Virchows Arch. A Path. Anat. and Histol. 371, 101—119 (1976)

Fluorescence Histochemical and Electron-Microscopical Observations on the Innervation of the Atrial Myocardium of the Adult Human Heart

Kimmo Kyösola, Seppo Partanen, Olli Korkala, Erkki Merikallio, Olli Penttilä, and Pentti Siltanen

Department of Anatomy, University of Helsinki, Department of Thoracic Surgery, University Central Hospital, University of Helsinki, Department of Pharmacology, University of Helsinki, and First Department of Medicine, Cardiovascular Laboratory, University Central Hospital, University of Helsinki, Finland

Received March 24, 1976

Summary. The existence of both adrenergic and cholinergic innervation of the atrial myocardium of the adult human heart was demonstrated by means of fluorescence induced by formaldehyde or glyoxylic acid and by electron microscopy.

The adrenergic fluorescing axons (1) followed the course of blood vessels as typical perivascular nerve plexuses, and (2) formed a three-dimensional fairly dense nerve net obviously not related to the blood vessels. The varicosities frequently came into close apposition on myocardial cells.

Several types of nerve terminals were differentiated at electron microscopy: (1) an "adrenergic" type containing small (diameter 450–700 Å) dense-cored vesicles and usually (in various proportions) small "empty" and/or large (900–1500 Å) dense-cored vesicles, (2) a "cholinergic" type containing small (ca. 500 Å) "empty" vesicles and occasionally also some large (mean diameter ca. 1200 Å) dense-cored vesicles, (3) a "pale" type containing only a few or no vesicles, (4) a "disintegrated" type containing degenerated mitochondria, autophagic vacuoles, and occasional normal-looking mitochondria, (5) nerve terminals containing a large number of mitochondria in addition to varying vesicle populations, and (6) a (possibly baroreceptive type of) nerve terminal containing myelinlike lamellated structures. The "disintegrated" and the "pale" types of nerve terminals possibly represent different stages of axonal degeneration, or may correspond to diminution in the transmitter substance concentration under certain pathophysiologic conditions, respectively. Nerve terminals crowded with mitochondria may be sensory and involved in mechano-or chemoreceptive functions.

In preliminary experiments convincing evidence was obtained that the glyoxylic acid-induced fluorescence histochemical method will be suitable for comparative studies on (human) clinical specimens, e.g., for analyzing the degree of the functional activity of the intrinsic adrenergic innervation of the myocardium under various pathophysiologic conditions. The modification which appeared most appropriate for such studies is described in detail, and is proposed for use as a standard method in other similar or related studies on human clinical series. The essential criteria for analyzing the specimens at fluorescence microscopy are suggested as well.

Key words: Human heart — Innervation — Fluorescence histochemistry — Electron microscopy — Catecholamines.

Introduction

Formaldehyde-induced fluorescence (FIF) of catecholamines (Eränkö, 1955, 1967; Falck, 1962; Falck et al., 1962) has recently been used for the demonstration and mapping of adrenergic nerves and cells containing monoamine in the

heart of various species. These studies have given confirmative morphologic evidence of the existence of adrenergic innervation in the atrial and ventricular myocardium, in the sino-atrial and atrio-ventricular nodes, in the conducting system, in the valves, and around blood vessels. Cells containing catecholamines have been observed in the atrial wall, within cholinergic ganglia, and in small clusters of cells considered to be typical paraganglia (Angelakos et al., 1963; Jacobowitz, 1967; Ehinger et al., 1968; Winckler, 1969; Schiebler and Winckler, 1971; Dail and Palmer, 1973; Yamauchi and Chiba, 1973; Partanen and Korkala, 1974; van der Zypen, 1974; van der Zypen et al., 1974). In the heart of the human fetus, no terminal adrenergic nerves could be demonstrated during the first half of pregnancy (Dail and Palmer, 1973; Partanen and Korkala, 1974). Only a few papers providing fluorescence histochemical evidence of the existence of functional adrenergic innervation of the human cardiac muscle have been published (Pearse, 1964; McLean, 1968; Sachs, 1969). Additional studies thus seem indicated.

A reduction of the tissue concentration of noradrenalin (NA) has been observed in the failing heart of both man and various experimental animals (Chidsey et al., 1963; Vogel et al., 1969; Penttilä et al., 1975). In the present study, one of the aims was to correlate different groups of patients suffering from heart disease using fluorescence histochemical methods. Most of the respective specimens from the same patients were also examined using a biochemical method in order to determine the tissue concentration of various catecholamines. The tissue concentration of noradrenalin was found to decrease in congestive myocardial failure and to be high in ischemic heart disease (Penttilä et al., 1975).

During the last few years, innervation of the heart has also been studied at the ultrastructural level in various species, including children aged 3–10 years (Chiba and Yamauchi, 1970). However, to our knowledge, no papers concerning the electron microscopy of the innervation of adult human heart have been published. The present paper is also limited in scope, dealing only with the innervation of right atrial tissue.

Patients and Methods

The series included 36 adult patients, randomly selected from the series of elective open-heart operations performed in the Department of Thoracic Surgery, Helsinki, University Central Hospital, between 1973 and 1975. The patients were divided into four groups. The first group comprised 10 patients with uncomplicated atrial septal defect (ASD). The second group comprised 11 patients suffering from ischemic heart disease (IHD); they had been selected for a revascularization operation on the basis of anamnestic, clinical, and coronary angiographic data. The third group comprised 9 patients with a left-sided univalvular or multivalvular heart disease (VHD) without symptoms or signs of cardiac incompensation. The fourth group comprised 6 patients who had experienced congestive heart failure (CHF) due to VHD prior to surgery. They showed a tendency to dyspnea even at rest, orthopnea, elevated central venous pressure, and increased body weight due to edema. The preoperative functional class of myocardial performance was assessed during effective medical treatment according to the clinical, laboratory, and radiologic findings and following the classification of the New York Heart Association (N.Y.H.A., 1964). Some clinical findings and the main cardiologic data of the present series are presented in Table 1. The usual daily medication with digitalis, diuretics, antiarrhythmic drugs, anticoagulants, nitroglycerin, and antihypertensive drugs was continued until the operation; any beta-adrenergic blocking agents, however, were withdrawn 10 days previously.

Table 1. Summary of the clinical and laboratory data in the different patient groups of ischemic heart disease (IHD), valvular heart disease (VHD), atrial septal defect (ASD), and congestive heart failure (CHF)

Group Number of patients in parentheses	Age (Mean ± SDM)	Degree of symptoms (NYHA criteria I–IV)	Heart volume (cc/m²) (Mean ± SDM)	Blood pressure (mmHg) (Mean ± SDM)	dp/dt LV_S (mmHg/sec) (Mean ± SDM)	Left ventricular end-diastolic pressure (mmHg) (Mean ± SDM)
IHD (11)	45.2 ± 8.7	II–III	470 ± 80	140/85 10 10	1,630 ± 450 (8)	16.0 ± 6.0 (8)
ASD (10)	27.2 ± 10.3	None or I	570 ± 140	120/75 10 10	1,530 ± 190 (7)	9.5 ± 4.0 (7)
VHD (9)	46.1 ± 9.2	II–III	820 ± 280	130/85 10 15	1,690 ± 800 (6)	19.5 ± 8.0 (6)
CHF (6)	47.8 ± 11.2	III–IV	920 ± 210	140/85 15 15	1,360 ± 340 (5)	18.5 ± 11.0 (5

Standard premedication (atropine + pethidine), induction (thiopentone), and anesthesia ($O_2 + N_2O$, pethidine or anileridine, alcuronium or pancuronium) were employed. Anesthesia was maintained during cardiopulmonary bypass with thiopentone and analgesics (pethidine or anileridine). In a few cases halothane was administered in addition, but only after resection of the specimens. (see below).

The technique of cardiopulmonary bypass was basically the same in all cases. Moderate hypothermia and hemodilution to a hematocrit level of 25 were used. A Kay-Cross disk oxygenator equipped with roller pumps and a heat exchanger was primed with heparinized fresh blood, Haemaccel, physiologic saline, 5% glucose in water, and isotonic sodium bicarbonate. In some cases mannitol was added. The priming volume was 3000–3500 ml. Heparin, 3 mg/kg of body weight, was administered before the caval cannulas were inserted.

A median sternotomy or right-sided thoracotomy was performed. In valve surgery and correction of congenital anomalies, the arterial return line from the oxygenator went through the femoral artery, but usually went through the ascending aorta in revascularization operations. The left ventricle was vented. Electrical fibrillation was maintained throughout the operation.

A purse-string suture was tied around the apex of the right auricular appendage, and either the right atrium or the caval veins were cannulated via an incision made in the middle of the purse-string suture. After a partial perfusion for 1–2 min, total extracorporeal perfusion was begun. At this moment a small wedge resection was made from the apex of the right auricular appendage at the site of insertion of the venous drainage perfusion cannule. The tissue specimen was thus obtained within a few minutes from the beginning of the perfusion, always in exactly the same manner. The specimen was thereafter immediately processed further (as will be described below).

Fluorescence Histochemistry

Specimens from 29 patients (ASD:8, IHD:9, VHD:7, CHF:5) were frozen by immersion in liquid nitrogen and subsequently freeze-dried for approximately 1 week at −40°C over phosphorus pentoxide *in vacuo*, warmed, removed from the freeze-drying apparatus, and exposed to hot formaldehyde vapor generated from paraformaldehyde powder equilibriated with 60% relative air humidity. The temperature of exposure was 60°C for 30 min and 80°C for 1 h (for further details, see Eränkö, 1967). Finally, 25 specimens were embedded in Epon or in a mixture of Epon and Araldite, cut with an LKB ultratome at 5 μ, partly in series, transferred dry to slides, and mounted in a mixture of Entellan (E. Merck, Darmstadt) and xylene. Every fifth section was stained with 1% alkaline toluidine blue for orientative light-microscopic studies. Four specimens were vacuum-embedded in paraffin and sectioned at 7 μ with a sliding microtome.

Fluorescence was examined and photographed using a Leitz Ortholux microscope equipped with an epi-illuminator (Ploem, 1971). An Osram HBO 200 high pressure mercury lamp

was used as the UV light source. The filter combination used was BG 38 (4 mm), 2×BG 3 (3 mm), TAL 405 (Schott & Gen., Mainz) as the primary filters and Leitz K 470 as the secondary filter. The specificity of the FIF was checked with 0.5% sodium borohydride (Merck) in 80% ethanol (Corrodi et al., 1964); in this procedure paraffin-embedded sections were used after deparaffinization of the sections with a small amount of xylene.

From four patients of the former group, myocardial strips were immersed in ice-cold physiologic saline solution immediately after resection, dried with blotting paper, stretched on microscope slides, dried in a desiccator over phosphorus pentoxide for 6 h, and gassed with paraformaldehyde, mounted, viewed, and photographed as described above.

From four other patients, thin myocardial tissue strips were incubated in ice-cold 2% glyoxylic acid monohydrate (Sigma) in 0.1 M phosphate buffer (pH 7.4) for 1 h, dried with blotting paper, stretched on microscope slides, dried further in a hot current of air from a hair-dryer for 15 min, heated in an oven at 100°C for 6 min, mounted ,viewed, and photographed as described above. (For further details of the procedure, see Lindvall and Björklund, 1974; Lindvall et al., 1974; Waris and Partanen, 1975).

Electron Microscopy

Small specimens from three patients were immersion-fixed in ice-cold 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 5 h, rinsed overnight in the same buffer solution, postfixed with 1% osmium tetroxide, dehydrated in graded series of ethanol, and embedded in a mixture of Epon and Araldite. Ultrathin sections were cut with a Reichert ultrotome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963), and viewed and photographed with a Philips 300 electron microscope operated at 60 kV.

Similarly, small specimens from three patients were fixed with 3% potassium permanganate in 0.1 M phosphate buffer (pH 7.0) at 0°C for 3 h, rinsed with the same buffer solution, and dehydrated, embedded, and sectioned as described above. The ultrathin sections were viewed both unstained and after staining with lead citrate (Reynolds, 1963), and photographed as described above.

Results

Fluorescence Histochemistry

Formaldehyde-Induced Fluorescence in the Freeze-Dried Specimens. The distinction of the specific formaldehyde-induced fluorescence from the strong autofluorescence of the connective tissue was very difficult. The color of the autofluorescence varied from yellow to blue-green, which was the color of the specific catecholamine fluorescence. A strong autofluorescence was observed in the reticulin, elastin, and collagen fibers in the endocardium and epicardium, between bundles of myocardial cells, and in the adventitia and internal elastic membrane of larger blood vessels. The thick bundles of collagen fibers exhibited a light-green fluorescence while the fluorescence emitted by the long wavy elastin fibers coursing individually was blue-green, and that of the fine fragmented network of reticulin fibers surrounding the myocardial cells was bright yellow, with the filter combination used. The strong autofluorescence of the connective tissue, observed in all specimens, was very disturbing in both covering and simulating the specific fluorescence. The myocardial cells showed a faint greenish "background" fluorescence, and in the sarcoplasm, varying amounts of autofluorescing orange to red granules and droplets were observed, mostly accumulated near the poles of the ovoid nucleus. These accumulations most probably represented lipofuscin (Fig. 1).

Specific Fluorescence. A loose-meshed net of fluorescing varicose nerves was observed in the myocardial layer, some of the nerves obviously coming into

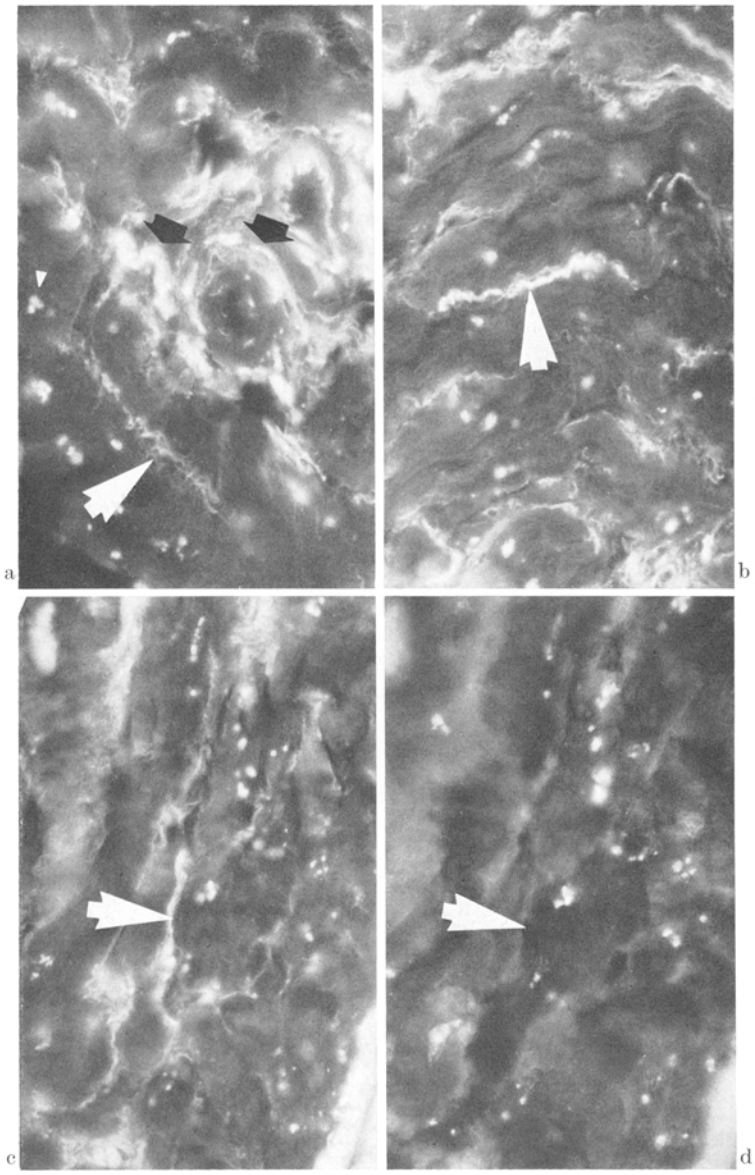


Fig. 1a—d. Formaldehyde-induced fluorescence (FIF) in a freeze-dried specimen. (a) Intense blue-green fluorescence seen in nerves (black arrows) around blood vessel. Connective tissue (e.g., filling interstices between myocardial cells, large white arrow) exhibits strong autofluorescence, as do lipofuscin aggregates (small white arrow). ×400. (b) Thick fluorescing nerve (arrow) between myocardial cells. ×400. (c) Great many fluorescing structures seen in this field (cf., Fig. 1d). ×400. (d) Same field as in Figure 1c, but after borohydride treatment. Intensely blue-green fluorescing beaded fiber, marked with arrow in Figure 1c, no longer visible, due to disappearance of (neural) catecholamine following incubation with sodium borohydride, whereas nonspecific autofluorescence of connective tissue and lipofuscin still visible. ×400. (Male, age 36 years, suffering from rheumatic aortic valve insufficiency necessitating replacement with aortic valve prosthesis. Clinically without overt congestive heart failure. N.Y.H.A. classification I–II. Heart volume 820 cc/m². RR 150/40-0. Digoxin and frusemide

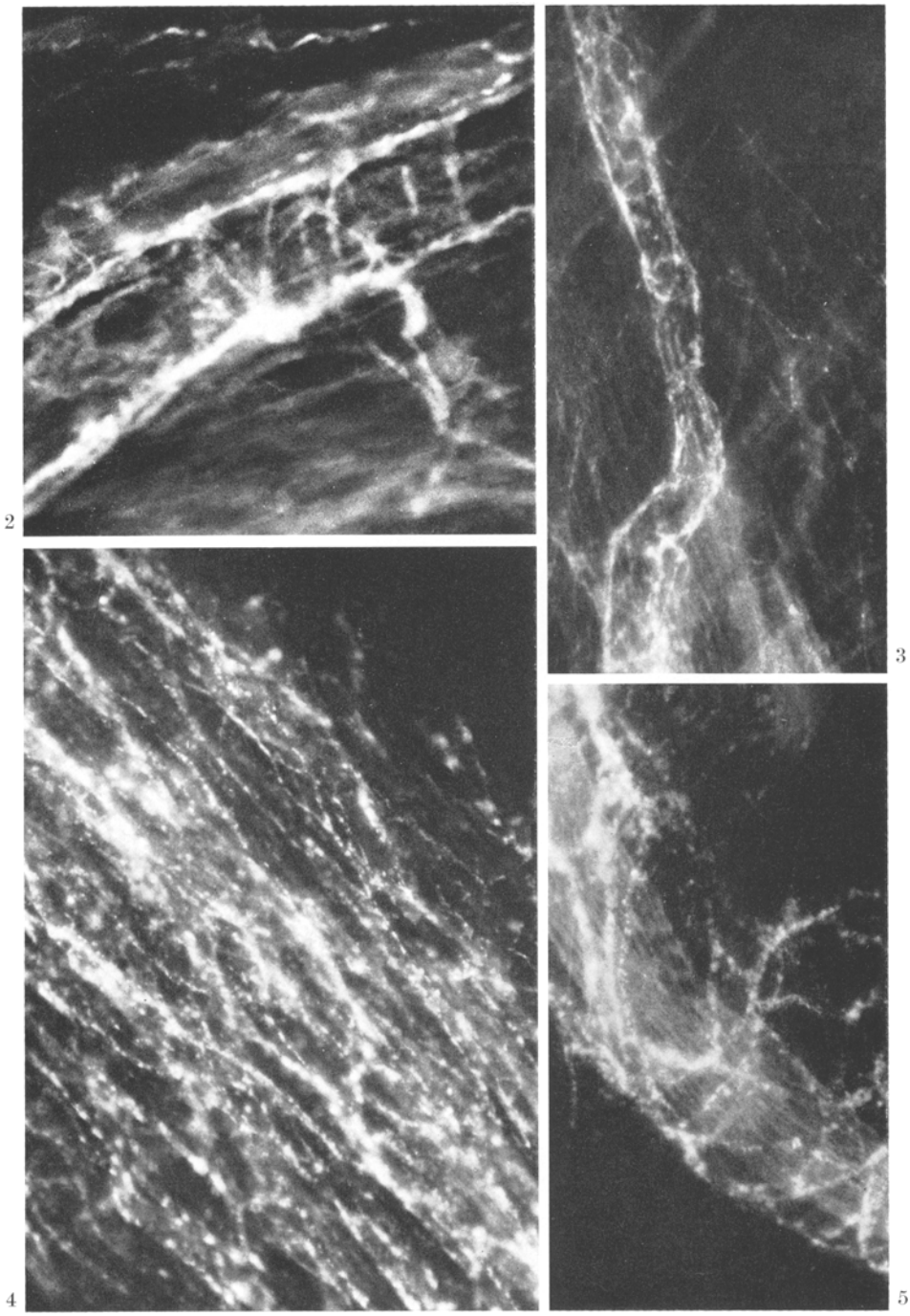


Fig. 2. Fluorescing perivascular nerve plexus surrounding arteriole in myocardial tissue. FIF, stretch preparation. ×250. (Female, age 29 years, with uncomplicated ASD of secundum type. Asymptomatic, routine chest x-ray suggestive of ASD. L-R shunt 5.4 l/min. Heart volume 450 cc/m². RR 120/80. No medication)

fairly close contact with the myocardial cells. The others probably followed the course of small blood vessels as single fluorescing ribbons. Larger blood vessels were surrounded by the typical adrenergic perivascular nerve plexuses. Because of the strong autofluorescence of the connective tissue, it is evident that only densely woven adrenergic nerve plexuses or individual axons containing an abundance of fluorescing amine in their synaptic enlargements ("varicosities") could be verified, and even these nerves only on some favorable occasions. Nevertheless, the existence of adrenergic nerves could be firmly established by comparing untreated and borohydride-treated sections: following borohydride treatment the fluorescence was abolished from structures which resembled noradrenergic nerves both morphologically and as regards the color of the fluorescence (blue-green). The autofluorescence of the connective tissue was unaffected by the borohydride treatment, and that of lipofuscin increased slightly, and its color became yellower. No cells containing catecholamine were observed (Fig. 1).

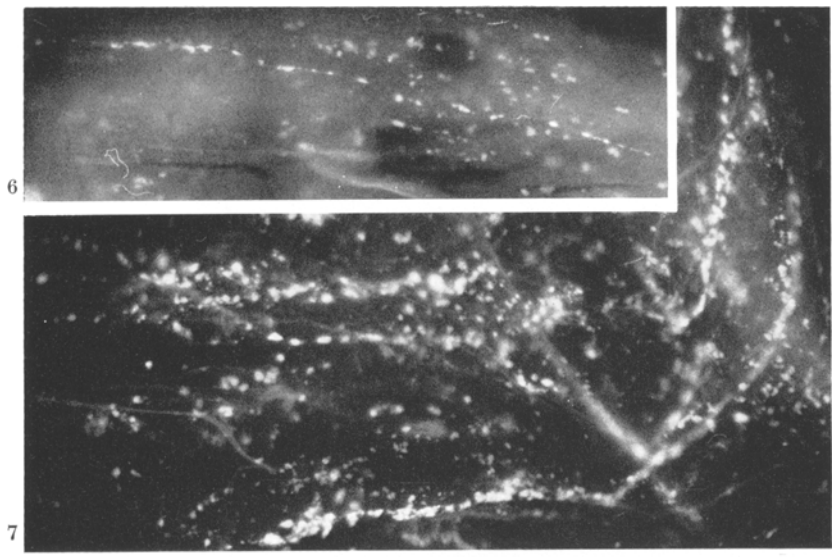
Because of the prominent autofluorescence of the connective tissue, no valid correlations between specimens obtained from different groups of patients could be made.

Glyoxylic Acid-Induced Fluorescence in Stretch Preparations. A fairly rich distribution of brightly blue-green fluorescing single varicose axons and small fascicles of them was observed in the myocardium. The nerves formed a three-dimensional nerve net obviously not related to blood vessels, and frequently coming into close apposition on the myocardial cells. In addition, the typical perivascular fluorescing nerve plexuses were observed around blood vessels. A few larger fluorescing nerve trunks were also seen. The difference between the colors, and especially in the intensity, of the autofluorescence of the connective tissue and the specific catecholamine fluorescence were by far more distinct than that seen in formaldehyde-treated specimens. No cells containing catecholamines were observed. Considerable individual differences in the specific nerve fluorescence were readily observed (see Figs. 3–7 and the respective legends).

Fig. 3. Fluorescing perivascular nerve plexus surrounding arteriole or small artery in myocardial tissue. Some very faint myocardial nerves seen among muscle cells. Glyoxylic acid-induced fluorescence histochemical method (GIF). ×125. (Female, age 30 years, with uncomplicated ASD of secundum type. Asymptomatic, routine chest x-ray suggestive of ASD. L-R shunt 13.8 l/min. Heart volume 530 cc/m². RR 130/90. No medication)

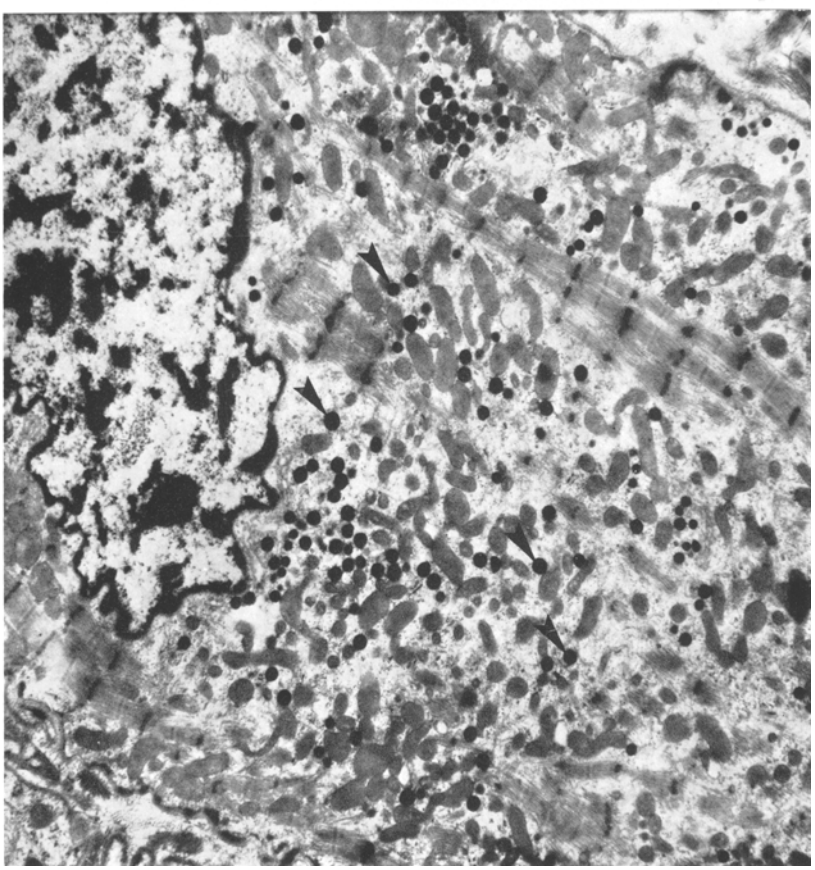
Fig. 4. Rich innervation of atrial myocardium with extremely intensely blue-green fluorescing adrenergic axons. Both perivascular and myocardial axons seen. Fluorescence histochemical appearance of this kind typical of IHD group. Intense diffuse fluorescence mainly nonspecific autofluorescence of lipofuscin, readily identified at fluorescence microscopy by its typical orange to red color (GIF). ×125. (Male, age 48 years, with IHD. N.Y.H.A. classification III. In coronary angiography significant stenoses in coronary arteries. No history of myocardial infarcts. Heart volume 390 cc/m². RR 130/80. Nitroglycerin, practolol [withdrawn 10 days before operation])

Fig. 5. Loose periarterial nerve plexus consisting of single fluorescing varicose axons and small fascicles of them supplies adjacent myocardial tissue with adrenergic nerves. Autofluorescence of connective tissue of blood vessel wall readily discernible. (GIF). ×250. (Female, age 24 years, with uncomplicated ASD of secundum type. Slight decrease in physical performance. N.Y.H.A. classification I. L-R shunt 7.1 l/min. Heart volume 430 cc/m². RR 115/75. No medication)



6

7



8

Figs. 6—8

Formaldehyde-Induced Fluorescence in Stretch Preparations. By using the conventional formaldehyde-induced fluorescence histochemical method on stretch preparations, a picture similar to the one described above for the glyoxylic acid-induced fluorescence was obtained. The autofluorescence of the connective tissue, however, did cause some difficulties; the most delicate single fluorescing varicose axons could not be identified with the same accuracy as when glyoxylic acid was used, whereas the fluorescing perivascular nerve plexuses were equally prominent. The maximum intensity of the specific fluorescence was somewhat lower than in the freeze-dried specimens, and some diffusion of fluorophores was usually observed. The colors of the connective tissue autofluorescence were about the same as in the freeze-dried specimens, but the intensity was lower (Fig. 2).

Electron Microscopy

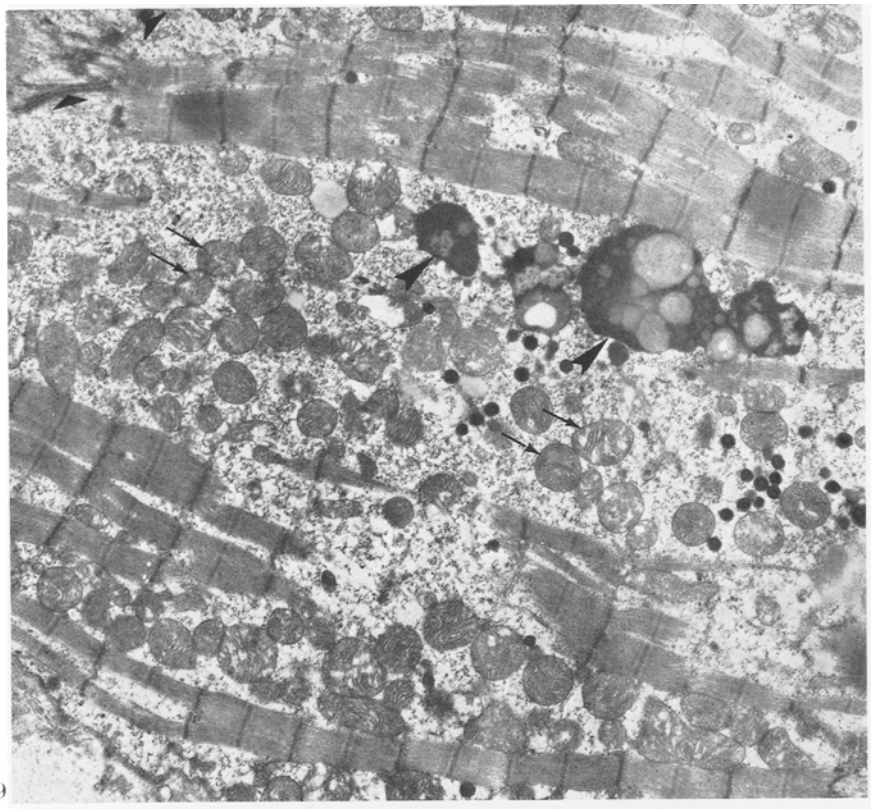
Glutaraldehyde fixation properly preserved the ultrastructure of the myocardial cells (Figs. 8 and 9) and of the various types of nerve terminals. However, a separation of the muscle fiber bundles from each other along the connective tissue filling the interstices between was regularly observed. In consequence, the individual nerve terminals, which approached the myocardial cells in the interstices filled with connective tissue elements, very often appeared to be torn off from the site of their termination on the muscle. This was probably at least partly due to the disordered contractions of the adjacent muscle fiber bundles during or before the fixation procedure (Fig. 10).

A rich distribution of nerve fibers embedded in Schwann cell cytoplasm either individually or gathered into bundles of various sizes, always unmyelinated, was observed. Within the bundles different types of nerve terminals were frequently in close apposition without intervening Schwann cell cytoplasm. No membrane specializations were discernible at these sites of close apposition suggesting direct mutual axo-axonal influence. (Fig. 10). In general, the nerve terminals and the myocardial cells were separated from each other by an intervening gap of the order of several thousands of Å, filled with connective tissue fibers embedded in electron-opaque homogeneous material ("basement membrane") surrounding the muscle. (Fig. 10). Frequently, additional "insulation"

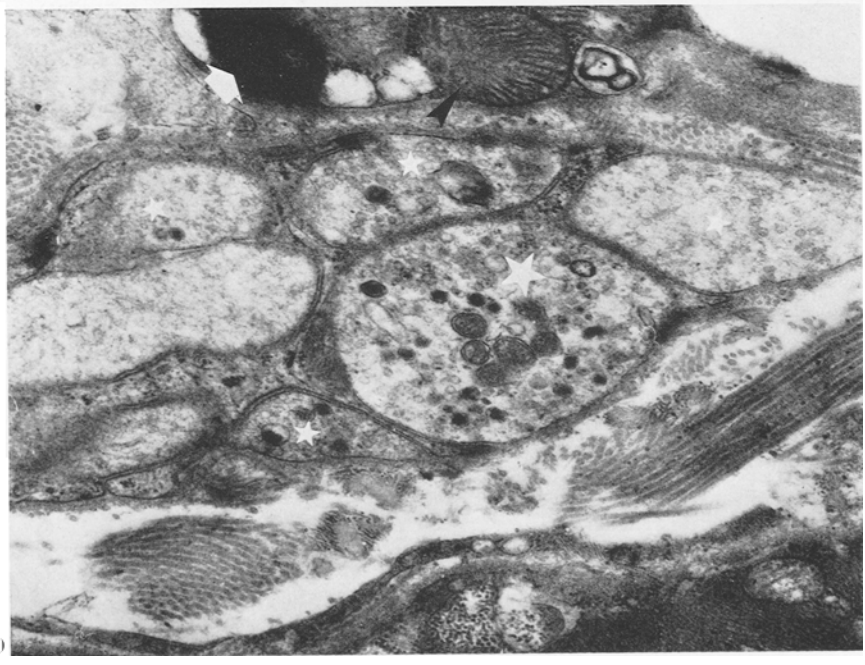
Fig. 6. GIF in stretch preparation. Long portion of adrenergic axon, showing varicosities of varying sizes, forms and fluorescence intensities, is seen coursing among myocardial cells. ×400. (Female, age 19 years, with uncomplicated ASD of the secundum type. Asymptomatic, routine chest x-ray suggestive of ASD. L-R shunt 13.8 l/min. Heart volume 545 cc/m². RR 125/80. No medication)

Fig. 7. GIF in stretch preparation. Intensely blue-green fluorescing varicose axons, both singly and gathered into small fascicles, seen coursing among myocardial cells. Connective tissue and lipofuscin exhibit moderate nonspecific autofluorescence. ×400. (Same patient as in Fig. 5)

Fig. 8. Well-preserved ultrastructure of myocardial cell (see also Figure 9). Specific atrial granules (arrows) typically concentrated near nucleus. ×5,000. (Female, age 47 years, suffering from ASD of the secundum type and grave aortic valve insufficiency due to endocarditis lenta. L-R shunt 9 l/min. Heart volume 1010 cc/m². RR 120/90. Clinically overt congestive heart failure. N.Y.H.A. classification III. Digoxin, hydrochlorothiazide, and amiloride)



9



10

Figs. 9 and 10

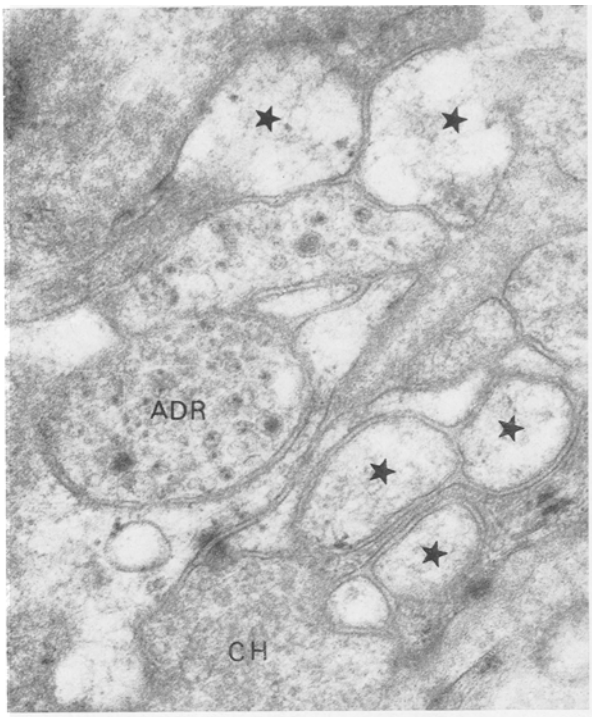
was provided by the Schwann cell covering of the terminals. However, closer contacts without intervening Schwann cell cytoplasm were occasionally observed between myocardial cells and nerve terminals (Fig. 16).

Within the nerve terminals, small "empty" vesicles, small dense-cored vesicles, large dense-cored vesicles, and mitochondria were observed in various combinations and in various relative proportions. No clear-cut rigid classification thus seems possible, and the terminals must be typed somewhat arbitrarily according to their main vesicle populations. If the presence or absence of the small dense-cored vesicles within a given terminal is taken as a conclusive criterion, then two main groups of nerve terminals can be identified. (1) An "adrenergic" type containing small (diameter 450–700 Å) dense-cored vesicles and usually (in various proportions) small "empty" and/or large (900–1500 Å) dense-cored vesicles (Figs. 10, 11, and 13), and (2) a "cholinergic" type containing small (ca. 500 Å) "empty" vesicles and occasionally also some large (mean diameter ca. 1200 Å) dense-cored vesicles (Figs. 10, 11, and 13). In addition, (3) a "pale" type containing only a few or no vesicles (Fig. 11), (4) a "disintegrated" type containing degenerated mitochondria, autophagic vacuoles, and occasional normal-looking mitochondria (Fig. 13), and (5) a [possibly baroreceptive (cf., Chiba, 1972) type of] nerve terminal, containing myelin-like lamellated structures (Fig. 16), were identified. (6) Some nerve terminals were crowded unusually rich in mitochondria, in addition to varying vesicle populations (Fig. 12). Such terminals were observed in the vicinity of, or even immediately beneath, the basement membrane of sinusoid-type blood vessels (relatively wide lumen lined only by the endothelium and the basement membrane). These terminals were probably sensory (cf., Chiba, 1972; Tranum-Jensen 1975).

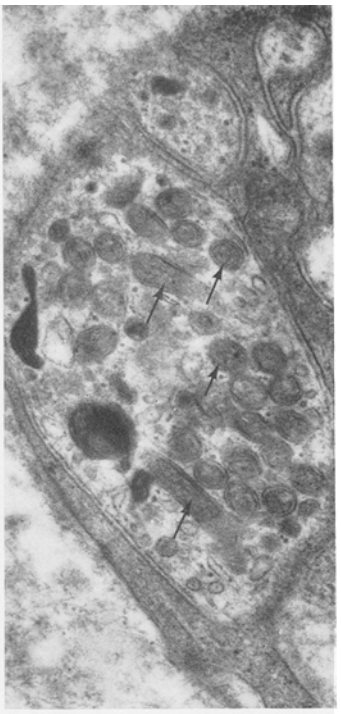
The small dense-cored vesicles, characteristic of adrenergic nerve terminals, were well preserved after glutaraldehyde fixation. A rich distribution of adrenergic nerve terminals among other types of nerve terminals was further confirmed with the potassium permanganate fixation technique, which reveals the small dense-cored vesicles even more reliably (Figs. 14 and 15). In many specimens, a large percentage of nerve terminals showed signs of axonal degeneration: the

Fig. 9. Well-preserved ultrastructure of myocardial cell. Globular mitochondria (delicate arrows) have long slender cristae. Within sarcoplasm, accumulations of pigment material (lipofuscin) in form of autophagic vacuoles (thick arrows) of varying sizes seen. Intercalated disk (triangle) observed in left upper corner of photograph. In spite of severe myocardial failure, basic ultrastructure well preserved, whereas that of many nerve terminals shows varying degrees of destruction (cf., Figs. 10 and 11). ×7,000. (Same patient as in Fig. 8)

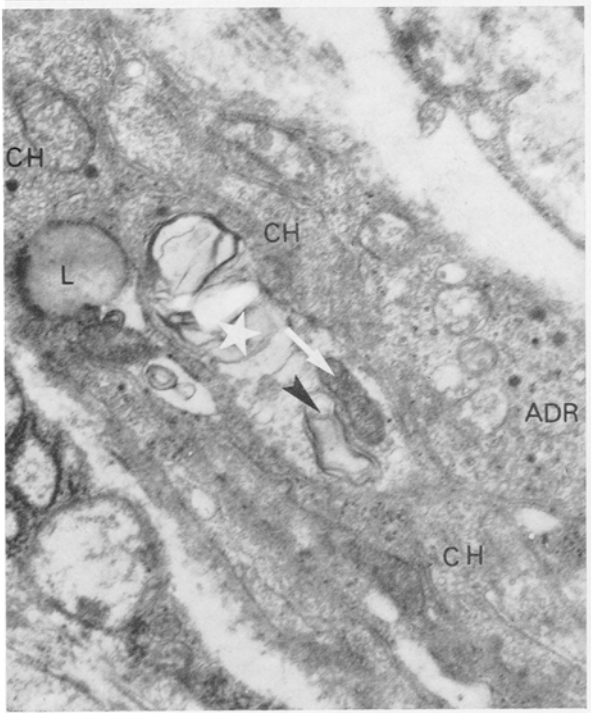
Fig. 10. Adrenergic nerve terminal (large asterisk) containing great many small dense-cored vesicles (in addition to small empty and large dense-cored vesicles) and several cholinergic nerve terminals (small asterisk) containing either exclusively small empty or both small empty and large dense-cored vesicles abutting intimately onto each other. In upper part of photograph, myocardial cell containing autophagic vacuoles (white arrow) (lipofuscin formation in process) and mitochondrion (black arrow) seen; "gap" between cell and nerve terminals filled with connective tissue. In lowermost portion of photograph, another myocardial cell partly seen, but some artificial separation in "gap" region has occurred, probably due to disordered contractions of muscle tissue before or during fixation procedure. ×14,760. (Same patient as in Figs. 8 and 9)



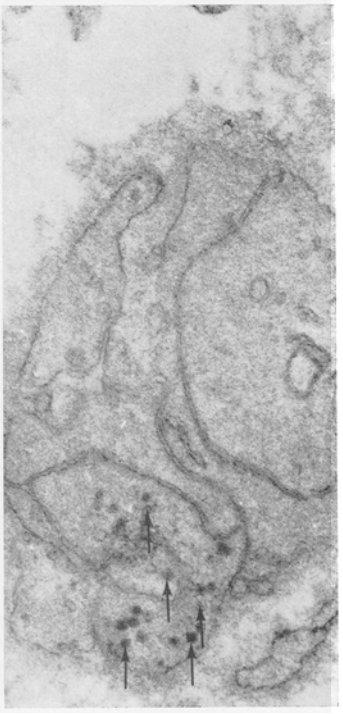
11



12



13



14

Figs. 11—14

terminals were filled with vacuolized, quite homogeneous, finely granular electron-lucent material, in which no vesicles or mitochondria could be identified. In the vicinity of such destructed nerve terminals, nerve terminals of the "pale" and "disintegrated" types were observed, as well as nerve terminals exhibiting only few ultrastructural degenerative changes (Figs. 10 and 11). Consequently, it is obvious that the morphologic alterations are due to the underlying disease, and not to technical errors. This view is supported by the observation that more destruction of nerve terminals was observed in specimens obtained from seriously ill patients with manifest congestive heart failure. It was clearly evident that, even in the same patient, adjacent myocardial nerve fibers could show different degrees of axonal degeneration.

Discussion

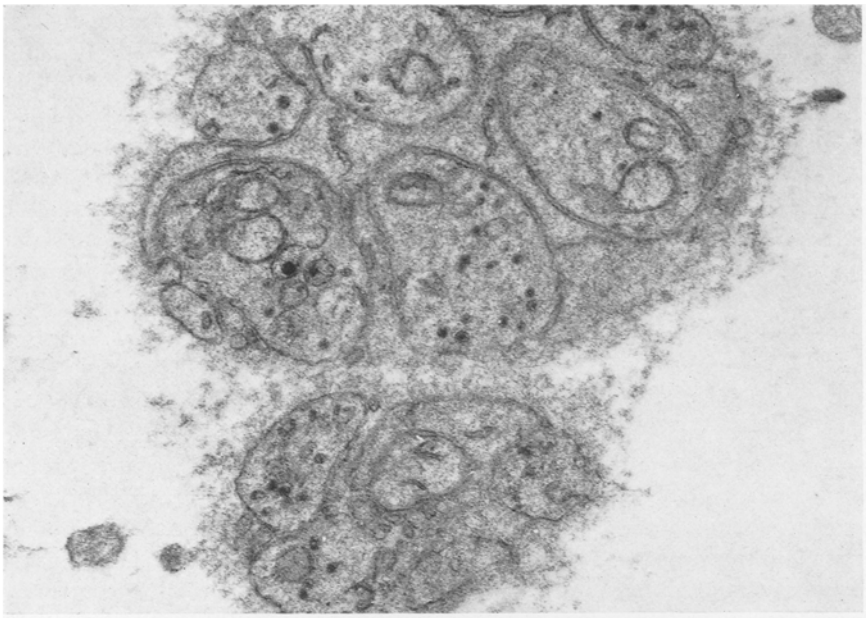
The intrinsic innervation of the human heart has been extensively studied with the classical neurohistologic staining techniques (for ref., see e.q., Stöhr Jr., 1957; Hirsch and Borghard-Erdle, 1961, 1962; van der Zypen, 1974), which, however, do not allow discrimination between the adrenergic and cholinergic nervous systems, because these techniques demonstrate neither the transmitter substances themselves, nor the related specific enzymes. On the contrary, the formaldehyde- and glyoxylic acid-induced fluorescence histochemical methods are based on the ability of these reagents to react with minute amounts of noradrenalin, the transmitter substance in the adrenergic nerves, in its physiologic storage sites, and the well-established chemical reactions lead to the formation of highly fluorescent dihydro-iso-quinolines (see Jonsson, 1967; Lindvall et al., 1974), reliably discernible at fluorescence microscopy. There is a great deal of convincing physiologic, pharmacologic, and clinical evidence of the adrenergic

Fig. 11. Adrenergic (ADR), cholinergic (CH), and "pale" type (asterisks) nerve terminals in nerve bundle coursing among myocardial muscle cells. "Pale" type terminals possibly reflect axonal degeneration and/or diminution in concentration of transmitter substance. ×34,440. (Same patient as in Figs. 8–10)

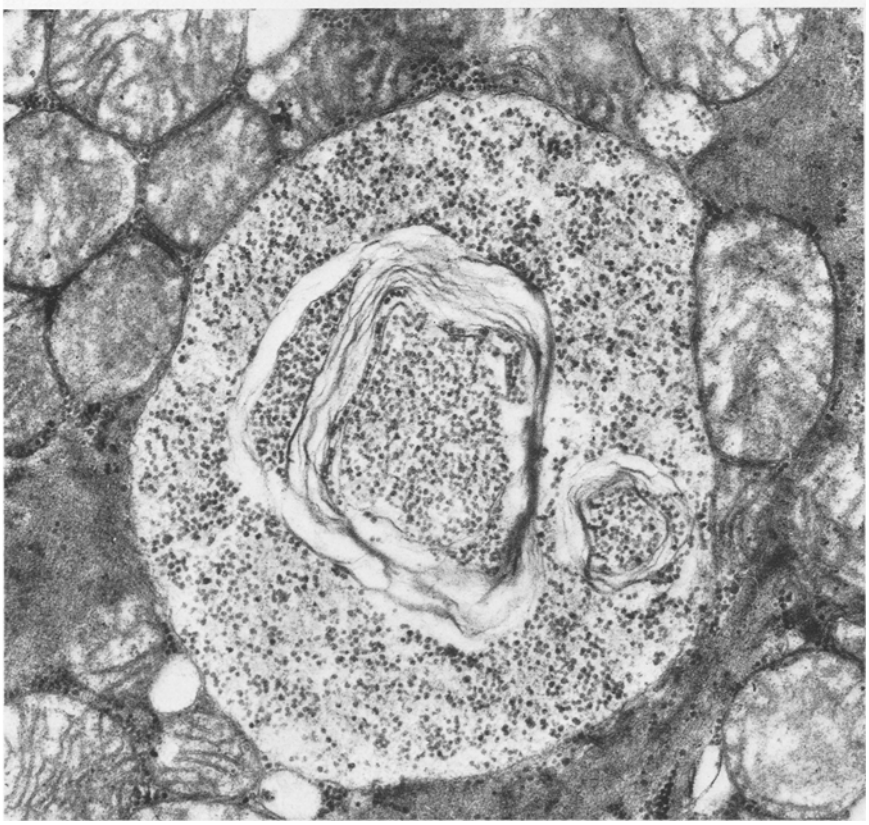
Fig. 12. Nerve terminal crowded with mitochondria (arrows), and thus possibly representing sensory nerve terminal in myocardial tissue layer. ×34,440. (Same patient as in Figs. 8–11)

Fig. 13. In middle of photograph, nerve terminal of "disintegrated" type seen. Terminal contains one normal (white arrow) and one degenerating (black arrow) mitochondrion, as well as lysosomal vacuolization products (autophagic vacuoles) (asterisk). Adrenergic (*ADR*) and cholinergic (*CH*) terminals are also observed, as well as a lipid droplet. (*L*). Some ultrastructural degeneration has also occurred in adrenergic (*ADR*) and cholinergic (*CH*) nerve terminals. ×18,000. (Female, age 57 years, suffering from combined rheumatic mitral stenosis and regurgitation, atrial fibrillation, and essential hypertension (WHO I) Clinically not overt congestive heart failure, N.Y.H.A. classification II. Heart volume 1350 cc/m². RR 180/100. Digoxin, frusemide, warfarin)

Fig. 14. Specific demonstration of adrenergic nerve terminals with potassium permanganate fixation technique. Small dense-cored vesicles (arrows) clearly identifiable. ×34,440. (Male, age 49 years, suffering from aortic stenosis due to unknown etiology and atrial fibrillation. Clinically overt congestive heart failure. N.Y.H.A. classification III. Heart volume 1,200 cc/m². RR 120/80. Digoxin, frusemide, spironolactone, verpamil)



15



16

Figs. 15 and 16

control of the human heart, but only little morphologic confirmatory evidence has been presented. While the heart of the human fetus is lacking in functional adrenergic innervation during the first half of pregnancy (Dail and Palmer, 1973; Partanen and Korkala, 1974), cells containing catecholamines were observed within the atrial myocardium of the human midterm fetus (Partanen and Korkala, 1974). McLean (1968), while referring to some earlier unsatisfactory experiments, gave an excellent demonstration of the presence of fine varicose fluorescing axons within the myocardial tissue of the human heart. At the electron-microscopic level, the intrinsic innervation of the heart has been studied in several lower animals (for ref., see e.g., van der Zypen et al., 1974), but to our knowledge, only one paper concerns the human heart: Chiba and Yamauchi (1970) found in their electron-microscopic investigation several types of nerves in the papillary muscle and the right auricular appendage of the heart of children. Further studies thus seemed indicated, especially to elucidate morphologic alterations associated with various patho-physiologic situations, which was one of the purposes of the present work.

The present study provides additional morphologic confirmation of the existence of an inbuilt intrinsic adrenergic nervous apparatus within the myocardium of the (adult) human heart by demonstrating fluorescing, borohydride-sensitive nerves (Corrodi et al., 1964; Eränkö, 1967), and nerve terminals containing small dense-cored vesicles (e.g., Burnstock and Iwayama, 1971). Although the present study was limited to the right auricular appendage, there is no reason to expect that the scheme of the intrinsic innervation (i.e., dual innervation by both adrenergic and cholinergic systems with extensive neuroneuronal mutual interaction) would be different in other parts of the heart.

The adrenergic innervation of the human right auricular appendage, as revealed by fluorescence microscopy, was similar in principle to that in the atria of the hearts of various lower mammals (for ref., see introduction). Thus, fluorescing nerves were seen surrounding blood vessels as perivascular nerve plexuses and also coursing "free" between the myocardial cells. The exact function of the adrenergic nerves in the atrial myocardium is unknown. It has been suggested that the adrenergic nerves could somehow regulate the tonus of the atrial wall or the metabolism of the atrial myocardial cells (Schiebler and Winckler, 1971).

It was clearly observed in the present study that the formaldehyde-induced fluorescence histochemical method as applied on freeze-dried specimens, although extremely sensitive in principle, is not suitable in its present form for exact mapping of the distribution of adrenergic nerves in the human heart, or for examining the changes in their noradrenalin content, because of the strong disturbing autofluorescence of the connective tissue, which is typical of human tissues and in-

Fig. 15. Analogical to Figure 14. ×34,440. (Same patient as in Fig. 14)

Fig. 16. Large nerve terminal containing granular material (obviously glycogen granules) and myelinlike lamellated structures protruding into invagination of sarcolemma of myocardial cell, sarcoplasm of which is richly crowded with mitochondria. Terminal possibly engaged in baroreceptive functions (see text). Intimate contact between nerve terminal and myocardial cell obvious. ×34,440. (Same patient as in Fig. 2)

creases with advancing age (Baumgarten, 1967). This difficulty naturally also concerns the FIF method applied on stretch preparations. Checking the specificity of the fluorescence with the borohydride test (Corrodi et al., 1964) leads to a great many practical difficulties, and thus is not appropriate for quantitative studies. In conclusion, the intrinsic adrenergic innervation of the human heart and the possible alterations in its activity cannot be studied adequately with the FIF methods.

On the contrary, the results of the present study showed that noradrenalin in the adrenergic nerves in the adult human heart can be properly demonstrated with the glyoxylic acid fluorescence method. This is possibly largely due to such a difference between the wavelengths of the lights emitted by the specific catecholamine fluorescence and the autofluorescence, which can easily be discerned by the human eye, in addition to the difference in the (relative) intensity of the specific and autofluorescence. The human eye is quite capable of discerning the difference of a few nanometers between the wavelengths of the lights (i.e., colors) emitted in the same histologic section, if the intensities of the lights are about the same (see Ritzen, 1967). It seems that the color of the autofluorescence of the connective tissue treated with aqueous glyoxylic acid at neutral pH is different from that in the formaldehyde-treated specimens, because the reported corrected emission spectras of noradrenalin-fluorophore have been reported to possess the peak of emission at 470–480 nm in both methods (Jonsson, 1967; Ritzen, 1967; Lindvall et al., 1974; Lindvall and Björklund, 1974).

It seemed that the maximal fluorescence intensities in the adrenergic nerves were of about the same magnitude after both formaldehyde and glyoxylic acid treatments. This is probably caused by the concentration-dependent quenching of the fluorescence. At the normal noradrenalin level in the peripheral and central adrenergic nerves a concentration-dependent quenching of the formaldehyde-induced fluorescence has been observed (Jonsson, 1969; Lidbrink and Jonsson, 1971). The fluorescence intensity is proportional to a certain critical level of endogenous noradrenalin, e.g., to 40% of the normal concentration in the adrenergic nerves of the iris of the rat; thereafter the fluorescence intensity does not increase in accordance with increasing noradrenalin concentration (Jonsson, 1969). The glyoxylic acid-induced fluorescence of catecholamines also shows the quenching phenomenon in model experiments (Lindvall et al., 1974) and, consequently, it might be justified to assume that the same phenomenon would be operating in the adrenergic nerves of the heart at normal noradrenalin levels. However, the glyoxylic acid-induced fluorescence histochemical method demonstrates obviously more efficiently than the formaldehyde method those axons in which the noradrenalin concentration is low, due to its greater sensitivity (cf., Lindvall and Björklund, 1974; Lindvall et al., 1974; Waris and Partanen, 1975). Thus, based on the results of the present study, the glyoxylic acid-induced fluorescence histochemical method is suitable for the demonstration of the intrinsic adrenergic innervation of the human heart, and also for studies on changes in noradrenalin loading under various pathophysiologic conditions which have been demonstrated biochemically (Penttilä et al., 1975). Thus, in addition to (1) the fluorescence intensity, (2) the density of the intrinsic adrenergic innervation, (3) the mean diameter of the synaptic enlargements (varicosities, "boutons"),

(4) the relative proportion of large varicosities, and (5) the relative proportions of visualizing myocardial ("free") and perivascular adrenergic axons, would serve as additional criteria in assessing the degree of the functional activity of the adrenergic neural control of the cardiac function.

The electron-microscopic investigation revealed a rich distribution of various types of axons within the wall of the auricular appendage. However, areas of close contact between a nerve terminal and a myocardial cell, suggesting "synaptic" transmission, were observed only occasionally. It is, however, possible that the transmitter substance released at the arrival of the electrical impulse can reach the target cell by diffusion across a "gap" of even several thousands of Å. On the other hand, it is also possible that the electrical excitation can be conducted from the "directly innervated" or "key" cells to adjacent myocardial cells by electrical coupling. This would suggest an extremely important general influence of the inbuilt intrinsic adrenergic nervous apparatus despite only a few areas of direct adrenergic innervation (close neuro-effector contacts) of the myocardium. In contrast, areas of close contact were frequently observed between nerve terminals of different types within nerve bundles embedded within a common covering of Schwann cell cytoplasm, as reported also e.g., by Nilsson and Sporrong (1970) and van der Zypen et al., (1974). Thus, there obviously exists a definite morphologic basis for an autonomous interneuronal link, which might function as an integrating regulative unit at the most peripheral level possible.

The nerve terminals containing a large number of mitochondria and located in the vicinity of, or immediately beneath, the basement membrane of sinusoidal blood vessels, would possibly be sensory terminals (cf., Burnstock and Iwayama, 1971; Chiba, 1972; Tranum-Jensen, 1975). Considering their location, their participation in mechanoreceptive or chemoreceptive functions is possible. This suggestion would be in accordance with that of Chiba and Yamauchi (1970), based on their studies on heart in children. Also those nerve terminals which contain myelinlike lamellated structures may be sensory, in analogy to the considerations of Chiba (1972) concerning the fine structure of the sensory baroreceptive nerve terminals in the carotid sinus of the dog, and of Tranum-Jensen (1975) concerning the ultrastructure of baroreceptors in the atrial endocardium of young pigs. The "pale" and "disintegrated" types of nerve terminals might possibly represent different stages of axonal degeneration. It is also possible that the relative occurrence of the "pale" type of terminals might correspond to diminution in the transmitter substance concentration under certain pathophysiologic conditions. Although no comparative studies could be performed because of the small number of electron-microscopic specimens, such a study might be of interest.

References

Angelakos, E. T., Fuxe, K., Torchiana, M. L.: Chemical and histochemical evaluation of the distribution of catecholamines in the rabbit and guinea-pig hearts. Acta physiol. scand. **59**, 184–192 (1963)

Baumgarten, H. G.: Über die Verteilung von Catecholaminen im Darm des Menschen. Z. Zellforsch. **83**, 133–146 (1967)

Burnstock, G., Iwayama, T.: Fine-structural identification of autonomic nerves and their relation to smooth muscle. Progr. Brain Res. **34**, 389–404 (1971)

Chiba, T.: Fine structure of the baroreceptor nerve terminals in the carotid sinus of the dog. J. Electron Microscopy **21**, 139–148 (1972)

Chiba, T., Yamauchi, A.: On the fine structure of the nerve terminals in the human myocardium. Z. Zellforsch. **108**, 324–338 (1970)

Chidsey, C. A., Braunwald, E., Morrow, A. G., Mason, D. T.: Myocardial norepinephrine concentration in man. Effects of reserpine and of congestive heart failure. New Engl. J. Med. **269**, 653–658 (1963)

Corrodi, H., Hillarp, N.-Å., Jonsson, G.: fluorescence methods for the histochemical demonstration of monoamines. 3. Sodium borohydride reduction of the fluorescent compounds as a specificity test. J. Histochem. Cytochem. **12**, 582–586 (1964)

Dail, W. G., Jr., Palmer, G. C.: Localization and correlation of catecholamine-containing cells with adenyl cyclase and phosphodiesterase in the human fetal heart. Anat. Rec. **177**, 265–288 (1973)

Ehinger, B., Falck, B., Persson, H., Sporrong, B.: Adrenergic and cholinesterase-containing neurons of the heart. Histochemie **16**, 197–205 (1968)

Eränkö, O.: Distribution of fluorescing islets, adrenaline and noradrenaline in the adrenal medulla of the hamster. Acta endocr. (Kbh.) **18**, 174–179 (1955)

Eränkö, O.: The practical histochemical demonstration of catecholamines by formaldehyde-induced fluorescence. J. roy micr. Soc. **87**, 259–276 (1967)

Falck, B.: Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. Acta physiol. scand. **56**, Suppl. 197 (1962)

Falck, B., Hillarp, N.-Å., Thieme, G., Torp, A.: Fluorescence of catecholamines and related compounds condensed with formaldehyde. J. Histochem. Cytochem. **10**, 348–354 (1962)

Hirsch, E. F., Borghard-Erdle, A. M.: The innervation of the human heart. I. The coronary arteries and the myocardium. Arch. Path. **71**, 384–407 (1961)

Hirsch, E. F., Borghard-Erdle, A. M.: The innervation of the human heart. II. The papillary muscles. Arch. Path. **73**, 100–117 (1962)

Jacobowitz, D.: Histochemical studies on the relationship of chromaffin cells and adrenergic nerve fibres to the cardiac ganglia of several species. J. Pharmacol. exp. Ther. **158**, 227–240 (1967)

Jonsson, G.: The formaldehyde fluorescence method for the histochemical demonstration of biogenic monoamines. M. D. Thesis, Stockholm 1967

Jonsson, G.: Microfluorimetric studies on the formaldehyde-induced fluorescence of noradrenaline in adrenergic nerves of the rat iris. J. Histochem. Cytochem. **17**, 714–723 (1969)

Lidbrink, P., Jonsson, G.: Semiquantitative estimation of formaldehyde-induced fluorescence of noradrenaline in central noradrenaline nerve terminals. J. Histochem. Cytochem. **19**, 747–757 (1971)

Lindvall, O., Björklund, A.: The glyoxylic acid fluorescence histochemical method: a detailed account of the methodology for the visualization of central catecholamine neurons. Histochemistry **39**, 97–127 (1974)

Lindvall, O., Björklund, A., Svensson, L.-Å.: Fluorophore formation from catecholamines and related compounds in the glyoxylic acid fluorescence histochemical method. Histochemistry **39**, 197–227 (1974)

McLean, J. R.: An improved technique for the demonstration of sympathetic nerves in human cardiac muscle. J. Histochem. Cytochem. **16**, 590–591 (1968)

Nilsson, E., Sporrong, B.: Electron microscopic investigation of adrenergic and nonadrenergic axons in the rabbit SA-node. Z. Zellforsch. **111**, 404–412 (1970)

Partanen, S., Korkala, O.: Catecholamines in human fetal heart. Experientia (Basel) **30**, 798–799 (1974)

Pearse, A. G. E.: In: Cardiomyopathies, Ciba Foundation Symposium (eds. G. E. W. Wolstenholme and M. O'Connor), pp. 132–171. London: J. & A. Churchill Ltd. 1964

Penttilä, O., Merikallio, E., Siltanen, P., Klinge, E.: Auricular catecholamine content in ischaemic heart disease. Abstract in VI International Congress of Pharmagology, July 1975, Helsinki

Ploem, J. S.: The microscopic differentiation of the colours of formaldehyde-induced fluorescence. Progr. Brain Res. **34**, 27–37 (1971)

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**, 208–212 (1963)

Ritzén, M.: Cytochemical identification and quantitation of biogenic monoamines. M. D. Thesis, Stockholm 1967

Sachs, C.: Uptake and accumulation in vitro of ^{3}H-noradrenaline in adrenergic nerves of human atrium. Histochemie **19**, 189–198 (1969)

Schiebler, T. H., Winckler, J.: On the vegetative cardiac innervation. Progr. Brain Res. **34**, 405–413 (1971)

Stöhr, Ph., Jr.: Mikroskopische Anatomie des vegetativen Nervensystems. In: Handbuch der mikroskopischen Anatomie des Menschen. Erg.-Bd. zu VII/4. und IX/1. Berlin-Göttingen-Heidelberg: Springer 1957

Tranum-Jensen, J.: Ultrastructure of baroreceptors (BR) in the atrial endocardium of young pigs. J. Ultrastruct. Res. **50**, 385 (1975)

Vogel, H. K., Jacobowitz, D., Chidsey, C. A.: Distribution of norepinephrine in the failing bovine heart. Correlation of chemical analysis and fluorescence microscopy. Circulat. Res. **24**, 71–84 (1969)

Waris, T., Partanen, S.: Demonstration of catecholamines in peripheral adrenergic nerves in stretch preparations with fluorescence induced by aqueous solution of glyoxylic acid. Histochemistry **41**, 369–372 (1975)

Watson, M. L.: Staining of tissue sections for electron microscopy with heavy metals. J. biophys. biochem. Cytol. **4**, 475–478 (1958)

Winckler, J.: Über die adrenergen Herznerven bei Ratte und Meerschweinchen. Entwicklung und Innervationsmuster. Z. Zellforsch. **98**, 106–121 (1969)

Yamauchi, A., Chiba, T.: Adrenergic and cholinergic innervation of the turtle heart ventricle. Z. Zellforsch. **143**, 485–493 (1973)

Zypen, E. van der: Über die Ausbreitung des vegetativen Nervensystemes in den Vorhöfen des Herzens. Acta anat. (Basel) **88**, 363–384 (1974)

Zypen, E. van der, Hasselhorst, G., Merz, R., Fillinger, H.: Histochemische und elektronenmikroskopische Untersuchungen an den intramuralen Ganglien des Herzens bei Mensch und Ratte. Acta anat. (Basel) **88**, 161–187 (1974)

Kimmo Kyösola, M. D.
Department of Anatomy
University of Helsinki
Siltavuorenpenger 20b
SF-00170 Helsinki 17
Finland