large lipid granules which were slightly prominent in some cells. The surface of the cells was irregular, often sending out long finger-like processes of variable length. Some fibrocytes contained intracellular collagen fibres which are presumably an indication of active collagen synthesis (Fig. 20).

The bulbus arterious showed a roughly concentric arrangement of the fibrocytes when cut in cross-section which could possibly be interpreted as a layer of muscle under the light microscope. The inside of the bulbus arterious was convoluted to form the ridges that occur in this chamber in teleosts.

3. DISCUSSION

The four chambers of the heart of the plaice have been described with the light and electron microscopes.

As in the frog ventricular myocardium (Sommer & Johnson, 1969) there was no coronary blood supply to the ventricular and atrial tissues of the plaice. In other fish such as the trout (Salmo trutta) and Cottus scorpius, coronary vessels run backwards from the ventral aorta to the ventricle in which there is an extensive capillary bed. This capillary bed is totally absent in the plaice. The few capillaries (or intercellular canals) that are found in the ventricle are similar in form to the vascular canals of cyclostome hearts (Leak, 1969) which allow the blood to flow through the myocardium, thus lessening the diffusion distance in thicker parts of the tissue. The endothelial lining of the intercellular canals of the plaice heart corresponds to that of the vascular canals. Thus, for the most part, as in the amphibian Necturus, the plaice myocardium receives its blood supply directly from the heart lumen at the intertrabecular spaces. The distribution of an extensive coronary supply in fishes is correlated with an outer compact or cortical layer of myocardium the presence of which is related to the body weight of the fish, rather than to its phylogenetic position (Ostadal & Scheibler, 1971).

The organisation of cardiac tissue in the plaice is not elaborate, the morphological arrangement of the trabeculae is not complex and the junctions between the heart chambers are mainly connective tissue. The invaginations of the endocardium into the trabeculae supports the myocardium and preserves its integrity together with the aid of connective tissue, formed

by fibrocytes, which becomes more extensive in older fish.

Connective tissue plays an important part in constituting the teleost heart. The sinus venosus is mostly composed of connective tissue whilst the bulbus arterious and the valves consist totally of it. As the age of the fish increases, the amount of connective tissue in the sub-epicardial space and alongside the endocardial invaginations greatly increases.

The overall fine structure of plaice myocardial tissue conforms to the pattern earlier described for the teleost heart (Kisch & Phillpott, 1963; Yamamoto, 1966; Kilarsky, 1967) and also conforms to the features of myocardial cells of small diameter (2-6μ) from lower vertebrates that have been investigated. There has been a considerable amount of work on the fine structure of the myocardial cells of all classes of lower vertebrates, viz: cyclostomes by Leak (1969); teleosts by Kisch and Phillpott (1963), Yamamoto (1966), Kilarsky (1967); amphibians by Scheyer (1960), Naylor and Merrillees (1964), Staley & Benson (1968), Howse et al. (1969), Sommer and Johnson (1969), Gros and Schrevel (1970), Baldwin (1970), Hirakow (1972) and Page & Niedergerke (1972); reptiles by Fawcett & Selby (1958), Yamamoto (1965), Leak (1967), Forbes & Sperelakis (1971) and Okita (1971). From these studies, several features common to all classes of lower vertebrate myocardial cells have emerged which are of significant physiological consequence. In comparison with mammalian cardiac tissue, the cells are small (2-6μ in diameter), lack a T-system (transverse tubular system), have a poorly developed sarcoplasmic reticulum (SR) and few, small areas of close apposition of adjacent cell

membranes. The above four points will be discussed in relation to the plaice myocardial cell.

Mammalian "working" myocardial cells are 10-15μ in diameter and are individually surrounded by an external lamina whereas those of lower vertebrates are considerably smaller (2-6μ) and often packed tightly together in bundles surrounded by an external lamina outside the bundle only. Thus the immediate extracellular space of many individual cells is considerably reduced in comparison with mammalian cardiac cells which are often separated from their neighbours by an extracellular space of as much as 0.5μ . It would therefore seem that the advantage of a small cell diameter, namely the reduced diffusion distance for ions and oxygen into the centre of the cell, are counteracted by this association of small numbers of cells into bundles. The significance of the geometry of muscle cell apposition is as yet obscure but it is conceivable that these smaller cells within a bundle are intimately related in view of their close apposition, even though no morphological correlates of cell -to-cell transmission have as yet been seen, and act as a single contractile unit, their unity making up for the paucity of myofibrils in individual cells. However, if there is a morphological correlate of cell-to-cell transmission in smaller cardiac cells, it has so far eluded detection. Therefore, it is impossible to do more than speculate on this problem. The relationship of associated cell bundles to one another within the functional syncitium of cardiac muscle is also unclear.

In mammals, the morphological correlate of cell-to-cell transmission through the cardiac "syncitium" is thought to be the nexus (fascia occludens) (McNutt, 1970) as in the

case of smooth muscle (Dewey & Barr 1964), another functional "syncitium". But not all cardiac tissue in which the cells can be demonstrated to be electrically coupled can be shown to possess nexuses. Indeed cardiac nexuses are commonly only found in mammalian and avian "working" fibres and in all Purkinje fibres and not in the cells of lower vertebrates (Sommer & Johnson, 1969, Hirakow, 1971). There is also a relationship between the small cell size of lower vertebrate myocardium and the absence of nexuses and therefore a consequent lack of a demonstrable morphological correlate of electrical transmission. If it is to be postulated that the nexus is the sole site of electrical coupling between cells, it must be shown that the amount of nexus connection between the cells is sufficient to perform such a function. This is obviously very difficult to measure qu ntitatively. The actual size of observed nexus contact between cells increases with increasing cell size. For example, small nexuses are found in the larger fibres of adult birds (Scott, 1971, Jewett et al. 1971) but they are very rarely observed in the cells of embryo chickens, which are 40% smaller (Sommer 1968). Likewise they are only rarely observed in amphibian (Sommer and Johnson, 1969; Baldwin, 1970; Hirakow, 1971) and have not been reported in teleost myocardial cells. Certainly they have not been observed in the present study of the plaice heart. Thus there is a gradient from larger "working" cells and Purkinje cells with large areas of nexus connections down to small "working" cells with no detectable nexuses. The plaice has small "working" cells and no observable nexuses and thus fits into this scheme. It is possible that this apparent relationship of nexus size with cell size is more significant than at first sight, and that this reflects a

primarily structural role. Intercalated discs are presumed to fulfil a structural role and it is within this structure that large extents of nexus occur between mammalian and avian "working" cells. Brightman and Reese (1969) have shown that those structures that have been termed "nexuses" are in fact gap junctions at which point the adjacent cell membranes are 20Å apart. Within this gap junction there is a hexagonal substructure revealed by lanthanum staining but it is as yet unclear to what extent this substructure reflects low resistance pathways. It should also be borne in mind that present fixation techniques may not preserve small areas of nexus. The evidence presented by Martinez-Palomo and Mendez 1971) for "gap" junctions in the goldfish myocardium does not solve the problem since the "gap" junction shown had an intercellular space of 70-80Å and bears no structural affinity to the gap junctions described by Brightman & Reese (1969) in which the intercellular space is of the order of 20Å. The "gap" junctions shown by Martinez-Palomo and Mendez are very similar to those described by Baldwin (1971) and Denoit-Mazet and Vassort (1971) in frog atrial cells which, in addition, show a certain amount of unidentified intercellular material at the site of the junction. Baldwin (1971) reports that on electrically uncoupling cells by injury, neither these "close" junctions ("gap" junctions) nor the "cardiac adhesion plates" (desmosomal-like structures often at the Z-line level) become disrupted and concludes that the idea that close membrane appositions are totally responsible for electrical coupling between cells is not tenable in tissues where they are only infrequently observed. Also that the other observed membrane specializations are probably not involved in electrical coupling

between frog cardiac cells. Thus the morphological basis for electrical coupling in cardiac cells that have no observable nexus remains undetected and it is likely that other cell membrane specializations fulfil a primarily structural rather than physiological role. Whilst the preparative procedures for electron microscopy could well disrupt intercellular connections particularly if they are small in area, it should be remembered that nexuses in smooth muscle are not labile when subjected to osmotic stress (Cobb & Bennett, 1969) and can be isolated intact from liver cell homogenates (Benedetti & Emmelot, 1968).

The intercalated discs (ID) of the plaice heart are very simple in construction compared with mammalian IDs. The plaice IDs are only slightly convoluted and do not extend for more than the width of a single cell. Often, at sites of bifurcation of the cells, the ID covers only the width of a single myofibrils. Those of the mammal are highly convoluted and this, when combined with their larger cell size, results in the interdigitations between cells at such a surface providing a very considerable surface area of connection between the cells. This is not so in the teleost heart whose IDs are more reminiscent in structure to those in some reptilian (Yamamoto, 1965) and amphibian cardiac tissue (Gros & Schrevel, 1970; Hirakow, 1971).

In common with other myocardial cells of 2-6μ diameter and Purkinje cells, a T-system is absent from the plaice myocardium. The diameter of "working" fibres of mammals which have T-tubules is at least twice that of those that do not, and it seems likely that fibre diameter is the determining factor for their presence or absence. Skeletal muscle cells are 45-90μ in diameter and thus a role for a T-system in

excitation-contraction coupling is easily envisaged in that it introduces the electrically excitable sarcolemma into the main body of the cell. Also, whilst it is possible that this may not apply with cardiac cells of 2-6μ diameter, it is pertinent to question the need for a T-system in a functional syncitium whose component cells are of such a small diameter.

As in the amphibian Necturus (Hirakow, 1971) the sarcoplasmic reticulum (SR) of the plaice heart cells is extraordinarily sparsely distributed within the cell. So much so, that it is not possible to construct a detailed description of the anatomy of this intracytoplasmic tubular system. Early work on the amphibian cardiac SR also revealed its sparseness (Scheyer, 1960, Staley & Benson, 1968, Sommer & Johnson, 1969) but recently Page & Niedergerke (1972) have described the anatomy of the SR in the frog heart, even though it is much reduced in comparison with its counterpart in the mammalian heart. Perhaps another species will offer better material for such studies on the teleost cardiac SR. There is now good evidence that the SR is the site of an ATP-dependent calcium pump that moves calcium into the cytoplasm against a gradient during the contraction-relaxation cycle (see Sommer & Johnson, 1969). Assuming a similar role for the plaice SR, the small amount in the myocardial cells could be either a very efficient type of SR in effecting calcium transport, or the very small extent of this system could be related to the small amount of myofibrils within these teleost cells or to their small diameter.

The similarity of the "light" cells of the myocardium to the mammalian and avian Purkinje or conducting tissue should be mentioned. The two types of cell in question are structurally very similar but it is obvious that the "light" cells do not

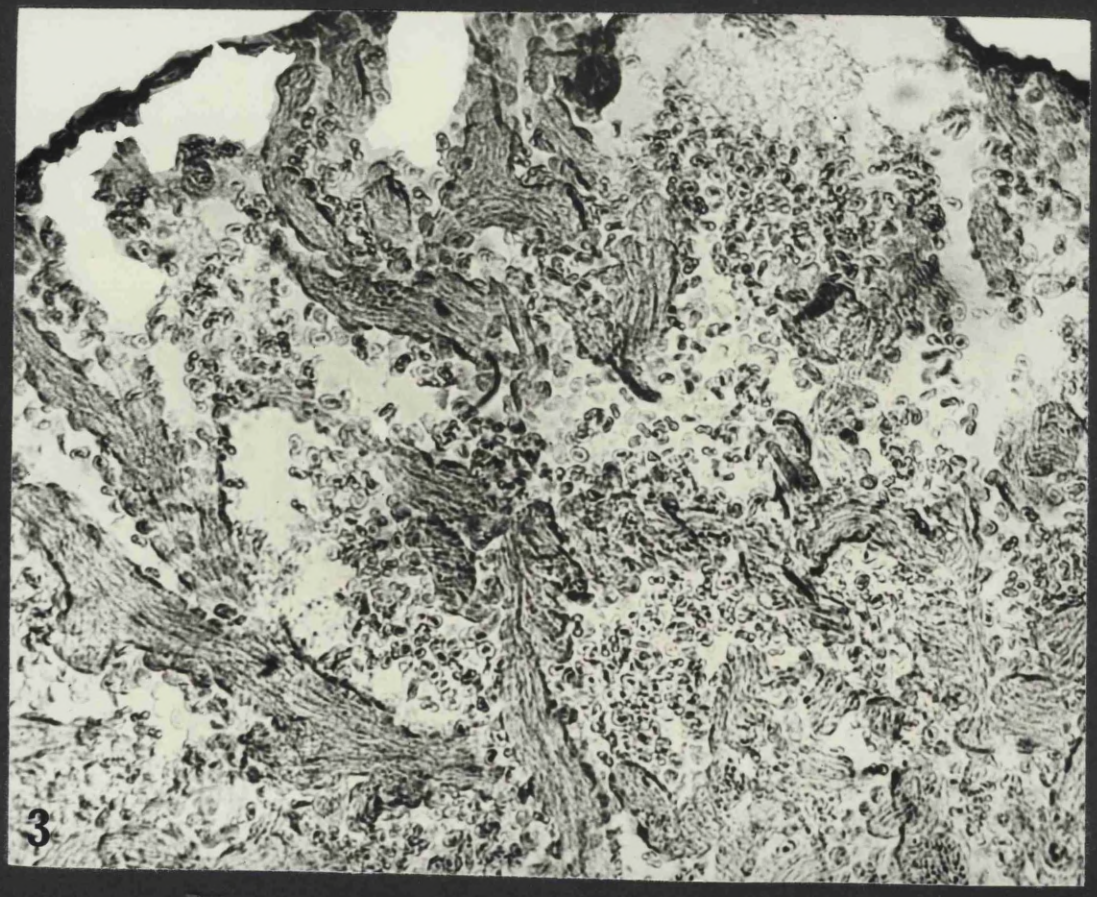
run in tracts as Purkinje cells do in a conducting system. Their distribution appears to be random. Another point of note is that the diameter of these "light" cells is in the same range as the plaice "working" myocardial cells and also that they are usually seen individually and not in bundles of closely apposed cells. Fibres of a large diameter have a faster conduction velocity than smaller ones and large mammals with correspondingly larger hearts have significantly larger diameter Purkinje fibres than smaller mammals (Sommer & Johnson, 1968). On this basis the plaice "light" cells do not have any selective morphological advantage favouring an increased conduction velocity over the "working" cells. Whilst it is hazardous to attempt to correlate morphological features of cardiac tissue with the heartrate, it could be tentatively suggested that in an animal with a small, slowly beating heart (40-50/min) like the plaice, there is no need for a special conduction system in the ventricle, and that electrical coupling of the cells is fast enough to make such a system redundant. Alternatively, the lack of a conduction system might be a primitive feature of lower vertebrate hearts. However, the effect of a conducting system in mammals with large hearts is to coordinate the contraction of the very large amount of ventricular muscle into an effective pump for the expulsion of blood from the heart. Perhaps the small size of the plaice heart and its slow rate of beat obviate the necessity for a conducting system with respect to this factor.

Figure 3.

Section of the atrium to show the loose network of trabeculae to which many erythrocytes have adhered. The darkly staining tissue is sub-epicardial connective tissue. Orange G and aniline blue stained. Magnification: X350.

Figure 4.

Section of the ventricle to show the dense packing of trabeculae of the ventricular myocardium. Note that there is no outer compact layer of myocardium in the ventricle, and that the trabeculae are juxtapposed to the sub-epicardial connective tissue. Orange G and aniline blue stained. Magnification: X420.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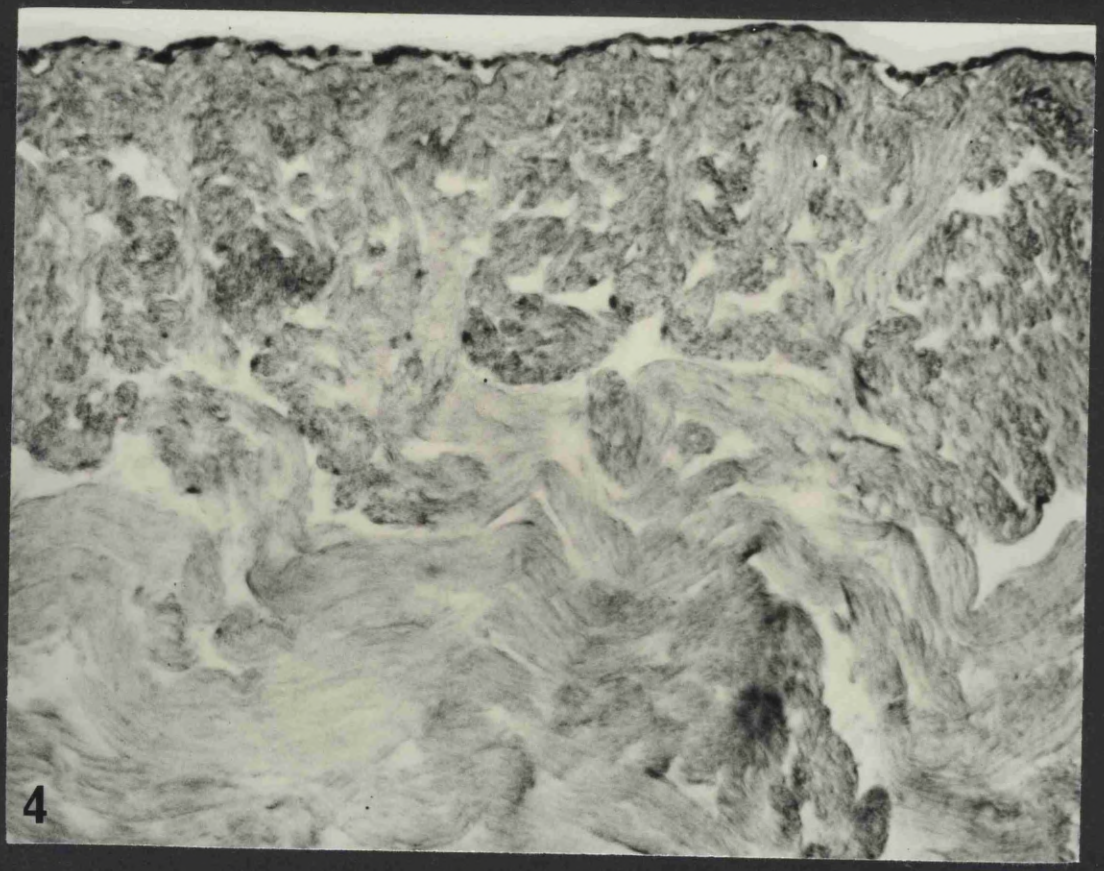
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Figure 5.

Longitudinal section through the sino-atrial junction illustrating the abundance of connective tissue (darkly stained) at this junction. SV, sinus venosus; A, atrium. Orange G and aniline blue stained. Magnification: X280.

Figure 6.

Section through the atrio-ventricular junction showing the connecting strand of cardiac muscle (asterisk) between the two chambers. A, atrium; V, ventricle; R, valve; Orange G and aniline blue stained. Magnification: X420.

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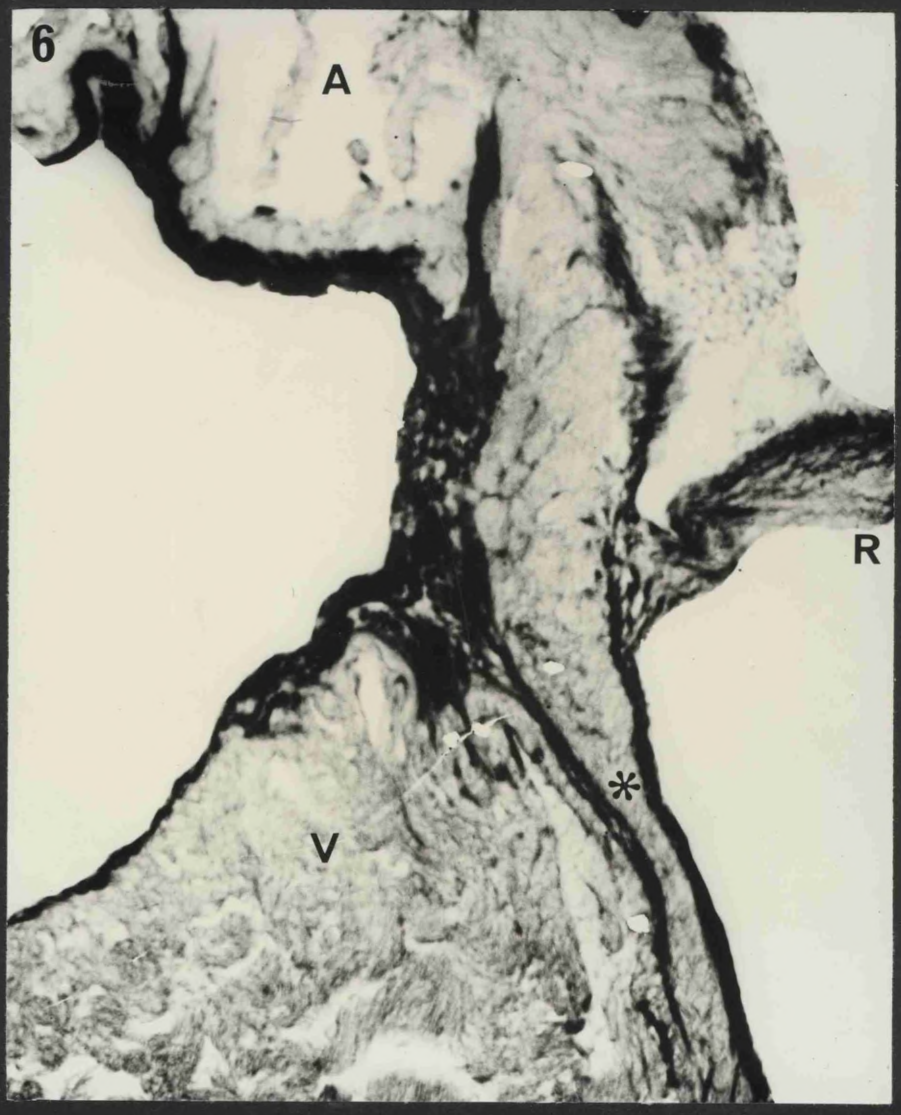
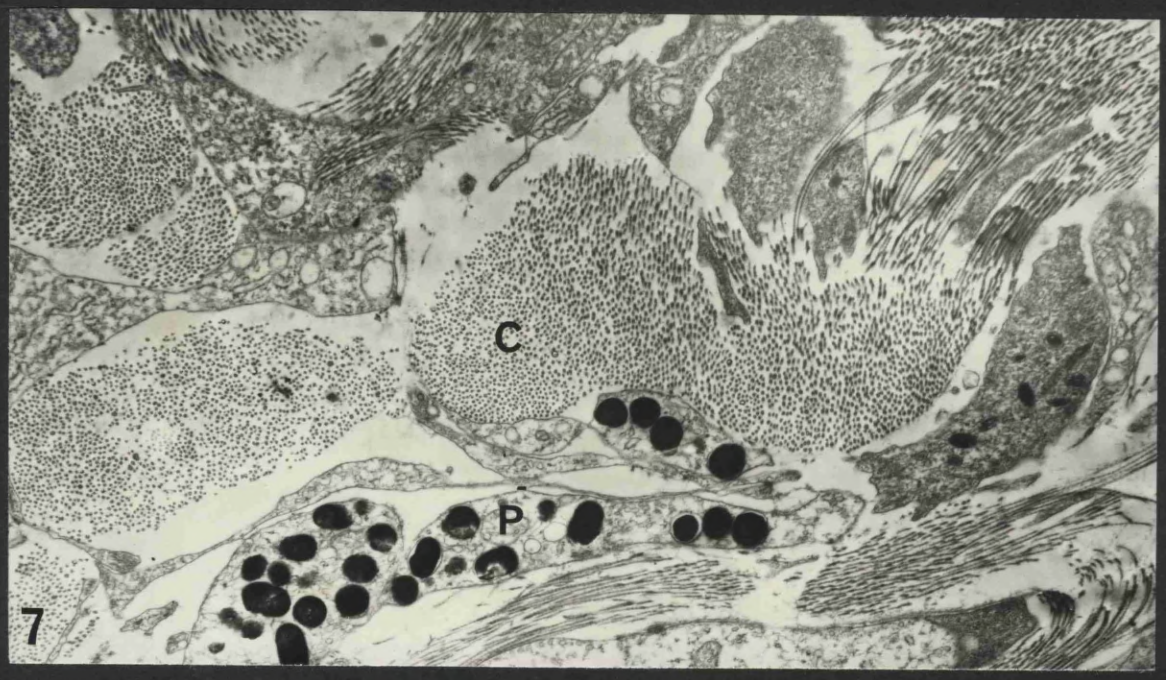


Figure 7.

A chromatophore (P), collagen bundles (C) and connective tissue cell processes in the sinus venosus. Magnification: X10,000.

Figure 8.

A small bundle of myocardial cells (M) in the sinus venosus. The muscle bundle is heavily innervated by unmyelinated nerves and is surrounded by connective tissue. The nerves on the left hand side are part of the cardiac plexus. Magnification: X11,000.


C
P
7

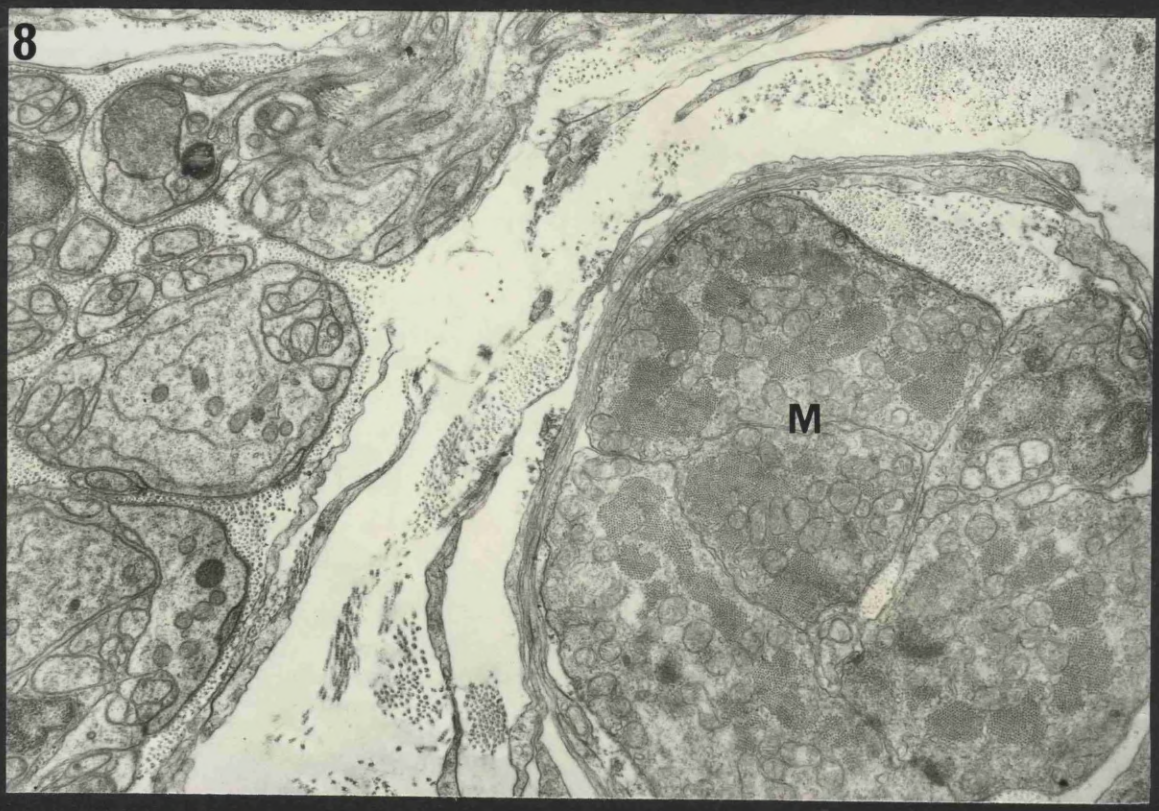

8
M

Figure 9.

Muscle cells and nerves in the sino-atrial region. The muscle cells are small in diameter (average 1.76μ) and are individually surrounded by an external lamina. Lightly myelinated axons (N) pass through this region. Magnification: X21,000.

Figure 10.

Diagram illustrating the structural arrangement of the atrium and ventricle. EC, epicardium; S, sub-epicardial space with connective tissue; T, trabeculae; M, myocardial cells; NC, endocardium; EI, endocardial invagination.

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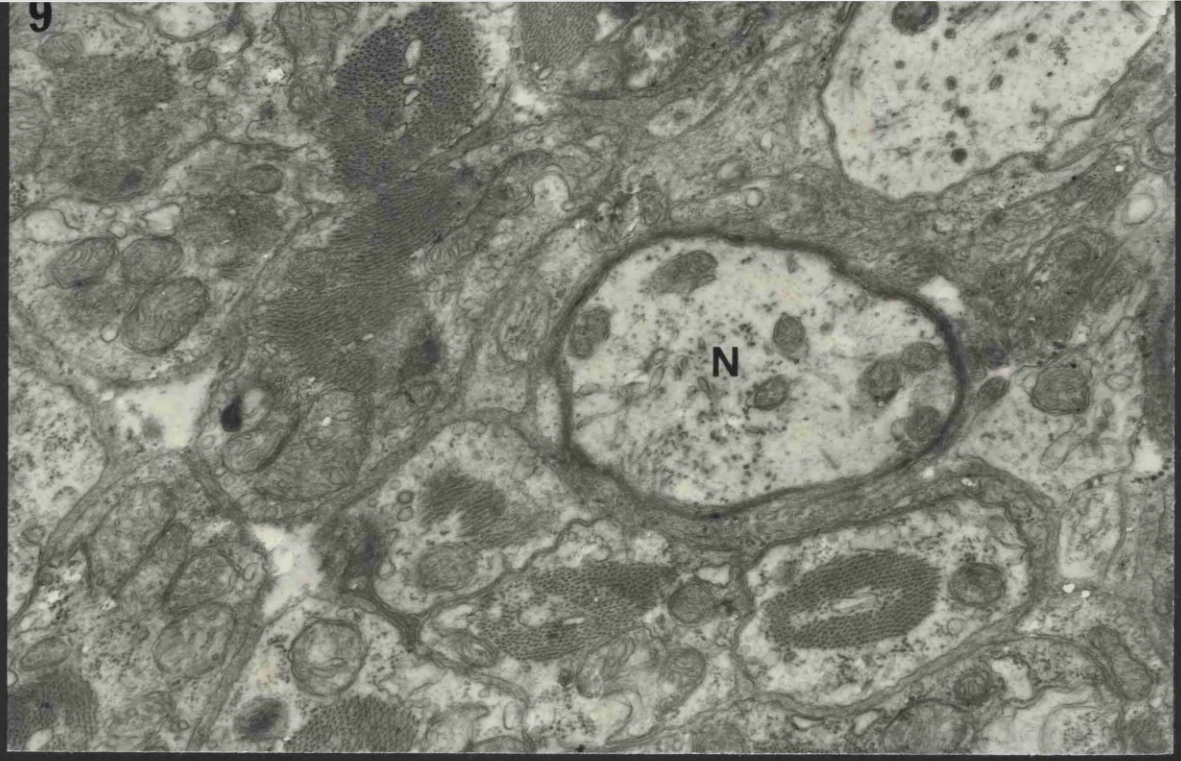

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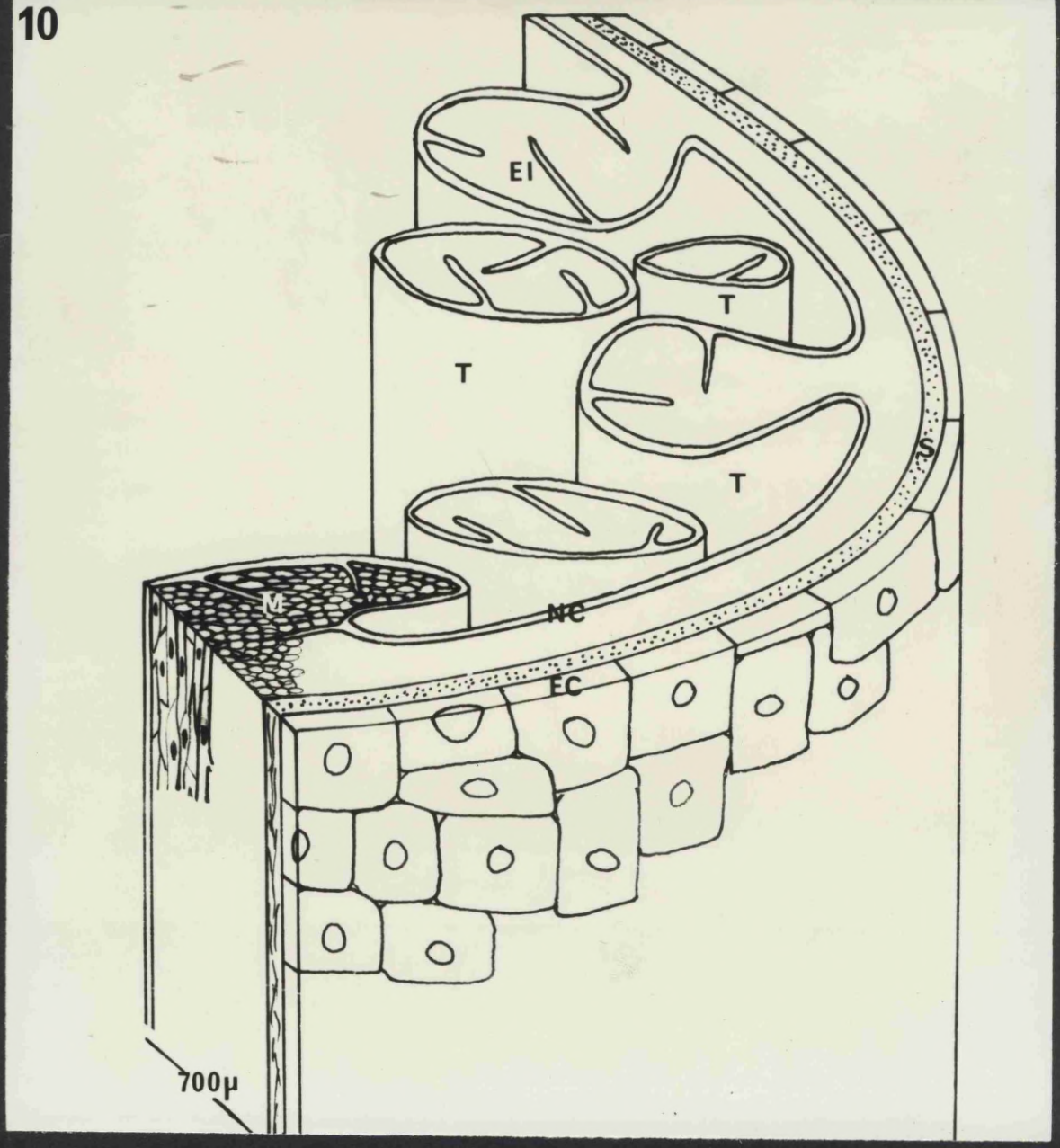


Figure 11.

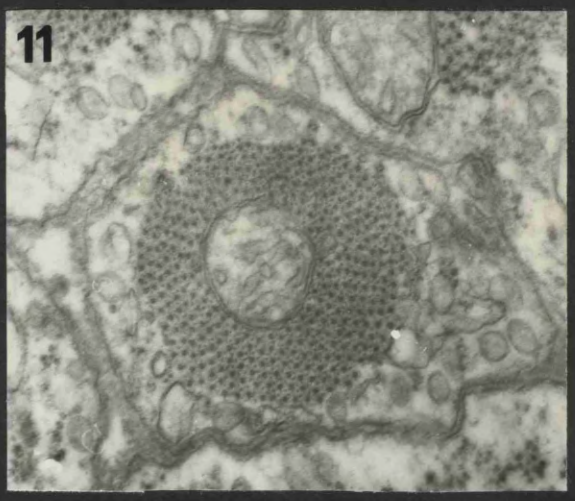
Section through an atrial myocardial cell to show a tube of myofilaments enclosing a mitochondrion. Note the external lamina around the sarcolemma. Magnification: X45,000.

Figure 12.

Myofilaments in an atrial cell. Note the standard array of six thin, actin filaments surrounding a single, thick myosin filament. Magnification: X70,000.

Figure 13.

Longitudinal section through an atrial myocardial cell showing the myofibrils (M) and closely packed mitochondria between them. The sarcolemma (S) is convoluted and, at certain points, intercalated discs (D) are visible. Note the aggregation of large granular vesicles (V) within the cytoplasm. Magnification: X24,000.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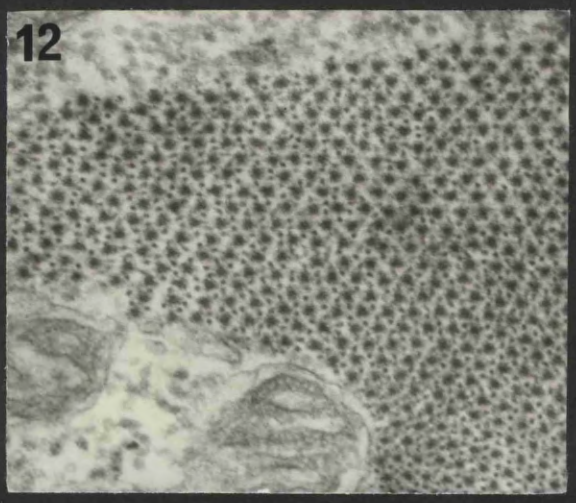
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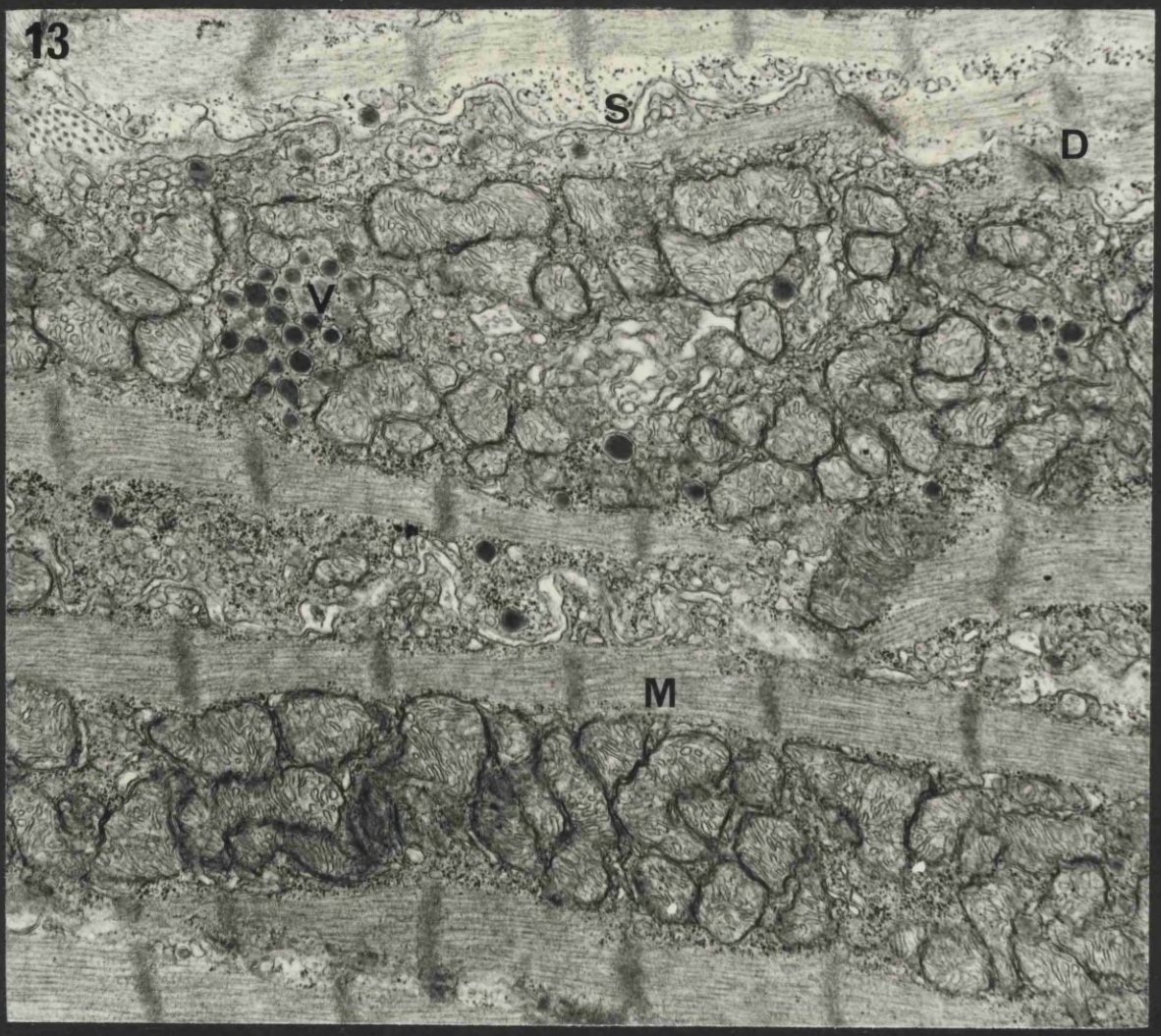
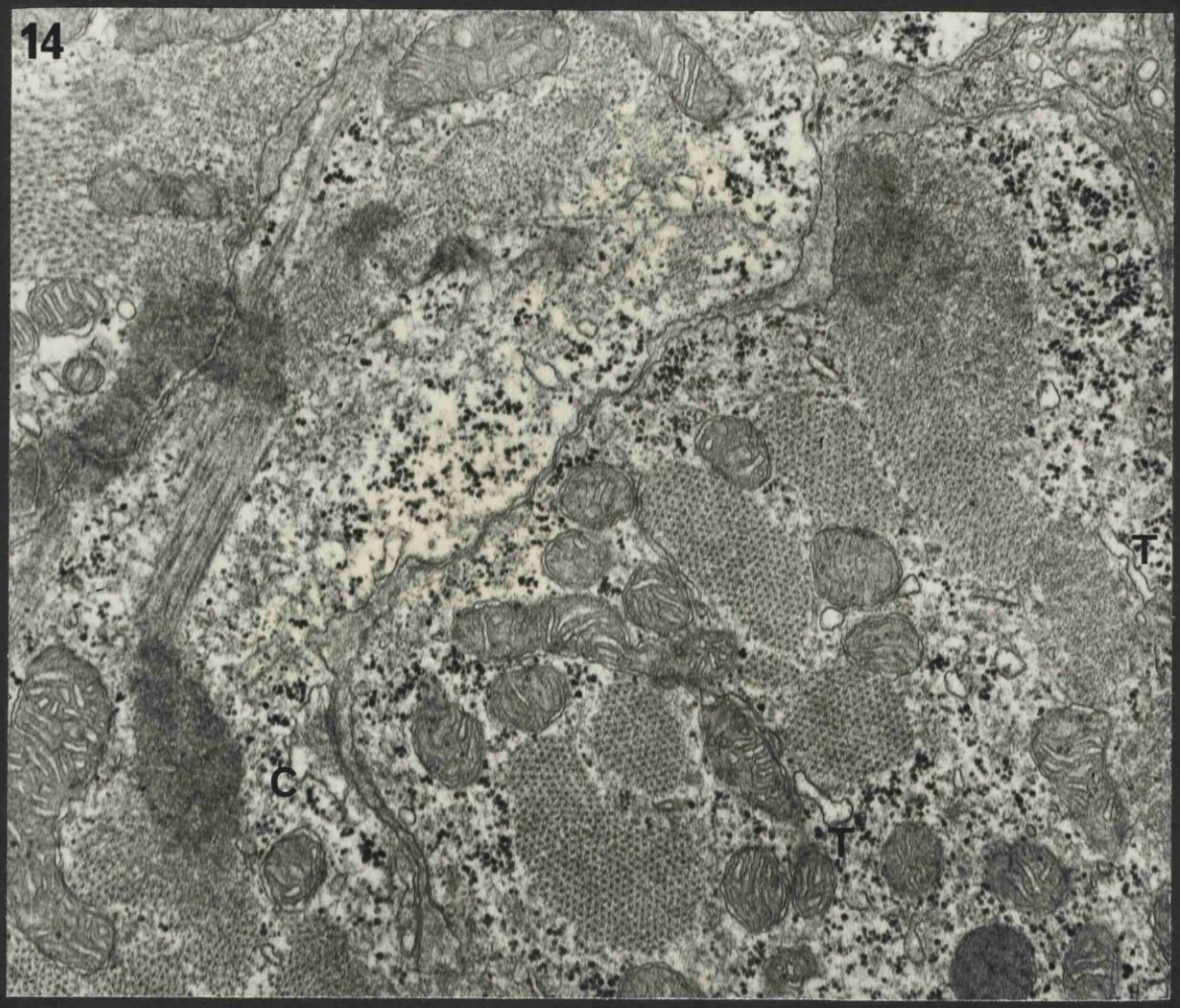

13
S
D
V
M

Figure 14.

Atrial myocardial cells. The cells are not completely filled with myofilaments and mitochondria and the remaining cytoplasm is filled with densely staining glycogen granules. Note the small, simple intercalated discs composed solely of fasciae adhaerentes. The very s parse sarcoplasmic reticulum is present as tubules (T) within the cytoplasm and as subsarcolemmal terminal cisternae (C). Magnification: X34,500.

Figure 15.

Ventricular myocardium. This electron micrograph illustrates the different cell types found in the ventricle. Most cells are tightly packed with myofibrils and mitochondria (cells marked F) whilst certain others have a "light" appearance due to the large amount of free cytoplasm (cells marked L). Such cells occur randomly throughout the myocardium and do not run in tracts. Magnification: X22,500.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T
C
T

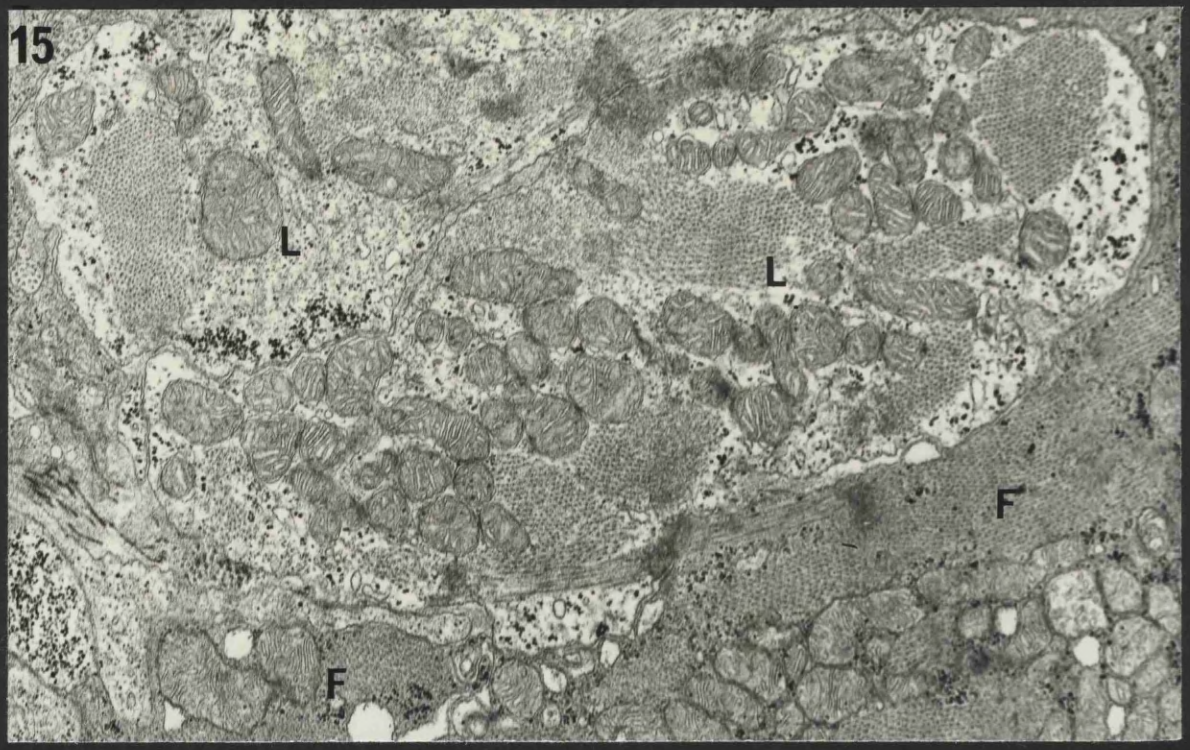
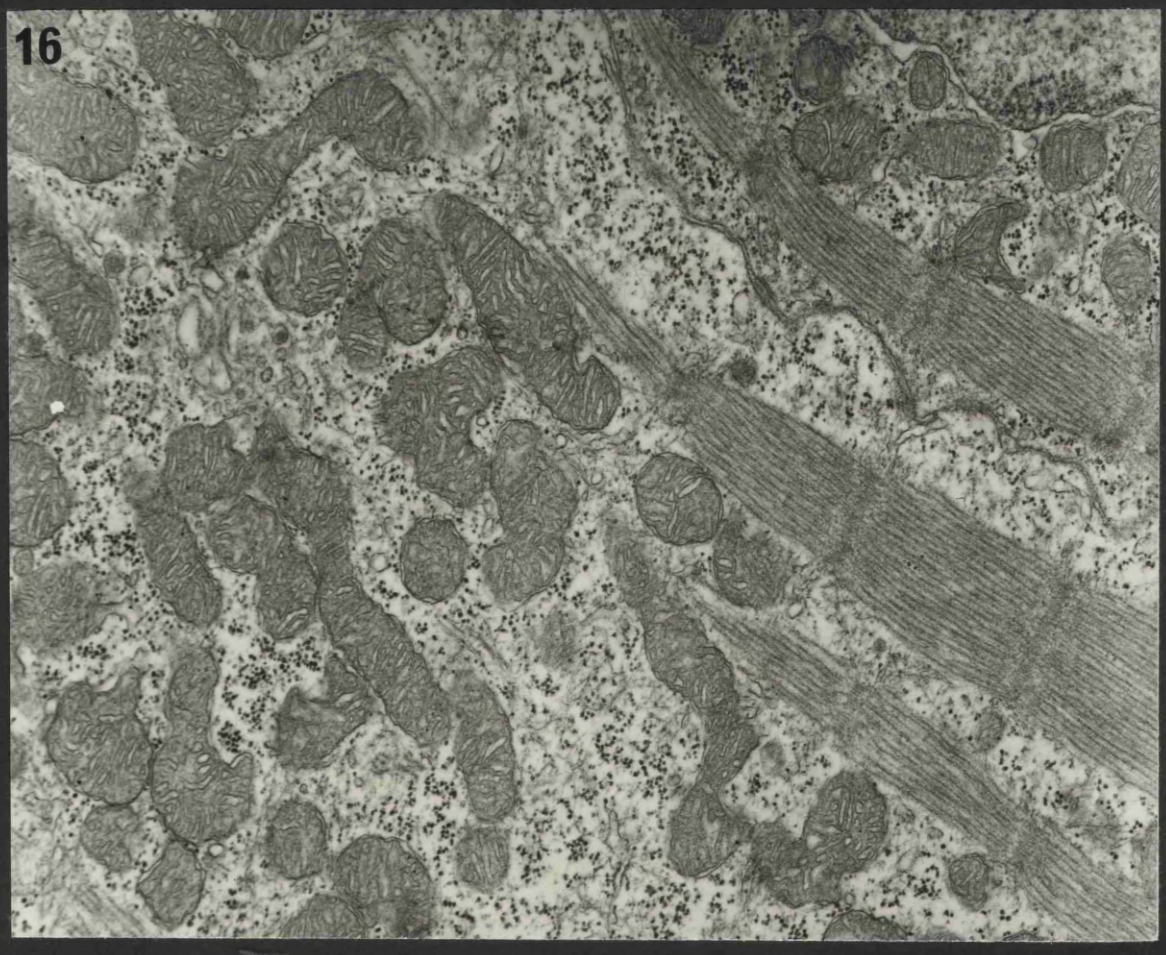

15
L
L
F
F

Figure 16.

Atrial myocardial cells. Note the small amount of myofibrils and mitochondria in this tissue compared with the ventricular cells in Figure 17. Magnification: X23,500.

Figure 17.

Ventricular myocardial cells cut obliquely. In comparison with the atrial cells in Figure 16, these cells are completely filled with myofibrils and mitochondria and also contain a large number of membrane limited vesicles which are probably lipid droplets. The glycogen is aggregated within the cytoplasm and also occurs between myofilaments. Magnification:X20,000.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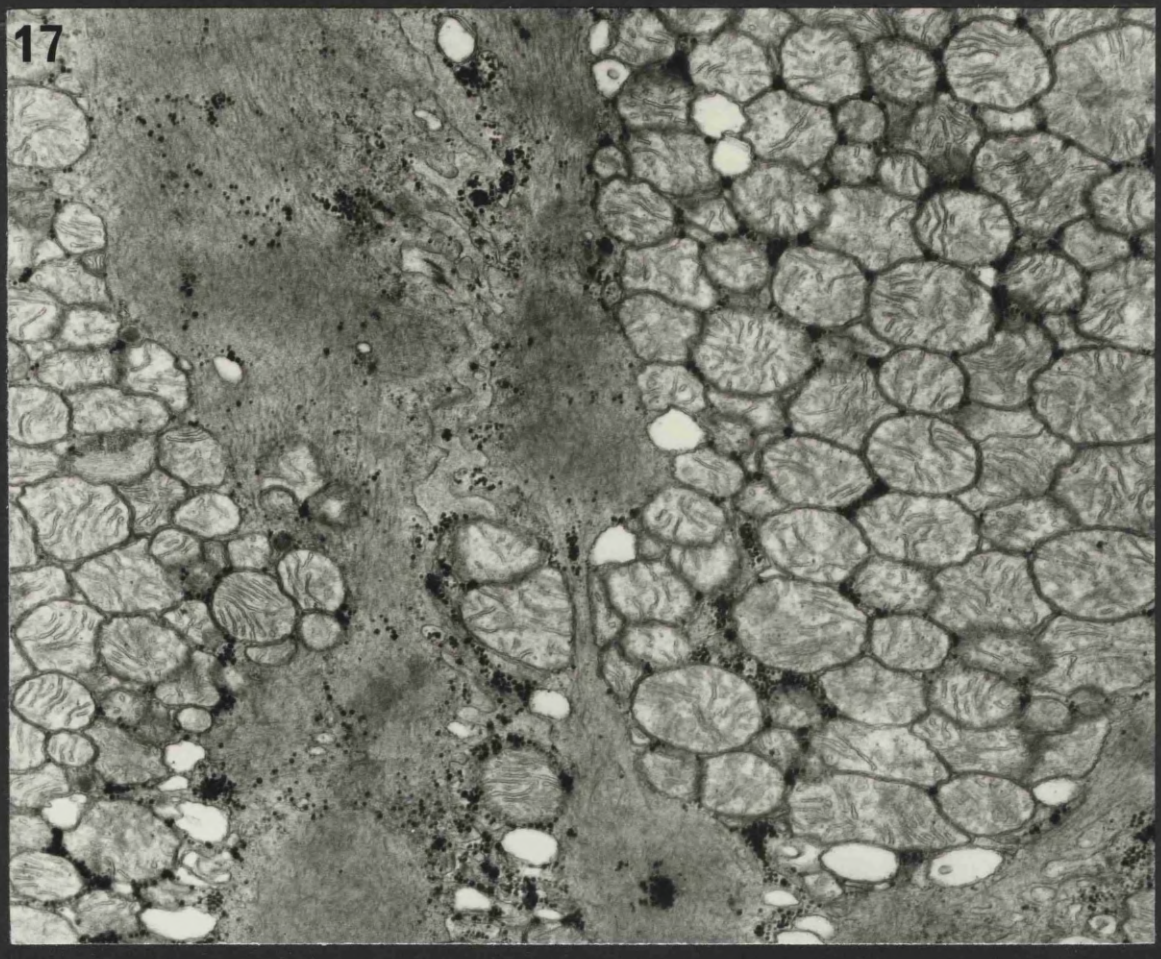
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Figure 18.

The atrio-ventricular junction showing the base of the A-V valve (V) and the profusion of collagen (C) in the sub-endocardial space. E, ventricular myocardium. Magnification: X11,500.

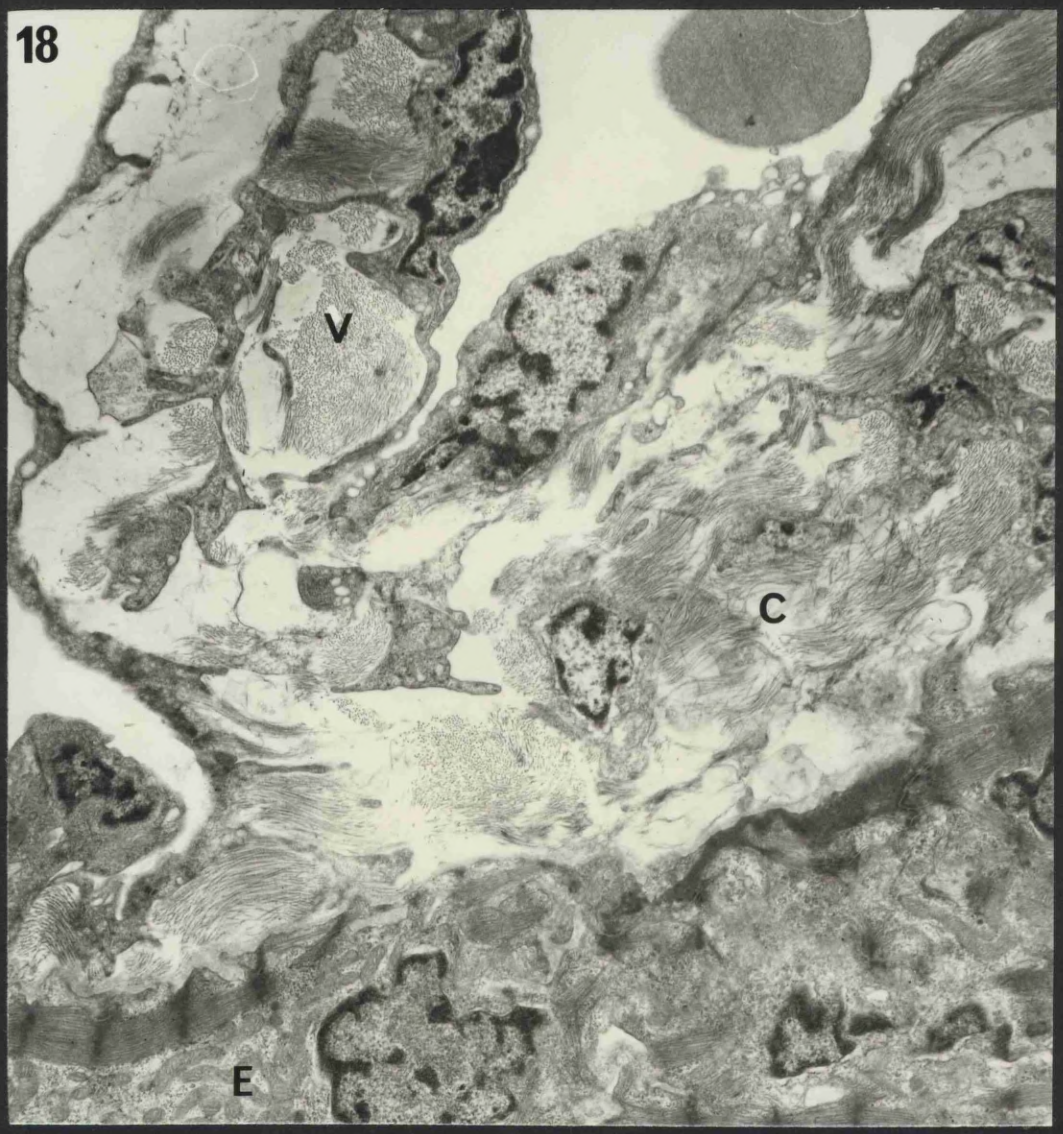

18
V
C
E

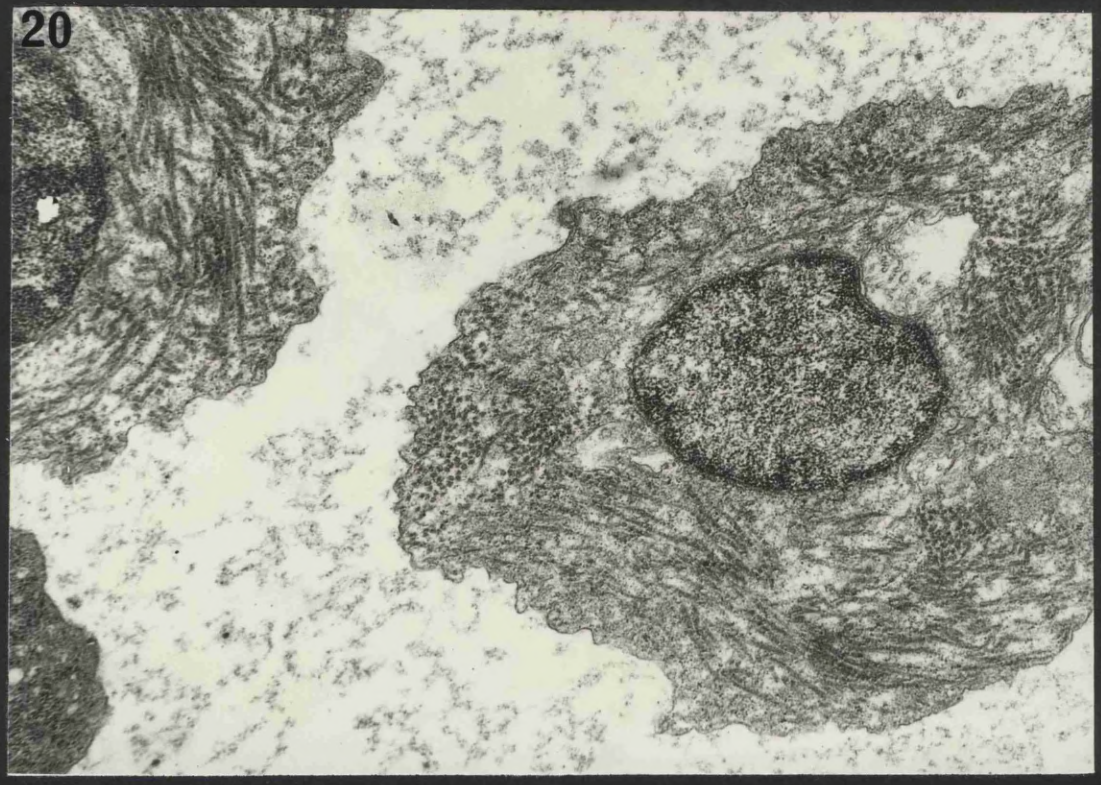
Figure 19.

Section of the bulbus arteriosus showing a matrix of elastin tissue (E) in which is seen many fibrocytes (F). The fibrocytes are concentrically arranged within the bulbus arteriosus, their alignment being indicated by the arrow. Magnification: X14,000.

Figure 20.

Cross-section of two fibrocytes showing lengths of collagen within the cytoplasm. Note the irregular contour of these cells. Magnification: X15,500


19
E
F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IV. DEVELOPMENT OF THE HEART

1. INTRODUCTION

i) Development of plaice

Plaice spawn from late February to mid-April in the North Sea. The fertilized egg hatches after 14 or 15 days into a pelagic larva which is entirely dependent upon its nutritive yolk-sac. During the succeeding ten days, the yolk-sac is resorbed and the larva subsequently feeds on planktonic crustacean larvae. At North Sea temperatures the larval life, which terminates upon the completion of metamorphosis, is about sixty-six days (Ryland, 1966). Throughout virtually all of their larval life, larval plaice are active midwater swimmers but towards the end of this stage in their life they lie upon the seabed whilst metamorphosis is completed. The deep body shape of the flatfish and the asymmetry of the eyes develop during the latter part of the midwater stage with the result that, when the larvae take to a benthic life, the left eye has migrated to the upper side of the fish. Metamorphosis is completed within the succeeding fortnight.

ii) Previous work

Apart from studies on myofibrillogensis in both skeletal and cardiac muscle, ultrastructural studies of cardiac development have not been numerous, most attention having been paid to mammalian (Muir, 1957; Pager, 1968; McNutt, 1970) and avian (Wainrack and Sotello, 1961; Olivo et al. 1964; Manasek, 1968, 1969, 1970) myocardia. Manasek's series of papers on the myoblast cytodifferentiation and histogenesis of the chick heart has provided a very detailed picture of cardiac development in the avian embryo. The ultrastructure of cardiac development, in lower vertebrates has been neglected apart from Huang's

(1967) paper on myofibrillogenesis in the frog heart.

There have been no ultrastructural studies on the development of the teleost heart which, though morphologically more simple, shows a similar histological organisation to the hearts of higher vertebrates. However Gros and Schrevel (1970) compared the ultrastructure of the ventricular myocardium of the urodele Ambystoma with that of its neotenic larva, the axolotl, and demonstrated that there are fewer myofibrils per myocardial cell and more glycogen in the axolotl than in Ambystoma, the inference being that the neotentic condition is manifested at an ultrastructural level as well as in the general morphology of the animal. Those axolotls that were artificially induced to metamorphose by thyroxine injection showed typically adult ultrastructural myocardial tissue when examined post-metamorphosis. The following study on the teleost heart was carried out in order to examine the structure the larval heart for comparison with the adult, to determine the time on onset of the adult condition and the temporal relationship of the development of the heart with the state of development of the fish.

Senior's (1909) light microscopical study of cardiac development in the shad (Alosa sapidissima) pays special attention to the origin and movements of the presumptive endocardial cells during formation of the heart-tube. Also, it is evident from studies on Fundulus heteroclitus (Armstrong & Child 1965) that the early stages in the process of heart formation differ temporally with respect to the number of somites present in the embryos of different teleosts.

iii) Development of the heart-tube

In order to provide a background to the present study, a summary of events leading up to the formation of the heart tube is given as follows.

The part of the mesoderm from which the endocardium is derived is a bilaterally symmetrical strip of cells on either side of, and immediately adjacent to the median borders of the lateral plates. The lateral plates grow inwards towards the ventral embryonic midline where they fuse. At the time of fusion the presumptive endocardial cells have moved to the edge of the advancing lateral plates so that their fusion brings the two presumptive endocardial cell populations together. This union of the lateral plates occurs first at the anterior end around the lateral and posterior circumference of the aortic root. Thus the bulbus end is formed first and, as the lateral plates fuse in a cephalo-caudal direction, the ventricular and atrial regions of the heart-tube form sequentially. The sinus venosus forms later. Hence the bulbus arteriosus has a thin lining of endothelium directly continuous with the endothelium of the ventral aorta.

The primitive heart tube forms from the presumptive endocardial cell mass, due to a lumen forming at the centre, lined by a single layer of endocardial cells. A second layer of cells forms concentrically on the outside of the endocardium which is the myocardial cell layer. This myocardium, initially a monolayer, soon increases in thickness to become several cells thick by the time the heart-tube has formed completely. Thus the primitive heart-tube is conically shaped, the thicker portion being at the bulbo-ventricular end. From the beginning, the endocardial and myocardial cell layers are separated by a wide extracellular space containing the cardiac jelly. This is a gelatinous substance predominantly consisting of sulphated

acid muco-polysaccharides which Manasek (1970b) has shown to be formed by the myocardium and not by the endocardium as had previously been suspected in the chick embryo. Thus the myocardium has a secretory as well as a contractile role in avian cardiac development.

By the time the heart-tube is almost complete, the first irregular contractions can be observed which soon become more regular. The embryo has grown sufficiently by this stage to lift the body clear of the yolk sac in order to allow room for the cardiac pulsations. Since the heart tube forms in a cephalo-caudal direction with the sinus venosus forming last, and since cardiac pulsations begin before formation of the sinus venosus, (that part of the heart to which the innervation grows) it is evident that heartbeat in fishes is of a myogenic origin.

This study concerns the development of the heart from the early "heart-tube" at the stage at which myofibrillogenesis has begun in the myoblast. The developing heart has been examined throughout the larval life of the fish as far as two months post-metamorphosis when the adult structure is attained. Studies on myofibrillogensis were not undertaken since there is much more suitable experimental material to be found in the chick (Manasek 1968a; Rash et al. 1970) in which the developing heart is larger and more accessable than in the fish embryo.

2. RESULTS

i) Gross morphology of development

On Day 9 (five days prior to hatching) the tubular heart was beating irregularly and was situated in the ventral midline just posterior to the eyes. By Days 10-11 the heart was markedly coiled to the left at its posterior (atrial) end. It consisted of a single myocardial cell layer and an inner single endocardial layer, the only difference between the two layers was that the myocardial cells are more closely packed than the endocardial cells. The two layers were widely separated by a layer of cardiac jelly, which gradually was reduced in width until, by Day 14 (hatching period) the two cellular layers were only narrowly separated (approx. 1μ apart) by the cardiac jelly. By Day 15 (stage 1a Ryland, 1966), the heart consisted of two apposed cell layers. At this stage the posterior (atrial) end of the tubular heart was only one cell thick whereas the ventricular end was 6-8 cells thick. The epicardium formed later during the 1d stage.

Growth of the heart was rapid, the ventricular end soon becoming distinctly thicker and by the 3a stage the heart had assumed an adult appearance, the initial coiling having resulted in the positioning of the atrium above the ventricle and formation of the bulbus arteriosus being complete. The first signs of trabeculae were visible in the ventricle at stage 2a when the luminal surface was seen to be convoluted. The apex of the ventricle was visible by the 2b stage. The mesenchymal cell component (the fibrocytes and their associated connective tissue) took much longer to ramify throughout the heart and it was not until the 4b stage that the atrioventricular valves formed. Initially subendocardial collagen appeared at

the 1d stage and subepicardial collagen at the 4b stage. By the 4c stage the heart was morphologically complete, the atrial and ventricular trabeculae being well developed. Subsequent growth of the tissue followed, together with a slight increase in the number of atrial trabeculae but this was completed at the end of stage 5, when metamorphosis of the larva was complete. The position of the heart gradually moved backwards until by stage 3c it assumed the adult position in the ventral midline immediately under the opercular slit close to the gills.

ii) Fine structure of the developing cardiac tissue.

The functional tubular heart on Day 13 (pre-hatching) consisted of myocytes tightly packed together resting on a basal lamina situated on the luminal side. This basal lamina separated the myocytes from the cardiac jelly (Davis, 1924) which filled the lumen of the tube and had a flocculent appearance (Fig. 21). The outer surface of the myocytes was slightly convoluted and uncovered. Myofibrils were present, though only a few sarcomeres in length, in greater numbers on the luminal side of the myocytes (Fig. 22) but they were not restricted to this region of the cells. Nash et al. (1970) pointed out that in the early embryonic chick heart the association of the first-formed sarcomeres with the sarcolemma is quickly obscured by the development of sarcomeres throughout the cell. Nevertheless it is worthwhile noting that in fish the most extensive and complete sarcomeres were peripherally situated within the cell at this stage. This was also indicative that the subsarcolemmal region is preferentially used for myofibrillogenesis than the more central portions of the myocytes. The myofibrils were narrow (0.4μ) inserting into

simple intercalated discs near the edge of the cells. According to Huang (1967) these intercalated discs may be derived from terminal bars of the foregoing myoblasts, but few terminal bars between adjacent myocytes were observed in this study. However, the most obvious feature of the myofibrils at this stage was their very random orientation throughout the cell. Myofibrils at right angles to each other were present at the luminal side whilst in the more central regions of the cell, their arrangement was totally random. The more mature myofibrils on the luminal side were presumably those upon which cardiac contractions rely at this stage. The cytoplasm of the myocytes contained mitochondria interspersed amongst the myofibrils, a few small vacuoles and many ribosomes in rosette form which can presumably be related to the continuing synthesis of contractile proteins. Golgi bodies were observed close to the nucleus but not related to any specific granule. Single cilia were observed occasionally projecting into the lumen of the heart-tube, but they were not as common as in comparable avian tissue (Manasek, 1968a). The myocyte nucleus was elliptical with an irregular contour and had diameters of approximately 2μ and 3μ, thus filling a greater proportion of the cell volume than in the adult myocardial cell (Fig. 25). The double nuclear membrane was often separated by a gap of up to 500Å to give a perinuclear space of that width (possibly a fixation artifact), but discontinuous due to the nuclear pores. The nucleoplasm had a homogeneous, granular nature with centrally placed clumps of chromatin material.

By Days 14-15 (stage 1a), immediately after hatching, the myocytes were in contact with the endocardium at the ventricular end but it was not until Day 19 (stage 1c) that the

whole of the luminal surface was lined with endocardium (Fig. 23). The cells of the endocardial monolayer had a large, centrally placed nucleus and cytoplasm containing small clear vesicles, smooth endoplasmic reticulum, multivesicular bodies and mitochondria. Endocardial cells at stage 1c contained centrioles and prominent Golgi apparatus. Beneath the luminal membrane of the endocardial cells were many small vesicles similar to those thought to be involved in the transport of macromolecules into the mammalian endocardium by pinocytosis (Shirahama & Cohen 1972). There was a very electron dense basal lamina on the luminal surface of the endocardial cells. Desmosomes were the only type of intercellular connections between these cells. In the adult myocardial cells there is a very sparse sarcoplasmic reticulum and whilst it is difficult enough to identify this subcellular system in the adult, to do so in the developing heart presented even greater difficulty. Even so, around stage 1a, short extents of irregular tubules with no attached ribosomes were seen in the myocyte cytoplasm, often located in the subsarcolemmal region and it is conceivable that these may be parts of the SR.

At stage 1a the myocardium was thicker and the myocytes interdigitated, in some cases to a considerable extent. In the thinner portion of the tubular heart the myocytes were closely packed together whilst at the thicker ventricular end, intercellular spaces were apparent. The myocyte profiles had an average diameter of 2.35μ which remained fairly constant throughout larval life but the myocytes had not assumed their final shape. The myofibrils were still randomly arrayed, even though many short lengths were attached at varying angles to intercalated discs which consisted of desmosomes and fasciae

adhaerentes and some of which were more convoluted than the adult state. A single 'Z'-centre (Weinrack and Sotello, 1961) often had sarcomeres radiating from it in more than one plane, a condition described as abnormal by Manasek (1970a) in the chick, but common in this teleost tissue (Figs. 25,27). Occasionally, intercalated discs were observed in conjunction with Z bands of myofibrils in two adjacent myocytes (Fig. 27), as in the adult condition. There was a proliferation of myofibrils within the myocytes but there still remained free cytoplasm filled with glycogen in rosette form. Pools of glycogen were not evident, the glycogen being evenly distributed throughout the cells. Desmosomes were the only other intercellular attachments. Centrioles were seen in the myocytes at this stage (Fig. 24) in agreement with Przybylski's (1971) observation of their presence in chick myocytes throughout differentiation as compared to their restriction to myoblasts in developing skeletal muscle. Manasek (1968b) and Rumyantsev & Snigirevskaya (1968) have shown that mitosis can occur in cardiac myocytes in which myofibrils can be observed ultrastructurally, and Pryzbylski & Chlebowski (1972) have shown mitosis and DNA replication, in actively contracting myocytes in vitro. Throughout the present study, no mitotic divisions were recognised in the developing myocardium despite the presence of centrioles. This absence of observable mitosis suggests that there may be very active periods of cell division of limited duration, with long interphase periods, as is seen in the developing chick gizzard (Bennett & Cobb, 1969b; Cobb and Bennett, 1970). These authors note very active periods of mitosis in the smooth muscle of the gizzard on day 6 and then not until pre- and post-hatching periods. Thus, if a similar

situation exists in the developing teleost heart, the active periods of mitosis could easily be missed in the tissue samples during the much longer teleost larval life.

By stage 1d the myocytes were packed with myofilaments interspersed with mitochondria. Glycogen granules filled up the remaining intracellular space sometimes forming pools. The myofibrils were now predominantly arranged in parallel within a cell although there were still occasional examples of stellar sarcomere configurations around Z-centres. In the ventricular tissue, there were large (1200Å) membrane-bound intracellular vesicles, typical of the mature myocardium and an increase in intercellular spaces.

The epicardium covered the heart as a monolayer of flat cells with prominent nuclei by the end of stage 1d (Fig.26). A subepicardial space approximately 1.5μ in width was filled with uneven fine flocculent matter but no subepicardial collagen was seen at this stage. Small extents of collagen were found in the subendocardial space but there was no mesenchymal cell component of the myocardium. There was no indication of the epicardium originating by de-differentiation of outer myocardial cells (Romanoff, 1960) and thus it would seem that this layer of cells is derived from a source other than the myocardium as in the avian embryo (Manasek, 1969a). The myocardium was still a homogeneous tissue consisting solely of myocytes, save for an occasional unmyelinated axon profile at the atrial end (see later).

Ten days later, at stage 2a, the myocardial cells themselves had changed little, save for the appearance of desmosomes as intercellular attachments. The Z bands often appeared double and very broad which was unusual for fully

developed cells. Occasionally myocytes in a primitive state were still seen which indicates that de novo differentiation of myoblasts as well as cell division (Manasek, 1968b) took part during the progressive growth of the teleost heart. It was at this stage that the luminal surface of the heart became slightly convoluted, this being the first indication of trabeculae formation.

By stage 4b-4c the heart was morphologically complete and it was interesting to note that there was very little increase in size of the heart from the beginning of stage 4a until metamorphosis was virtually completed, despite the continuing growth of the larva itself. However there were considerable changes still to occur at the cellular level of the myocardium. After the end of stage 3c, there was an advance in the organization of the myocardial cells in that the majority of the myofibrils were now well aligned and peripherally situated within the cells. There was a proliferation of mitochondria which now filled the centre of the cells and approached a mature arrangement of organelles. The width of observed myofibrils had also increased, in some cases to twice that of the first-formed myofibrils (approx. 0.7μ) and H-bands were evident (Fig. 28).

The most noticeable advance at the 4a-4b stages was the loss of homogeneity of the myocardium due to invagination of the endocardium amongst the myocardial cells. This invagination seemed not to be prompted by the formation of trabeculae since the onset of trabeculation was a prior event, and also trabeculae lacking an endothelial covering were seen at this and later stages. A mesenchymal cell component of the myocardium was also present in the form of many so-called

phagocytic cells characterised by large vacuoles, comparatively electron-dense cytoplasm and a highly irregular cell contour. They were seen in inter-trabecular spaces and intercellular channels (Fig. 29). Whilst the plaice heart does not have a coronary blood supply there was a very diffuse system of intercellular capillary-like channels in the ventricle, often lined by endocardium and it was often in these channels that many phagocytes occurred. The epicardium did not invaginate into the myocardium in the teleost heart but remained distinct, separated from it by a thin layer of collagen and occasionally fibrocytes and nerve axons in the subepicardial space.

Large granular vesicles of 1200Å approx. diameter become more evident at the 4b stage. They were present in both the atrial and ventricular myocardia and closely resembled the atrial bodies described by Jamieson and Palade (1964) and those occurring in most vertebrate myocardial cells. They have been implicated in a secretory role of developing myocardial cells (Manasek, 1969c). Whilst it appeared that in general, development of the heart is a gradual process throughout the embryonic and larval life of the fish, immediately following metamorphosis there was a period of markedly increased cellular activity in the myocardium. In the later larval stages the morphological arrangement of the heart was almost complete and, at a cellular level, the myocardial cells showed the adult organization of cellular components. However, before reaching the totally adult condition, further changes took place during the two months succeeding metamorphosis which are perhaps analogous to the neonatal state of higher vertebrates. First, more intercellular contacts were

made: there was an increase in the number of desmosomal contacts and also an increase in the number of intercalated discs between the lateral sarcolemmae of adjacent cells (Fig. 32). These 'lateral' intercalated discs contained regions of both desmosome and fasciae adhaerentes. It should be mentioned that throughout this study of cardiac development, no nexuses (fasciae occludentes) were observed in conjunction with the intercalated discs or at any other point between adjacent sarcolemmae. Nexuses have not been seen in the adult plaice heart but they do occur between both embryonic and adult myocardial cells of the chick (Spira, 1971) and the mammal (McNutt, 1970).

Second, morphological changes in the mitochondria occured, the most obvious of which was change to the oval shape and an increase in the number of the mitochondrial cristae. Thus, for a period of approximately six weeks following metamorphosis the mitochondria assumed the appearance of those of the rodent heart (Fager, 1968; Hagopian and Tennyson, 1971). There was also an increase in the number of mitochondria per unit area (Fig. 30). At the same time as this increase in the number, some mitochondria were observed exhibiting a dumb-bell shape (Fig. 31). The shape of mitochondria in the heart of newly-metamorphosed fish was oval as opposed to the cigar-shaped mitochondria of the adult.

Third, there was a great increase in the amount of glycogen in the myocardial cells which occurred abundantly in all areas of free cytoplasm and also amongst the myofibrils. (Fig. 31).

Fourth, there was an increase in cell diameter during the two months post-metamorphosis. Throughout larval life, the

average myocardial cell diameter was 2.35μ with a range of diameters (taken over 35 measurements) of between 1.6μ - 4.0μ. In the post-metamorphosis heart, the average cell diameter was 3.35μ with a range of diameters from 2.0μ - 5.0μ which was very close in value to that of the adult heart myocardial cells of 3.2μ. No cell lengths could be measured.

iii) Development of the Innervation

Whilst the first contractions of the heart were seen on days 8-9, no ultrastructural indications of an innervation were observed until ten days after hatching at day 24 (stage 1c). At this stage, individual profiles of cholinergic type axons approximately 0.7μ in diameter and containing many small agranular vesicles occurred at the atrial end of the developing heart. (Fig. 33). These axons were in close (200Å) apposition to the muscle cells and thus already showing an adult relationship with these cells. No granular vesicles of the type (600-800Å in diameter) seen in axons of the adult atrial innervation were observed in the larval heart.

Throughout larval life, the innervation of the atrial myocardium was very sparse, axons being observed only rarely and in no instances were neuromuscular junctions seen. After metamorphosis, the density of innervation of the atrial myocardium increased and more axons were observed either individually or in small bundles (3-5 axons) (Fig. 35). It was only in these small bundles that any Schwann cell processes around the axons occurred, all individual axons remaining uncovered. The density of innervation of the adult atrial myocardium was also sparse, save for the S-A region where most cells are

individually innervated and by two months after metamorphosis, the innervation was complete. As in the adult, the larval ventricle was aneural.

The time of establishment of the cardiac ganglion was not discovered.

3. DISCUSSION

Whilst bearing in mind that the adult ultrastructure of the chicken and teleost myocardia differ in many respects, the overall pattern of development of the heart chambers of the two animals is essentially similar, despite the more primitive phylogenetic position of the fish. The description of the myocardium of neonatal mice by Edwards and Challice (1958) resembles closely that of the adult plaice with particular reference to the paucity of myofibrils in these tissues. Neonatal mice show a larger complement of mitochondria in myocardial cells as is seen in those of newly-metamorphosed plaice. The fully adult nature of the plaice heart is not achieved until about two months after metamorphosis during which time there is a great reduction in the concentration of mitochondria within the cells. Throughout this period it is observed that a considerable growth in the size of the heart takes place and perhaps the post-metamorphosis proliferation of mitochondria is accounted for by this period of growth. Certainly, during this post-metamorphosis period there was an increase in the diameter of individual cells to a value corresponding to that of the adult heart. In their study of the postnatal development of the smooth muscle of the mouse vas deferens, Yamauchi and Burnstock (1969a) noted a similar increase in cell dimensions in the neonate. Therefore, sudden periods of rapid growth are not restricted to developing cardiac tissue and can perhaps be related to the restricted periods of active cell division. The artificially induced metamorphosis of the axolotl into the adult Ambystoma shows changes in the myocardial organization of which the main one is a great increase in the number of myofibrils in the myocardial cells (Gros & Schrevel, 1970).

In this case there is no mention of any increase of mitochondria as seen in teleosts and in mammals but merely an intracellular rearrangement of these organelles. This is a diversion from the pattern seen in other vertebrates but it must be remembered that the metamorphosis was experimentally induced, and that the animal was therefore in an abnormal physiological state.

In comparison with the work on the development of the chick heart as described in Manasek's series of papers, certain observations can be made pertaining to teleost cardiac histogenesis. The basic pattern of ultrastructural development is similar despite the greater length of time taken by the plaice to achieve an adult heart compared with the chicken (21 days) in which an adult heart is present at hatching. Manasek's (1969a) proposal that the epicardium is formed from an extramyocardial source rather than by dedifferentiation of the outer myocardial cell layer is supported by this study on the plaice heart, albeit the evidence is negative, in that there are no observations to contest this proposal. There is however, no direct evidence that the embryonic myocardium assumed a secretory function in the production of cardiac jelly since there are few observable Golgi complexes and associated granules at the earliest stages examined in the plaice, large granular vesicles not being observed until stage 1d. Manasek's (1970b) study on the chick and that of Pager (1968) on the rat demonstrate clearly the production of cardiac jelly by the myocardium of the tubular heart and it is more likely that the absence of studies on myofibrillogenesis and of the earliest stages of heart formation in the plaice have precluded any results in support of their findings.

It appears that in contrast to the chick embryonic heart, myocardial outgrowth preceeds endocardial ingrowth in the formation of trabeculae. Indeed endocardial ingrowth does not occur until stages 4a-4b by which time the luminal side of the myocardium is showing convolutions and some complete trabeculae. In the chick (Manasek, 1970a) the formation of the epicardium occurs at approximately the same time as endocardial ingrowth. Therefore, setting aside the temporal differences in formation of the teleost and avian hearts, it is evident that some events are not sequentially equivalent.

The apparent abundance of Z-centres with radiating lengths of sarcomeres in more than two directions in the teleost tissue compared with their relatively scarce occurrence in the developing chick (Manasek, 1970a) is indicative that, whilst cardiogenesis is an essentially similar process in all vertebrates, that seen in the chick has perhaps been refined to a greater extent than that of the fish. Certainly, Pager (1968) makes no mention of such findings of such fibrillar configurations in her study of the early stages of development of the rat heart.

The present study on the development of the plaice heart shows that the whole process of cardiogenesis bears little relation to the state of the larva or to the young fish itself. At the time of hatching, merely a tubular heart is present whose beat is effective in providing a circulatory system as the animal changes from cutaneous to branchial respiration. Likewise, throughout the ensuing larval life the heart matures both morphologically and ultrastructurally, but by the completion of metamorphosis, the heart is as yet incompletely developed. As discussed above, other visceral

tissues are incompletely formed at equivalent stages in the life of vertebrates. Since the process of development of the teleost heart continues right up to its completion some two months after metamorphosis, it is difficult to nominate definitive stages concurrent with the state of development of the fish itself. That is to say it is difficult to define the "larval heart" as opposed to the "adult heart". It has been convenient during this study to refer to the larval heart in two stages: the "early larval heart" is not complete until it is a three-layered organ (Fig. 26) consisting of the myocardium bounded internally by endocardium and externally by epicardium at Day 24 (stage 1d ten days after hatching), the process of hatching being irrelevant to its formation. This condition exists until 4a stage with the onset of endocardial ingrowth which is the criterion distinguishing the "late larval heart". The "late larval heart" lasts throughout metamorphosis of the larva itself and then gradually assumes total adult characteristics by about two months following metamorphosis.

It is becoming evident that the development of the innervation of vertebrate visceral organs follows a definite pattern as revealed by work on several different organs. An initial but very sparse innervation is laid down early in embryonic life (or larval life in the case of teleosts). Later on even in the neonate (or after metamorphosis), there is a second period of growth of the innervation so that, within a period of up to six months, the innervation is complete. For example, the innervation of the mouse vas deferens is very sparse at birth, but is complete by six-months postnatal, since individual cells are then innervated (Yamauchi & Burnstock 1969b):

the human S-A node is innervated early on in embryonic life, but the atrium and A-V node not until the sixth month of gestation (Yamauchi, 1968). The innervation of the plaice heart follows the same pattern. The only previous report on the establishment of the cardiac innervation in teleosts was by Armstrong (1931) who reported that the heart of Fundulus beats for several days before there is any physiological indication that the vagus nerve has reached the heart and it is not until the eight day (or five days after the initiation of heartbeat) that any cardiac nerves can be revealed by methylene blue staining (Armstrong, 1935). The plaice has a longer larval life than Fundulus and a correspondingly longer period during which the heart remains aneural, as revealed by electron microscopy.

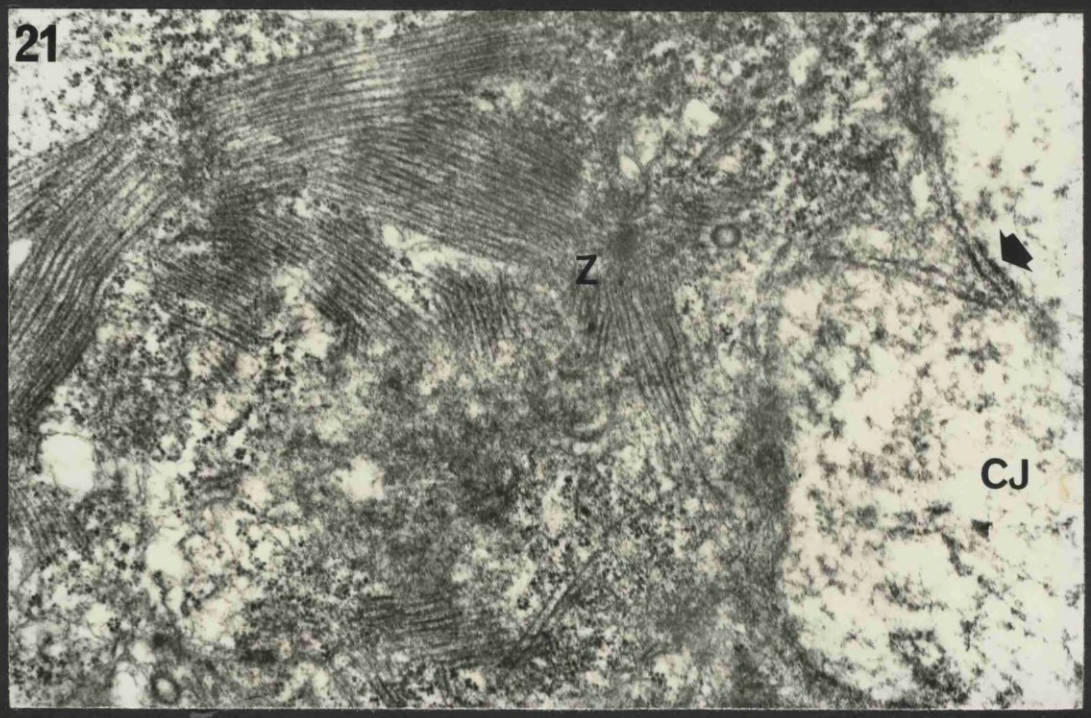
By the time that the innervation reaches the heart, the heart tube has not only coiled, but has begun to assume an adult shape which indicates, as in Fundulus (Armstrong, 1931), that differentiation of the heart is not dependent upon the presence of an innervation. It has also been shown by Manasek & Monroe (1972) that in its early stages, the developmental process of the chick heart is not dependent upon function. The coiling of the heart-tube continues unimpaired when the functional contractions are suppressed by high potassium media. Thus from all these studies it is evident that, in the early stages, the presence of an innervation is not essential for successful cardiac function. It would be very interesting to know when the observed innervation becomes physiologically effective.

Figure 21.

Day 13 (pre-hatching) myocyte showing a random orientation of myofibrils in varying stages of completion. Note the stellar configuration of myofibrils radiating outwards from a single Z-centre (Z). The myocyte is separated from the cardiac jelly (CJ) by a basal lamina (arrowed). Magnification: X29,000.

Figure 22.

Day 14 (stage 1a) myocyte showing the preferential location of completed sarcomere lengths in the subsarcolemmal region. The basal lamina (B) separates the myocyte from the cardiac jelly which has a flocculent appearance. Magnification: X21,000.


21
Z
CJ

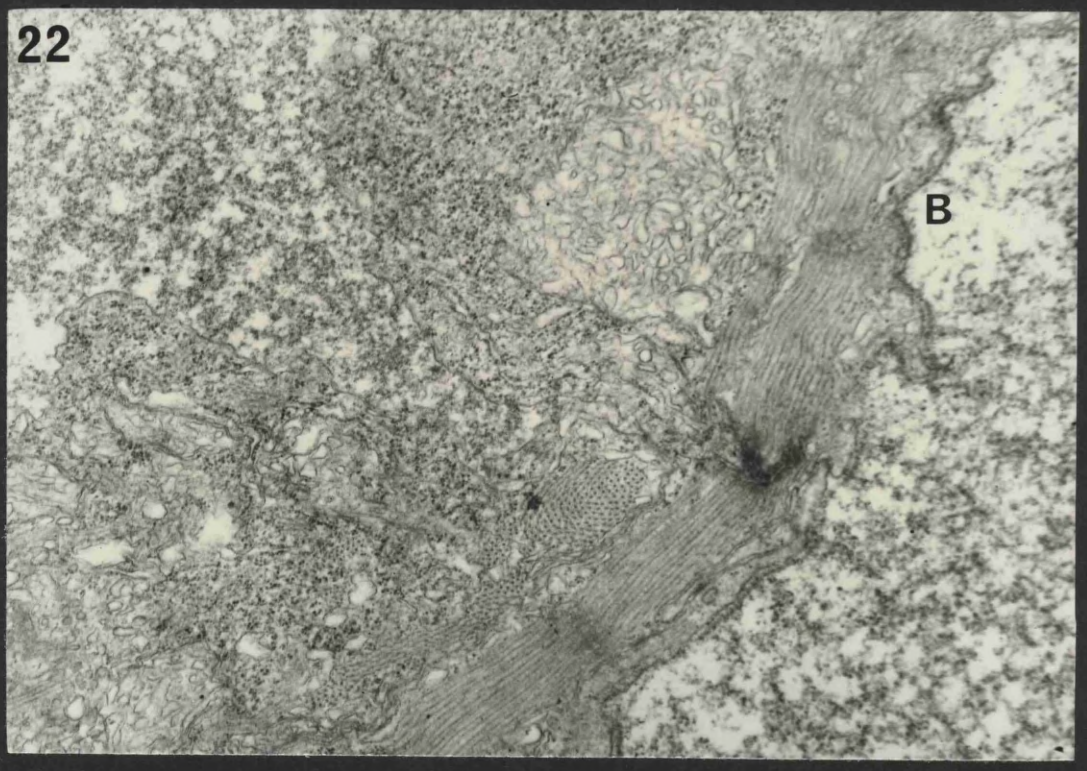

22
B

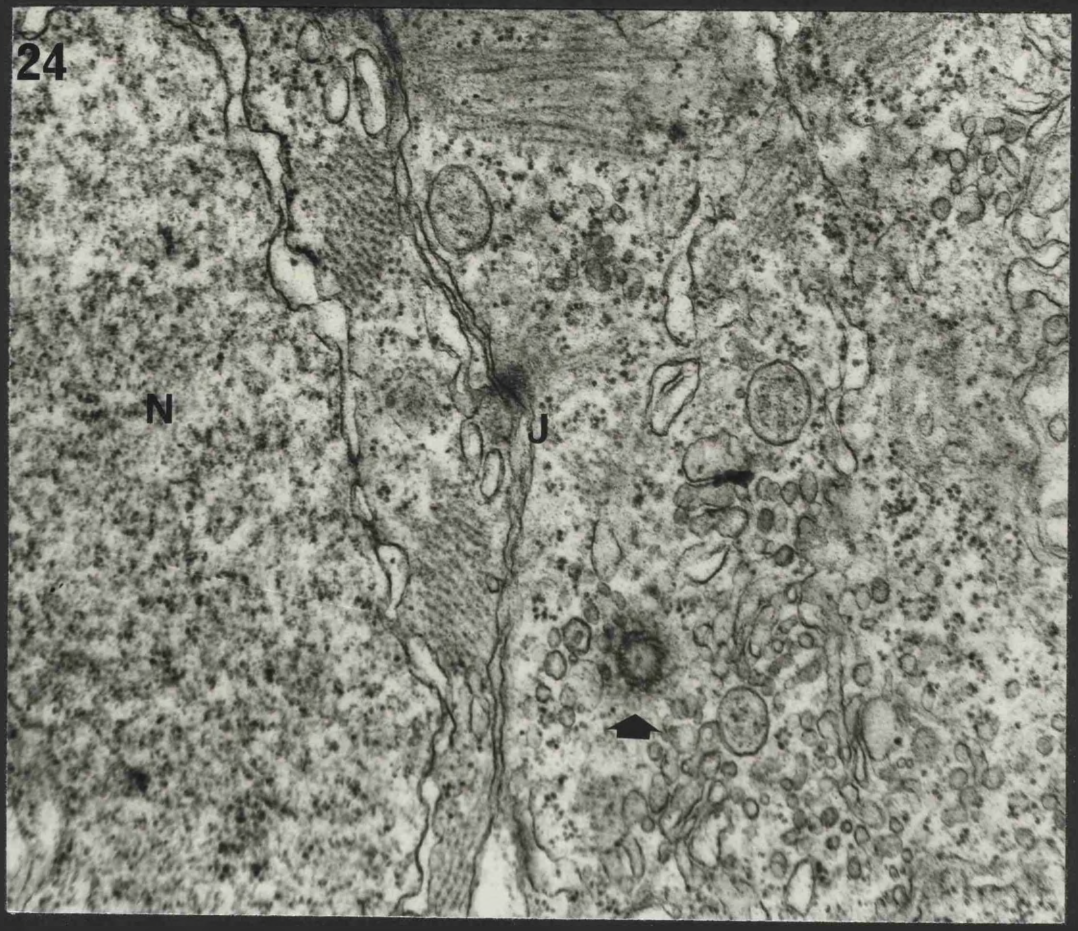
Figure 23.

Day 15 (stage 1a). The atrial end of the tubular heart consists of a single layer of myocytes in which the myofibrils have become slightly more regularly arranged, particularly near the cell surface. An endocardial monolayer (E) is situated very close to the myocytes, separating them from the lumen (L) of the heart. Magnification: X19,250.

Figure 24.

Day 15 (stage 1a). Two adjacent myocytes showing a desmosomal (J) attachment. A centriole (arrowed) is present in the right hand cell along with incompletely formed myofibrils. Note the large nucleus in the left hand cell (N) and also the nuclear pores. Magnification: X40,000.


23
E
L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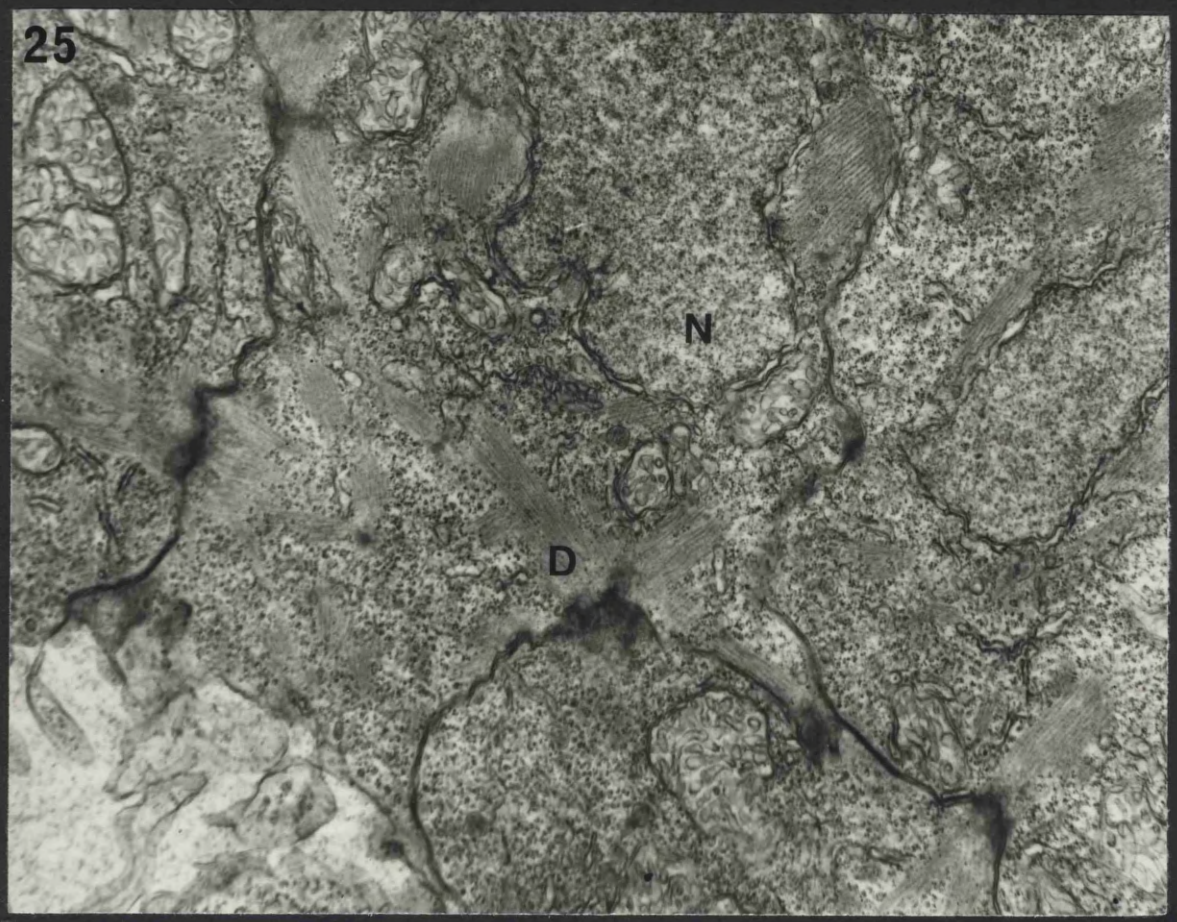
N
J

Figure 25.

View of the myocardium at stage 1a (Day 15) showing the large nuclei (N), intercalated discs (D) and randomly arrayed myofibrils. The cytoplasm of the myocytes contains much glycogen. Z, Z-centre. Magnification: X18,000.

Figure 26.

Stage 1d. Trilaminar structure of the 'early larval heart.' The myocardium (M) is lined on the luminal side by the endocardium (EN) and on the outside by the single cell layer of epicardium (EP). Note the large nuclei of the epicardial cell. The myocardial cells are filled with mitochondria and myofibrils and show prominent intercellular spaces. Magnification: X27, 500.


25
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D

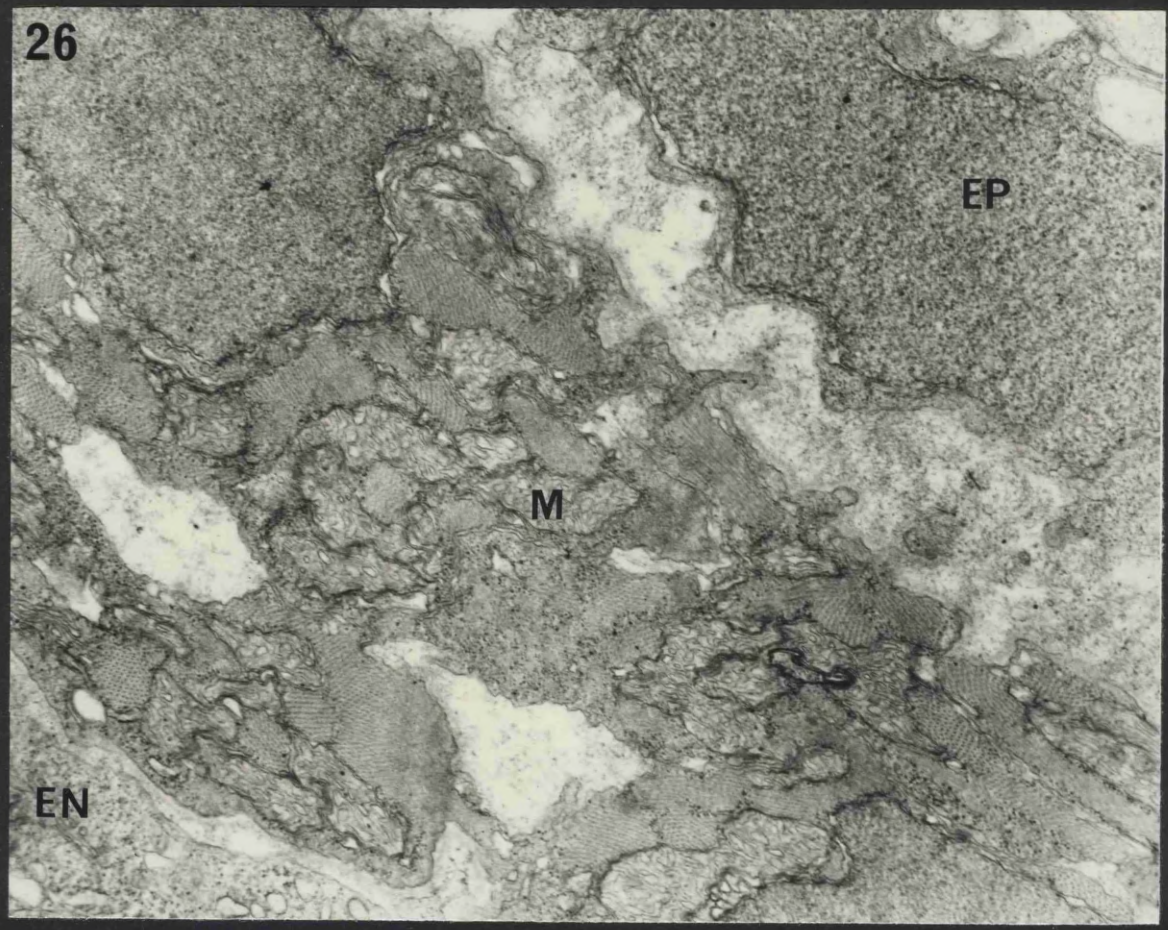
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Figure 27.

Stage 1c material showing that already intercalated discs link Z-bands of sarcomeres in adjacent myocytes. However stellar configurations of myofibrils around Z-centres are still evident. Magnification: X28,000.

Figure 28.

Stage 4b. Ventricular muscle cell showing a mature sarcomere (1.5μ in width) limited at both ends by intercalated discs. Note also the concentration of granular vesicles (V) in the muscle cell cytoplasm. Magnification: X25,000.

Figure 29.

Stage 4b. Mesenchymal cells (probably phagocytes characterised by large vacuoles and dark cytoplasm (M) are found in intercellular channels within the myocardium at this stage, thus making the myocardium a heterogeneous tissue. Magnification: X16,000.


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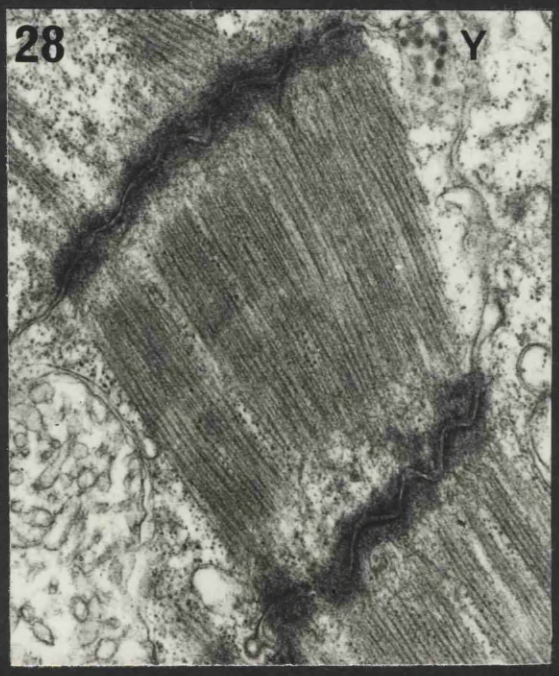
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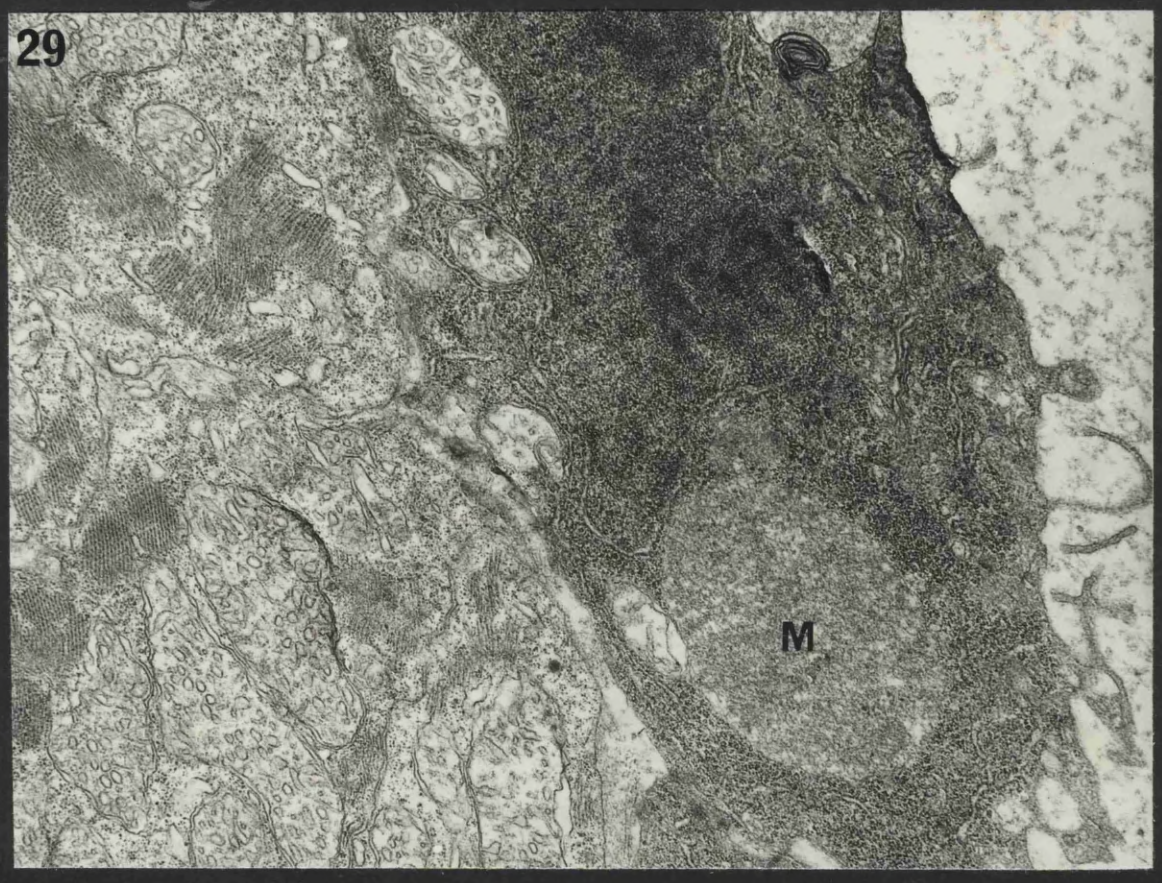
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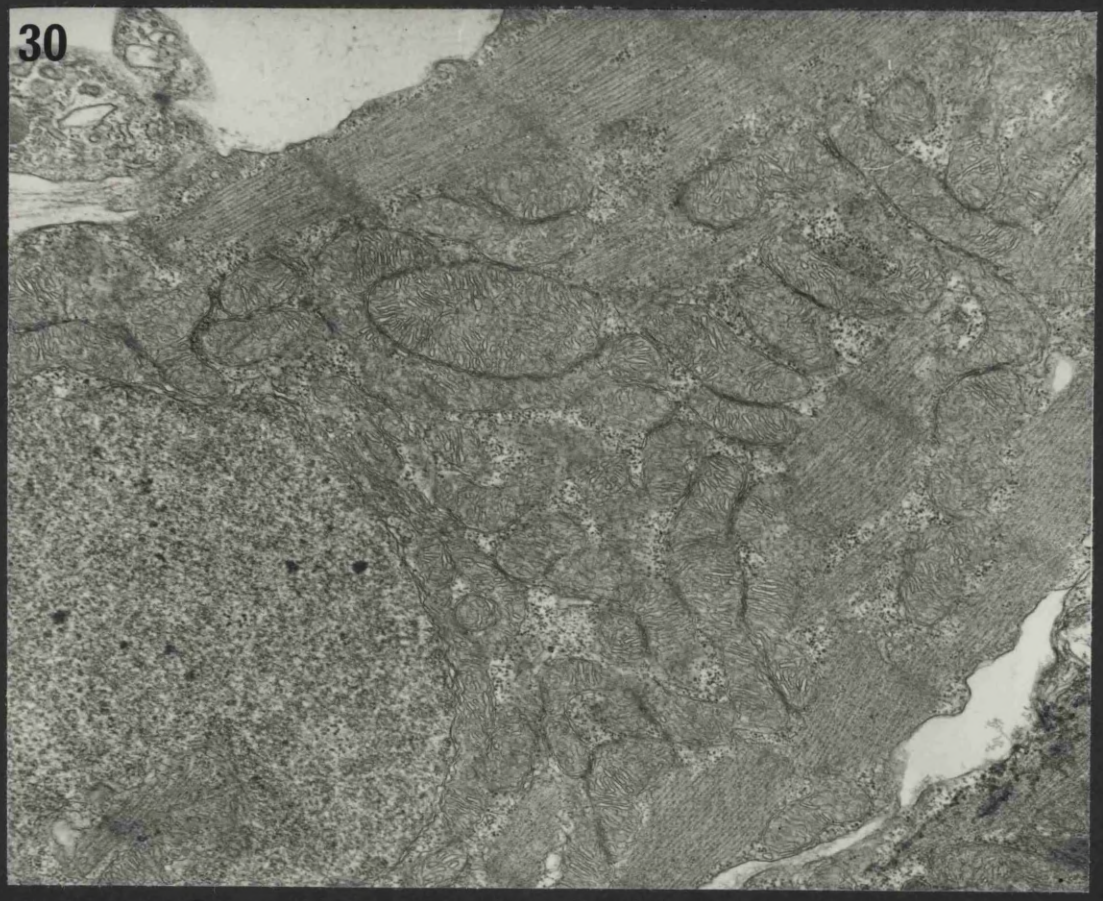
Figure 30.

One month postmetamorphosis. Atrial muscle cell showing the proliferation of mitochondria that occurs at this stage. Note the abundance of cristae within the mitochondria. Magnification: X22,000

Figure 31.

Mitochondria exhibiting a dumbell shape (asterisk) in an atrial cell of the heart one month post-metamorphosis. Note also the abundance of glycogen. Magnification: X55,000.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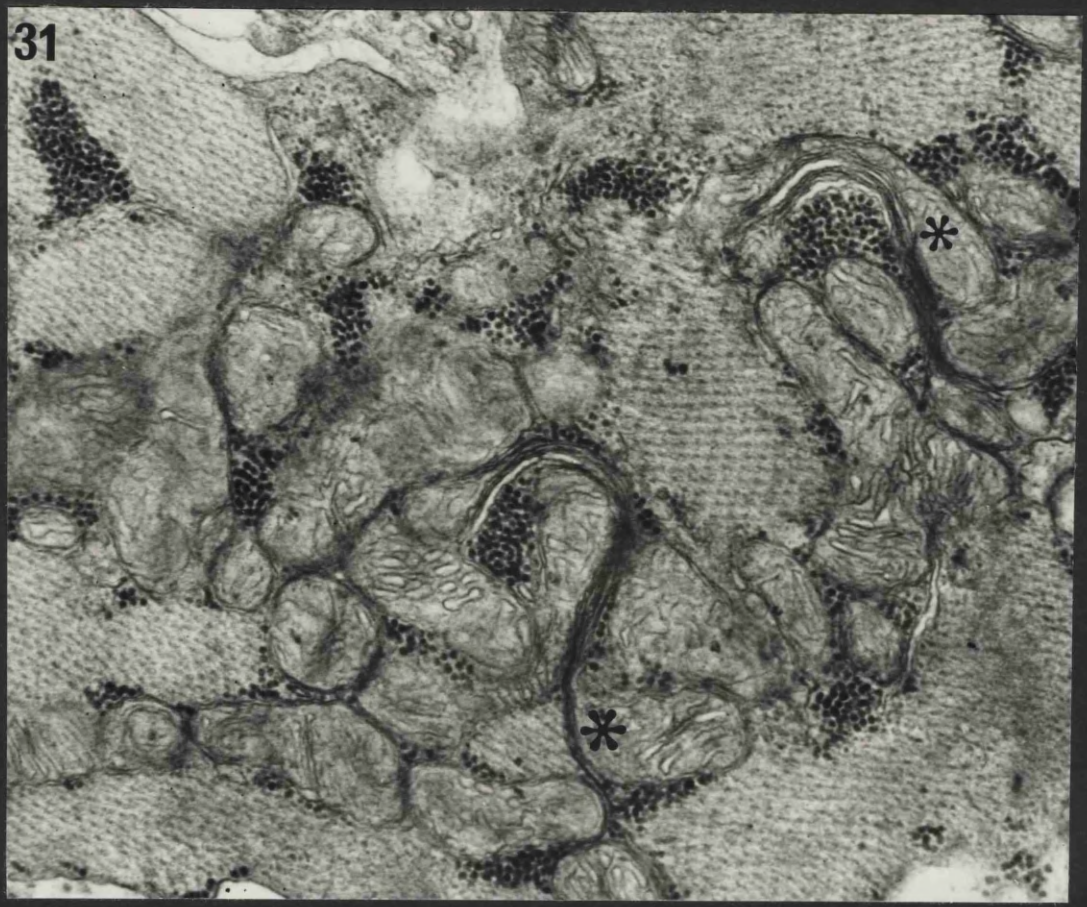
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Figure 32.

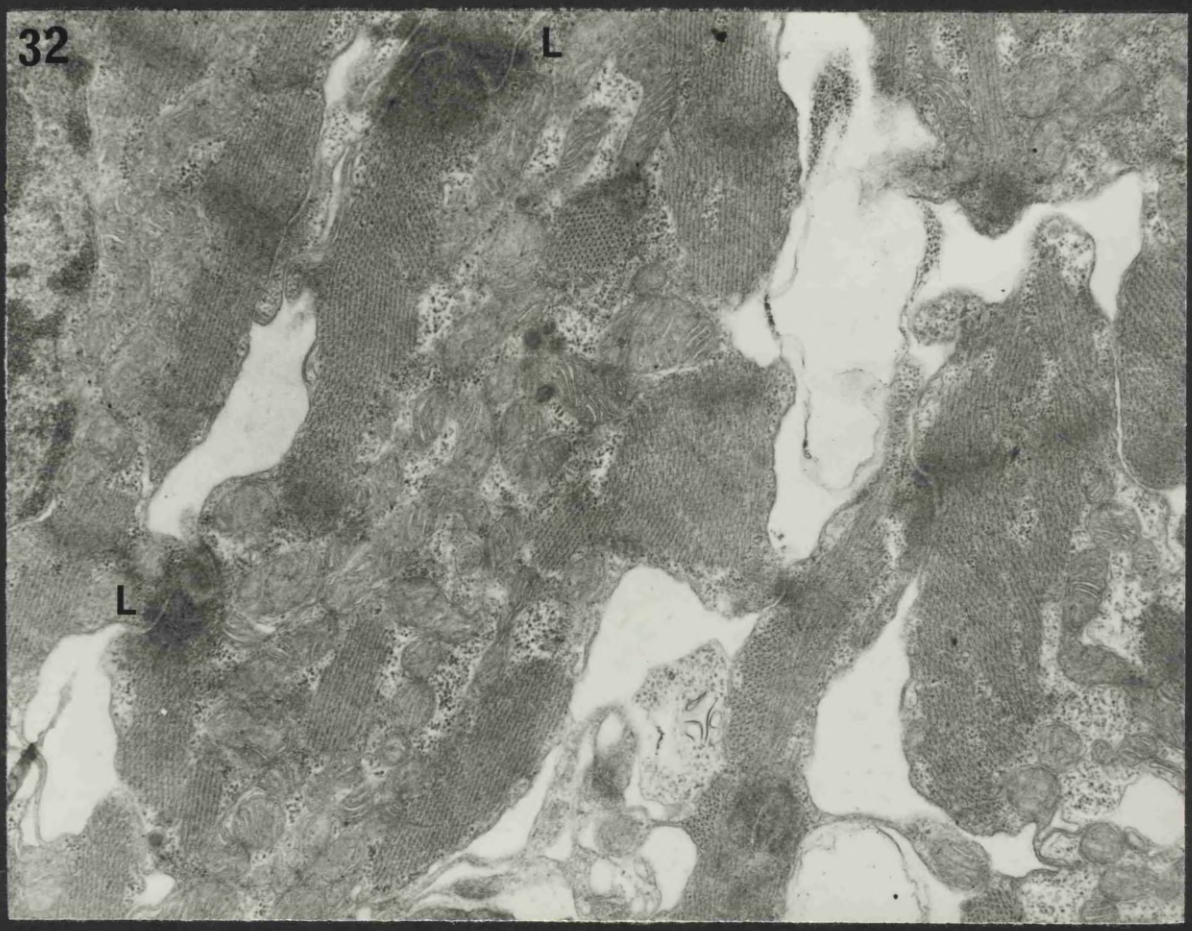
View of the atrial myocardium two months post-metamorphosis. The cells are showing an adult arrangement and complement of organelles and are connected to each other by lateral intercalated discs.(L). Magnification: X20,500 .

Figure 33.

Day 24 (stage 1d). A single unmyelinated axon containing a large number of small agranular vesicles in close contact (100Å) with two myocardial cells. Magnification: X40,000.

Figure 34.

One month post-metamorphosis. A small group of three unmyelinated axons in close contact with two atrial cells. Magnification: 40,000.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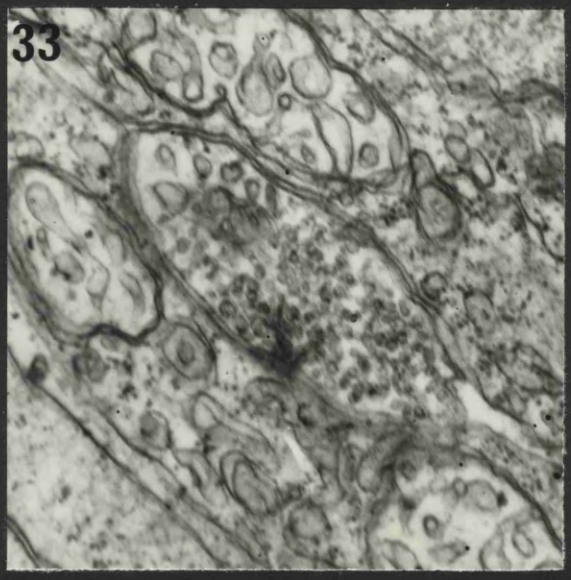
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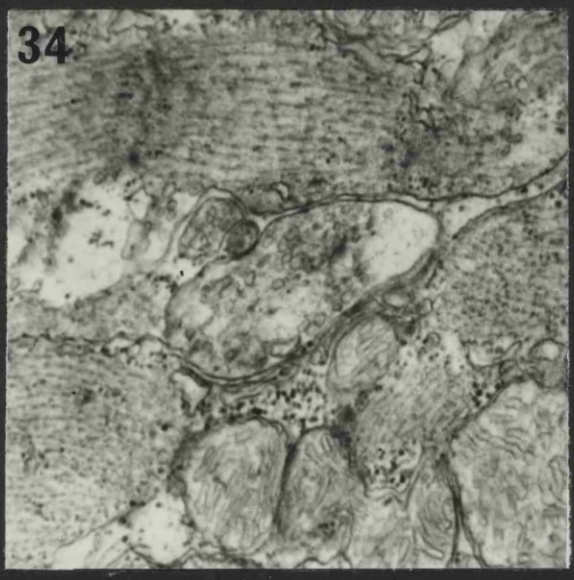

34

V. INNERVATION OF THE HEART

1. INTRODUCTION

Many physiological investigations demonstrate the vagal inhibitory innervation of the teleost heart. (see Campbell 1970) and until recently, it was assumed that the teleost heart received only this vagal inhibition innervation. Gannon & Burnstock (1968) and Gannon (1971) have presented evidence of a sympathetic adrenergic excitatory innervation of the trout heart. Electron microscopic studies of the cardiac innervation of any teleost are confined to these by Couteaux & Laurent (1957) and Yamauchi & Burnstock (1968). The latter authors described the sinoatrial region which is the presumed site of pacemaker activity, and also the innervation of the atrium and ventricle of the trout. In the sinoatrial region they showed ganglion cells and also each myocardial cell to be in close (200Å) contact with at least one axon profile and that this number decreased along the length of the atrium. The incidence of axon profiles in the ventricle is very low in the trout.

Light microscope studies such as that of Laurent's (1962) on the innervation of the heart of a number of teleosts combining light microscopy and degeneration studies showed that there was a correlation between the patterns of degeneration of myelinated axons in the heart and their diameters. This enabled him to describe the anatomy of the afferent and efferent pathways of cardiac innervation. He also described two receptor systems in the heart. Firstly, a subepicardial plexus at the atrio-ventricular region and secondly, a loose unmyelinated plexus in the ventricular subepicardium both of which form part of the afferent innervation and are seen to degenerate following

peripheral vagotomy.

Due to the lack of information on the cardiac innervation of teleosts, it is necessary to conduct an extensive study before any correlations can be made with physiological data.

2. RESULTS

1) Light microscopy

The vagal parasympathetic innervation of the heart comprises two small cardiac branches of the vagus nerve which run along the ducts of Cuvier to the sinus venosus. The cardiac nerve itself contains about twenty myelinated nerve fibres of varying diameter and is ensheathed with connective tissue. It runs between the outer limiting membrane of the duct of Cuvier and the connective tissue surrounding the vessel. At a point approximately 1mm from the S-A junction the cardiac nerves enter the matrix of the wall of the SV and from there on are contained within it. Between the point of entry of these nerves into the wall of the SV and the S-A junction, the nerve breaks up and divides extensively to form a diffuse plexus which is part of the cardiac ganglion. Ganglion cells are located individually within the plexus and also in groups often at the points of bifurcation of the nerves and along the length of nerve branches. No ganglion cells were seen along the length of the undivided cardiac nerves as reported by Laurent (1962) in other teleosts. The main body of the ganglion is situated at the S-A junction at which many tracts of both myelinated and unmyelinated nerves are seen as well as many ganglion cells (Fig. 35-37). A considerable amount of connective tissue surrounds the myelinated nerve tracts and also a 10μ layer separates the ganglion cells from the atrial myocardium. The ganglion cells are fairly uniform size, ranging from 15-25μ in diameter and are unipolar. From the S-A region nerves leave the cardiac ganglion and are seen in the atrial myocardium, and in a few fish, a single large nerve ran almost to the A-V junction without branching for most of its length. No nerves were seen at the A-V junction

by light microscopy and there is no A-V node, comparable to Bidder's ganglion in the frog heart. There were no ganglion cells in the atrium and no subepicardial or subendocardial receptor plexuses were apparent. Thus the cardiac ganglion is a very diffuse ganglion extending over a wide area on the dorsal surface of the S-A junction and not as localised as some autonomic ganglia of the gut of higher vertebrates.

The two cardiac branches of the vagus are the only pathways for the extrinsic innervation. There is no coronary blood supply to the heart and therefore no blood vessels along which a secondary extrinsic nerve pathway might run. The branch of this nerve on the dorsal (ocular) side of the fish contained more myelinated nerves and formed a bigger and more complex side of the plexus than its counterpart on the ventral (eyeless) side. Thus the ganglion was somewhat asymmetrical. It was not possible to estimate the relative numbers of ganglion cells with respect to this asymmetry. Whether this asymmetry is a consequence of the general asymmetry of the fish is unknown, but it is a consistent feature of the extrinsic cardiac innervation.

ii) <u>Electron microscopy</u>

a) Cardiac ganglion

The nerves constituting the plexus of the cardiac ganglion were mixed, containing both unmyelinated and myelinated axons in varying numbers. There were between 3 and 10 myelinated axons within a nerve. The numbers of unmyelinated axons varied greatly within a nerve, ranging from 20-200 approximately. The unmyelinated axons were grouped into fascicles containing up to 20 axons associated with a single Schwann cell. As many as 10

of the fasicles occurred in a nerve. The nerves were all bounded by a connective tissue sheath and within the nerve, each myelinated axon and fasicles of unmyelinated axons are bounded by collagen, which thus separated them from eabh other. Ganglion cells occurred frequently along the length of nerves of the plexus, incorporated within the outer limiting connective tissue. These ganglion cells were usually smaller than those found at the bifurcations of nerves, but otherwise identical in structure (Fig. 38) (see later). Two types of synaptic association were seen within the nerves (Figs. 40-45). First there were synaptic terminals on the ganglion cells and second, synapses were formed between unmyelinated axons enclosed within the same Schwann cell (Fig.45). Axon-axon associations were seen elsewhere in autonomic plexuses as in Auerbach's plexus of amphibia (Rogers and Burnstock, 1966b) but these authors showed no synaptic contact, merely a very close (90Å) apposition between such axons. As mentioned above, there were bundles of myocardial cells within the cardiac plexus which are heavily innervated. These are to be contrasted with the individual myocardial cells which were incorporated within the nerves close to the S-A junction. These cells occurred singly, each one surrounded by an external lamina. Close association between the axons within a nerve and these myocardial cells were observed, but their significance remains obscure.

The ganglion cells of the cardiac ganglion (Figs. 46,47) ranged in diameter from 15 -25μ. The cell soma appeared oval with occasional projections and the cells were unipolar. The ganglion cell somata were enveloped by satellite cell processes over most of their surface and the individual ganglion cells within a group were separated from one another by interstitial

collagen. The origin and structure of autonomic interstitial cells has been examined by Rogers & Burnstock (1966a) who conclude that it is very difficult to identify separate cell types in the autonomic ground plexus. The nucleus of the satellite cells was often prominent. The cytoplasm of the ganglion cell somata contained many mitochondria, Golgi bodies, rough endoplasmic reticulum (ER) which often appeared swollen, ribosomes, electron-dense lipid droplets and also multivesicular bodies. The rough ER was identical to the Nissl bodies described by Taxi (1965) in mammalian enteric ganglia. The ganglion cell soma cytoplasm was usually more electron-dense than that of neighbouring Schwann cells. The nucleus was centrally placed and had a homogeneous nucleoplasm with no obvious nucleoli. The axon hillock of the ganglion cell was characterised by extentions of smooth ER. The axon hillock was associated with many unmyelinated axon profiles, none of which were observed to make synaptic contact with the ganglion cells. Closely associated with the ganglion cells was much collagen formed by fibrocytes which had the same standard role and relationship with the ganglion cells as in the nerves of the plexus. Another interstitial cell type was seen close to the ganglion cell somata which had a very large nucleus and many concentric layers of external lamina around the cell. Throughout the ganglion the external lamina was obvious around nerves, fibrocytes and ganglion cells. A few capillaries supplied the cardiac ganglion.

Synapses were seen within the cardiac ganglion occasionally between unmyelinated axons in the nerve plexus (Fig. 45) but more commonly between presumed preganglionic axons and the ganglion cell soma or projections from it. Most of these synapses were on the surface of the ganglion cell (Fig. 40)

though a few were indented into the perikaryon (Fig. 41). All presynaptic axon terminals were unmyelinated and lay between the ganglion cell soma and the surrounding satellite cell processes. The synaptic regions were distinguished by electron-dense thickenings of the pre- and post-synaptic cell membranes and usually by a distinct cleft about 200Å across. The only post-synaptic specialization that was observed were densities directly continuous with the membrane thickening extending for about 500-600Å into the post-synaptic cell. The synaptic clefts appeared to be without structure. At the pre-synaptic region there was always an aggregation of small agranular vesicles of 300-400Å diameter. Elsewhere in the presynaptic axons there were similar agranular vesicles less densely distributed and also larger granular vesicles of 500-700Å diameter. In pre-synaptic axons which synapsed on ganglion cells, the proportions of granular and agranular vesicles varied greatly from an almost complete absence of granular vesicles (Fig. 42) to some axons in which they comprised about 30% of the vesicle population (Fig. 44). In the axon-axon synapses in the plexus nerves , the pre-synaptic axon contained only small agranular vesicles. No axon profiles resembling classical adrenergic type axons (Hokfelt, 1968) containing large numbers of granular vesicles were seen anywhere in the ganglion. Mitochondria are also very numerous in the pre-synaptic terminals.

As well as the nerves of the plexus in the SV itself there are large tracts of nerves surrounding the S-A junction (Fig. 48) from which intramural axons pass to the myocardium. In these tracts many myelinated and unmyelinated axons are seen. In contrast to the myelinated axons of the cardiac nerve and many of the large myelinated plexus nerves, the myelin sheath

around those of the smaller plexus nerves and of the S-A region was thinner and they were not individually enclosed in collagen. The myelinated nerves contained many mitochondria and neurofilaments. Unmyelinated axons contained mitochondria, neurofilaments, neurotubules, glycogen granules and, in unmyelinated preterminal regions of axons there were considerable numbers of large granular vesicles interspersed with the neurotubules.

b) Innervation of the myocardium.

The density of the intrinsic innervation of the teleost myocardium was not as great as that of higher vertebrates. Unmyelinated intramural axon profiles mostly occurred individually and only rarely in small bundles. Most of the axon profiles observed were not associated with a Schwann cell, but some axon profiles had a very thin wrapping (800Å) around them (Fig. 51). No myelinated axons were seen in the atrium except in the few fish in which a large tract of nerves traversed the atrium almost to the A-V junction. This nerve consisted of 15-20 myelinated axons and about 20 small fascicles of unmyelinated axons. The significance of this nerve is not known. The characteristic subepicardial nerve networks of higher vertebrates were absent in the plaice heart, most nerves accompanying and entering the trabeculae. The density of innervation of the plaice atrium decreased towards the anterior (ventricular) end as it does in that of the trout (Yamauchi & Burnstock, 1969). The myocardium at the sinoatrial region were densely innervated, most cells being in contact with at least one axon profile. No specialised neuromyocardial junctions were seen but many axons approach within 100Å of muscle cells, some

of them lying in grooves in the cell surface (Fig. 49) like those noted in the frog heart by Thaemert (1966). The axon profiles in the myocardium varied in diameter from 0.3-2μ and contained small, round mitochondria, neurofilaments, neurotubules extending for lengths of up to 1μ as seen in longitudinal section, electron dense glycogen granules, large numbers of small (300-450Å) agranular vesicles and much smaller numbers of larger granular vesicles of 600-800Å diameter. Those axon profiles containing large granular vesicles were similar to those suggested by Yamauchi and Burnstock (1969) to represent the adrenergic innervation of the trout heart, but it should be emphasised that such vesicles do occur in cholinergic nerves (Taxi, 1965; Tranzer et al., 1969) as in the preterminal endings seen in the plaice cardiac plexus where they occur in large numbers. It was possible to localise AChE on the membranes of axons containing some large granular vesicles amongst the larger population of agranular vesicles (see later) and thus it was unlikely that these profiles were adrenergic, but represented the cholinergic postganglionic innervation. No profiles typical of adrenergic nerves containing large numbers of small (about 450Å) granular vesicles were seen in any part of the heart.

No axon profiles of any type were seen in the ventricle which is therefore aneural. This anatomical finding is in agreement with the observation of the lack of effect of vagal stimulation on the teleost ventricle noted by several authors (McWilliam, 1885; Skramlik, 1935; Jullien & Ripplinger, 1957; Gannon, 1971).

iii) Localisation of cholinesterases

In both electron and light microscopical studies, AChE

activity was observed when acetylthiocholine was used as substrate with 10^{-5} iso-OMPA present as inhibitor of butyrylcholinesterase. No AChE activity was seen when the substrate acetylthiocholine was omitted, or the specific AChE inhibitor BW284 (at 10^{-5} M) was present along with the substrate. No enzyme activity was observed when butyrylthiocholine was used as substrate with BW284 present as AChE inhibitor.

a) Light microscopy

Stretch preparations of the sinus venosus and atrium showed AChE localised throughout the extrinsic innervation of the heart (Figs 52-55) in the nerves of the cardiac plexus, ganglion cells and in the cardiac nerve itself. The sites of AChE activity were associated with nerves of widely varying diameter and also those in which myelinated nerves could be clearly distinguished. Many nerves at the S-A junction were intensely stained for AChE as were many throughout the atrium, though in greater numbers at the sinus end. These nerves passed in a random fashion throughout the atrial myocardium presumably related to the arrangement of trabeculae. There was no extensive plexus of nerves revealed by AChE localisation comparable to that seen in the frog heart by Woods (1970). The comparatively sparse AChE positive innervation of the plaice atrium is in agreement with the sparse intrinsic innervation of the myocardium as seen with the electron microscope. AChE was localised in ganglion cells throughout the cardiac plexus, in many cases in what appeared to be a higher concentration in the nucleus than in the ganglion cell perikaryon (Figs. 54,55). A similar situation exists in the rat caudate nucleus (Bloom & Barrnett, 1967), but as in the

present light microscopical study it is difficult to discern whether the observed dense staining is in the nucleus or the nucleolus. However in most other autonomic ganglion cells AChE is predominantly seen in the perikaryon with the light microscope.

b) Electron microscopy

For electron microscope histochemistry, the use of the Karnowsky (1964) method enables the accurate localisation of the AChE reaction product as it forms in the tissue.

In the cardiac ganglion AChE was localised between the ganglion cell soma membrane and the surrounding satellite cell membrane (Figs. 56,57), between the unmyelinated axon membrane and Schwann cell membrane (Fig. 59) and at the axon membrane of uncovered unmyelinated axons (Fig. 58). Also, within the cardiac nerve and the plexus nerves there was AChE activity associated with both unmyelinated and myelinated axon membranes but it was very difficult to determine the precise reaction site of AChE in the latter. In the guinea-pig atrium AChE is localised between the axon membrane and the myelin sheath of myelinated axons (Hirano & Ogawa, 1969). Many of the unmyelinated axon profiles showing AChE activity contained numbers of agranular vesicles and were situated close to the ganglion cells. It is suggested that these are preterminal regions of preganglionic vagal fibres, by definition cholinergic, but no actual synaptic contacts were seen in these experiments. There is an inconsistancy in the AChE localisation in that not all axons within a bundle showed AChE activity, as seen in other autonomic preparations (Robinson & Bell, 1967).

There was no AChE localisation within the endoplasmic reticulum of the perikarya or in the perinuclear region of the ganglion cells as seen by Erzin et al. (1966) in frog sympathetic

ganglion cells. Likewise, no reaction product was observed in the axoplasm of any nerves in the plaice cardiac ganglion.

Many intramural unmyelinated axon profiles in the atrium show AChE activity which was always located on the outside of the axon membrane whether or not the axon was surrounded by a Schwann cell process. AChE was never seen in the axoplasm. Most of the axons around which AChE was localised contained small (300-450Å) agranular vesicles in large numbers, but in some axons, large granular (500-700Å) vesicles occurred in varying numbers alongside the agranular vesicles (Fig. 58).

There was AChE activity on the sarcolemmae of some muscle cells within the atrial myocardium (Fig. 60). Most of these AChE positive cells were on the outer edge of the trabeculae which at first sight might reflect the uneven diffusion of reactants into the centre of the trabeculae. However, centrally placed axons showed AChE activity. No AChE activity was noted within the myocardial cells.

iv) Fluorescence histochemistry

The absence of any ultrastructural evidence of adrenergic nerves in the plaice heart made it necessary to investigate the presence or absence of such an innervation by histochemical methods.

Fluorescence histochemistry of both freeze-dried sections of the ventricle and stretched preparations of the sinus venosus and the atrium failed to reveal any typical catecholamine fluorescence of nerve fibres in any part of the heart or in the cardiac nerve. Some of the tissue was exposed to formaldehyde for 3 hours in order to reveal the presence of adrenaline, the excitatory neurotransmitter in the amphibian heart (Falck et al. 1963), and that present in the pronephros of the plaice (Grove et al.

1971), but no fluorescent fibres were observed.

The catecholamine content of adrenergically innervated teleost tissues is approximately 10% of their counterparts in mammals (D.J. Grove: personal communication) and thus it is necessary to increase the intensity of fluorescence of such tissues. Read & Burnstock (1969) increased the weakly fluorescing human foetal gut by loading the tissue with α-methyl-noradrenaline prior to freeze-drying. Also, when such low quantities of catecholamine are present, it is vital to preserve as much as possible for the fluorescence technique. This can be effected by incubating the tissue in Krebs Ringer with 100μg/ml of nialamide, a monoamine oxidase inhibitor, for 2 hours, prior to incubation with 10μg/ml α-methyl-noradrenaline in plaice Ringer for 1 hour. Since no fluorescence was detected using the routine method, the above procedure was used before drying the tissue which was subsequently exposed to formaldehyde for 1 or 3 hours.

No specific fluorescence of nerves was seen in any part of the plaice heart or in the cardiac nerve itself using the above preparative procedure. Also none of the above procedures revealed any "point" fluorescence which might correspond to intracellular storage of catecholamine within muscle cells, or to the groups of granular vesicles seen in the muscle cells. The background fluorescence was not too high to preclude observation of fine specific fluorescence in the heart. Control sections of tissue from plaice skin and small intestine showed fluorescent nerve fibres only after 3 hours exposure to formaldehyde. These results confirm those of von Mecklenberg (1966) who also applied the fluorescent histochemical technique to the plaice heart but observed no specific fluorescence.

v) Drug Studies

a) Reserpine (Serpasil) at 5mg/kg injected intravenously 12 hours before sacrifice produced no changes in the vesicle content of the extrinsic or intrinsic innervation of the heart. The frequency and distribution of the large (500-700Å) granular vesicles remained unaltered.

b) 6-hydroxydopamine (6-OHDA) causes a highly selective chemical sympathectomy of adrenergically innervated tissues (Thoenen & Tranzer 1968) by depletion of the noradrenaline content and by rapid degeneration of the nerve terminals (Furness et al. 1970). Injection of 100mg/Kg 6-OHDA followed by electron microscopy produced no signs of either "loading" or degeneration of any nerves in the heart examined two hours after administration of the drug. Ultrastructural changes are visible in sympathetically innervated tissues after such a time lapse (Bennett et al. 1970). There was no change in the appearance and distribution of large granular vesicles within axons following 6-OHDA injection (see Fig. 50).

3. DISCUSSION

Laurent's (1962) very detailed light microscopical description of the anatomy of the teleost heart was derived from studies on the tench, catfish, eel and carp. He described a complex innervation of all heart chambers which included both afferent and efferent components. The structure of the plaice heart is uncomplicated compared with other teleosts, one of the main features of which is the lack of a coronary blood supply associated with the absence of an outer compact layer of atrial and ventricular myocardium. Coronary blood vessels provide another pathway by which nerves can reach the heart, other than the cardiac nerve itself, and it is Laurent's contention that the nerves running with the coronary arteries are afferent and are those of the ventricular subepicardial plexus. Gannon and Burnstock (1968) suggest that the adrenergic supply to the trout ventricle reaches the heart along the coronary vessels. In other teleosts such as Cottus scorpius, small bundles of unmyelinated axons do accompany the blood vessels to the ventricular myocardium but it is more likely that these are innervating the blood vessels themselves (Womersley 1973). No comparable subepicardial plexus has been seen with the electron microscope in the plaice ventricle and this might therefore be a consequence of the lack of a suitable pathway along which the nerves could run. Laurent also notes that the innervation of the ventricular myocardium itself is very poor. That of the plaice is completely aneural.

Degeneration studies to determine the location of the afferent and efferent pathways within the innervation were not undertaken due to Home Office Regulations and it has also not been possible to identify the sensory endings in the plaice heart.

However there are a considerable number of axons with thin myelin sheaths of varying diameter in the S-A region which could correspond to Laurent's distinction between larger diameter afferent fibres and smaller efferent fibres. No intramural myelinated fibres were seen in the myocardium except at the S-A region. Like all the fish studied by Laurent the main cardiac vagus of the plaice nerve contains about twenty myelinated axons as well as numerous unmyelinated fibres. Ganglion cells were not seen in the plaice cardiac nerves in contrast to Laurent's result which indicated, as a result of degernation studies, that some of the unmyelinated axons within the cardiac nerve are efferent post-ganglionic, arising from ganglion cells higher up the nerve.

The plaice cardiac ganglion is typical in form of other vertebrate parasympathetic ganglia in that it is situated close to the organ that it innervates. Also, unlike the more compact sympathetic ganglia (Pick, 1970), vertebrate parasympathetic ganglia are much more diffuse, comprising a number of ganglion cells located within a nerve plexus, and in this respect, the plaice cardiac ganglion conforms to this arrangement. The presence of many ganglion cells along the length of the nerves of the cardiac plexus is reminiscent of the ganglionated nerve trunks of Auerbach's Plexus in birds (Bennett & Cobb, 1969c) and here it has been developed to a much greater extent. Like the plaice, the eel cardiac ganglion is situated at the S-A region but that of the tench, carp and catfish is more extensive extending almost as far as the A-V junction on the ventral side of the atrium (Laurent, 1962).

In summary, it is evident that the innervation of the plaice heart is not as extensive as in those fish studied by

Laurent (1962) and certainly does not approach the situation described in the pike by Kazem-Beck & Dogiel (1882) in which "groups of nerves" are seen in the SV, the S-A junction and at the A-V junction. Such an arrangement is similar to that of amphibia. The considerable discrepancies in innervation pattern between that of the plaice heart and that of those fish studied by Laurent is remarkable, even taking into account possible interspecific differences in teleosts. Most of the light microscope studies undertaken by Laurent (1962) used the Bielschowsky-Gros silver impregnation technique to stain the nerves. This technique is notoriously capricious (see Richardson, 1960) and it is possible that certain nonspecifically stained tissues could be mistaken for nervous elements. Fibrocyte processes on atrial and ventricular trabeculae are very similar in appearance to axons when the Bielschowsky-Gros technique is slightly altered and it is necessary to confirm their identity by other histological stains. Subepicardial and subendocardial connective tissue elements could also very easily be mistaken for nerve plexuses and this should be borne in mind when assessing earlier light microscopical studies of autonomic innervation.

Since the first example of an excitatory, adrenergic innervation of a teleost heart by fluorescence histochemical and pharmacological methods in the trout (Gannon & Burnstock, 1968; Gannon, 1971) it became important to determine whether such an innervation supplied the plaice heart. In this respect, the occurrence of many granular vesicles as well as agranular vesicles in intramural axon profiles was of possible significance as it has been suggested that these are the storage sites of noradrenaline in peripheral autonomic axons (Hokfelt, 1968). In

the present study it has not been possible to degenerate axons containing these vesicles with 6-OHDA, to deplete the vesicle content by reserpine injection nor to identify ultrastructurally axon profiles with large numbers of small agranular vesicles typical of adrenergic neurones (Hokfelt, 1968, Nilsson and Sporrong, 1970). AChE has also been localised around these axons. The "adrenergic" axon profiles of the trout heart (Yamauchi & Burnstock, 1968) are almost identical to the profiles in the plaice heart discussed above and these authors did not describe classical adrenergic axon profiles in their study. It has been shown by Esterhuizen et al. (1968) and by Graham et al. (1968) that the ability of axons innervating peripheral mammalian smooth muscle to accumulate ^{3}H-noradrenaline was exclusive to AChE-negative axons, and that these axons always contained large numbers of small (300-500Å) granular vesicles. These results of the present study, do not present any evidence for an adrenergic innervation of the plaice heart. It is also suggested that there is insufficient evidence to support the claim that those axons seen in the trout heart by Yamauchi & Burnstock, (1968) are indeed adrenergic.

The aggregations of small (300-500Å) agranular vesicles are always associated with the synaptic regions of the preganglionic terminals upon ganglion cells, there are also a variable number of larger (500-700Å) granular vesicles distributed throughout these terminals almost always away from the synaptic region. The contents and function of these vesicles is unknown but their distribution in preganglionic terminals is widespread throughout the vertebrates (Grillo, 1968; Tranzer et al., 1968). Similar preganglionic profiles in mammalian sympathetic ganglia are, as in the plaice cardiac ganglion,

AChE-positive but the former also have the ability to accumulate ^{3}H-noradrenaline (Graham et al., 1971). This would suggest the possibility of an adrenergic modulation of ganglionic transmission. The absence of catecholamine fluorescence from the plaice cardiac ganglion together with the lack of effect of 6-OHDA and reserpine upon the vesicle content of the pre-ganglionic terminals makes this idea unlikely in the plaice. Adrenergic involvement has also been suggested in mammalian parasympathetic ganglia (see Norberg & Sjoqvist, 1966).

Thus, the evidence presented above is indicative of only a parasympathetic innervation of the plaice heart in which all the extrinsic and intrinsic nerves are cholinergic in nature and in which transmission through the cardiac ganglion is also cholinergically mediated.

In contrast to Karnowsky's (1964) work on the localisation of AChE in adult cardiac muscle of the rat in which the enzyme was seen along the SR, in the A-band and around the nuclear membrane, no intracellular localisation of AChE is reported in the present study. Only an uneven distribution of the enzyme at the outer edge of the sarcolemma is noted. Also, Nagopian and Tennyson (1971) localised BuChE at similar sites in the rabbit heart including also the terminal sacs of the SR adjacent to the T-tubules. Obviously the lack of a T-system and the very sparse SR in plaice myocardial cells reduces the possible number of sites for enzyme activity, but the total lack of intracellular enzyme activity is not understood.

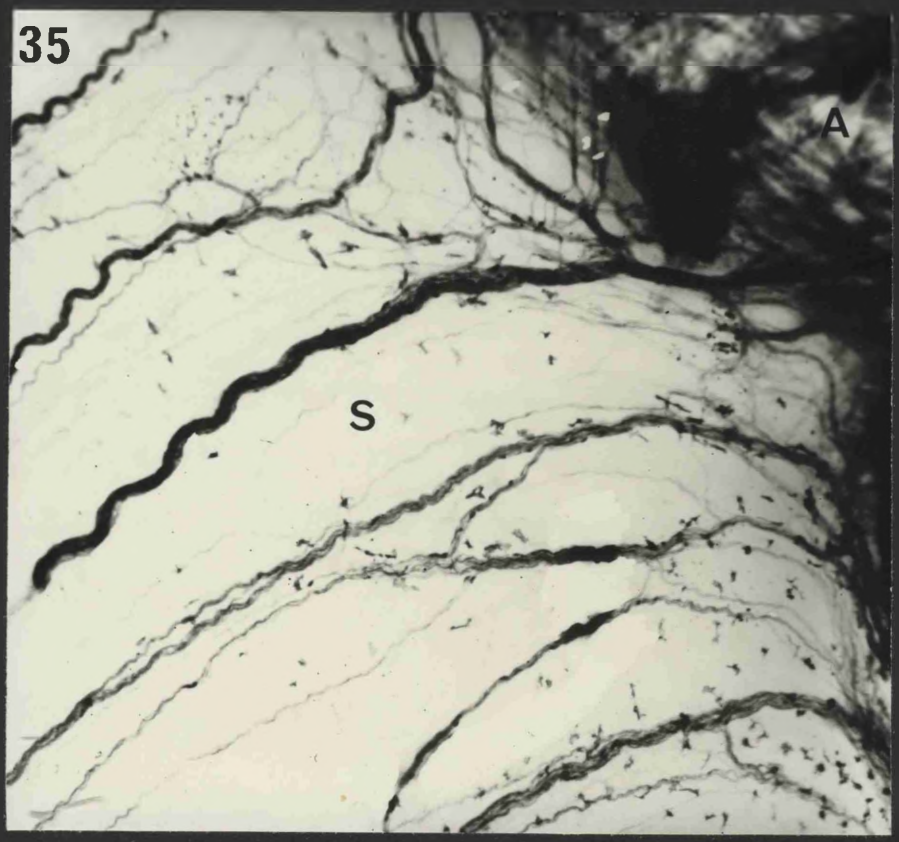
Figure 35.

Osmium stained stretch preparation of the sino-atrial junction to show nerves of the cardiac plexus running towards the atrium (A). Note the difference in size and composition of individual nerve branches and the considerable amount of branching of the nerves to form a plexus. Many chromatophores are seen in the sinus venosus (S). Magnification: X140.

Figure 36.

Osmium stained stretch preparation of the sino-atrial junction to show the plexus of nerves, some of which are seen passing into the atrium whilst others encircle the junction. Magnification: X450.

INSET. Electron micrograph of a myelinated axon from a plexus nerve to show the individual collagen sheath surrounding it. Magnification: X18,750.


35
A
S

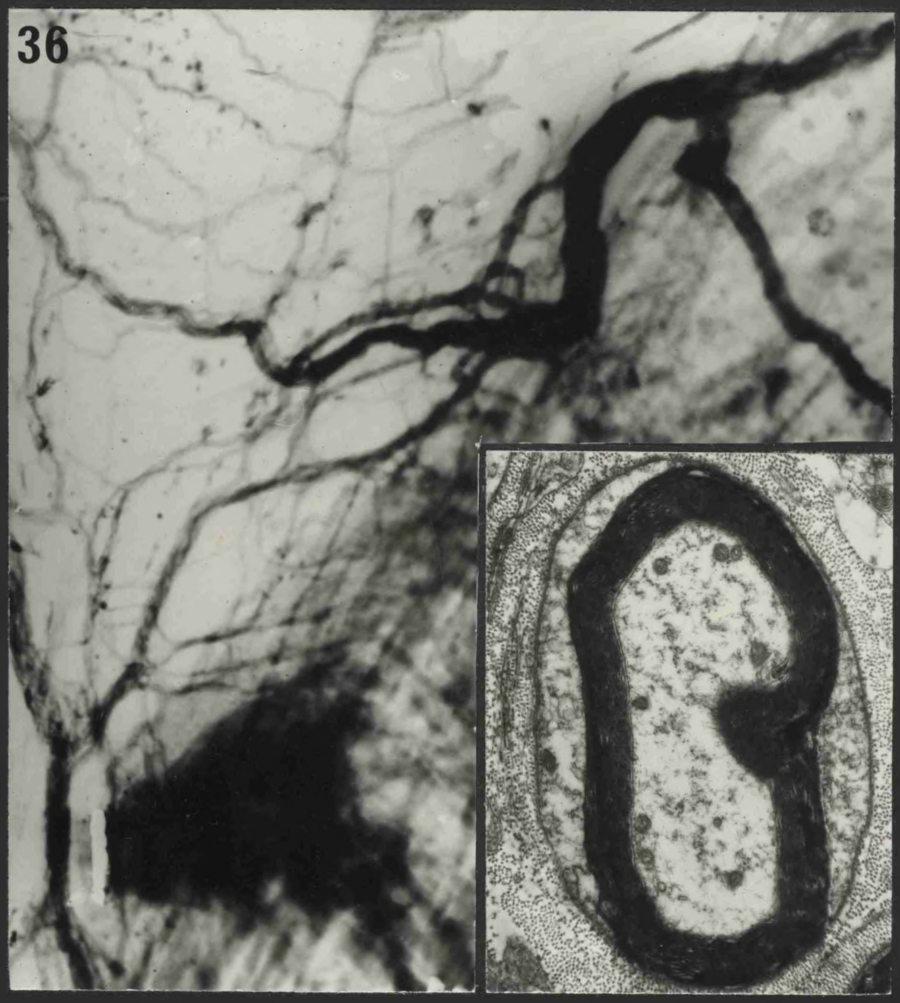

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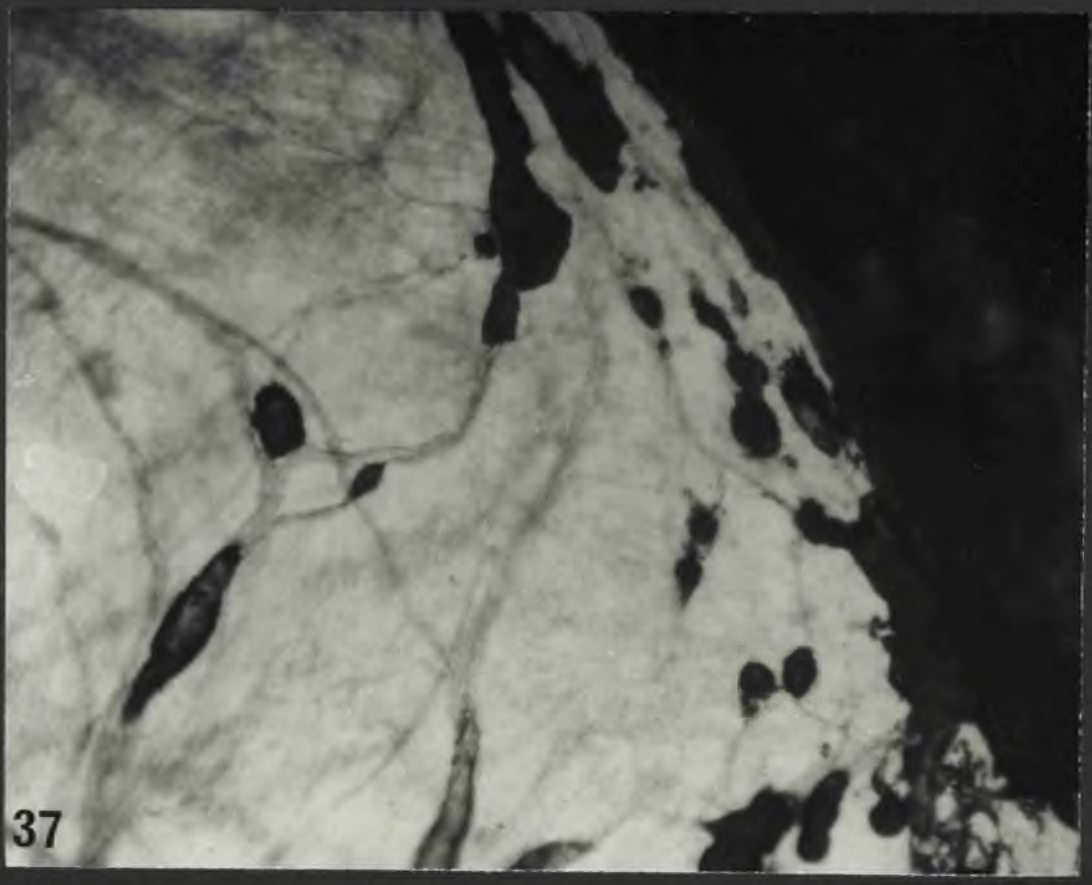
Figure 37.

Stretch preparation of the sinus venosus incubated for AChE for 40 mins. to show cholinergic nerves and ganglion cells (G) of the cardiac plexus. Magnification: X260.

Figure 38.

Transverse section of a nerve of the cardiac plexus containing fascicles of unmyelinated nerves (F), myelinated nerves (M) and Schwann cells (S). Two ganglion cells included within the nerve are cut at the axon hillock region (H) and are closely associated with small unmyelinated axons. Note also the axon-axon synaptic contacts (A) within the nerve. Magnification: X24,000.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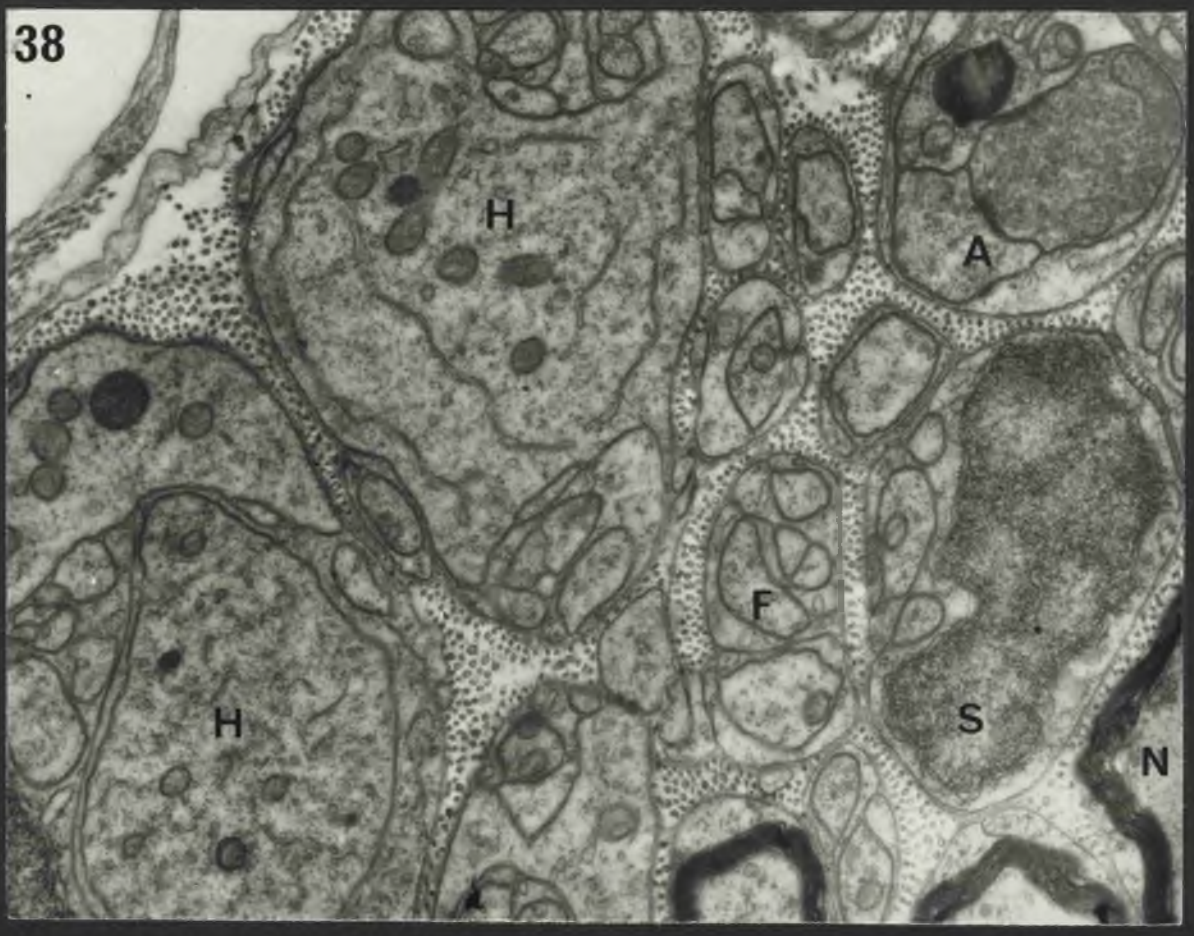

38
H
A
F
H
S
N

Figure 39.

Myocardial cells of the sinus venosuss in the region of the cardiac plexus. Myofibrils and mitochondria are randomly oriented within the cells which are enveloped by connective tissue processes. There is a small bundle of unmyelinated axons some of which are in close contact with the myocardial cells (A), and other axons are seen within the muscle bundle. S, Schwann cell. Magnification: X19,000.

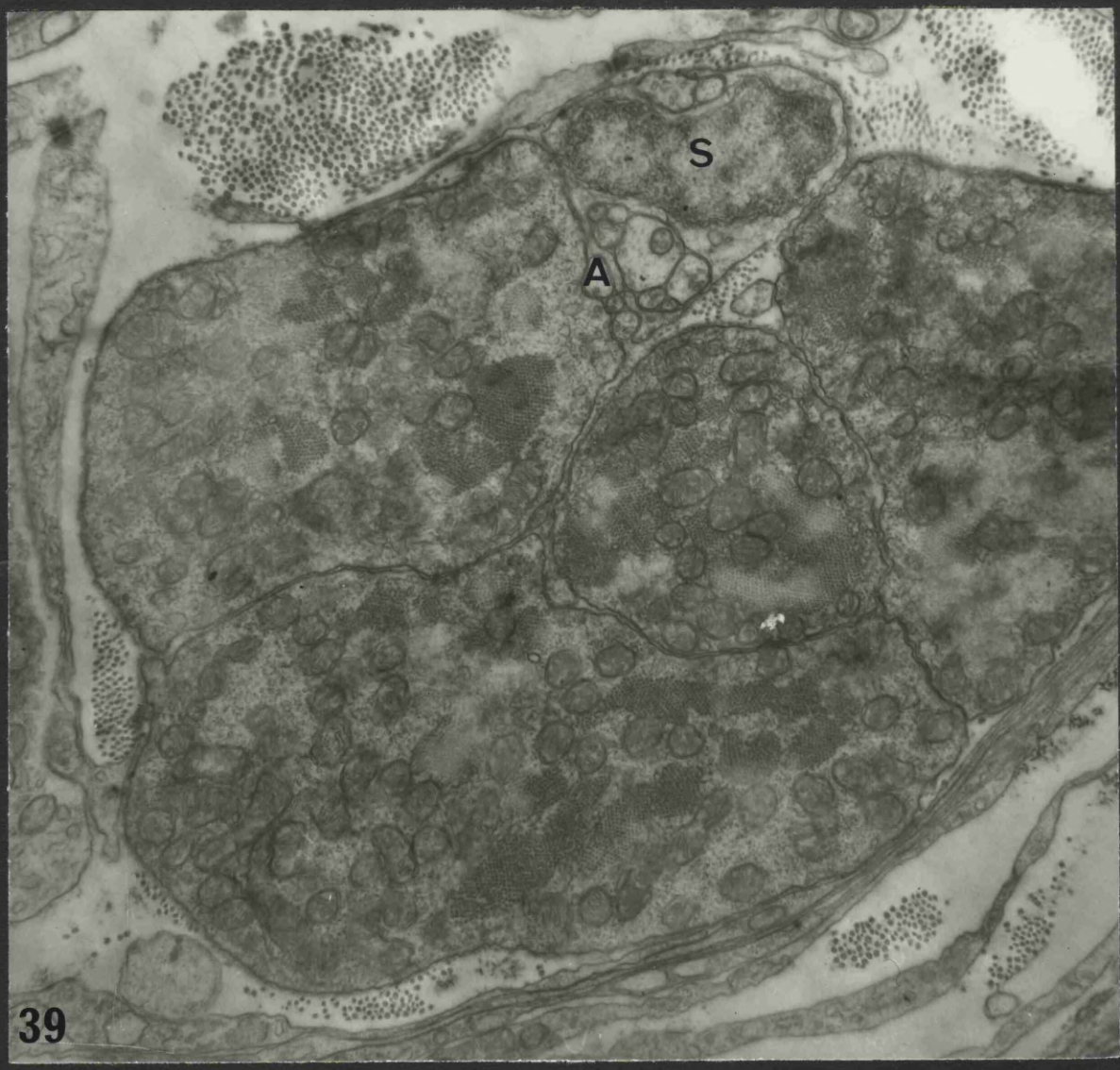
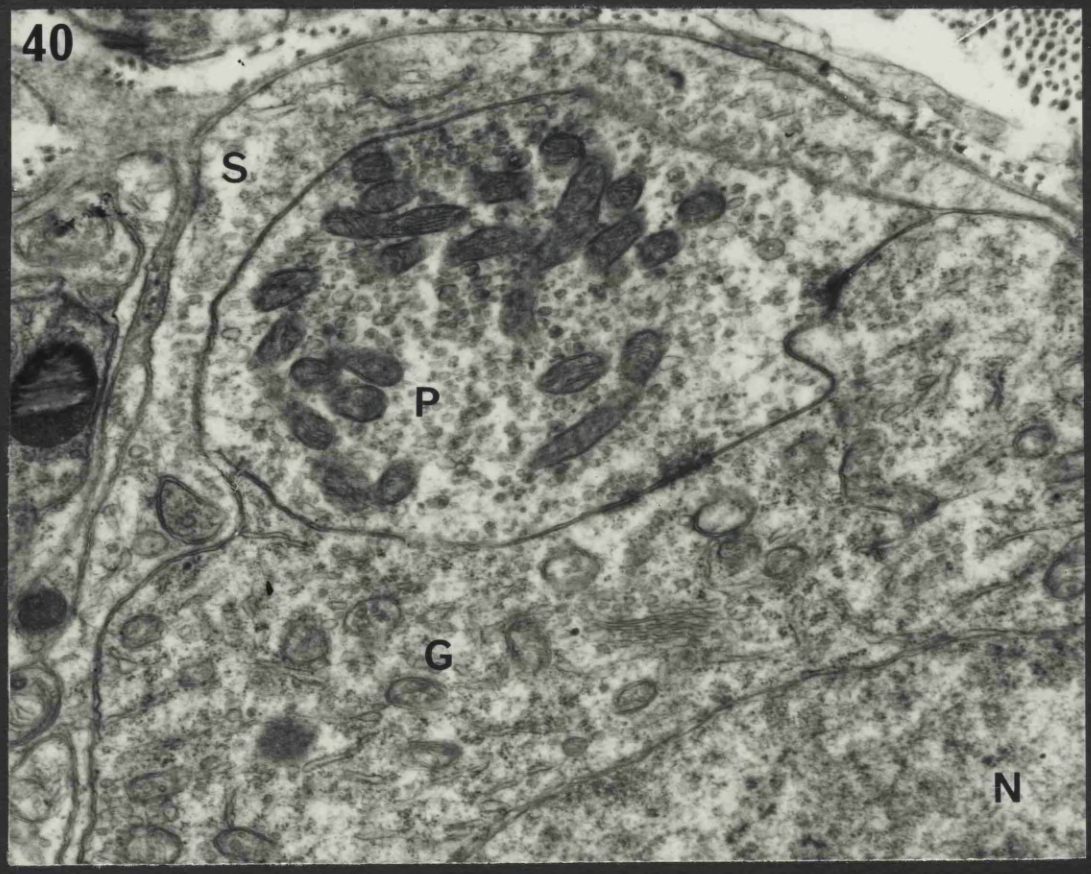

S
A
39

Figure 40.

Presumed preganglionic axon (P) making synaptic contact on the surface of a ganglion cell (G) of the cardiac ganglion. The axon contains many mitochondria and a large population of small (350-450Å) agranular vesicles. A few larger granular vesicles are scattered amongst the agranular vesicles. Note that there is an aggregation of agranular vesicles at the points where thickenings of the membranes indicate a point of synaptic contact. S, satellite cell process; N, ganglion cell nucleus. Magnification: X26,000.

Figure 41.

A preganglionic axon (P) indented into the perikaryon (K) of a ganglion cell and making synaptic contact. Note the expanded endoplasmic reticulum of the ganglion cell (E). N, nucleus. Magnification: X29,000.


40
S
P
G
N

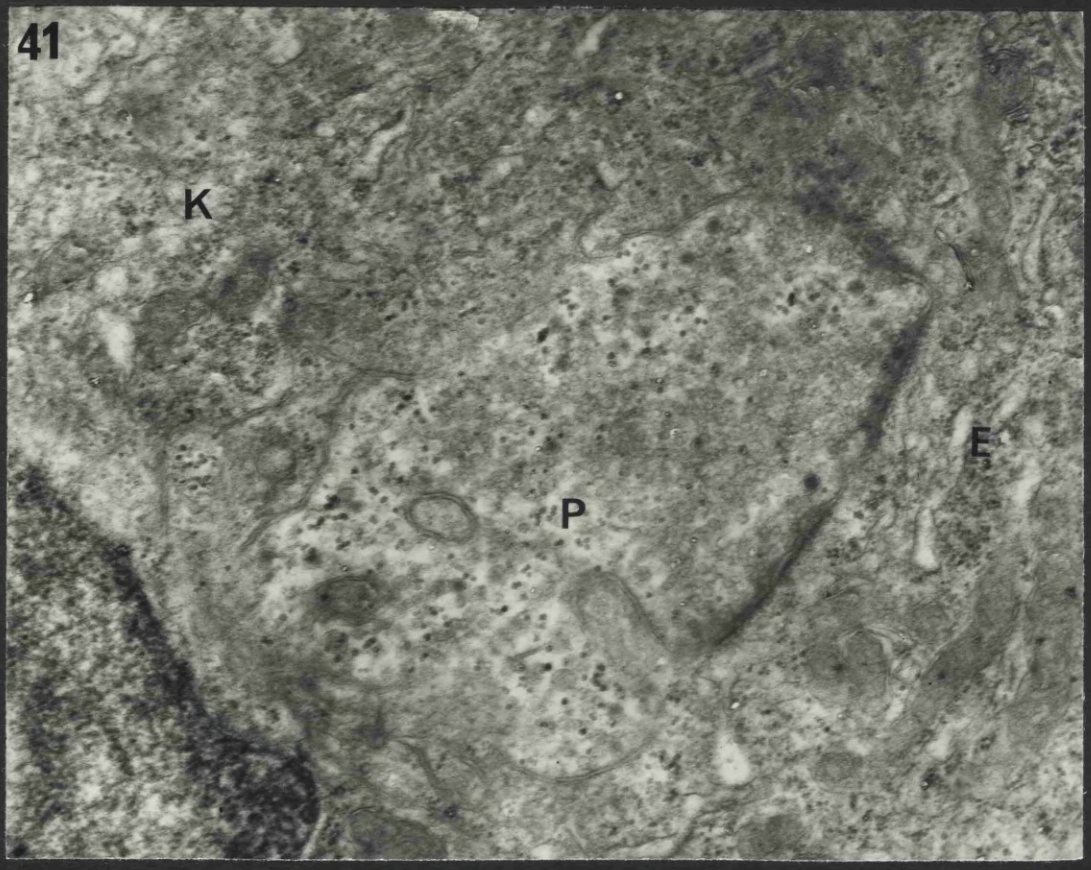

41
K
E
P

Figure 42.

Preganglionic axon (P) making synaptic contact with a ganglion cell (G). Note that there are very few granular vesicles in the axon profile compared with the axon in Figures 41 and 44. Magnification: X22,500.

Figure 43.

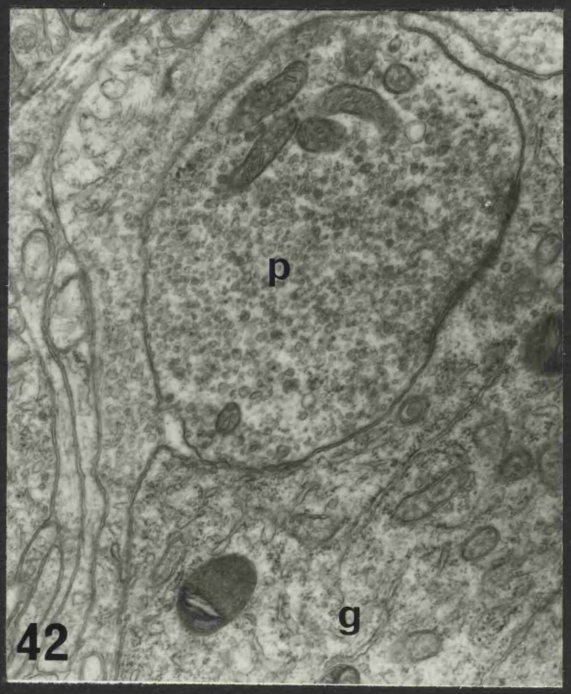
Preterminal region of a preganglionic axon (P) in close association, but not in synaptic contact with, a process from a ganglion cell (G). There is a very high proportion of large granular vesicles in this axon profile. Magnification: X29,000.

Figure 44.

Synapse between two adjacent axons at the sino-atrial region. This material was treated with 6-OHDA (100 mg/Kg for 2 hours) before fixation but the granular vesicles in the presynaptic axon (P) show no loading, nor does this terminal show any signs of degeneration. Magnification: X30,000.

Figure 45.

Synaptic contact between axons in a nerve of the cardiac plexus. The presynaptic axon (P) is filled with small agranular vesicles. Magnification: X29,500.



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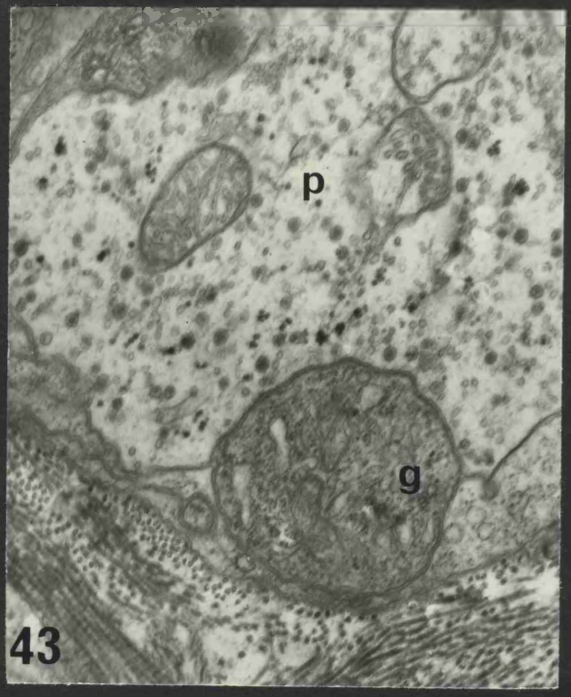

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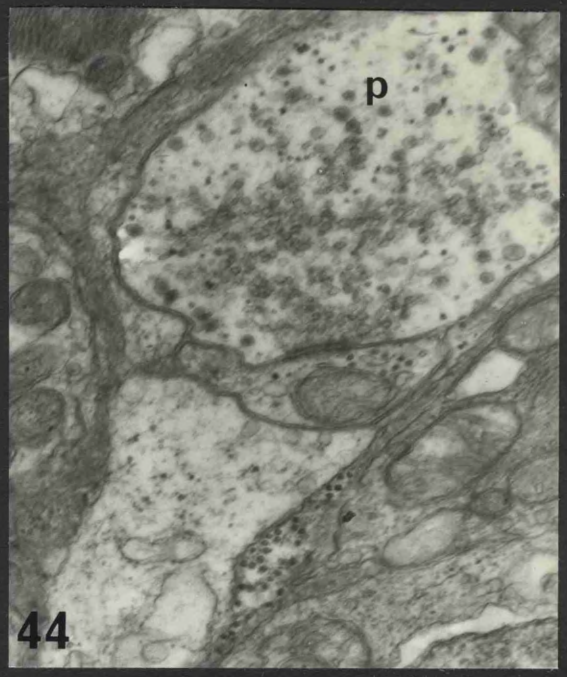

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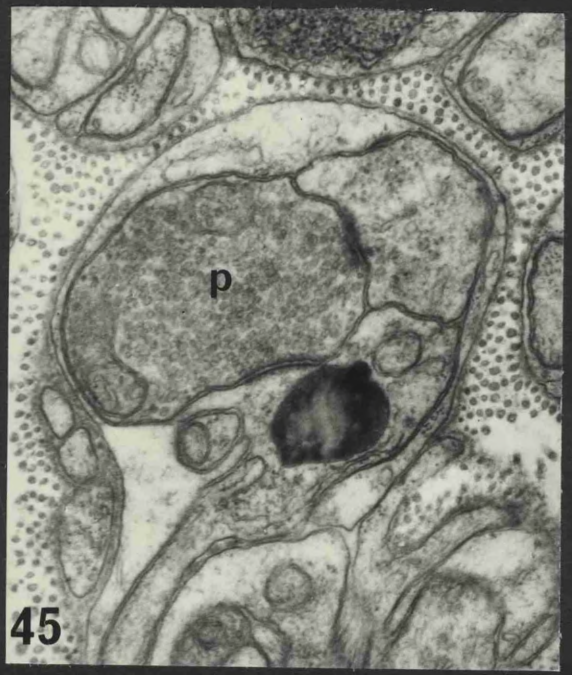


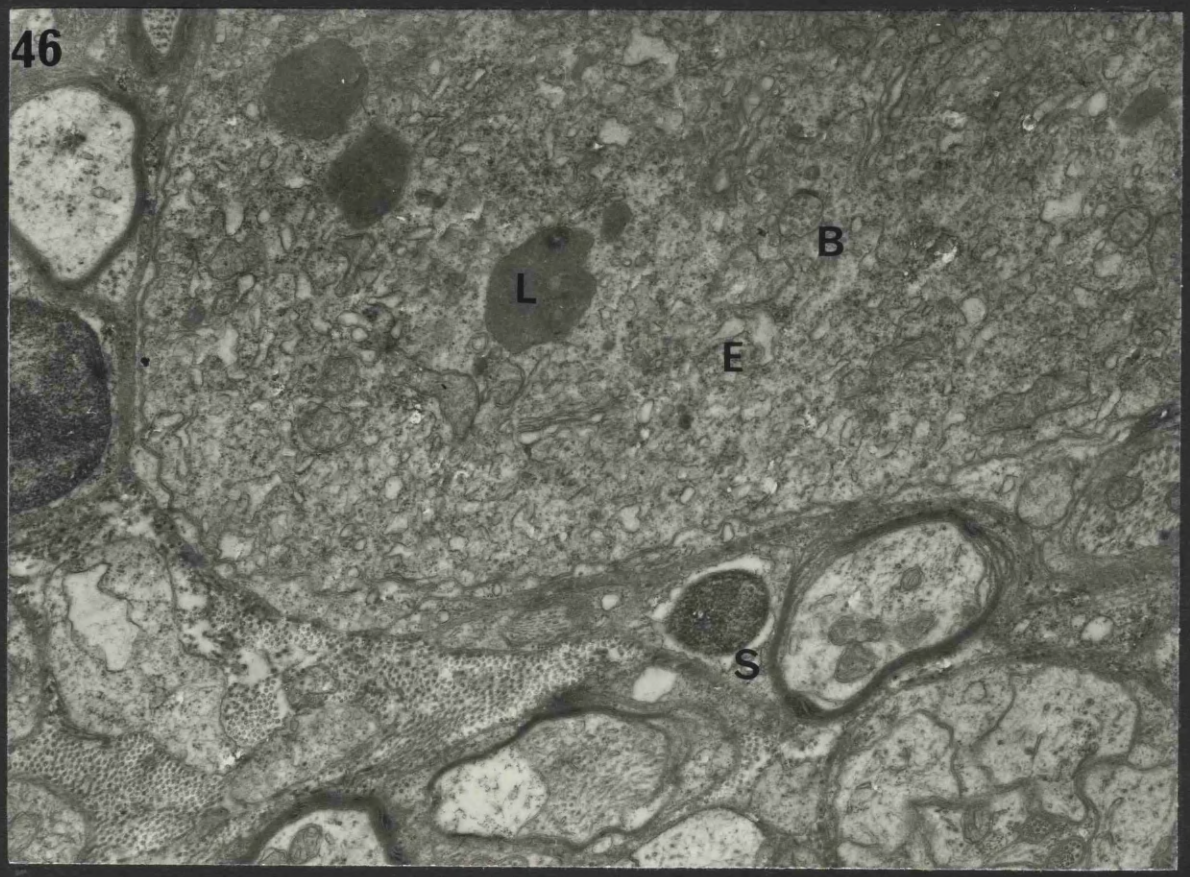
45

Figure 46.

A ganglion cell soma in the sino-atrial region. The perikaryon of the ganglion cell contains electron-dense lipid droplets (L), expanded endoplasmic reticulum (E), multivesicular bodies (B) and many small mitochondria. S, Schwann cell nucleus. Magnification: X18,500.

Figure 47.

A ganglion cell in the sino-atrial region. This ganglion cell is characterised by many long, thin mitochondria within the perikaryon. Note the abundance of concentric external lamina around the interestitial cell (C). Magnification: X15,500.


46
L
B
E
S

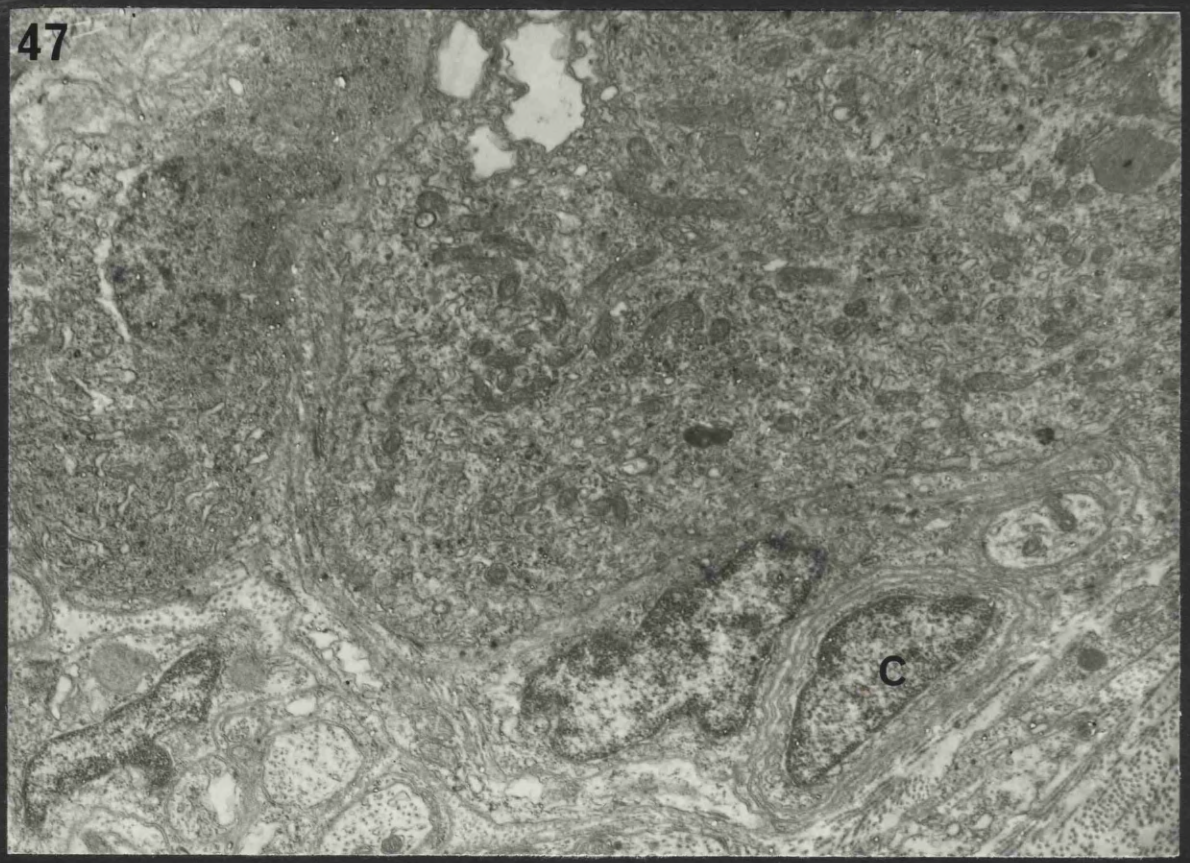

47
C

Figure 48.

Longitudinal section through part of the sino-atrial region to show the large tracts of nerves surrounding this junction. Many Schwann cell nuclei (S) are present in association with lightly myelinated axons which contain many mitochondria. Fasicles of unmyelinated nerves are also present (F). G. ganglion cell soma. Magnification: X11,000.


S
F
48

Innervation of the myocardium

Figure 49.

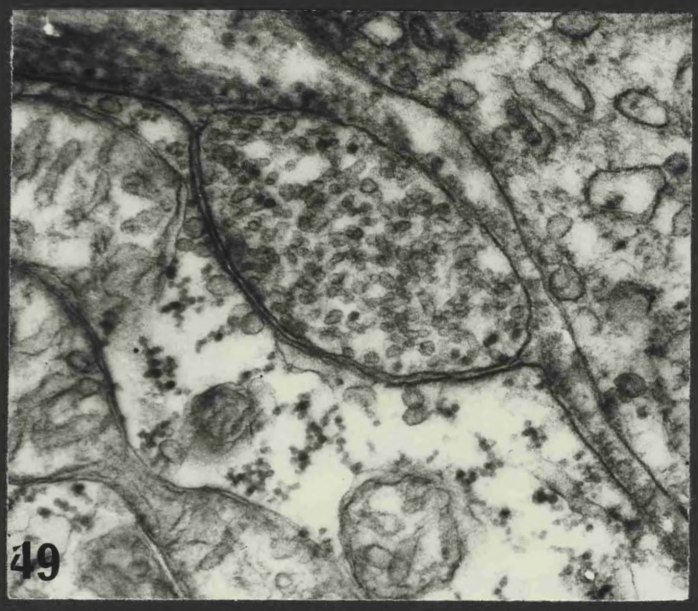
A single intramural unmyelinated axon is indented into the surface of an atrial muscle cell. The gap between the axon and the muscle cell is constant in width (approx. 200Å) but no specialised contact is observed. Magnification: X56,000.

Figure 50.

Intramural axon profile closely apposed to two muscle cells in the atrium. The axon contains small mitochondria (R), very electron-dense glycogen granules and a mixed population of granular and agranular vesicles. Note also the absence of any Schwann cell wrapping around the axon (cf. Figure 51). Magnification: X47,500.

Figure 51

Longitudinal section of an intramural axon passing between two muscle cells in the atrium. The axon (X) contains neurotubules (t) and neurofilaments (f) and is surrounded by a thin Schwann cell wrapping (W). Magnification: X70,000.



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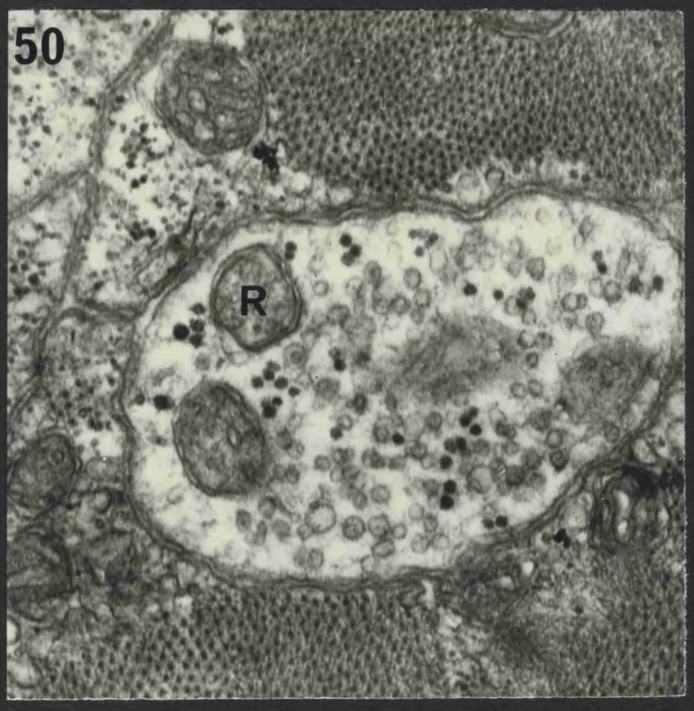
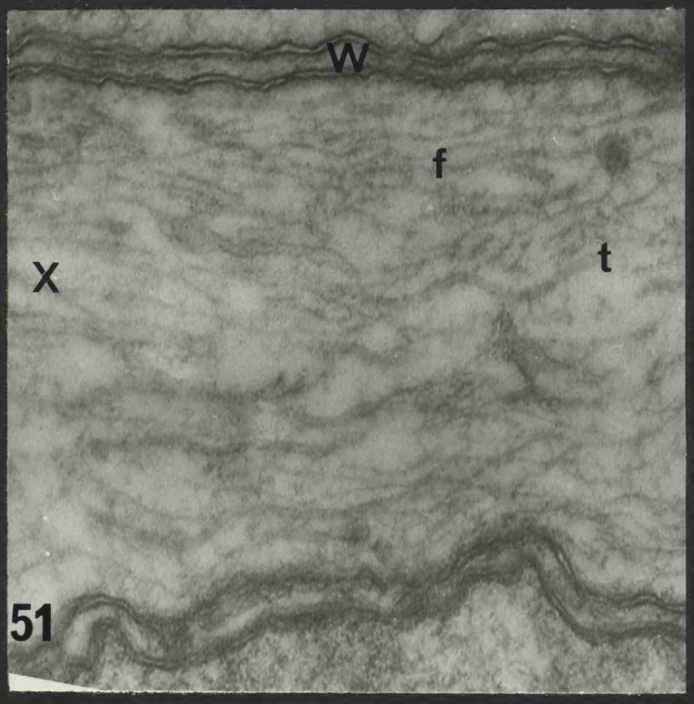




Light microscopical localisation of Acetylcholinesterase

Figure 52.

Stretch preparations of the sinus venosus stained for AChE (40 mins). Reaction product is seen in the ganglion cells and also in the nerve branches of the cardiac plexus. Note the location of certain ganglion cells at the points of bifurcation of the nerve branches. Magnification: X650.

Figure 53.

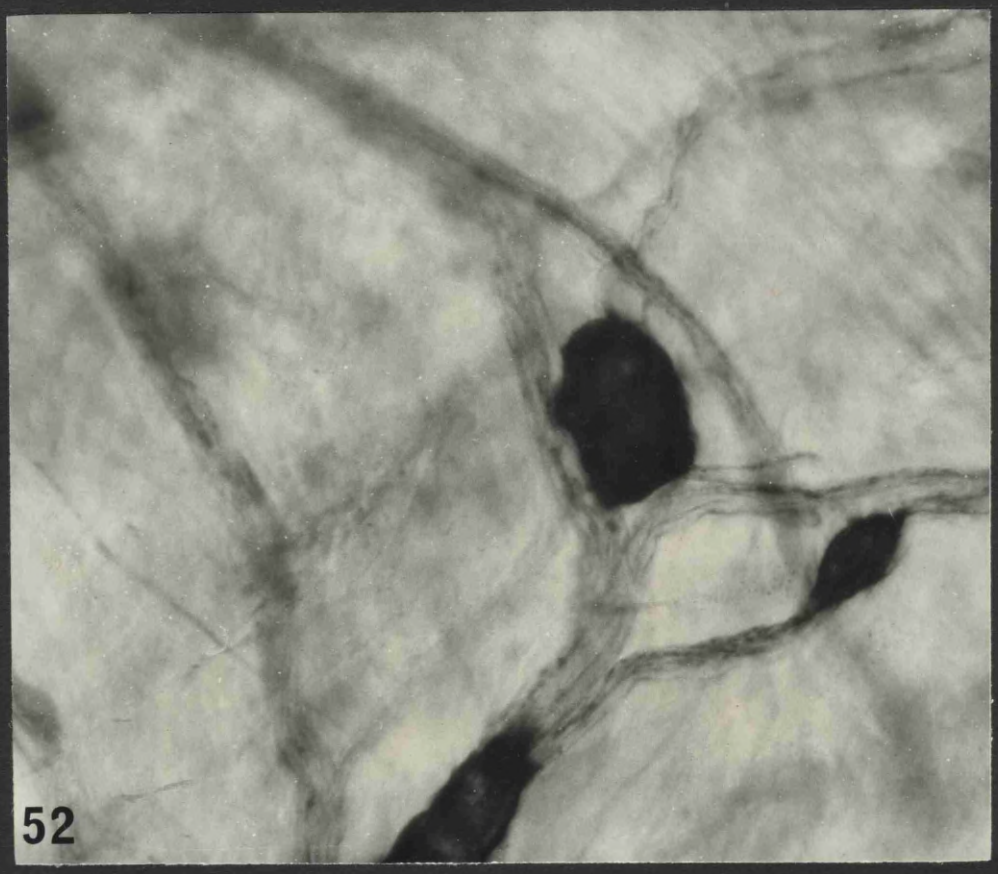
Ganglion cells at the sino-atrial junction stained for AChE (40 mins). SV, sinus venosus; A, atrium: Magnification: X220.

Figure 54.

Groups of ganglion cells in the sinus venosus stained for AChE (30 mins). Note the heavily stained nucleus. Magnification: X140.

Figure 55.

High power detail of ganglion cells stained for AChE (30 mins). The reaction product is seen at the surface of the cells and also in the nucleus. Magnification: X620.



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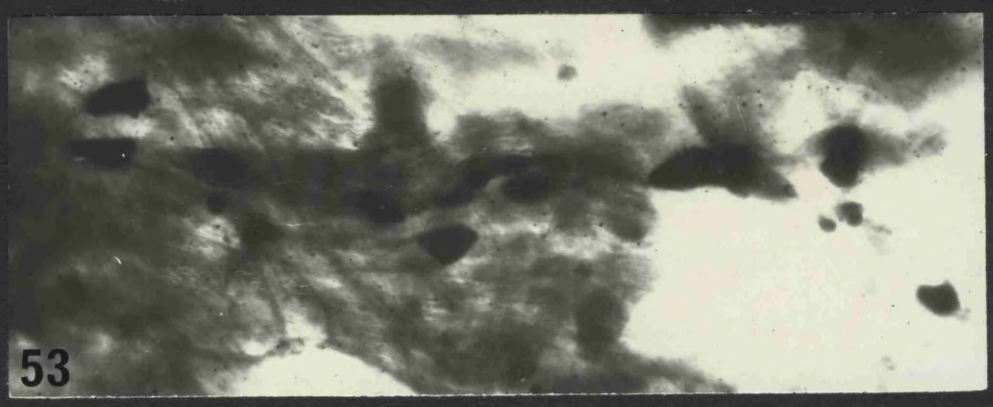

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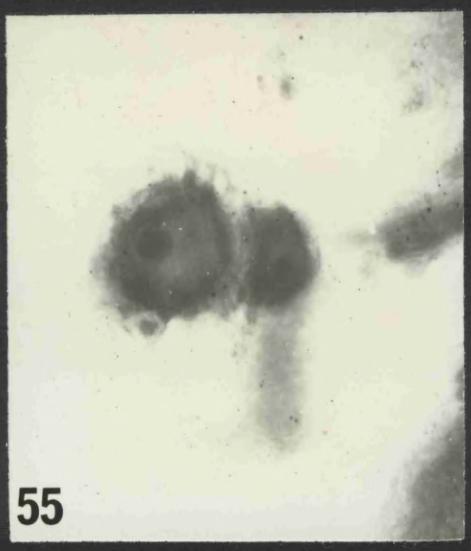

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Figure 56.

Sino-atrial region. AChE is localised on the ganglion cell soma membrane, between the axon membrane of unmyelinated axons and their Schwann cells (arrow) and on some Schwann cell membranes. No reaction product is seen within the ganglion cell perikaryon nor on the axon membrane of the myelinated axon. Incubation for 60 minutes. Magnification: X21,000.

Figure 57.

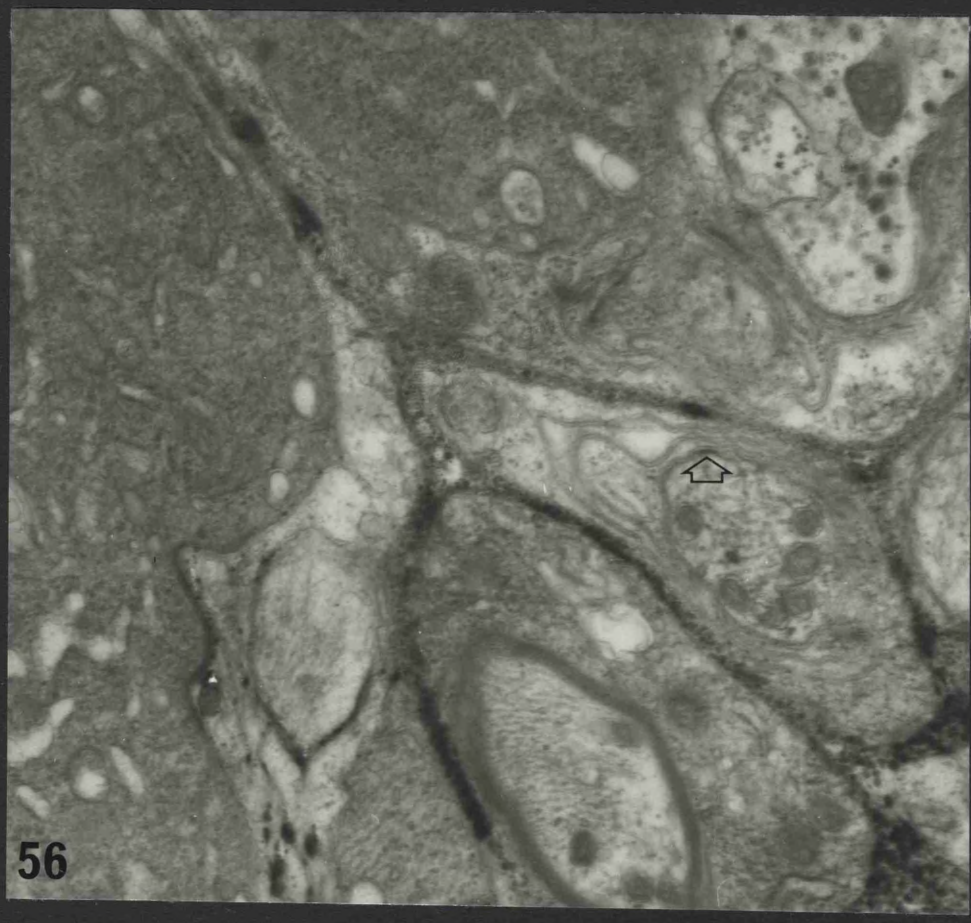
Detail of AChE reaction product between the ganglion cell membrane and the satellite cell membrane. Incubation for 60 minutes. Magnification: X30,000.

Figure 58.

AChE activity is present on the membrane of an intramural axon profile in the atrium. This profile contains a few granular vesicles amongst the population of agranular vesicles. K, muscle cell. Incubation for 30 minutes. Magnification: X38,500.

Figure 59.

A group of three intramural axons in the atrium. The reaction product is seen between the axon membrane and the associated Schwann cell membrane. Magnification: X37,000.



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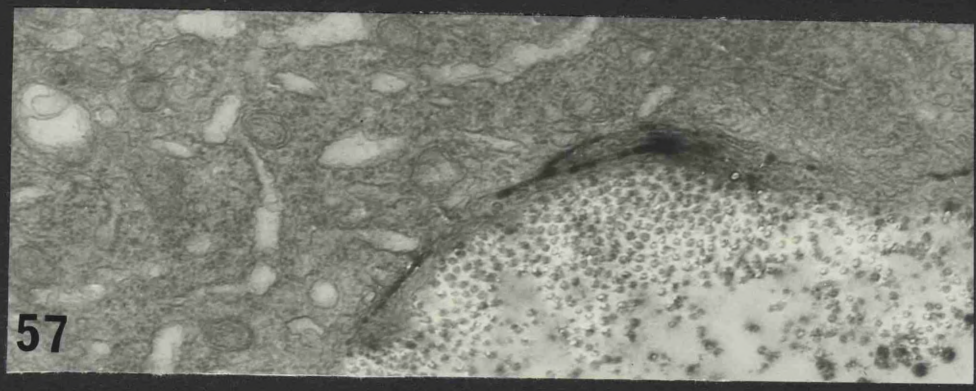

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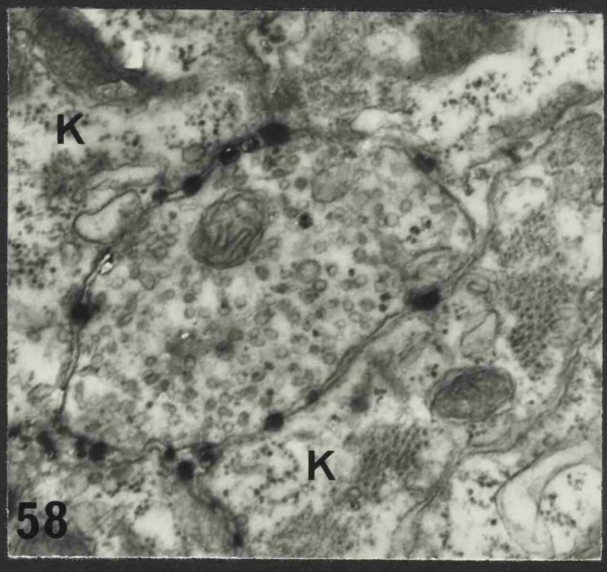

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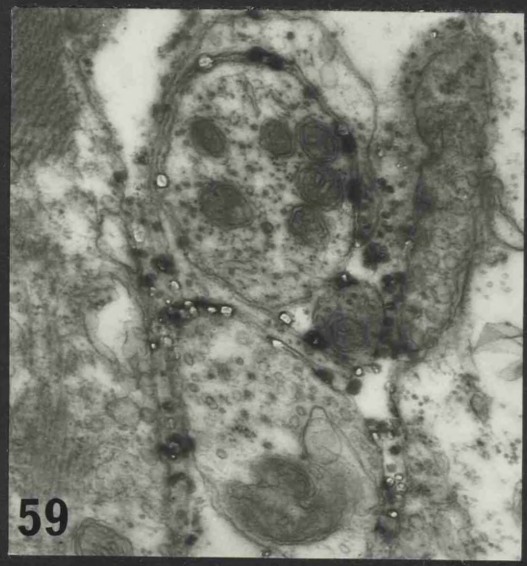

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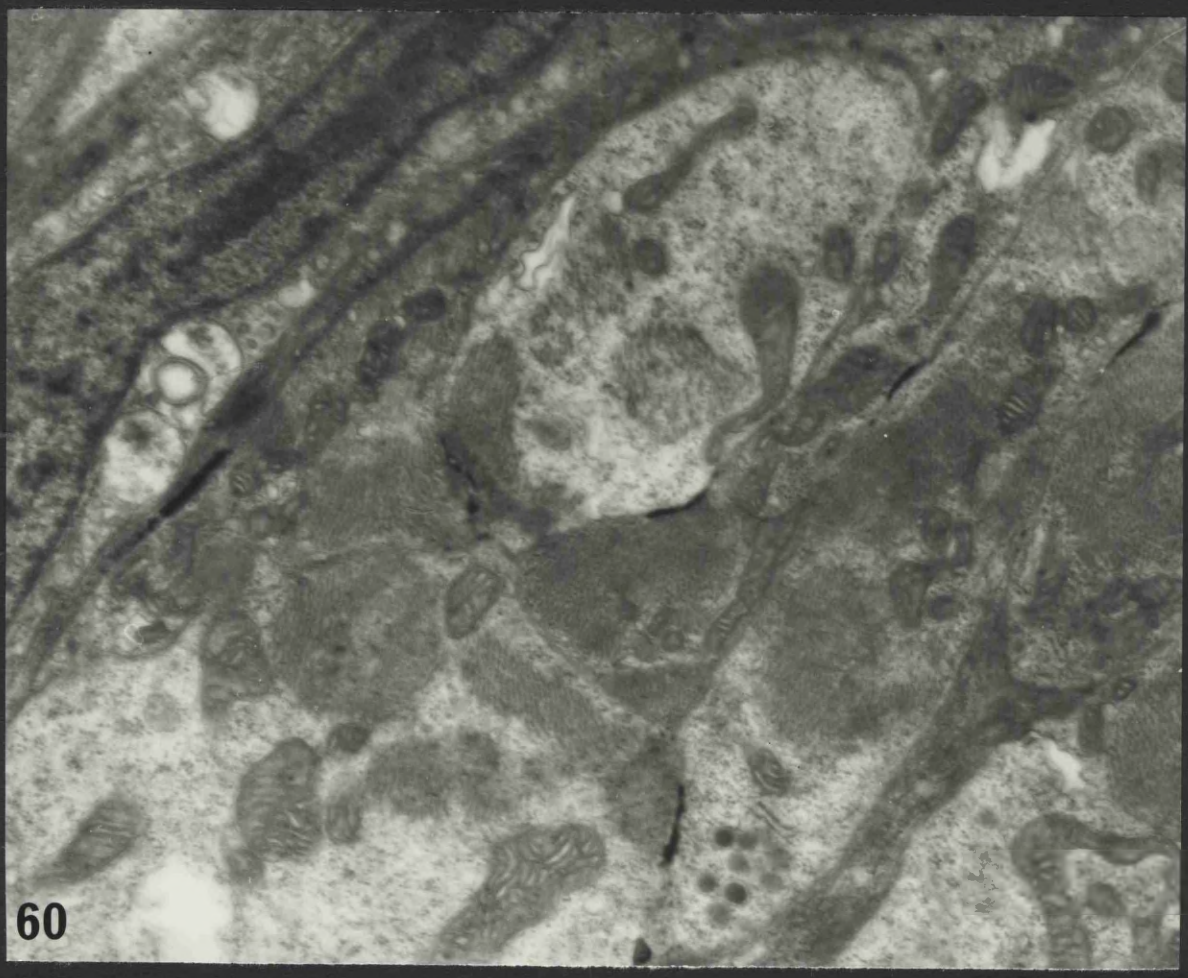
Figure 60.

AChE activity between atrial muscle cells. The reaction product is not associated in particular with any special feature of these cells and mine is seen in an intracellular location. Magnification: X24,000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VI. PHYSIOLOGY AND PHARMACOLOGY

1. INTRODUCTION

There has been little electrophysiological work on innervation of the teleost heart compared with higher vertebrates. Apart from the work by Govyrin & Leonteva, 1965; Gannon & Burnstock, 1968; Otsuka & Tomisawa, 1969; and Gannon, 1971, who report an adrenergic innervation of some teleost hearts demonstrated by fluorescence histochemistry the only general feature that has been demonstrated is that there is a parasympathetic inhibitory innervation (Skramlik, 1935; Jullien & Ripplinger, 1957; Laurent, 1962; Randall, 1966) of the heart. Stimulation of the vagus nerve in teleosts causes a bradycardia which can be blocked by atropine and these effects can be mimicked by exogenous acetylcholine. Kulaev (1957) believed that, besides the characteristic inhibitory response to vagal stimulation, the observable positive chronotropic effects of heartrate due to vagal stimulation at different parameters resulted from "active vagal influences" in the pike.

Perhaps the lack of information on the physiology of the fish heart is due to the small size of the muscle cells compared with amphibian and mammalian counterparts which are easier to impale with microelectrodes. Jensen (1965) made intracellular recordings from the muscle cells of the aneural hagfish heart, and cardiac action potentials of the trout cardiac cells were shown by Randall (1968) to be essentially the same as those of higher vertebrates. The only work reporting the intracellular recording of the interaction of vagal stimulation and pacemaker cells in teleosts is by Saito (1973) in the carp (Cyprinus carpio) heart. Isolated vagus nerve-heart preparations were used by Gannon and Burnstock (1968) for extracellular

recording and it should be noted that they could isolate cardio-inhibitory and cardio-excitatory responses to vagal stimulation from each other by differential stimulation using different stimulus parameters.

Receptor studies on isolated teleost hearts have been undertaken only by Gannon & Burnstock (1968) on the trout and by Falck et al. (1966) on the plaice heart although there are several reports on the pharmacology of isolated cyclostome hearts (Augustinsson et al., 1956; Falck et al., 1966; Lukomskaya & Michelson, 1972; see Fange, 1971).

The following study was undertaken in order to examine the types of cardiac action potential, the interaction of the vagus nerve with heart rate, to identify the transmitters involved in the vagal control of the heart and to re-investigate the effects of drugs on the isolated heart.

2. RESULTS

1) Electrophysiology

Intracellular recording from atrial cell types

The frog and all higher vertebrates have discrete regions of pacemaker cells at the sino-atrial region of the heart and an examination of the S-A region and atrium of the heart of the plaice was made in an attempt to locate a similar discrete region. The initial experiments were carried out recording from the dorsal surface of the heart close to the S-A junction. It is in this region that one of the two cardiac branches of the vagus breaks up to form the cardiac plexus. No concentration of cells showing characteristic pacemaker responses was found. In at least 90% of the cells impaled, the action potentials recorded showed a fast depolarisation rising from a steady resting potential at a rate of about 6.5 volts/second. This is the atrial cardiac action potential (Fig. 61). The shape of this atrial action potential is similar to that of certain other higher vertebrate atrial action potentials. After the rapid depolarisation, the initial rapid repolarisation is absent (unlike mammals) and there is no distinct plateau component of the repolarisation which is a simple decline with no inflections (Hoffmann & Cranefield, 1960). The small value of the rate of depolarisation is more comparable with that of the frog atrial fibres (Hoffmann & Cranefield, 1960) than with the much faster mammalian types. The resting potential averaged between 75-78 mV and the spike potential was 85-90mV with an overshoot of as much as 15mV. Scattered in an apparently random fashion throughout the atrium was a minority of cells showing two different types of potential. The first type, only rarely seen, was a typical pacemaker potential showing a slow diastolic

depolarisation between characteristic action potentials. The second type arose from a steady resting potential with each spike proceeded by a junction potential-like depolarisation similar to those seen in frog conducting fibres (Denoit-Mazet & Vassort, 1971) (Fig. 61), but in this case probably representing electronic spread from a neighbouring cell. All of the recorded action potentials had a repolarisation as much as twenty times slower than the depolarisation. The duration of all action potentials was 200-250 msec. Potentials with these characteristics were obtained from impalements of atrial cells in situ in five pithed animals, and from hearts dissected from fifteen animals.

A further survey was undertaken to sample cells of the whole atrium. Hearts were pinned out with either the dorsal or ventral surface uppermost. In other preparations the tube formed by the sinus venosus and atrium was split open so that cells could be impaled from the luminal side of the heart. These studies have not revealed a localised region of cells showing either type of interspike depolarisation and are in accordance with the earlier suggestion that the pacemaker system in fish cardiac tissue may be diffuse throughout the atrial myocardium (Laurent, 1962; Jensen, 1965), but it is very difficult to imagine how such an arrangement could function efficiently. A further study may however show that such a localised area exists, as suggested by Wardle (1962) situated at the median surface of the S-A junction in the flounder (Platichthys flesus) and as shown electrophysiologically in the carp (Cyprinus carpio) to be at the base of the S-A valves.

Extracellular recording during and after vagal stimulation

There is a cardiac branch of the vagus passing over the

dorsal (ocular) side of the sinus venosus which either remains intact or branches into a double tract as far as the pericardial membrane. Inside the pericardium this branch of the vagus splits up into a number of large and small tracts running over the sinus venosus before they reach the atrium. This plexus of cardiac nerves also contains the parasympathetic ganglion cell bodies (see above). The stimulating suction electrode was placed over the cardiac nerve where it initially branched over the sinus venosus. The pericardium was opened by a longitudinal slit and a second recording suction electrode was placed on the atrium. Heartbeat could always be completely blocked by applying stimuli of 0.5 volts for 0.03 msec duration at frequencies of 7 Hz and incompletely blocked by frequencies of 4-7 Hz (Fig. 62). The same results are achieved using a stimulating voltage as high as 40 volts. On cessation of stimulation the heartbeat returned after a delay of approximately 1.0-1.5 secs. (Fig. 63). The first and subsequent extracellular potentials were smaller in size than those at resting rate but increased in size until, after 4-5 beats they had returned to control values (Fig. 63). An intracellular recording of this phenomenon is shown in Fig. 63d. After the cessation of inhibitory stimulation the heart rate increased above the original resting rate for a short period before returning to the resting rate of that particular heart (Fig. 62a).

In a minority of experiments (about 1 in 5) low frequency stimulation (2 Hz), using the same stimulus parameters, produced an increase in the heart rate above normal (Fig. 62b). Fifty fish were used for the above *in vivo* study.

These experiments were repeated with whole nerve-heart preparations of forty fish dissected out, *in vitro*.

These studies have made it clear that the critical factor which determines excitation and inhibition due to vagal stimulation is the heart rate of the individual preparation itself. In whole animal experiments the heartrate after pithing remains high (1 per second or more) for long periods, but in dissected preparations it slows to 0.5/sec or slower, sometimes becoming irregular after about 30 minutes. In these preparations which were beating at a slow rate it was always possible to increase the rate of low frequency vagal stimulation of 3.1 Hz and at even slower frequencies to drive the heart on a 1 for 1 basis (1.2 Hz). (Fig. 64), whereas this was more difficult to achieve in whole animal preparations. Measurement of the stimulus parameters indicated that a change in frequency from 4 Hz to 4.5 Hz caused a change from excitation to partial inhibition and a change from 3.1 Hz to 6 Hz caused a change from maximum excitation to total inhibition. Maximum excitation was maintained from 3.1 Hz to 2 Hz but below this frequency the heart rate slowed until directly driven at a frequency of 1.2 Hz. Thus, as in the trout (Cannon & Burnstock, 1968), excitatory and inhibitory effects of vagal stimulation can be shown by differential vagal stimulation.

In some isolated preperations where the heartbeat had ceased it was possible with low frequency stimulation (2 Hz and below) to restart contractions which continued for the duration of the applied stimulus. A higher frequency stimulation applied to such quiescent preparations always caused at least one heartbeat on cessation of the stimulus, and sometimes several.

Intracellular recording during and after vagal stimulation

Impalements of atrial cells with microelectrodes were carried out on seven hearts *in situ*, and on forty *invitro* heart preparations pinned out in a preparation dish. The vagus nerve was again stimulated via a suction electrode. Each pulse applied to the vagus produced a hyperpolarisation of 2-3 mV/ with a duration of approximately 200 msecs. Higher frequency stimulation produced a summation of the hyperpolarisations which eventually produced a steady potential 6 mV below resting potential. The value of this steady potential is approximately half that noted by del Castillo & Katz (1955) in frog SV pacemaker cells and by Saito (1973) in carp pacemaker cells and it is likely that decremental electrotonic spread of conduction would account for this. Responses to different frequencies are shown in Fig. 65.

In preparations where low frequency (2 Hz) stimulation caused a speed-up of heart rate each hyperpolarisation was followed by a spike (Fig. 66). It has not proved possible in these recordings to separate the depolarisation from the hyperpolarised state into two phases; the spike appears to arise straight from the hyperpolarisation. In virtually all recordings, even from previously quiescent hearts, after high frequency stimulation had caused a summated hyperpolarisation, a single spike, and sometimes two or three, was initiated on cessation of stimulation (Fig. 66b). This excitatory effect was observed after vagal stimulation of the heart which raises the possibility that the action potentials observed are a secondary effect caused by a set of intramural excitatory nerves. Alternatively, the "rebound" effect may be an over-compensatory response of the muscle cell membrane resulting from the preceding hyperpolarisation due perhaps to an increase in sodium permeability.

In order to solve this problem, hearts were stimulated with current from external electrodes, in the presence of atropine (10^{-6} g/ml) and bretylium (10^{-5} g/ml) with the result that any nerves to the heart were effectively blocked. The effects of the nerve blocker tetrodotoxin on the teleost heart are as yet unknown but this drug sometimes effects the cardiac muscle potentials of higher vertebrates (Narahashi, 1972). For this reason tetrodotoxin was not used to block the cardiac nerves. In ten fish, after an applied hyperpolarising current to the atrium was stopped, a single action potential was almost always seen but in a few recordings as many as three occurred. This implies that the rebound effect is not nerve-mediated.

Effects of adrenergic and cholinergic blocking agents.

With the whole heart and vagal branch dissected out, a stimulating suction electrode was applied to the vagus nerve and a similar recording electrode to the atrium. When the stimulation electrode had been correctly placed and the heartbeat could be inhibited at 7 Hz., the preparation was allowed to achieve a relatively low rate of beating. When the heart was beating, stimulation of the vagus caused excitation at a frequency of 3 Hz. and total inhibition at 7 Hz with stimulus parameters of 0.03 msecs and 0.5V.

The effect of drugs was tested on hearts in this experimental condition. Atropine at 10^{-6} g/ml blocked both excitatory and inhibitory effects of vagal stimulation without altering heart rate (Fig. 67). Neither the adrenergic neurone blocker, bretylium tosylate nor the adrenergic β-blocker pronethalol at concentrations up to 10^{-5} g/ml had any effect on the excitatory or inhibitory responses to vagal stimulation. (Fig. 68). Also, the transient postvagal increase in heart rate was not

affected by the application of bretylium or pronethalol. Neither drug altered spontaneous heart rate.

ii) Perfusion studies and the effects of drugs on isolated hearts

Acetylcholine and cholinomimetics.

ACh at concentrations ranging from 10^{-6} g/ml - 10^{-12} g/ml was applied to the hearts (Fig. 69). Low concentrations (10^{-10} - 10^{-12} g/ml) produced negative chronotropic and inotropic responses whilst higher concentrations (10^{-6} - 10^{-9} g/ml) totally inhibited the heartbeat in diastole, the duration of inhibition lasting longer at the higher concentrations. A post-inhibitory rebound always occurred consisting in the first instance of a positive inotropic response quickly followed by a positive chronotropic response. Normal heart rate ensued after a period of time varying with the earlier applied concentration of ACh. With high concentrations (10^{-6} g/ml) of ACh, normal heart rate did not return for as long as 2 minutes after washing out of the drug. These effects of ACh were blocked by the muscarinic blocker atropine (10^{-7} g/ml) but not by the nicotinic blocker curare (10^{-7} g/ml). Differential application of ACh to the heart chambers indicated that these results were due entirely to its effect on the atrium, the ventricle being insensitive to exogenously applied ACh, but excited by spread of excitation from the atrium. Carbachol (carbamyl choline chloride) mimicked the effects of ACh but the threshold for carbachol was higher (10^{-7} g/ml). At the concentration of 10^{-6} g/ml, the effect of carbachol was much more potent than for an equivalent concentration of ACh, lasting up to 7 minutes after washing out. The post-inhibitory rebound following carbachol administration was not markedly different to that seen after ACh. The effect

of carbachol was blocked by atropine (10^{-6} g/ml) but not by hexamethonium or curare (10^{-6} g/ml). Responses to ACh were potentiated by the presence of eserine (physostigmine) (10^{-7} g/ml) in the perfusate.

The positive inotropic and chronotropic responses after ACh administration were not affected by either bretylium or pronethalol (10^{-6} g/ml).

Ganglion stimulants

Nicotine at 10^{-5} g/ml inhibited the heart, but this drug was effective on only 50% of hearts. The effect of nicotine was blocked by hexamethonium at 10^{-5} g/ml (Fig. 69).

Catecholamines

Noradrenaline and adrenaline at 10^{-5} g/ml always produced positive inotropic and chronotropic responses, the heart being about 10 times more sensitive to adrenaline. The three hold for response was at 10^{-8} g/ml and 10^{-9} g/ml for noradrenaline and adrenaline respectively.

These responses were blocked by the adrenergic β-receptor blocker pronethalol (10^{-6} g/ml) but not by the adrenergic neuron blocker bretylium at 10^{-6} g/ml previously added to the perfusate (Fig. 70). Both atrium and ventricle were sensitive to catecholamines.

Tyramine:

Tyramine at 10^{-4} g/ml had no effect at all on the heart rate.

5-Hydroxytryptamine:

5-HT at concentrations up to 10^{-4} g/ml was applied to ten hearts, five in July 1972 and 5 in December, 1972. In July, 5-HT elicited a transient positive chronotropic response lasting for about 45 seconds but in December, 5-HT up to 10^{-2} g/ml

did not elicit any response at all (Fig. 70). A similar seasonal variation in the sensitivity of the frog heart to 5-HT also exists (M.S. Laverack, pers. comm.). Both atrium and ventricle are sensitive to 5-HT.

3. DISCUSSION

The apparent diffuse occurrence of pacemaker cells throughout the sino-atrial region has made the examination of the effect of the inhibitory innervation upon them a difficult problem. Recently Saito (1973) has recorded transmembrane potentials from pacemaker cells at the S-A junction in Cyprinus carpio and also the effect of vagal stimulation upon these potentials. As in the frog sinus venosus (del Castillo & Katz, 1955; Hutter & Trautwein, 1956), and rabbit S-A node (Toda & West, 1965), vagal stimulation causes the slope of the diastolic depolarisation to be decreased until bradycardia occurs and an increase in stimulation frequency causes a hyperpolarisation of the pacemaker cell membrane which reaches a maximum amplitude of 18mV at 100Hz. Whilst the magnitude of this hyperpolarisation is similar to that seen in frog sinus fibres, a frequency of only 10Hz was used. The strength of the shock was not reported (del Castillo & Katz, 1955). The two types of cells showing interspike depolarisation have been previously described: the slow depolarisation between action potentials representing the true pacemaker potential (Hutter & Trautwein, 1956), and the more rapid pre-spike depolarisation being considered as characteristic of conducting fibre cells in the frog (Denöit-Kauet & Vassort, 1971). It seems more likely that electrotonic spread from propagated action potentials in neighbouring cells would produce identical effects to the latter type of recording.

The anatomical localisation of a region of pacemaker cells in the plaice remains obscure but it is difficult to conceive how diffusely placed pacemaker cells could operate as efficiently as a single pacemaker region at the S-A junction,

In contrast to all previous work on the effects of vagal stimulation of vertebrate hearts, the vagus-induced hyperpolarisation is seen in impaled cells in wide regions of the atrium of the plaice as well as at the S-A junction. In the mammal (Hoffman & Suckling, 1953), frog (Hutter & Trautwein, 1956) and carp (Saito 1973), such hyperpolarisations are restricted to the pacemaker cells whilst the diastolic membrane potential of atrial cells remains constant during vagal stimulation. A combination of two factors could explain this. First is that either all or at least a considerable percentage of atrial cells are latent pacemaker cells having the capacity to respond passively to vagal stimulation and second that the inhibitory innervation is very extensive. This latter condition might reflect the absence of any S-A nodal tissue in the plaice heart. Such a system of latent pacemakers could function effectively if a dominant region existed, probably at the S-A junction. The resting potential of frog pacemaker cells is less than that of the atrial cells (Hutter & Trautwein, 1956) which is about -70mV. Therefore the equilibrium potential of the inhibitory hyperpolarisation of the pacemaker cells would approach but not exceed the atrial cell resting potential which would thus not show a hyperpolarisation during inhibition of the heart. On the other hand, if a system of widespread latent pacemakers exists, one could predict that they would all have a similar resting potential and would hyperpolarise during inhibitory stimulation.

The observation of a very subtle difference in vagal stimulation frequency causing a change between excitation and inhibition of the heart was also reported by Kulaev (1957) in

heart of the pike. Kulaev achieved cardio-acceleration in response to vagal stimulation but there is no mention of stimulus parameters used in his experiments. This does not permit the extrapolation of the results of the present study to cardio-regulation in the pike and be able to state that this is also effected by rebound excitation. The phenomenon of vagus-induced excitation and inhibition of the heart does not only apply to fish. Udelnov (quoted in Kulaev, 1957) demonstrated in both intact and sympathectomized frogs that there were similar vagal effects in responses to differential stimulation and that these effects are determined by the number of vagal impulses that "impinge simultaneously on the heart". Heartrate is accelerated in response to few impulses and inhibited by a large number of impulses. An important consequence of direct vagus-induced cardio-acceleration in fish is that it casts doubt upon the suggestion of von Skramlik (1935) and Jullien & Ripplinger (1957) that the post-ganglionic cell bodies are tonically active and held under an inhibitory control of the vagus. By this means, it would not be possible to excite the heart directly, but only to alter the degree of inhibition upon the ganglion cells, which would cause cardio-acceleration. If this were the case, it should be possible to achieve a gradual increase of heartrate as the frequency of stimulation was reduced, but the observed changes of heartrate in response to different frequencies of vagal stimulation are very sudden, for small frequency changes. The use of blocking drugs demonstrates that in the plaice both the excitatory and inhibitory effects during vagal stimulation are mediated cholinergically and not by an adrenergic innervation and that the vagus is not a "mixed" nerve.

The transient increase in heartrate following inhibitory vagal stimulation has been reported many times in mammalian cardiac physiology under the names of postvagal tachycardia, cardiac escape and post-stimulus excitation. The mechanism responsible for this phenomenon has been suggested to be one of several types: sympathetic nerve stimulation that outlasts vagal stimulation (Warner & Russell 1969); release of catecholamines from vagally innervated intracardiac stores (Kottegoda, 1953; Copen et al., 1968); ACh depolarisation of intracardiac sympathetic endings (Leaders, 1963); an "inherent myogenic property of cardiac muscle" (Raper & Wale, 1969).

However, the only experimental analysis of this postvagal tachycardia has been by Burke & Calaresu (1972) on the <u>in situ</u> cat heart and the isolated rabbit vagus-atria preparation. These authors showed primarily that the occurrence of postvagal tachycardia was dependent on a preceeding vagal bradycardia and that the size of the two responses are correlated. They showed also that the post-vagal tachycardia was not due to any adrenergic mechanism whether direct or indirect, nor was it due to a simple cholinergic reflex. Intracellular recordings of the pacemaker cells in the rabbit preparation during postvagal tachycardia showed that the maximum diastolic potential was greater due to a hyperpolarisation and that the slow diastolic prepotential was steeper than in the control heart rate. Thus the suggestion by Raper & Wale (1969) that an inherent property of the muscle cell membrane is involved seems the most likely answer. Misu & Kirpekar (1968) also indicate that postvagal tachycardia lasting for as long as two minutes after the cessation of

vagal stimulation is due to a direct effect of vagal stimulation on the muscle cells in the cat atrium.

All cells impaled in the plaice sino-atrial region showed a hyperpolarisation to a single pulse vagal stimulation and it would therefore seem likely that there is an extensive inhibitory innervation in this region. Anatomical and histochemical findings agree with this suggestion with regard to the sinus end of the atrium.

In the present report the excitatory effect of stimulation at low frequencies and also following the cessation of prolonged inhibitory stimulation (postvagal tachycardia) results from a rebound phenomenon arising from the hyperpolarisation as described in smooth muscle by Bennett (1966) which involved an over-compensation of the muscle cell membrane as a result of the inhibitory hyperpolarising action of ACh on the innervated cells which is electrotonically propagated throughout the myocardium. It is possible that the membrane potential rises slightly above its normal level due, perhaps to an increase in sodium permeability and that this accounts for the increased rate of action potential firing.

Also, pharmacological studies indicate that, in the plaice heart, applied catecholamines do not effect this response and that the observed "rebound" is a response of the muscle cell membrane to the preceeding vagus-induced hyperpolarisation. Further support to this hypothesis is provided by the fact that the "rebound" response persists in preparations electrically hyperpolarised in the presence of both atropine and bretylium. Therefore the rebound is unlikely to be a secondary nerve-mediated excitation. Similar rebound excitation

is seen in various smooth muscle preparations (Bennett 1969; Furness, 1970) and in all cases is due to a myogenic mechanism, although the ionic basis of this phenomenon is not known.

It is therefore suggested that the mechanism underlying postvagal tachycardia in the plaice is an "inherent myogenic property of cardiac muscle" (Raper & Wale, 1969) due to a hyperpolarisation of the muscle cell membrane from which it rebounds, (perhaps due to an increased sodium permeability) and in so doing, overcompensates to increase the resting membrane potential above the control rate with a resultant increased rate of firing. This mechanism would also apply to direct excitatory vagal stimulation (see below). It may well be that this mechanism is that which causes the observed postvagal tachycardia in some mammalian hearts.

The critical threshold between inhibition and excitation can be explained by reference to the time course of the vagus-induced hyperpolarisation. High frequency stimulation (7 Hz and above) leads to summation of the inhibitory hyperpolarisation as seen by del Castillo & Katz (1955) in the frog heart and by Saito (1973) in the carp heart, whilst lower frequency stimulation of the atrial cells gives rise to a rebound spike from the hyperpolarisation before the next inhibitory stimulus. Clearly the refractory period of the muscle after each spike potential is also involved since it is only at frequencies below 1-2 Hz that the heart can be directly driven. It must be emphasised that it is not yet known whether the rebound excitation is the mechanism whereby the heart rate is speeded up in the intact fish and, in this context, Udelnov's (1955) results are of great interest. A study of the heart rate in intact animals and in those under stress may help to solve this problem.

It is interesting to note that the intensity of stimulation but not the frequency or duration used by Gannon & Burnstock (1968) is more than ten times greater than that used in the present study in order to detect the same effects of varying stimulus frequency on the heartrate. Using stimulation parameters of up to 40 volts, identical results to those with lower stimulating voltages were achieved. This would preclude the possibility of the lower (0.5V) stimulating voltage failing to excite non-myelinated sympathetic efferent fibres, should they be present. The effects of atropine and of the adrenergic blockers were identical at higher voltage stimulation. However, Gannon & Burnstock (1968) and Gannon (1971) using field stimulation combined with blocking drugs have shown that the inhibition is cholinergically mediated and the excitation adrenergically mediated in the trout. But it is also conceivable that their results are due to the sympathetic transmitter being released from the adrenergic perivascular plexuses around the coronary blood vessels by the high (40-80V) stimulating voltage and acting upon the myocardium endogenously. The significance of the very precise stimulus frequency dependence of heartrate using two different neural mechanisms to achieve the same result is not clear and supports the idea that this may be due to vascular overflow. The fact that Gannon & Burnstock (1968) also showed an increase above normal heart rate after inhibition may itself indicate a change in the threshold of pacemakers or a rebound phenomenon to inhibitory stimulation.

There is a change in the height and duration of the first few action potentials following inhibition. Such a phenomenon is widespread during and following partial vagal inhibition in vertebrate sino-atrial tissue (Hutter & Trauwein, 1956; Toda &

West, 1965; Saito, 1973). It may be that the gradual increases in size of action potentials represent active events in cells other than the impaled cell, not being propagated, but spreading electrotonically through low resistance intercellular junctions. Possibly the spike potential is initiated within a group of latent pacemaker cells but is only propagated over restricted but increasing distances for the first few spikes. An alternative explanation is that the increased rate of the falling phase of these initial post-inhibitory action potentials is perhaps due to a transient permeability increase of the sarcolemma of all cells to potassium ions during inhibition which outlasts the permeability changes causing the hyperpolarisation and inhibition itself. It is generally assumed that the effects of cardiac vagal inhibition and those of applied acetylcholine are the same and that they cause an increase in permeability of the pacemaker cell membrane to potassium ions (Burgen & Terroux, 1953; Pappano, 1972).

In general the present report concerning the cholinergic and aminergic receptors of the heart is in agreement with that by Falck et al., (1966) on the plaice heart although these authors did not indicate which of the chambers of the heart were responding to the applied drugs.

Both the atrium and ventricle of the plaice heart were sensitive to biogenic amines as in the mammalian heart. The seasonal variation in sensitivity of the heart to drugs occurs in frogs (Hutter & Trautwein, 1956) is not restricted to 5-HT. This seasonal factor might explain the insensitivity of the trout heart to 5-HT (Gannon 1971) but the positive effect of this drug on the bulbus arteriosus of this fish (Klaverkamp & Dyer, 1971).

The responses of the plaice heart to catecholamines can be blocked by the adrenergic β-blocker pronethalol which indicates that the plaice adrenergic receptors are of the same type as mammals. With respect to the possibility of a sympathetic adrenergic innervation of the plaice heart, it should be noted firstly, that the exogenous application of tyramine had no effect upon the heartrate. Tyramine is an amino-acid that displaces catecholamines from intraneuronal and other intracellular stores. Were any catecholamines present in the heart, their release by tyramine would produce a positive inotropic and/or chronotropic response. The sensitivity of the plaice heart to 10^{-8} g/ml and 10^{-9} g/ml of noradrenaline abd adrenaline respectively means that even if tyramine were to release only minute amounts of catecholamine, it would stimulate the heart. Second, β -blockers have no effect on the post-inhibitory rebound after ACh application which precludes the possibility of an ACh-stimulated secondary excitatory nerve response.

The ventricle is not sensitive to ACh as is the case for higher vertebrates, although the atrium is very sensitive to this drug, responding to a concentration as low as 10^{-12} g/ml. The ACh receptors seem to be of the atropine and hexamethonium type as in mammals. The lack of sensitivity of the ventricle to ACh is interesting since it is tempting to correlate this with the lack of a parasympathetic innervation and to say that the development of ACh receptors is dependent upon the presence of an innervation as inferred by Armstrong (1935) in the Fundulus heart. However, in isolated embryo chick hearts, the heart is sensitive to ACh even though the ionic response to ACh

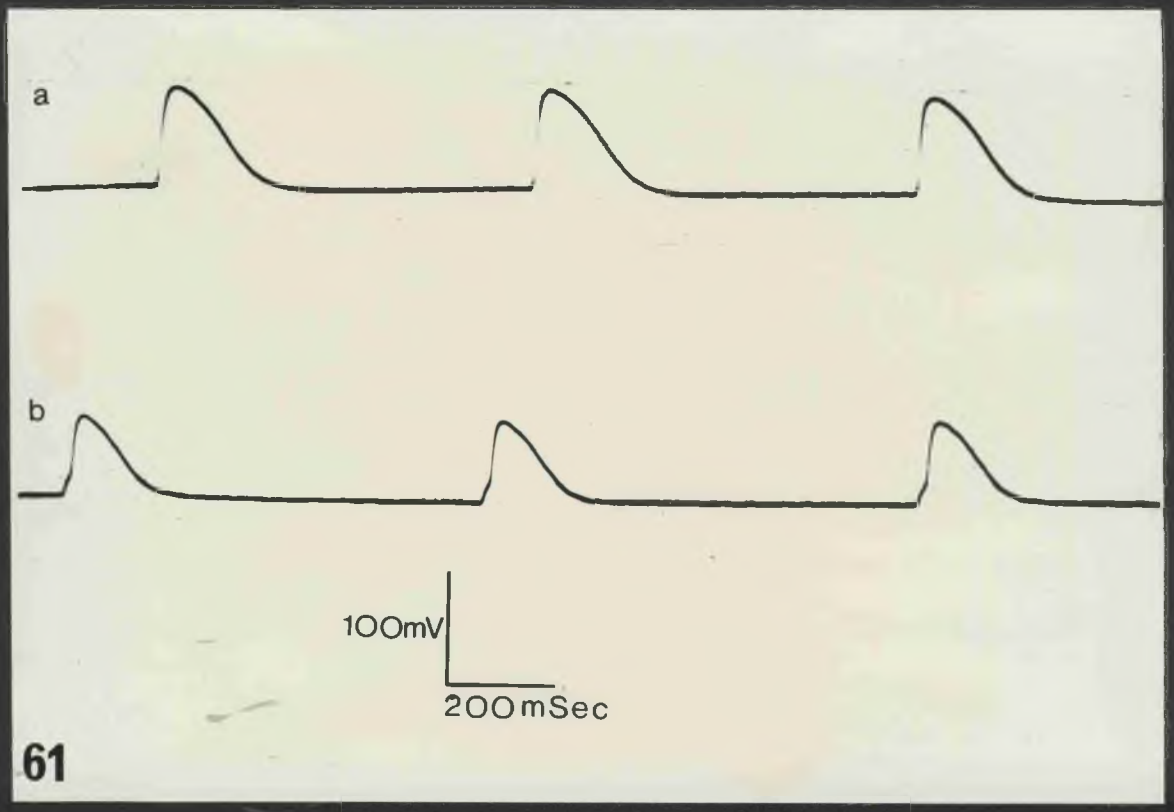
stimulation is sodium-dependent rather than the potassium dependent response of the innervated heart (Pappano, 1972). Thus one can conclude that ACh receptors never developed on the ventricular myocardium of the plaice for a reason unknown or that the correlation between ACh receptors and an innervation is not fortuitous and might be a primitive characteristic of the teleost heart.

Figure 61.

(a) Normal intracellular spike observed in most impaled atrial cells. (b) Record showing slow pre-spike depolarisation. It is proposed that this slow depolarisation is due to electrical events in neighbouring cells failing to propagate between coupled cells but manifesting themselves by electrotonic spread to the impaled cell.

Figure 62.

(a) Partial inhibition of heartbeat during vagal stimulation of 5Hz. (b) Excitation and total inhibition to vagal stimulation of 2 and 5 Hz. respectively. Stimulus parameters: 0.5 volts, 0.03 msec duration.



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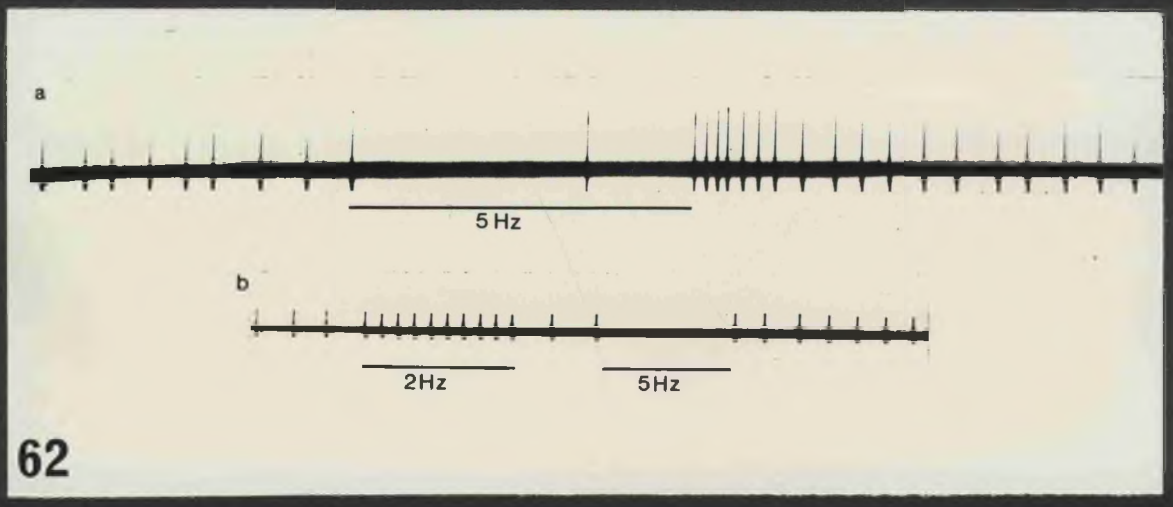


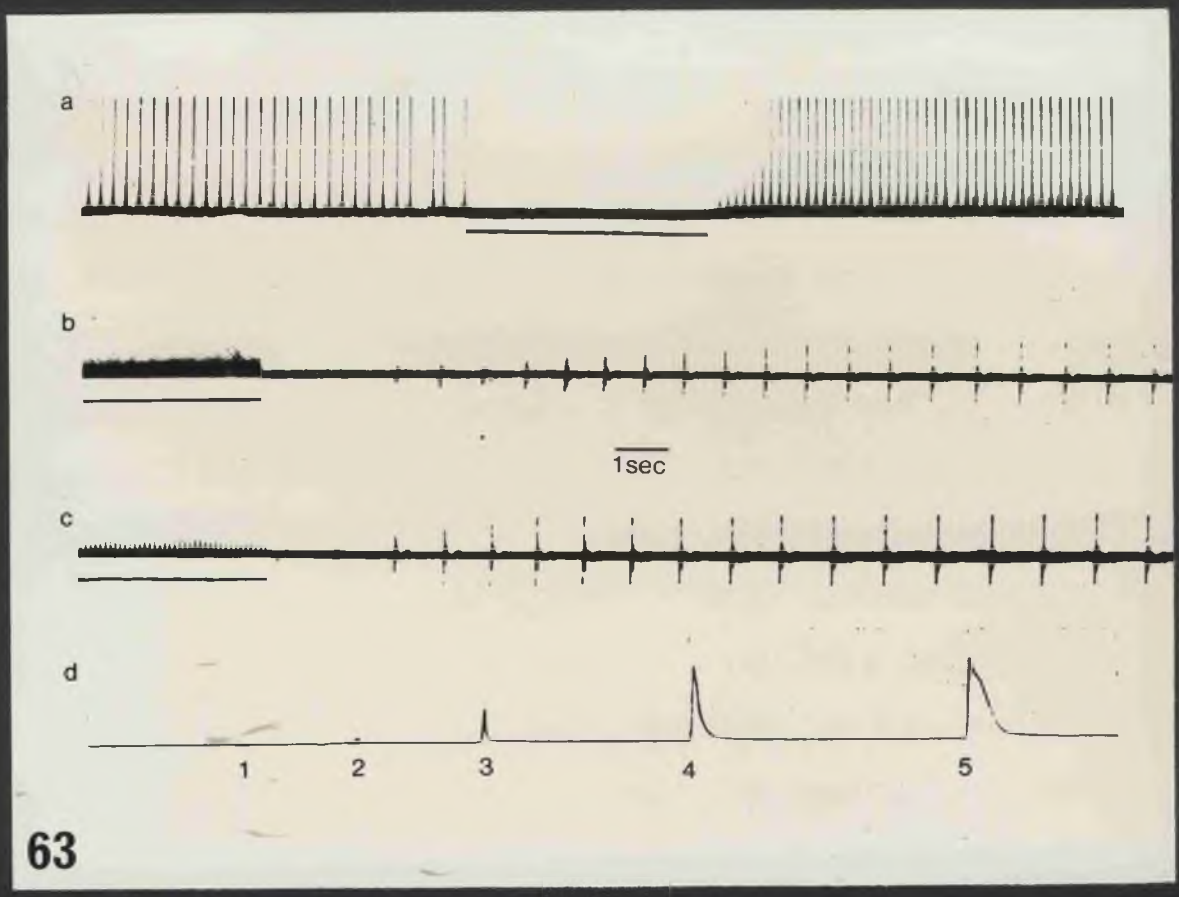
62

Figure 63.

(a) Intracellular recording of electrical activity in an atrial cell following cessation of stimulation at 10 Hz. Note the similar increase in the size of the first few post-stimulus depolarisation as seen with extracellular recording. Bar marks stimulus duration. (b) Extracellular recording of the delayed return to normal of the heartbeat following cessation of stimulation at 30 Hz. (c) Similar record to that in 3(b) but following cessation at 10 Hz stimulation. Note the shorter delay before the return of the heartbeat and the more rapid return to normal spike size. (d) Intracellular recording from an atrial cell following vagal stimulation of 10 Hz for 5 secs. Note the gradual return to normal spike profile. The difference in the falling phase between events 4 and 5 may be due to long lasting permeability changes in the atrial cell membrane following prolonged inhibition. The gradual increase in size of events 1 - 4 is explicable by proposing that events 1 - 3 are pacemaker potentials in relatively distant cells failing to propagate actively and being conducted decrementally by electrotonic spread through coupled cells. The distance actively propagated increases with each distant pacemaker event until it reaches the impaled cell at event 4. Stimulus parameters for all Fig 3 records are 0.5 volts and 0.03 msec duration.

Figure 64

(a) Heart driven by vagal stimulation of 1.2 Hz. (b) Control heartrate following cessation of driving stimulus. Stimulus parameters: 0.5 volts and 0.03 msec duration.



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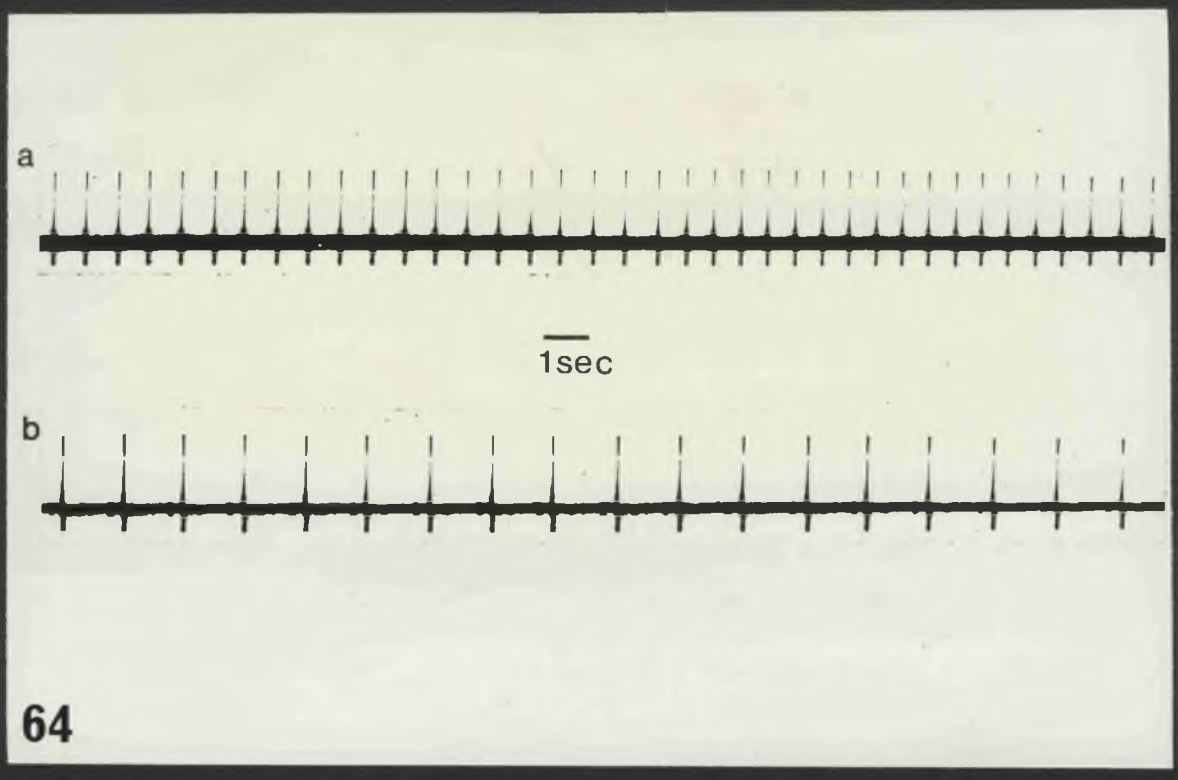
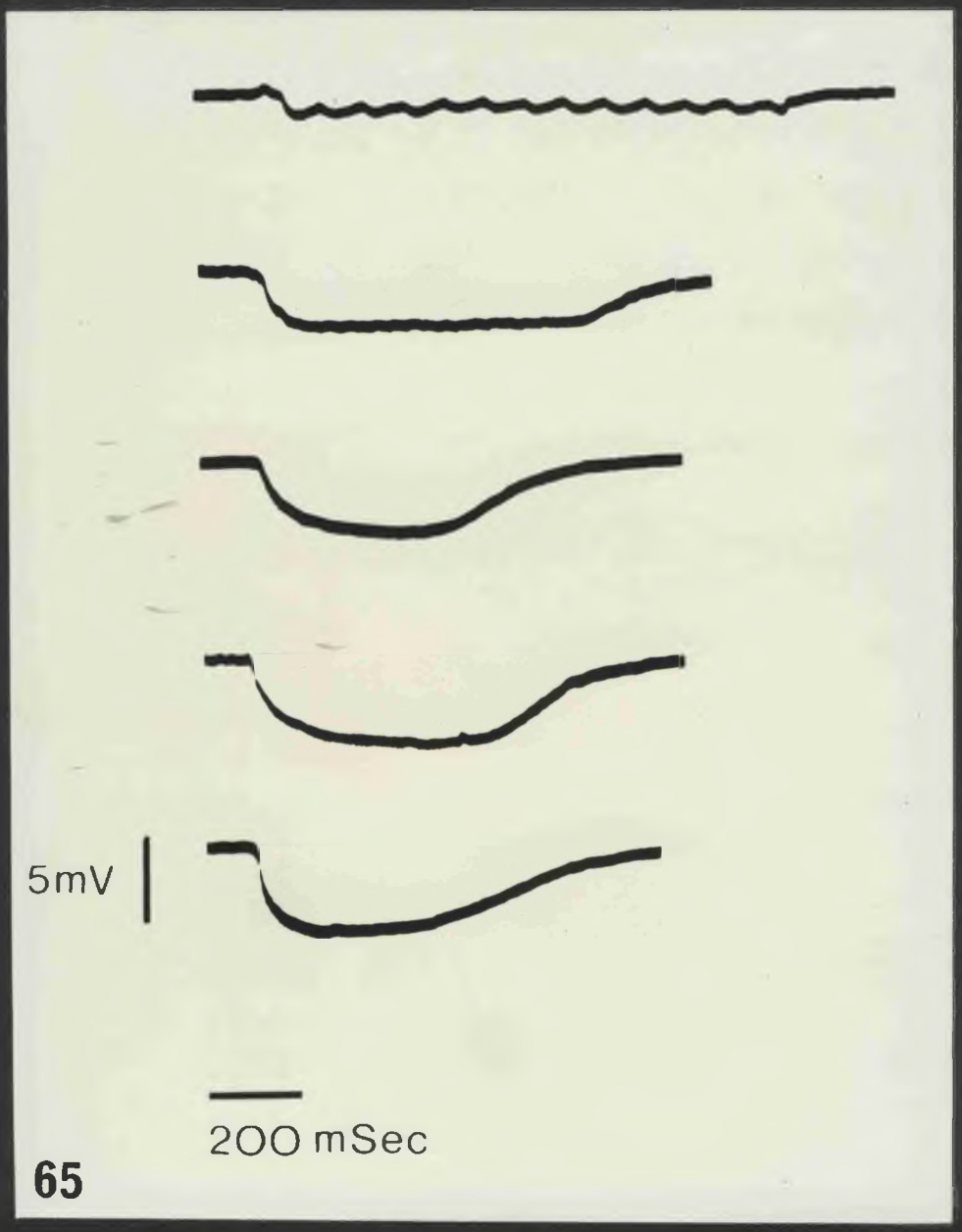


64

Figure 65

Hyperpolarisation of atrial cell resting potential in response to vagal stimulation at increasing frequencies (10, 20, 30, 35, 40 Hz respectively from top to bottom). Note the increase in the rate of hyperpolarisation and the increased amplitude of the hyperpolarisation with increased frequency. Stimulus parameters: 0.5 volts, 0.03 msec duration.



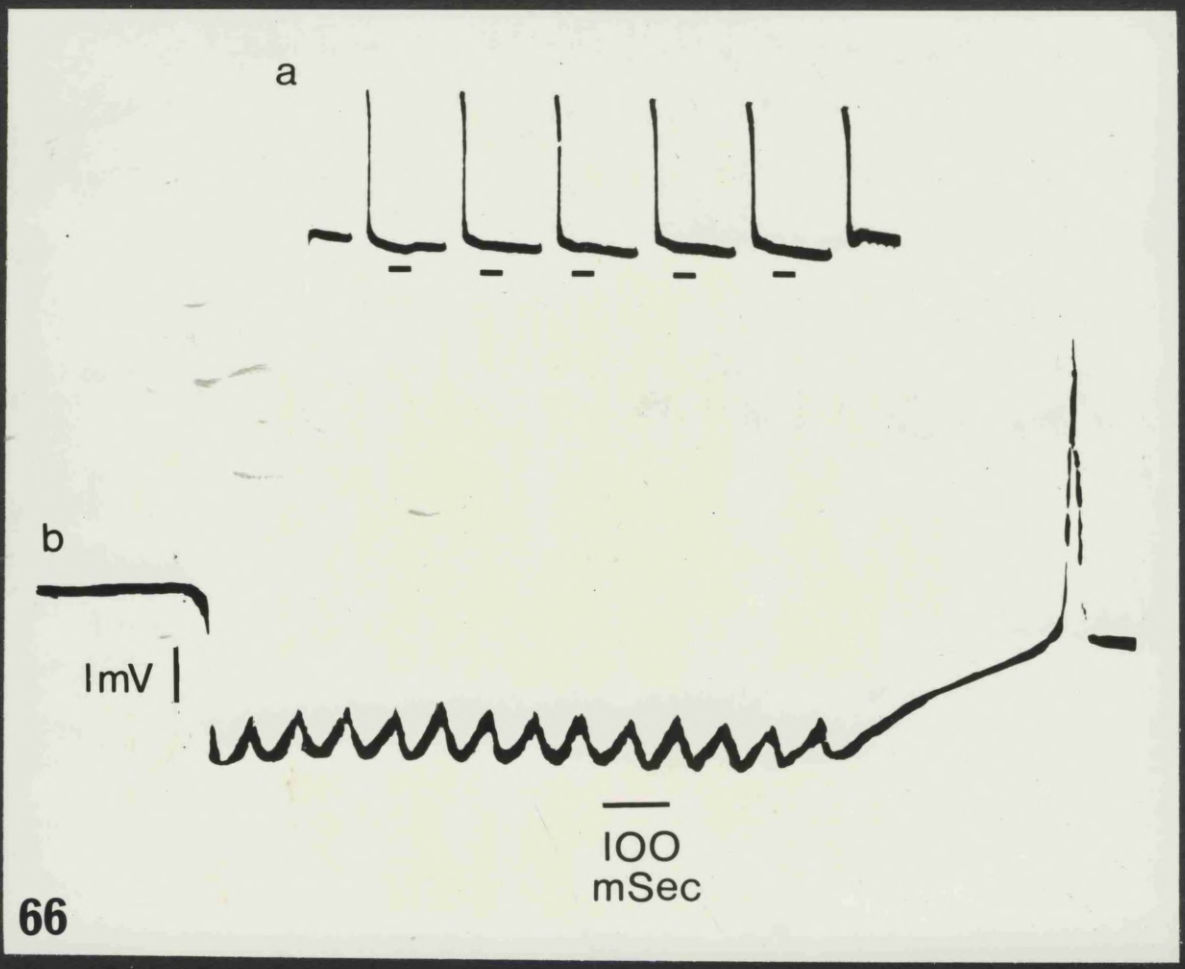
65

Figure 66.

(a) Intracellular recording from an atrial cell showing action potentials following each vagal stimulus (marked by short bar) at low frequency (1 Hz). Hyperpolarisation at this gain is not readily visible.

(b) Intracellular record from an atrial cell showing individual hyperpolarisations to individual vagal stimuli at 10 Hz. Note the depolarisation on cessation of the stimulus giving rise to a further rapid depolarisation but not to a standard action potential. This presumably represents a propagated event in another region of the atrium manifesting itself in the impaled cell by electrotonic spread through coupled cells.

Fig. 66b retouched.



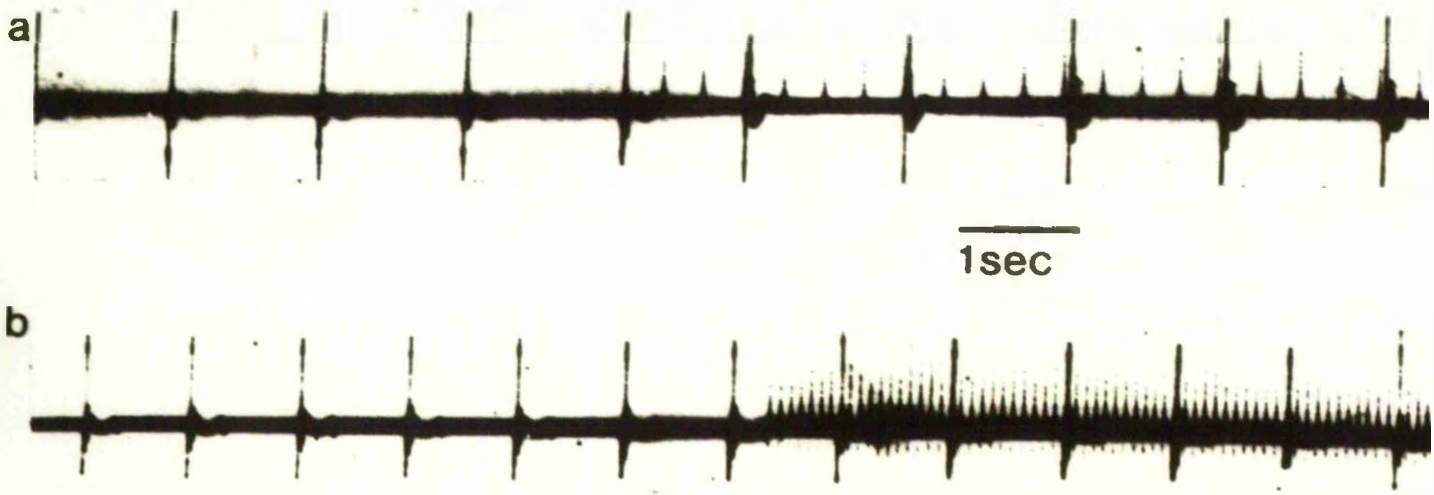
66

Figure 67.

Blockade of responses to excitatory (a) and inhibitory (b) frequencies of stimulating by atropine (10^{-6} g/ml). The frequency in (a) is 3Hz and that in (b) is 12 Hz. Stimulus parameters: 0.5 volts, 0.03 msec duration.

Figure 68

Illustrates the lack of effect of bretylium tosylate (10^{-5} g/ml) on the responses of the heart to differential vagal stimulation of 5 Hz in (a) and 2 Hz in (b). Note that the post-inhibitory excitatory response is also unaffected. Stimulus parameters:).5 volts, 0.03 msecs duration.



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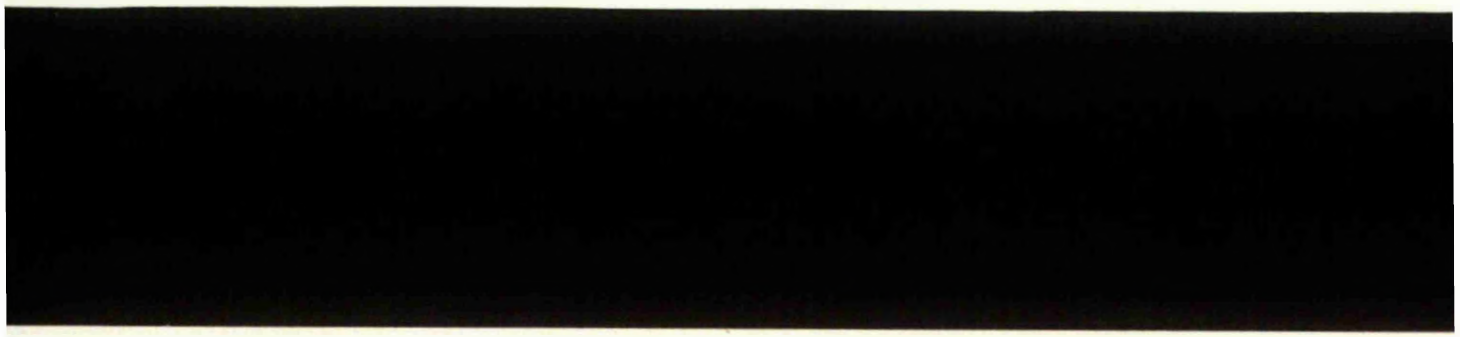

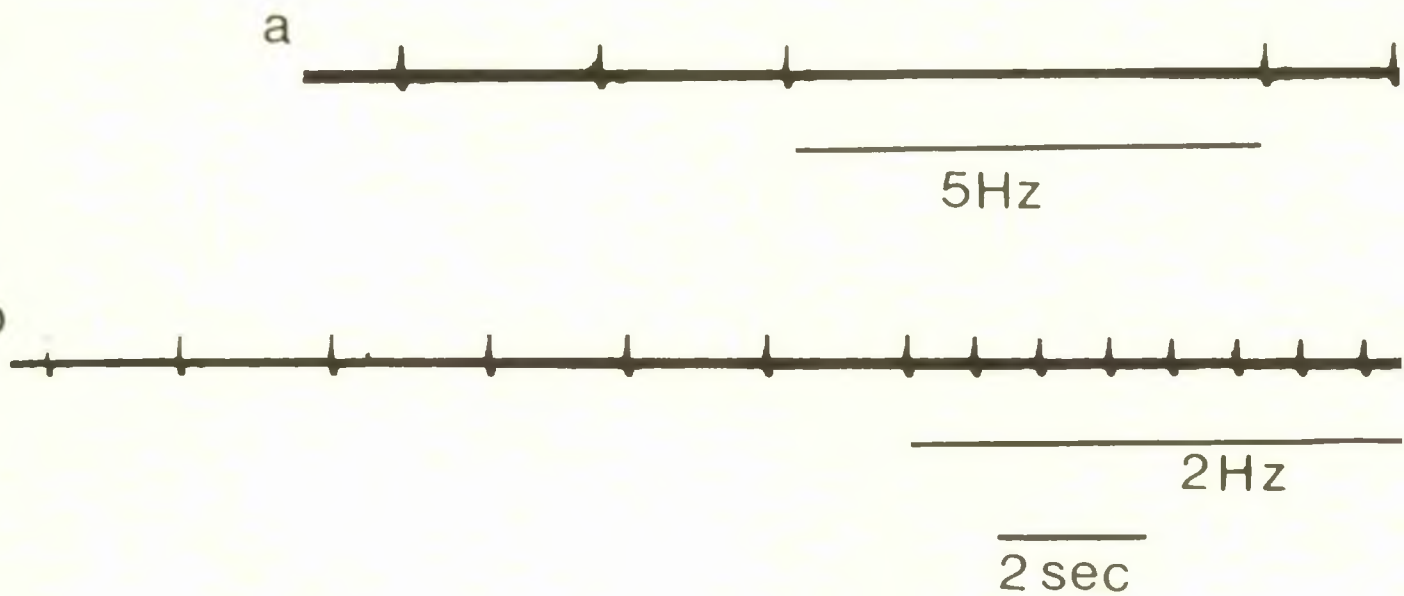

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Figure 69.

Kymograph records of the exogenous application of drugs to the isolated heart.

A. Inhibitory effect of ACh (10^{-6} g/ml). Note long-lasting post-inhibitory effects. A positive inotropic response is followed by a long positive chronotropic response.

B. Inhibitory effect of ACh (10^{-10} g/ml).

C. Failure of tubocurarine (10^{-7} g/ml) to block the response to ACh (10^{-6} g/ml).

D. Blockade of the response to ACh (10^{-6} g/ml) by atropine (10^{-6} g/ml).

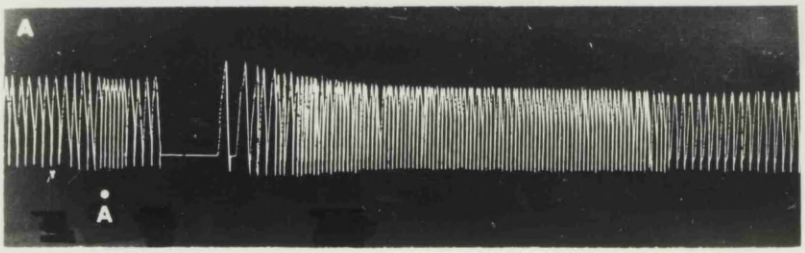
E. Long-lasting inhibitory effect of carbachol (10^{-6} g/ml)

F. Comparative effects of equivalent concentrations of ACh and carbachol (both at 10^{-6} g/ml). Inhibitory effect of atropine (10^{-6} g/ml) on the response to carbachol.

G. Inhibitory effect of nicotine (10^{-5} g/ml). Note the post-inhibitory response which is positively inotropic, and chronotropic.

H. Blockade of the response to nicotine by hexamethonium (10^{-5} g/ml).

a = acetylcholine; t= tubocurarine; at = atropine; c = carbachol; n= nicotine; h = hexamethonium.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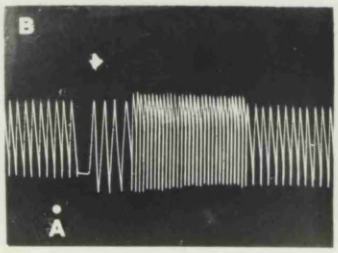
A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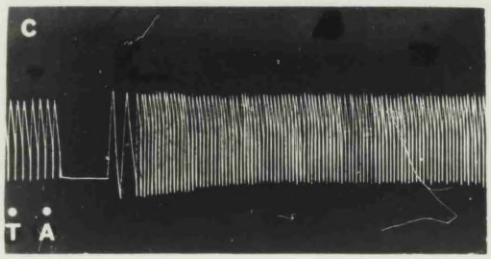
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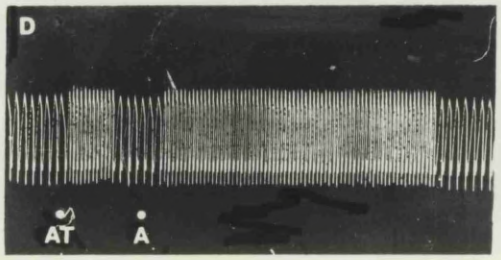
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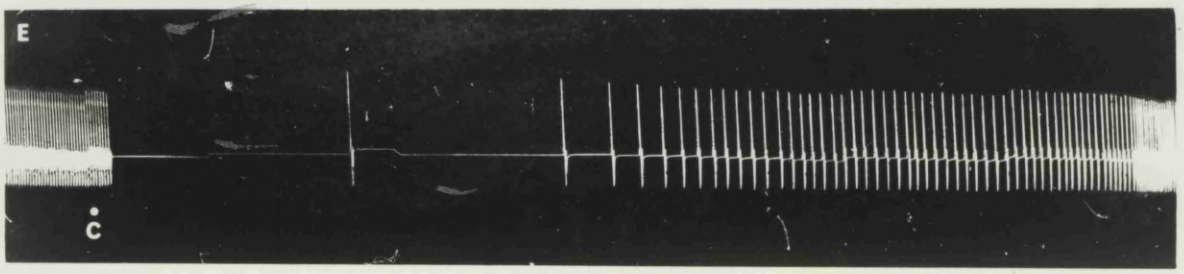
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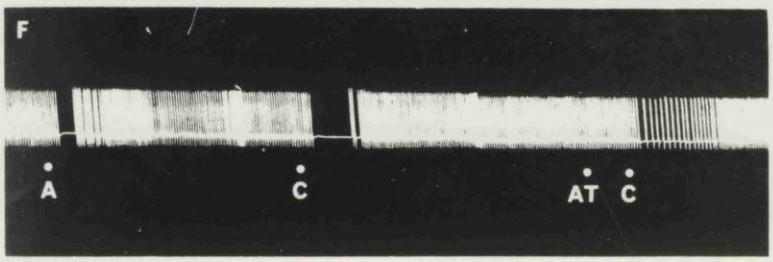
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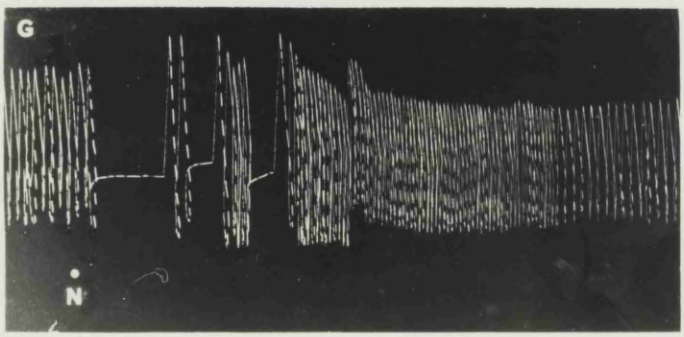
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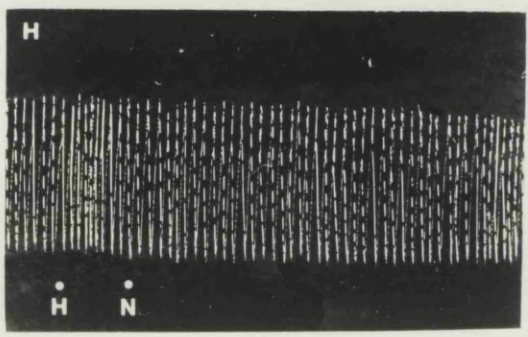
H
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Figure 70.

Kymograph records of the exogenous application of drugs to the perfused, isolated heart.

A. Positive inotropic and chronotropic effect of noradrenaline (10^{-6} g/ml).

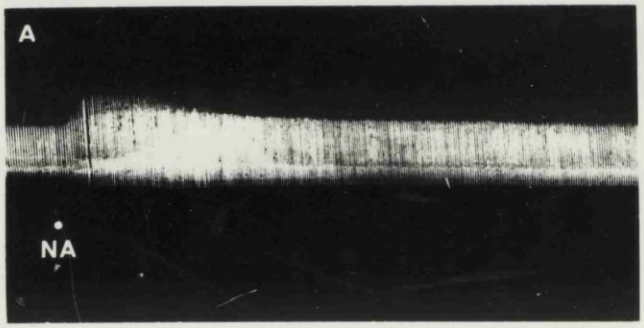
B. Blockade of the effects of noradrenaline by pronethalol (10^{-6} g/ml).

C. Failure of bretylium (10^{-6} g/ml) to block the effects of noradrenaline.

D & E. Comparative effects of adrenaline in (D) and noradrenaline in (E) both at 10^{-6} g/ml on the same heart. Note, the greater sensitivity to adrenaline.

F & G. Seasonal effect of 5-hydroxytryptamine. Record F was made in July 1972 and shows a positive chronotropic response. Record G was made in December 1970 and shows a complete lack of response.

H. Lack of effect of tyramine (10^{-4} g/ml).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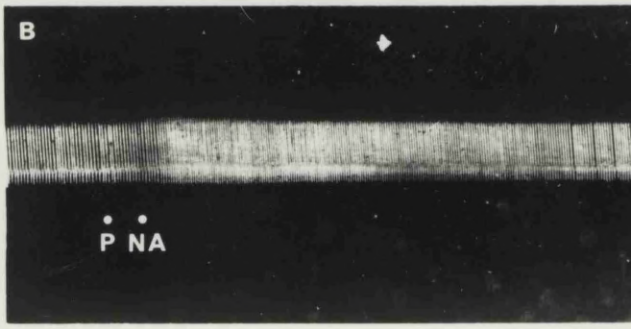
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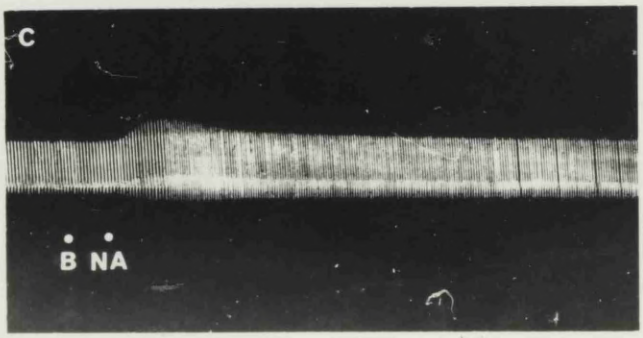
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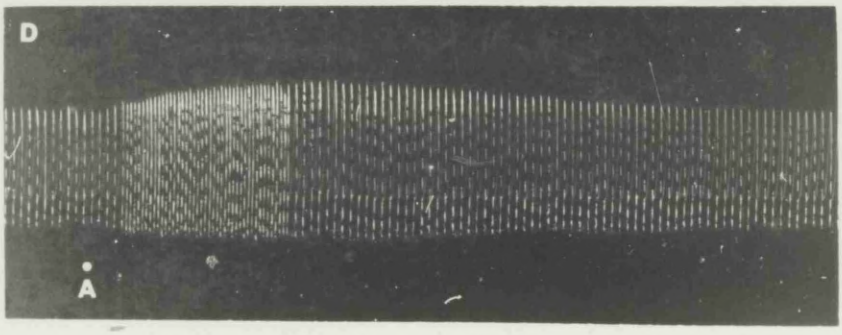
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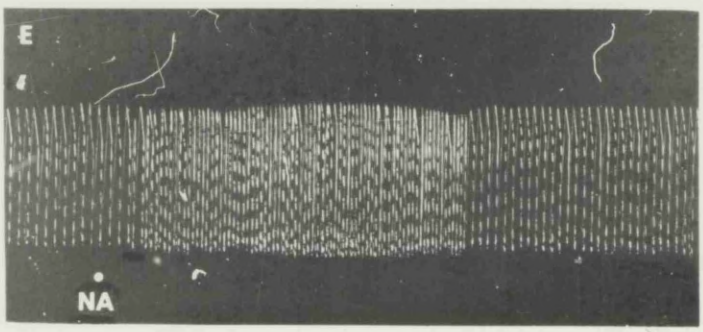
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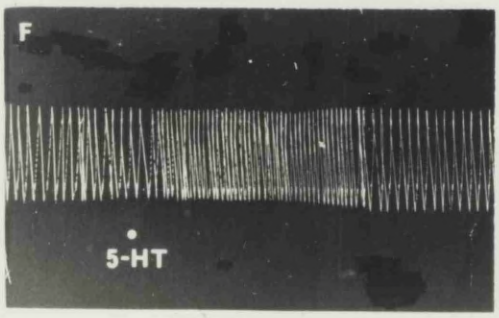
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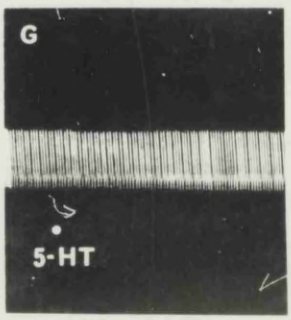
5-HT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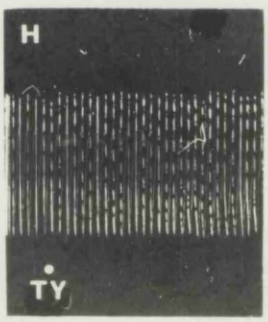
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VII. SUMMARY OF RESULTS

1. FINE STRUCTURE

The sinus venosus is a thin walled chamber between 60μ -90μ thick consisting of a connective matrix in which are situated the plexus of the parasympathetic cardiac ganglion and small localised bundles of myocardial cells. The myocardial cells do not form a continuous layer but are associated in particular with the region of the cardiac ganglion and are innervated by it. A few small muscle cell bundles occurred in the sinus venosus.

The sino-atrial junction has hitherto been described as a pacemaker region but the myocardial cells in this region are identical in morphology to myocardial cells in other parts of the heart but slightly smaller. There is a large complex of nerves, derived from the cardiac plexus, that runs around the junction before branching to innervate the atrium.

The myocardial tissues consist of a layer of myocardium forming the wall of the heart and a profusion of trabeculae. The endocardium invaginates into the endocardium to divide up the cells into populations of approximately 25 cells in profile. There is no classical coronary blood supply although capillaries are occasionally seen. The myocardial cells themselves are small in diameter (average 3.2μ) and show features which are typical of small diameter cardiac cells: a short sarcomere length (1.4 -2.0μ), a very sparse sarcoplasmic reticulum, no T-system, no nexuses and simple intercalated discs. In the atrial myocardial cells in particular, there are many groups of 1500Å membrane-bound, granular vesicles of unknown function. Ventricular cells contain more myofilaments and mitochondria than do atrial cells and have many lipid vesicles

of 0.1μ-0.3μ diameter. Certain cells within the myocardium had a very large amount of free cytoplasm, little glycogen and a few randomly distributed myofibrils. These "light" cells resembled mammalian Purkinje cells but did not run in tracts throughout the myocardium.

Connective tissue is very evident in the plaice heart, being an integral part of the sinus venosus and the atrio-ventricular junction and being the sole constituent of the atrio-ventricular valve and the bulbus arterious.

2. DEVELOPMENT

The process of development of the heart is essentially the same as that in higher vertebrates but there are certain minor sequential differences with comparable chick tissue.

By day 24 (ten days after hatching) the "early larval" heart has formed which is a trilaminar structure - a layer of myocardium bounded internally by endocardium and externally by epicardium. This condition lasts until the 4a (Ryland) stage with the onset of endocardial invagination into the myocardium which is the criterion distinguishing the "late larval" heart. The "late larval" heart lasts throughout metamorphosis of the larva and until two months post-metamorphosis when the total adult heart structure is assumed. Thus the process of cardiogenesis continues irrespective of hatching and of metamorphosis.

This study supports the concept that the epicardium is derived from an extramyocardial source. No results are presented concerning the theory that, in its earliest stages, the myocardium has a secretory function in the production of cardiac jelly, or of myofibrillogenesis in the teleost myoblast. Stellar configurations of short lengths of newly formed sarcomeres commonly radiate out from Z-centres in early myocytes and it is

suggested that this is a primitive feature of teleost myogenesis. There is also a proliferation of mitochondria within the myocardial cells at metamorphosis which may be connected to the subsequent fast growth of the heart in the succeeding two months.

Axon profiles are first observed in close contact with muscle cells soon after hatching. Throughout larval life the innervation of the atrium is sparse, but increases at the S-A end after metamorphosis. The larval ventricle is aneural.

3. INNERVATION

The only pathway for the extrinsic innervation of the heart are the cardiac branches of the vagus which run over the duct of Cuvier and sinus venosus where they form a plexus extending into the sinoatrial region. Many ganglion cells are scattered throughout this plexus upon which are seen cholinergic-type terminals at which AChE can be localised. It is therefore concluded that this is the parasympathetic cardiac ganglion.

The intrinsic innervation of the atrium is dense at the sinoatrial end but decreases towards the ventricle. The ventricle and BA are aneural, possibly a consequence of the lack of a coronary blood supply to act as a pathway for an innervation of this chamber. Most of the intramural axon profiles contain large numbers of small agranular vesicles and are cholinergic, AChE being localised at their membranes. Other profiles contain small numbers of larger granular vesicles amongst the agranular vesicles but these are unlikely to be adrenergic since these vesicles are not depleted by reserpine, do not load with 6-OHDA, and AChE can be localised at the membranes of the profiles.

No adrenergic-type profiles were seen in any part of the heart or cardiac nerve nor were any fluorescent, catecholamine-containing fibres observed.

4. PHYSIOLOGY AND PHARMACOLOGY

Vagal stimulation at 7 Hz totally inhibits heartbeat whilst stimulation at 2-3 Hz accelerates it. Both these effects are blocked by atropine (10^{-6} g/ml). Bretylium (10^{-5} g/ml) and pronethalol (10^{-6} g/ml) have no effect upon either response to vagal stimulation.

On cessation of prolonged inhibitory vagal stimulation there is a marked increase in the heart rate, and in quiescent hearts one or two beats are initiated after stimulation.

Vagal stimulation gives rise to a hyperpolarisation in atrial cells. It is proposed that all the excitatory effects of vagal stimulation are due to rebound excitation from an inhibitory hyperpolarisation. At high frequencies the hyperpolarisations summate to give rise to total inhibition. At lower frequencies of stimulation the heartbeat is increased to rates dependent on interaction between the time-course of the hyperpolarisation and the refractory period of the heart. Both effects are cholinergically mediated.

The rebound excitation in response to vagal stimulation (postvagal tachycardia) persists in response to stimulation of the atrial myocardium in the presence of atropine (10^{-6} g/ml) and bretylium (10^{-5} g/ml). It is therefore suggested that this is a response of the muscle cell membrane to vagal stimulation and is not nerve mediated.

The atrium is sensitive to ACh (10^{-12} g/ml) but the ventricle is not. Both chambers are sensitive to applied catecholamines. Tyramine has no effect on the heart. Nicotine produces

a long lasting inhibition which is blocked by hexamethonium. 5-HT has a positive chronotropic effect that is seasonal.

VIII. GENERAL DISCUSSION

1. MORPHOLOGY & DEVELOPMENT

In the present study of the plaice heart it has been shown that the ultrastructure of the adult heart and the process of cardiac development are fundamentally similar to those of higher vertebrates that have been studied. Apart from certain minor sequential differences the development of the heart follows the same overall pattern as the chick (Manasek, 1968; 1969; 1970). There are indications that the development of the chick heart is a slightly more ordered process. For example, sarcomeres radiating outwards in an apparently disorganised fashion from Z-centres are common in the developing teleost heart but are described as being a rare anomaly in the chick by Manasek. Nevertheless no aberrations which could be attributed to this feature of the developing tissue have been observed in the arrangement of sarcomeres in the adult plaice heart.

The development of the heart continues irrespective of the state of the developing fish and no pronounced changes are observed that are associated with hatching or the completion of metamorphosis. Due to this continuous process it has not been possible to categorically differentiate between the larval and adult heart. However, it is vital that when the change from cutaneous to branchial respiration occurs, the heart is adequately functional and also innervated.

The demonstration by Manasek & Monroe (1972) that the formation of the initial loop of the early embryonic chick heart continues uninterrupted if cardiac contractions are inhibited by high potassium media indicates that the developmental process is independent of cardiac function. If this very early and

important morphological event is an expression of the determination of the development process then it is not altogether surprising that it is not affected by hatching or metamorphosis. The latter is a slow process continuing over several days and unlike the tadpole metamorphosis involves few gross physiological changes.

If it is valid to say that the newly-metamorphosed plaice is in a state which is analagous to the neonatal mammal, it is fair to indicate and compare the changes that occur in the succeeding few months of life. The plaice heart does not attain the adult structure until about two months after metamorphosis is completed and this is comparable to certain mammalian organs that have been studied in which it is evident that the final structure may not be reached until three months post-natal. The intrinsic innervation of an organ may not be completely established six months post-natal in mammals. At metamorphosis, the plaice heart was morphologically complete but ultrastructural changes continue thereafter. More inter-cellular contacts were made, the mitochondria increased in number, more glycogen was laid down and lastly there was an increase in the diameter of individual muscle cells. A similar increase in cell dimensions is seen in the vas deferens of the post-natal mouse (Yamauchi & Burnstock, 1969a) and both in this example and also in the plaice heart, there is an associated period of growth of the organ. Edwards & Challice (1958) report that the heart of the neonatal mice is not mature since the myocardial cells contain few randomly placed myofibrils and large numbers of mitochondria with many cristae, as seen in the newly metamorphosed plaice heart. However, the adult condition is

is observed within a week after birth.

The neonatal mouse heart is almost identical to the adult plaice heart but the important difference between the two animals is that the mouse heart undergoes further changes to attain maturity. Therefore it might be possible to explain the comparative simplicity of the ultrastructure of the teleost heart and its receptors by proposing that these are consequences of the primitive phylogenetic position of the fish. Laurent (1962) notes that the type of receptor seen in adult teleosts is simple and comparable to those of the neonatal mammal. The simple (or primitive) features of the teleost myocardial cells are considered to be their small size (2-6μ), the lack of a T-system, a sparse sarcoplasmic reticulum (SR) and the absence of nexus connections between cells.

At this point in the discussion it is worth making reference to reports on the ultrastructure of certain invertebrate cardiac tissues. Layton & Sonnenblick (1971) and Sperelakis (1971) studied the fine structure of the heart of the horseshoe crab Limulus and reported myocardial cells of 25-65μ (Layton & Sonnenblick) and 6.4-26μ (Sperelakis) diameter, intercalated discs, a T-system, an extensive sarcoplasmic reticulum (SR) and sarcotubular couplings. There were also differences in sarcomere length from vertebrates and in the configuration of myofilaments. No nexus connections were observed and this feature is presumed to account for the lack of electrical coupling between the cells (Parnas et al. 1969). Therefore, Limulus myocardial cells are just as complex as the mammalian type apart from the lack of certain membrane specialisations.

It is apparent that the most significant feature of myocardial cells whether in invertebrates or vertebrates is that

a T-system, an extensive SR and many well-ordered myofibrils occur in the cells of a larger diameter. Studies on the ultrastructure of "working" myocardial cells are very numerous and demonstrate the fact that cells of about 12μ and above contain these subcellular systems which are absent from cells of very small diameter. The SR is present in a very rudimentary form in the plaice and amphibian (Hirakow, 1971; Page & Niedergerke, 1972) myocardial cells.

It is therefore suggested that it is as a consequence of the small diameter of these cells that they have a simple ultrastructure. However it may be that the larger cells and more complex cardiac structure found in higher vertebrates result from the more complex function ascribed to the hearts of such animals. Such features as a double circulation and a highly coordinated ventricular stroke are features of avian and mammalian hearts. Thus the evolution of function and not phylogenetic evolution may determine cardiac ultrastructure.

Purkinje fibres may have a diameter of up to 90μ in some large mammals (whales and ungulates) but T-tubules are never seen in these cells. Skeletal muscle cells of comparable diameter to the Purkinje fibres have an extensive T-system but the great functional difference is that these cells actively contract. It is generally accepted that the T-system in skeletal and cardiac muscle acts to introduce the electrically excitable sarcolemma into the depth of the cell as a first step in excitation-contraction coupling. However it is likely that cell diameter is the determining factor for the presence of a T-system in striated muscle cells since there is a very considerable

difference in cell diameter of most skeletal muscle cells and invertebrate myocardial cells (< 90μ) and vertebrate "working" myocardial cells (<15μ). The diameter of vertebrate "working" myocardial cells are of a much more restricted range yet some possess a T-system whereas others do not. Whilst all these cells are small in comparison with vertebrate skeletal muscle , the critical diameter above which a T-system becomes necessary is not clear. Certain echinoderm skeletal muscle cells have a diameter of only 1.3μ yet have an extensive T-system (Cobb, 1967). It should also be mentioned that, in "working" myocardial cells, the SR becomes progressively more extensive with cell diameter and it is conceivable that this may reflect the volume of myofilaments within the cell.

Comparison of invertebrate and vertebrate myocardial tissues questions the criteria determining the occurrence of nexuses which are the presumed morphological correlate of cell-to-cell transmission. In vertebrates, all of whose cardiac cells are electrically coupled it is possible to relate cell size to the size of the observed nexus contact by examination of electron micrographs. Large mammalian cardiac cells have long lengths of nexus connection but they have not yet been observed in fish. In the mollusc heart where the cells average 8μ in diameter, approximately 10% of the cell surface is estimated to be occupied by nexuses which are 0.3-0.5 μ in length (Irasawa et al. 1973). These cells are also electrically coupled. In Limulus no nexuses are observed (Sperelakis, 1971; Leyton & Sonnenblick, 1971) and there is a concomitant absence of electrical coupling (Parnas et al. 1969) whereas in another arthropod heart, (lobster: Anderson & Smith, 1971) there are no nexuses between the 15μ diameter myocardial cells which are also electrically coupled.

From these results one can only conclude that a) the cell diameter does not determine the occurrence of nexuses in cardiac muscle; b) certain electrically coupled cardiac cells are devoid of observable nexuses.

It is not possible to say that nexuses are necessary for cell-to-cell transmission as inferred by the work on Limulus since this situation occurs in functional syncitia where nexuses have not been demonstrated. In such cases, the other obvious candidate for low resistance junctions in cardiac tissue is the intercalated disc. There is a certain amount of evidence for this (see Weidmann 1969) but no definite conclusion can be reached as yet. It should also be remembered that whilst nexuses provide a very tempting solution to the problem of low resistance junctions in electrically coupled cells, the evidence for this is purely empirical.

2. INNERVATION

The recent demonstration of an adrenergic innervation in the heart of certain teleosts by fluorescence microscopy (Govyrin & Leonteva, 1965; Otsuka & Tormisawa, 1965; Gannon & Burnstock, 1968; Womersley, 1973) upset previous ideas on the mechanism of the control of heartrate. Prior to this it was assumed that in all teleosts only a vagal inhibitory control of the heart was involved and that cardio-acceleration was effected by an alleviation of vagal inhibition or by circulating catecholamines.

In the present study on the plaice heart it was necessary to determine whether a sympathetic component of the innervation was present before attempting any speculation or investigation on the control of the heart. All the evidence suggests that the plaice heart lacks an adrenergic innervation. The results supporting this statement will be summarised:-

1. No catecholamine fluorescence is seen anywhere in the heart or cardiac nerve after 1 or 3 hours incubation with HCHO, preceded by nialamide treatment and/or loading with α-methyl-noradrenaline.
2. No axon profiles in the SV or atrium show any loading or degeneration after treatment with 6-OHDA, even if they contain small numbers of granular vesicles.
3. Vesicles in such axons are not depleted by reserpine treatment, nor are the large granular vesicles in atrial cells.
4. AChE can be localised around all axon profiles whether or not they contain any granular vesicles.
5. No axon profiles containing granular vesicles in large quantities are seen by electron microscopy.

6. Endogenously applied tyramine does not cause any positive inotropic or chronotropic responses to the isolated heart.
7. Endogenously applied catecholamine effects are blocked by the β-receptor blocker pronethalol whilst excitatory stimulation is not affected by pronethalol or bretylium.
8. Both excitatory and inhibitory responses to vagal stimulation are blocked by atropine.
9. Stimulation at higher voltages (<40V) does not alter the results of 7 or 8 above. This voltage would be sufficient to stimulate small diameter unmyelinated efferent fibres should they be present.
10. Rebound excitation is not affected by pronethalol or bretylium.

It has not been possible to potentiate the effects of ACh with anti-cholinesterases with regard to the excitatory response to vagal stimulation due to the fundamentally inhibitory effect of ACh, nor to show the release of ACh following excitatory vagal stimulation.

Two pathways have been suggested by which the adrenergic innervation might reach the heart in teleosts. First that there is a connection between the anterior sympathetic chain and the vagus nerve to form a mixed vago-sympathetic cardiac nerve. Such a connection has been demonstrated in some teleosts by Stannius (1849) and Young (1930) but the very small size of this ramus and the difficulty in dissecting the teleost sympathetic chain make the possibility of stimulating it very remote. In Uranoscopus, a connection between the vagus and the sympathetic chain contained only myelinated fibres (Young, 1930) and comparison with the

cardiac vagus revealed only myelinated fibres in this nerve, so leading Young to conclude that the ramus in question could not provide any sympathetic fibres to the heart. However, an electron microscope study might revise this notion. In their detailed study of the anatomy of the plaice, Cole and Johnstone (1901) do not report any connection between the vagus and the sympathetic chain whereas Gannon (1971) reports the presence of such a connection in the eel and trout, both of which have been shown to have an adrenergic innervation of the heart (Gannon & Burnstock, 1968). This is also the situation in the tench (Gannon, pers. comm.). Therefore, in some teleosts the cardiac nerve is a mixed vago-sympathetic nerve but the central origin of the sympathetic component remains obscure. It has also been suggested that those fluorescent fibres seen in the cardiac nerve and SV are those responsible for fluorescent nerves seen in the atrium of the heart (Gannon & Burnstock, 1969; Womersley, 1973).

Unmyelinated fibres are also present in the cardiac nerves of teleosts but it is not known what function they serve. Laurent (1962) who reports ganglion cells within the cardiac nerve suggests that the unmyelinated fibres that degenerate after peripheral vagotomy (outside the pericardium) are the post-ganglionic axons of these ganglion cells and are not sympathetic nerves. Peripheral vagotomy of post-ganglionic sympathetic nerves would not cause degeneration of the whole axon, merely of the short distal region. Similar unidentified unmyelinated nerves are seen in the plaice cardiac nerve but, as mentioned above, Home Office regulations have precluded any degeneration experiments. The possibility of ganglion cells situated high up the cardiac nerve should perhaps be kept open.

The second possible origin of adrenergic fibres to the heart is along the coronary vasculature. These vessels arise from the efferent epibranchial arteries and reach the heart along the ventral aorta and are themselves innervated by an extensive adrenergic plexus (Gannon & Burnstock, 1969; Womersley, 1973). However, it is significant that a coronary supply occurs only in teleosts that have an outer cortical layer of ventricular myocardium. The presence of this layer depends on the ratio of heart size to body weight, occurring only in fish where this ratio is high and consequently a larger stroke required of the ventricle (Ostadal & Scheibler, 1971). Govyrin & Leonteva (1965) and Womersley (1973) note that when adrenergic nerves are seen in the ventricle, they are restricted to the perivascular plexuses and to the outer cortical layer of myocardium. Even so, their distribution is sparse in this latter region. Gannon & Burnstock (1969) show a fluorescence micrograph of a nerve bundle leaving the perivascular plexus to innervate the cortical myocardium which confirms this as a source of the adrenergic innervation of the ventricle. In this respect the plaice lacks a suitable pathway for an adrenergic innervation of the ventricle. In no teleosts have fluorescent nerves been observed crossing from the atrium to the ventricle.

Thus, an adrenergic innervation of the teleost heart does occur but not in all teleosts. The teleosts are therefore unique amongst the vertebrates in having a variable cardiac innervation, in some cases the sympathetic component being absent.

It is pertinent to try and define the factor that determines the participation of the sympathetic nervous system in the cardiac innervation but too few teleosts have been

investigated with respect to this problem to make any generalisation from either morphological or phylogenetic standpoints. It is tempting to try and show that, as the teleosts diverged away from the main vertebrate stock, the extent of the sympathetic innervation of the viscera has diminished. The presence of an adrenergic innervation of the Cottus heart (Womersley, 1973) and its absence in the plaice is interesting not only because both fish belong to recently evolved groups of teleost (Greenwood et al. 1966) but because this exemplifies the problem of trying to make any correlation between the teleost sympathetic nervous system and the phylogenetic position of a particular species or family within the class. It seems likely that the flatfishes as a group (Heterosomata) all lack an adrenergic cardiac innervation since fluorescence histochemistry of the hearts of the flounder (Platicthys flesus) and dab (Limanda limanda) reveals no catecholamine fluorescence. Conversley, the eel, trout and tench are comparatively primitive teleosts and have a very extensive adrenergic innervation of the heart and viscera (Gannon pers. comm.).

However, all flatfish that have been examined lack an outer cortical layer of ventricular myocardium which could therefore be the determining factor concerning a possible adrenergic innervation. Further examination of more teleost hearts may demonstrate such a relationship to be more than fortuitous but it is also possible that a combination of morphological and phylogenetic factors may be involved. The occurrence of a ventricular adrenergic innervation may result indirectly from a perivascular coronary innervation that has ramified into the myocardium secondarily and be of little physiological consequence.

If the effect of an adrenergic innervation is positively inotropic or chronotropic or both as reported for the trout (Gannon, 1971), then the important functional component will be that part innervating the S-A junction. In all teleosts in which a cardiac adrenergic innervation has been reported, fluorescent fibres have always been seen at the S-A junction and SV, in perivascular plexuses and in the cortical layer. Gannon & Burnstock,(1968) and Gannon (1971) report that its effects are directly excitatory in the eel and trout, presumably by increasing the rate of diastolic depolarisation of the pacemaker cells. There are reports of adrenergic modulation of synaptic transmission at mammalian parasympathetic ganglia (Norberg & Sjoqvist, 1966). Such a mechanism might account for the alteration of post-ganglionic inhibitory tonus causing cardio-acceleration in teleosts as suggested by Jullien & Ripplinger (1957). Alternatively, if the correlation shown by Ostadal & Scheibler (1971) that a cortical layer of myocardium is a result of a high Heart/body weight ratio is indeed significant, then an excitatory innervation might well be required in these fish to increase heartrate during excercise, for example. There have been no reports of fluorescent fibres passing from the atrium to the ventricle, or innervating the A-V region in teleosts, the presence of which would be more easily interpreted as a functional pattern of innervation.

Therefore, in the light of the present small amount of information on this subject it seems more likely that the sympathetic component of the cardiac innervation in teleosts is determined by the morphology of the heart rather than by the phylogenetic position of the fish in question.

The role of catecholamines in cardioregulation in teleosts is therefore variable in the form it takes. First there is the direct excitatory adrenergic innervation of the heart. There is evidence in support of this, but it is possible that the pharmacological study by Gannon (1971) could be explained by catecholamines being released from the perivascular plexuses in the trout by the very high stimulating voltage (<80V) and causing a secondary effect on the myocardium. Second is the possibility of adrenergic modulation of ganglionic transmission, but there is no evidence in support or against this idea in fish. Third, it is known that catecholamines are present in teleost blood (see Randall, 1970) and therefore its release by chromaffin tissue in response to stimulation could excite the heart of interaction with β-receptors. This situation is similar in principle to the mode of action of catecholamines on cyclostome and elasmobranch hearts. In cyclostomes, adrenaline and noradrenaline are released from intramural chromaffin stores in the heart (Bloom et al. 1961; see Fange, 1972) which minimizes the diffusion distance for the released catecholamines. The secretion of catecholamine is probably continuous and is increased during stress. Recently Gannon et al. (1972) have described cholinergically innervated chromaffin bodies that may release noradrenaline into the posterior cardinal sinus of a shark. Here again there is a short distance between the site of release and the effector. These authors have also described a very few fluorescent fibres in the SV of Heterodontus but this the only such report for an elasmobranch and there are no reports for cyclostome hearts.

As well as the variety of mechanisms by which catecholamines can influence the fish heart, the nature of the

the catecholamine itself varies. For example, the catecholamine of the trout heart is probably noradrenaline (Gannon & Burnstock, 1969) whilst that of Gottus (Womersley, 1973) is adrenaline. Elasmobranch and cyclostome chromaffin bodies release both adrenaline and noradrenaline but those of the plaice release only adrenaline (Grove et al. 1972). A review of the catecholamine content of teleost tissue can be found in Grove et al. (1972) which demonstrates great interspecific variety in teleosts.

It is apparent that the pattern of innervation of the plaice heart as revealed by electron microscopy is very different from that described in other teleosts by light microscopy. Laurent (1962) studied the eel, catfish, carp and tench and described features of the innervation that are absent from the plaice. These include ganglionated nerve trunks in the SV and atrium (extending as far as the A-V junction in the carp, tench and catfish), an extensive atrial innervation, a sparse ventricular innervation, innervation at the A-V junction, subendocardial and subepicardial receptor plexuses and a sparse innervation of the BA. The only other electron microscope study of the innervation of the teleost heart is that of Yamauchi & Burnstock, (1968). These authors note that the extensive innervation of the trout SA region decreases considerably along the length of the atrium, and that the ventricle is extremely sparsely innervated. The same is true for the plaice sinus and atrium, but the ventricle and BA are both aneural. No subepithelial plexuses as described in Laurent's work are evident. It must be remembered that Laurent's results were obtained by light microscopical examination of Bielschowsky - Gros silver impregnated sections and that, in the light of Richardson's (1960) work, such results derived from this silver technique may not

be as reliable as at first thought. There is a considerable amount of connective tissue in the plaice heart which, under certain conditions, has a very high affinity for this stain.

Clearly, the pattern of innervation of the plaice heart is very simple and, together with the absence of a demonstrable localised pacemaker area or any S-A nodal tissue, makes the problem of the control of the heartrate very intriguing. It is possible that the control of heartrate could be deceptively simple, relying basically on the extensive innervation of pacemaker cells (as in higher vertebrates). The problem of whether all the atrial cells are latent pacemakers would not assume such significance if those that were innervated were, in some way, dominant over the others. Even so, the concept of a diffuse pacemaker system functioning efficiently is difficult to conceive. Since Saito's (1973) discovery of pacemaker potentials in cells at the base of the S-A valves in the carp, the possibility of a similar region in the plaice cannot be ruled out as a potential pacemaker site. In the present study there has been no evidence that the A-V junction has a pacemaker role, and there is a corresponding lack of innervation at this region.

The pattern of innervation of the teleost heart varies almost as much as that of cyclostomes. All teleosts are innervated by the vagus at the S-A junction (Stannius, 1849) and there is always a pacemaker region in this area. No further generalisation can be made since it has been shown that sympathetic innervation, ventricular innervation and A-V pacemakers also occur (von Skramlik, 1935) in teleosts. The same situation also applies to elasmobranchs. The variation of innervation of cyclostome hearts ranges from the aneural heart of the hagfish (Hoffmeister et al. 1961) to the vagally innervated

lamprey heart, although the pharmacological properties of the latter are more akin to those of vertebrate skeletal muscle since the vagus effect is excitatory.

It is Laurent's (1962) suggestion that the very simple receptor system seen in the adult catfish are similar to those observed in the hearts of neonatal mammals and are a primitive type. Mammalian cardiac receptors become more complex in structure by three months postnatal. Also the pattern of cardiac innervation of the adult plaice is similar to that of the 6-month human foetus in which the A-V node and ventricle are not innervated whereas the S-A region is (Yamauchi, 1965).

The discovery that vagal stimulation causes a hyperpolarisation of many atrial muscle cells and that there is a rebound action potential following the hyperpolarisation can explain the mechanisms of some of the events seen with extracellular recording. First, differential stimulation of the plaice cardiac vagus produces cardio-acceleration at low frequency and cardio-inhibition at high frequency, both of which are cholinergically mediated. This follows a similar discovery in the trout by Gannon & Burnstock (1968), but in this fish, the acceleration is sympathetically mediated. Second, on the cessation of inhibitory stimulation, there is always postvagal tachycardia.

Postvagal tachycardia is a phenomenon observed in many mammalian preparations. Despite implications that the sympathetic innervation is the cause of this response in cardiac tissue, the consensus of opinion has been that the basis of this event is a property of the muscle cell membrane itself (Burke & Calaresu, 1972). It is suggested that in the

plaice heart, postvagal tachycardia is a rebound excitation of the muscle membrane as a direct consequence of the preceeding inhibitory hyperpolarisation. It is possible to extend this explanation to the mammalian heart. Rebound excitation has not been described in cardiac tissue before although it has been described in many enteric smooth muscle preparations in response to inhibitory stimulation. It is usually seen as a post-inhibitory contraction or "secondary excitation" (Furness, 1971) and has been reported in the guinea-pig taenia coli (M. Bennett, 1966), avian gizzard (T. Bennett, 1969) and the guinea-pig colon (Furness, 1970; 1971). In non-enteric preparations, an increase in firing following inhibitory stimulation has been seen in the hippocampus (Kandel & Spencer, 1961) and in the crustacean stretch receptor (Kuffler & Eyzaguirre, 1953). In the plaice heart, the persistance of rebound excitation to transmural stimulation in the presence of atropine and bretylium is strong evidence for this event being mediated by the muscle cell membrane.

Excitation and inhibition of the plaice heart by differential vagal stimulation is also a consequence of a vagus-induced hyper-polarisation. At high frequency the hyper-polarisations summate to give total inhibition, but at lower frequencies (1.2 - 3Hz) the heart rate is increased to rates dependent upon the time course of the hyperpolarisation and the refractory period of the muscle. At low frequency the membrane potential has had sufficient time to depolarise to threshold and fire an action potential before the next incoming inhibitory hyperpolarisation. The possibility that these dual effects of vagal stimulation are a result of two types of ACh

receptor in plaice cardiac muscle which are stimulated by differing ACh concentrations produced by differential stimulation is unlikely. The only response to a single vagal stimulus is a small hyperpolarisation of constant rate which is independent of stimulus frequency or voltage. A depolarisation from a steady resting potential is never seen in this situation. The hyperpolarisation is monophasic, unlike the biphasic hyperpolarisation of certain molluscan cerebral neurones in which each of the two components is the result of a distinct ACh receptor causing selective permeability increases to either potassium or chloride ions (Kehoe, 1972).

Kulaev (1957b) and Rodinov (1959) report reflex cardio-acceleration in the pike to stimulation of branchial and visceral afferent fibres. Low frequency stimulation excited the heart in this case, and high frequency stimulation inhibited it. These authors are essentially saying that cardio-acceleration is caused by the arrival of few impulses at the heart whereas Jullien & Ripplinger (1957) say that this effect is due to the increase of vagal impulse frequency on the "tonically active inhibitory ganglion cells." Similarly Randall (1966) reported a reflex increase in efferent vagal activity and cardio-inhibition on passing deoxygenated water over the gills of the tench until a synchrony of heartrate and respiration rate ensued. He also found no direct correlation between heartrate and efferent activity but noted a spontaneous "tonic" component. He suggested that it is the abolition of this spontaneous activity that is responsible for the observed cardio-acceleration on section of the vagus nerve or on the administration of atropine into the bloodstream. Therefore the discrepany might be resolved by causing a reflex cardio-acceleration and recording

efferent vagal impulses in the cardiac nerve and also intracellular events from the SA (or a pacemaker) region. Laurent (1962) recorded efferent vagal activity to the heart but was unable to discern any clear relationship between the heartrate and the observed efferent activity. The vagal impulse frequency increased in response to stimulation of the gills, and cardioinhibition resulted. Laurent attached little significance to his results since the experiments were performed on anaesthetised and dissected animals. Rebound excitation of the heart muscle from a hyperpolarisation could explain the observation that fewer impulses "impinging on the heart" could cause excitation in the pike (Kulaev, 1957a,b) and that a higher frequency causes inhibition.

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X. PUBLICATIONS

Much of the work reported in this thesis has been published in the form of the following articles:-

1. Cobb, J.L.S., & Santer, R.M. 1972. Excitatory and inhibitory innervation of the heart of the plaice (Pleuronectes platessa) : anatomical and electrophysiological studies. H. Physiol. 222, 42-43P.

2. Cobb, J.L.S. & Santer, R.M. 1973. Electrophysiology of cardiac function in teleostsL cholinergically mediated inhibition and rebound excitation. J. Physiol. in the press.

3. Santer, R.M. 1972. Ultrastructural and histochemical studies on the innervation of the heart of a teleost, Pleuronectes platessa. Z. Zellforsch. 131, 519-528.

4. Santer, R.M. 1972. An electron microscopical study of the development of the teleost heart. Z. Anat. Entwickl., 139, 93-105.

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