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Immunohistochemical change of actin in experimental myocardial ischemia. Its usefulness to detect very early myocardial damages

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Summary. Pathomorphological diagnosis of acute myocardial infarction has many problems in human autopsy materials less than 4 to 6 hours after clinical onset and in rats 2 to 3 hours after experimental coronary occlusion. Since immunohistochemical reaction depends on the antigen determinant site of the material, changes in the reaction may reflect alterations at the molecular level in myocardial fibers. With this consideration in mind, the effectiveness of diagnosing infarction at the earliest (possible) stage, and the changes of actin filaments were investigated through experiments, using immunohistochemical methods involving anti-actin antibodies produced from chicken gizzards in our laboratory. The left coronary arteries of rats were ligated to produce ischemia. Dehydrogenases were shown to be still present by triphenyltetrazolium chloride (TTC) reaction, but the anti-actin antibody reaction had disappeared in areas corresponding to the ischemic sites. However, on electron microscopic examination of these sites, actin fibers were clearly revealed. In the case of ischemia lasting for more than 6 hours, the anti-actin antibody reaction had disappeared, corresponding to the disappearance of the TTC reaction. At this stage, myocardial actin fibers were revealed by electron microscopic examination. These results indicate that ischemia induces some type of biochemical degeneration at the molecular level of myocardial actin, most likely the change of actin polymerization. Moreover, they show that the anti-actin antibody technique is capable of detecting such very early degenerative and ischemic changes proving itself to be better suited for determining the range and degree of early infarction.

Key words: Actin - Ischemia - Infarction - Myocardium - Immunohistochemistry

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Introduction

In the cases of a very early stage of clinical myocardial infarction, a pathological diagnosis of ischemic fibers based on morphological changes has many problems (Kent and Diseker, 1955; Shnitka and Nachlas, 1963; Fine et al., 1966; Jennings, 1969; Anderson et al., 1979), and a macroscopic diagnosis is dependent on the detection of dehydrogenase (Lie et al., 1975), which is impossible until 4 to 6 hours after onset. Another disadvantage of this enzyme-histochemical technique is that it is only applicable to un-fixed specimens.

Numerous studies (Mallory et al., 1939; Bryant et al., 1958; Kaufman et al., 1959; Jennings et al., 1965; Jennings and Ganote, 1974) have led to a light and electron microscopic compilation of the histological changes occurring in this condition. This compilation includes a number of findings that become manifest approximately 4 hours after the onset of myocardial necrosis; i.e. homogenization and acidophilic change, indistinctness and disappearance of cross striations in the sarcoplasm, nuclear pyknosis and karyorrhexis.

A number of methods for detecting ischemic changes and early infarction have been introduced; i.e. the detection of fuchsinophilia (Poley et al., 1964; Lie et al., 1971; Nayar and Olsen, 1974), lipid (Martín, 1963; Sakurai, 1977) and periodic acid shiff (PAS)-positive substances that are not digested by diastase (Kent and Diseker, 1955) and the disappearance of reactions for phosphorylase (Fine et al., 1966), lactate dehydrogenase (LDH) (Jennings et al., 1957), succinate dehydrogenase (SDH) (Wachstein, 1955; Jennings et al., 1957) and adenosin triphosphatase (ATPase) (Caulfield and Klionsky, 1959; Morales and Fine, 1966). Recently the immunohistological detection of LDH-1 (Herscher et al., 1984), myoglobin (Block et al., 1983; Kent, 1982), -1-acid glycoprotein (Siegel et al., 1985), myosin (Nolan et al., 1983) and mitochondrial aspartate aminotransferase (Siegel et al., 1984) have also been applied.

We have focused our attention on actin, one of the

contractile proteins of myocardium, and have produced polyclonal anti-actin antibodies using chicken gizzard as an antigen. In the present study, antigenic changes were regarded as changes in myocardial actin occurring in association with acute ischemic changes and infarction. It was investigated whether or not these changes were useful for diagnosis of very early infarction. Furthermore, using an immunohistochemical technique, we attempted to determine the stage of the infarction actin, which plays a leading role in contraction, when degeneration begins to occur as a result of ischemia.

Materials and methods

Preparation of Experimental Myocardial Infarction and Staining Methods.

A total of 106 male Wistar rats weighing 250-300 g were used. After anaesthetizing the rats with Nembutal and connecting them to respirators, their chests were opened. The left coronary ateries were ligated with No. 8-0 nylon thread according to the method of Olson and Johns (Johns and Olson, 1954; Selye et al., 1960) and the rat vascular distribution (Halpern, 1957). The vessels were exposed for gross confirmation. Coronary occlusions lasting for 5, 15 and 30 minutes, 1, 3, 6, 12 and 24 hours and 3, 10, 15 and 30 days were produced. Animals other than those with 5- and 15-minute occlusions were switched to spontaneous respiration after the ligation and closure of the chest. The left coronary artery could be seen during surgery as a thin, bright red streak beneath the pericardium under strong illumination (Johns and Olson, 1954). The status of occlusion by ligation of the coronary vessels was judged by confirming the change of the myocardium into pallid color.

Production of this ischemic model was successful in all except for 5 rats. As controls, we prepared 10 rats with sham operations by opening the chest and adapting the thread as described above. However, these produced no occlusion. At specified time intervals after occlusion, the heart was removed and divided into three parts, as shown in Figure 1. The central ischemic region, 2 mm in thickness, indicated by the points in the figure was used exclusively for electron microscopic specimens. The upper and lower parts were fixed in formalin after triphenyltetrazolium chloride (TTC) reaction (Sandritter and Jestadt, 1958; Jestadt and Sandritter, 1959), followed by routine preparation of paraffin sections. These serial paraffin sections were stained with hematoxylin and eosin stain (HE), and were reacted with anti-actin antibodies. In order to determine whether or not actin fibers existed in muscle fibers which were negative to anti-actin antibodies, electron microscopic examination was performed. A specimen which was taken from a slice exactly opposite to that which was negative for anti-actin antibodies was minced into 1-2 mm cubes, fixed in cacodylate buffer solution containing 2% glutaraldehyde and 2% paraformaldehyde, allowed to stand in 0.5% osmium at 4 C for one hour, and embedded in Epon 812.

Preparation of Actin Antiserum

Actin was isolated from fresh chicken gizzards according to the procedure described as above. Acetone-dried powder was prepared from minced chicken gizzard muscle according to the method by Suzuki (Suzuki et al., 1978).

Purified actin (MW, 42 K dalton) was extracted from the above powder as described by Spudich and Watt (1971).

The actin preparation was checqued by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophroresis according to Weber and Osborn (Weber and Osborn, 1969). The actin band was then cut from the polyacrylamide gel, and used as an antigen. Antisera against actins were produced in rabbits. Approximately 500 μg of the antigen conjugated with Freund's complete adjuvant was injected intracutaneously 3 times every week. After this, the antigen conjugated with Freund's incomplete adjuvant was used, and injected 3 times every 2 weeks.

Blood was collected 10 days after the last injection and the serum was clarified by centrifuging at 10,000 rpm.

The gamma-globulin fraction was partially purified twice by precipitation with 40% saturated ammonium sulphate. It was then dialyzed into 0.01M PBS (ph 7.4).

The actin antibodies were prepared by affinity chromatography on actin bound to CNBr-activated Sepharose 4B. These mono-specific antibodies were checked for reaction only with the actin band by immunoblotting test (Towbin et al., 1979).

These sera were diluted 1:100 in PBS before use.

Immunohistochemical Reaction with Anti-Actin Antibodies

The tissue had been routinely embedded in paraffin after fixation in 10% buffered formalin.

5 μm thick sections were cut and attached to slides, dried overnight at 37 C, deparaffinized through xylene and immediately immersed in methanol containing 0.03% H_2O_2 to inhibit endogenous peroxidase activity. These tissue sections were rehydrated through graded alcohols and equilibrated with phosphate-buffered saline (ph 7.4).

The avidin-biotin-peroxidase complex method (Hsu et al., 1981) was performed to visualize the binding of anti-actin antibodies on tissue sections attached to glass slides as follows.

Nonspecific staining was minimized by incubating the tissue sections for 30 min in PBS containing 10% normal goat serum at room temperature. After rising in PBS, the sections were reacted overnight at 4 C with anti-actin antibody. After washing, each specimens was layered with biotinized goat anti-rabbit IgG antibody (diluted 1:200 in PBS containing 10% NGS) for 30 min, washed in PBS and then treated with avidin-peroxidase.

Peroxidase activity was revealed using DAB-H_2O_2 method.

As a control reaction, normal rabbit serum (NRS) was applied to all the tissue sections.

Fig. 1. Heart of a rat in which the arteries have been made visible by injection of colored material. The heart was divided into three parts according to the lines shown in the figure. The central ischemic region represented by the points was exclusively used for electron microscopic specimens. The upper and lower parts were fixed in formalin after the triphnyltetrazolium chloride (TTC) reaction, following routinely prepared paraffin sections.
LM: light microscopic examination
EM: electron microscopic examination

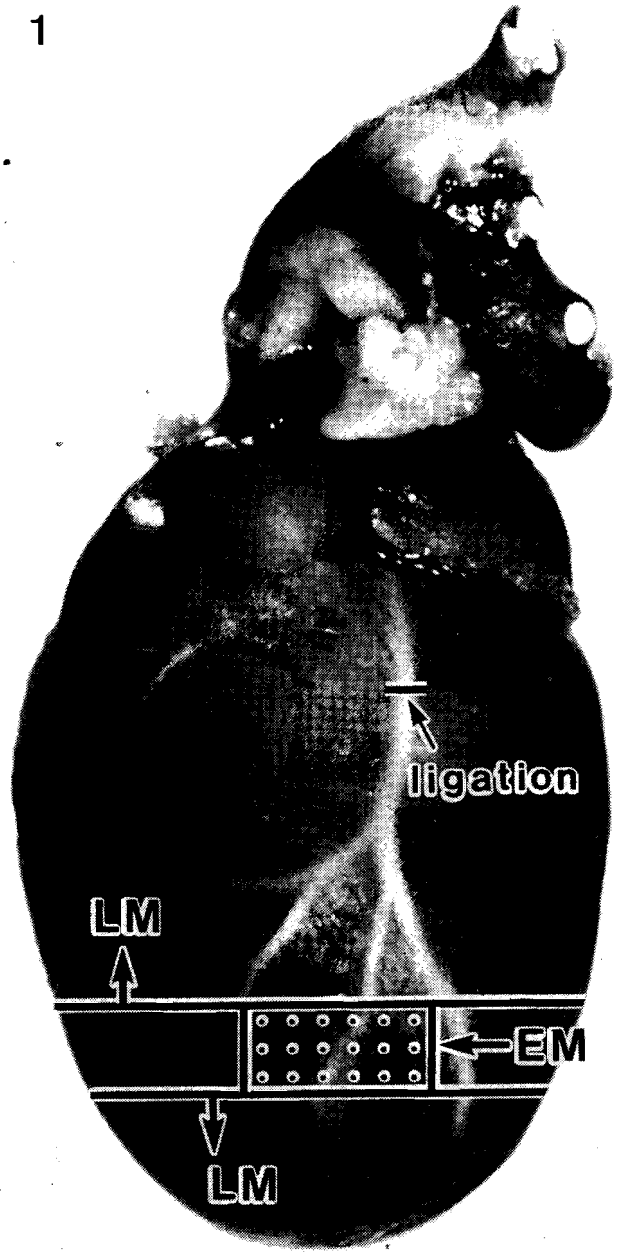


Fig. 2A. Transverse slice of a rat heart subjected to 5 minutes of left coronary artery occlusion. Heart slice was incubated in TTC for 5 minutes. Noninfarcted tissue appears darkly stained. No unstained area is shown. Bar=5 mm

Fig. 2B. Photomicrograph of anti-actin antibody reacted tissue in the ischemic site from TTC-stained myocardium after 5 minutes of coronary artery occlusion. Using the anti-actin antibody and ABC method, partial disappearance of immunohistochemical staining is evident. Bar=100 μm

Fig. 2C. Photomicrograph of hematoxylin and eosin staining in the serial section of Figure 2b, most of the fibers presenting clear cross-striations and no eosinophilia. Bar=100 μm

Fig. 3A. Left: Gross findings of ischemic heart wich TTC reaction following 30-minutes occlusion with TTC reaction. Loss of activity is not evident.
Right: Gross findings of paraffin section from the same tissue as that shown in left. Section was reacted with anti-actin antibodies. Reaction has disappeared in the wide area corresponding to the ischemic site. Bar=5 mm

Fig. 3B. Light microscopic findings of Figure 3A. Anti-actin antibody reaction has almost disappeared in the ischemic area. Bar=50 μm

Fig. 3C. Light microscopic findings with hematoxylin and eosin staining in the serial section of Figure 3C. Muscle fibers show no remarkable nuclear changes and slightly eosinophilic cytoplasm in the ischemic area. Bar=50 μm

Fig. 4. Electronmicrograph of tissue from a rat heart after 30 minutes of coronary artery occlusion. Mitochondria (Mt) appear swollen. There is a wide I band, and actin fibers (arrow) are clearly shown. (Uranyl acetate and lead citrate stain) Bar=1 μm

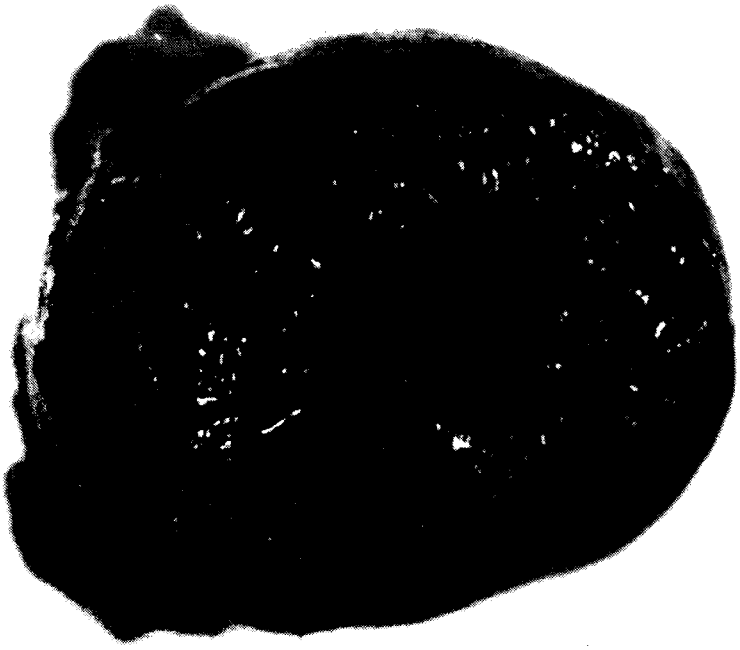
Fig. 5A. Left: Transverse slice of a rat heart subjected to 6 hours of coronary artery occlusion. Heart slice was incubated in TTC. Infarction appears as a pale area. Noninfarcted tissue appears darkly stained.
Right: Macroscopic findings of a paraffin section of the same tissue shown in figure 5A. Section was reacted with anti-actin antibodies. Reaction has disappeared in a wide area corresponding to the site of disappearance of the TTC reaction. Bar=5 μm

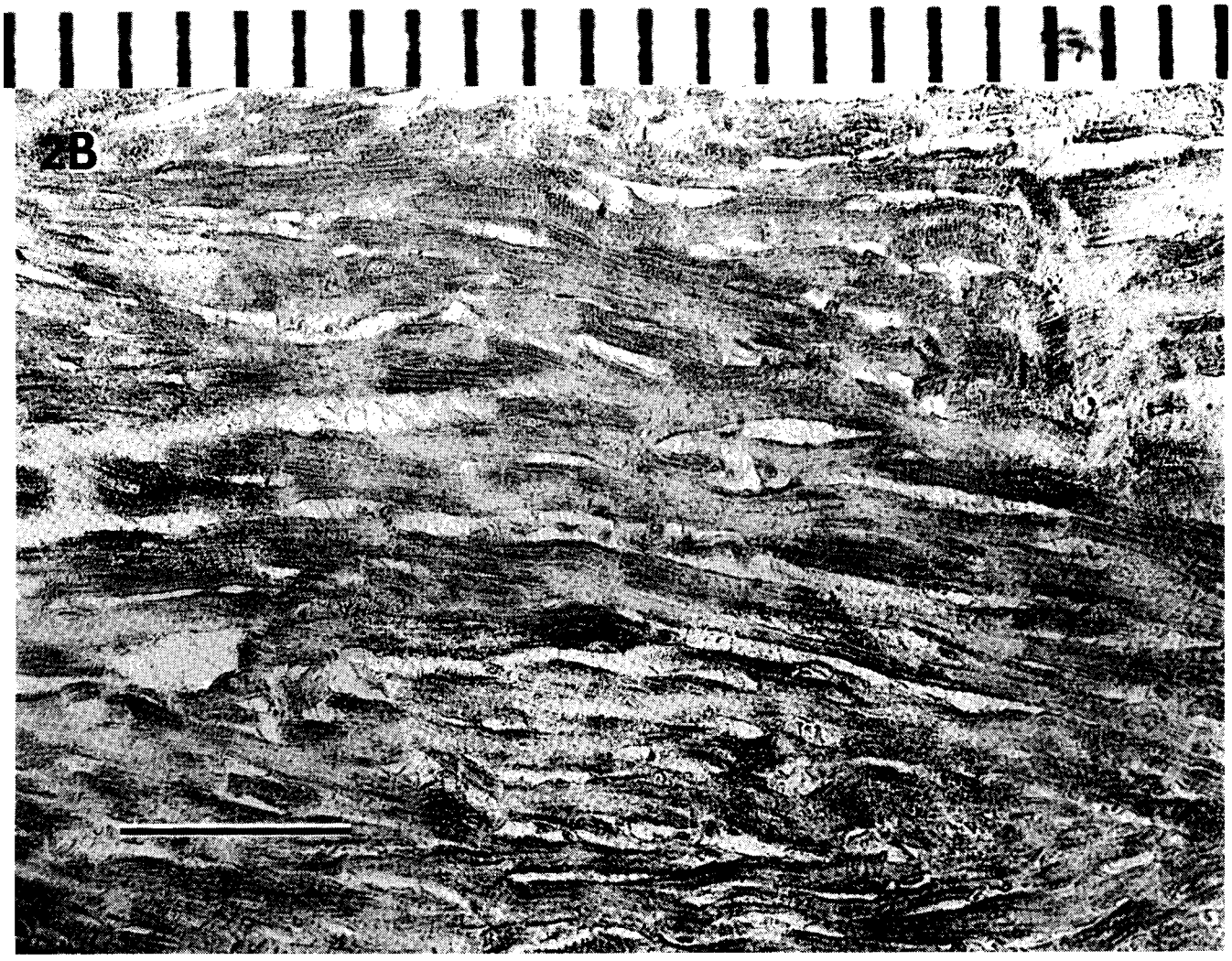
Fig. 5B. Photomicrograph of Figure 5A. Anti-actin antibody reaction has almost disappeared in the ischemic area. Bar=200 μm

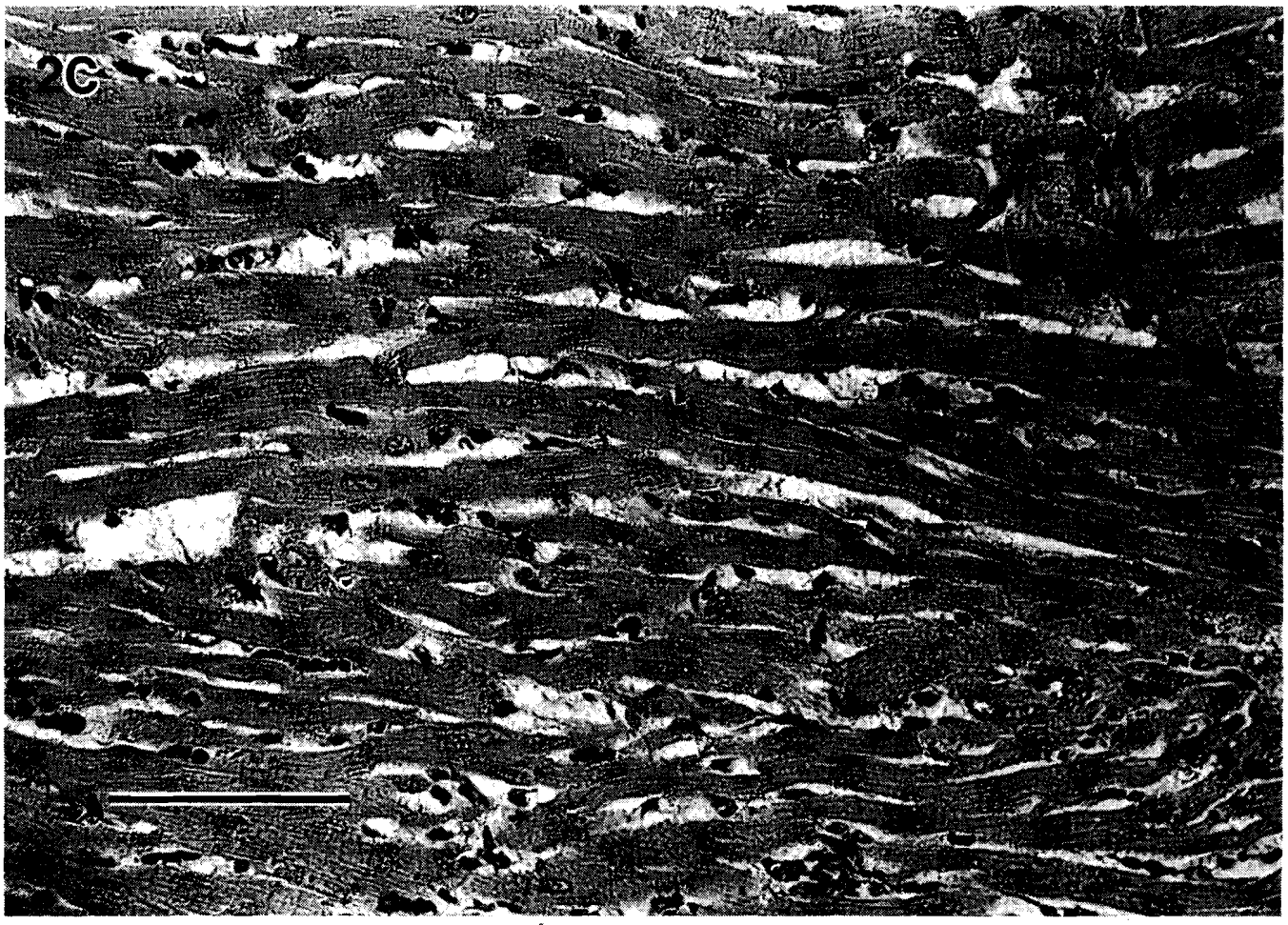
Fig. 5C. Photomicrograph of hematoxylin and eosin staining in the serial section of Figure 5B. There are many fibers with significant eosinophilic degeneration (darkly stain) and sporadic nuclear pyknosis. Bar=200 μm

Fig. 6. Electron micrograph of tissue from a rat after 6 hours of coronary artery occlusion. Mitochondria (Mt) are swollen, disrupted, and contain amorphous densities (triangles). Wide I bands and diminution of actin fibers (arrows) are evident. Bar=1 μm

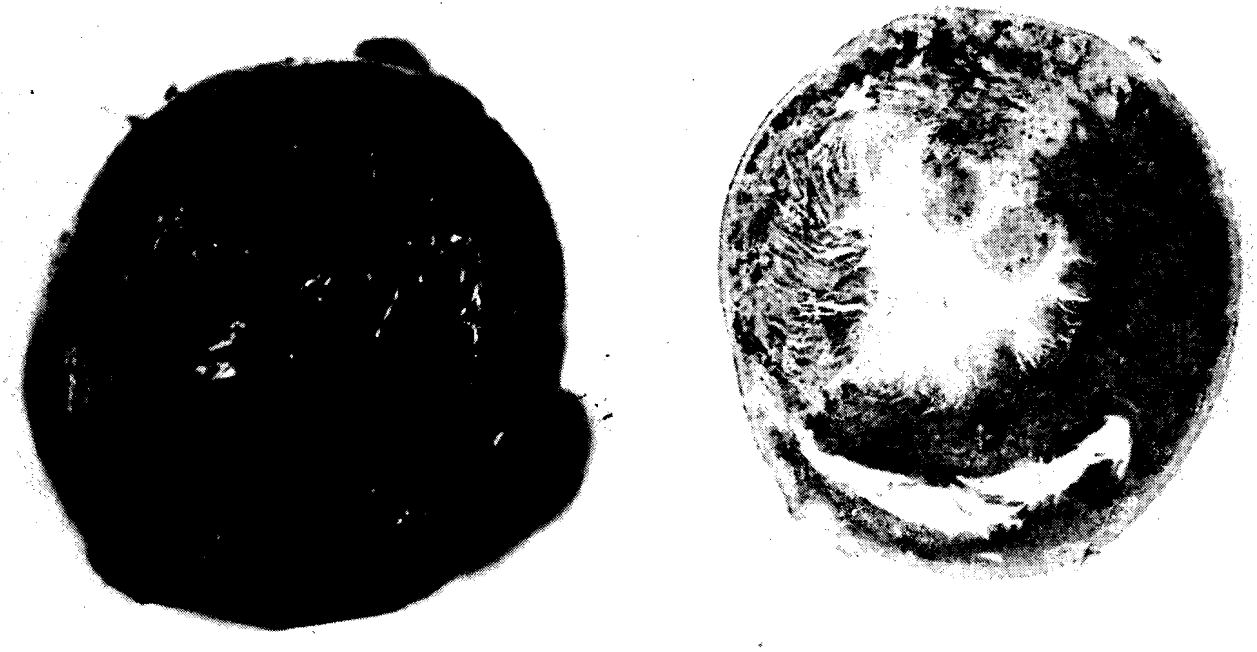
2A

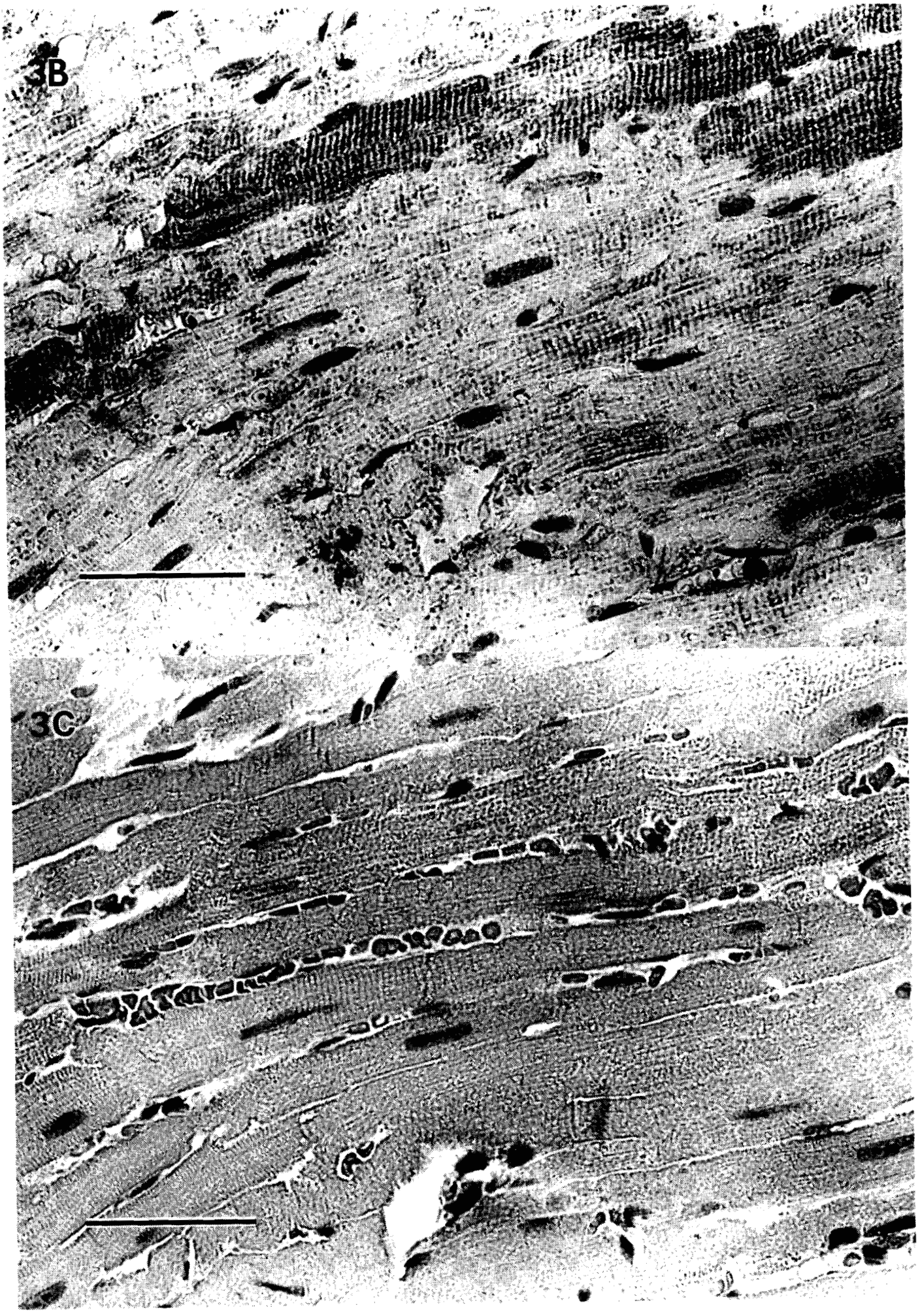


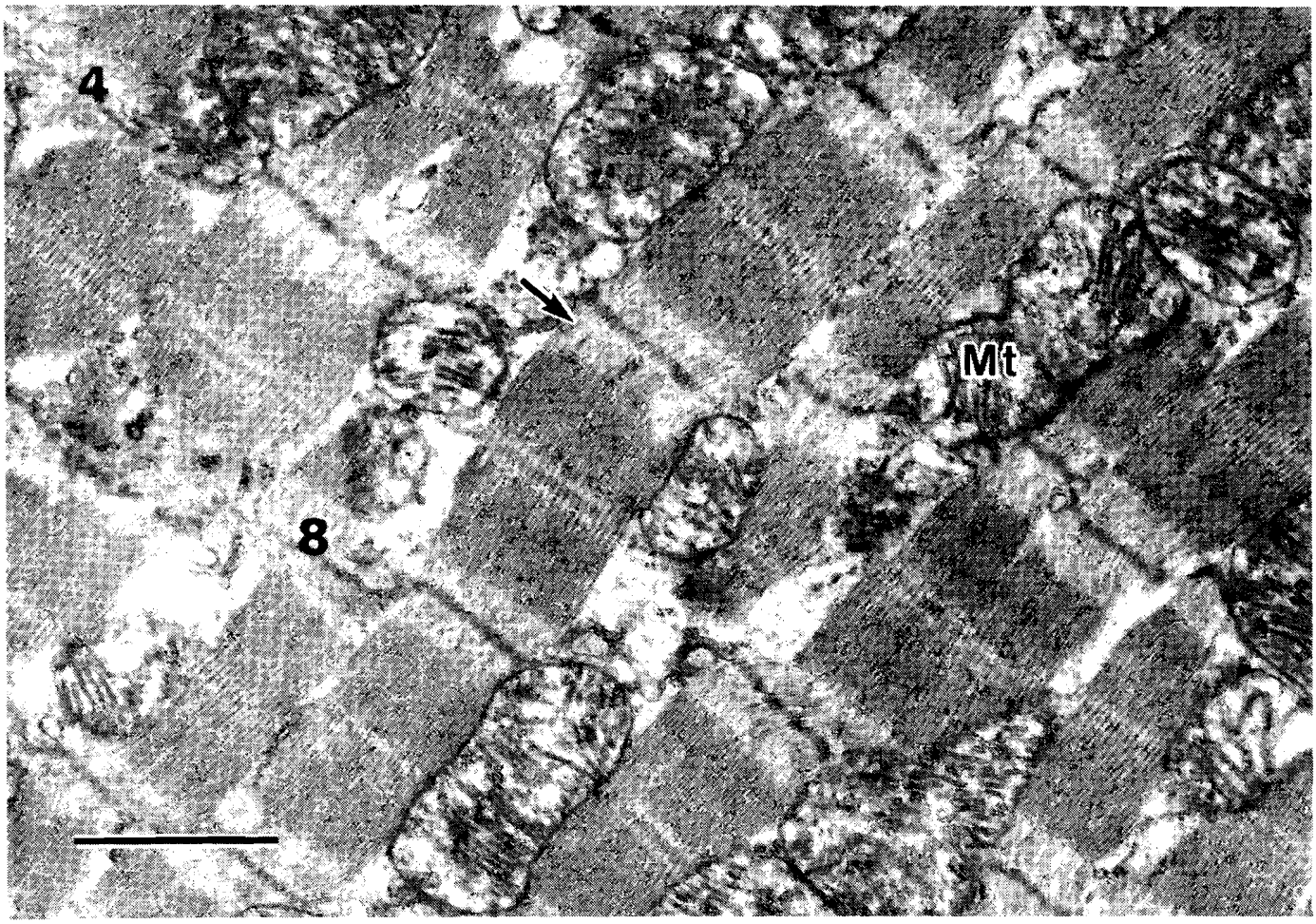




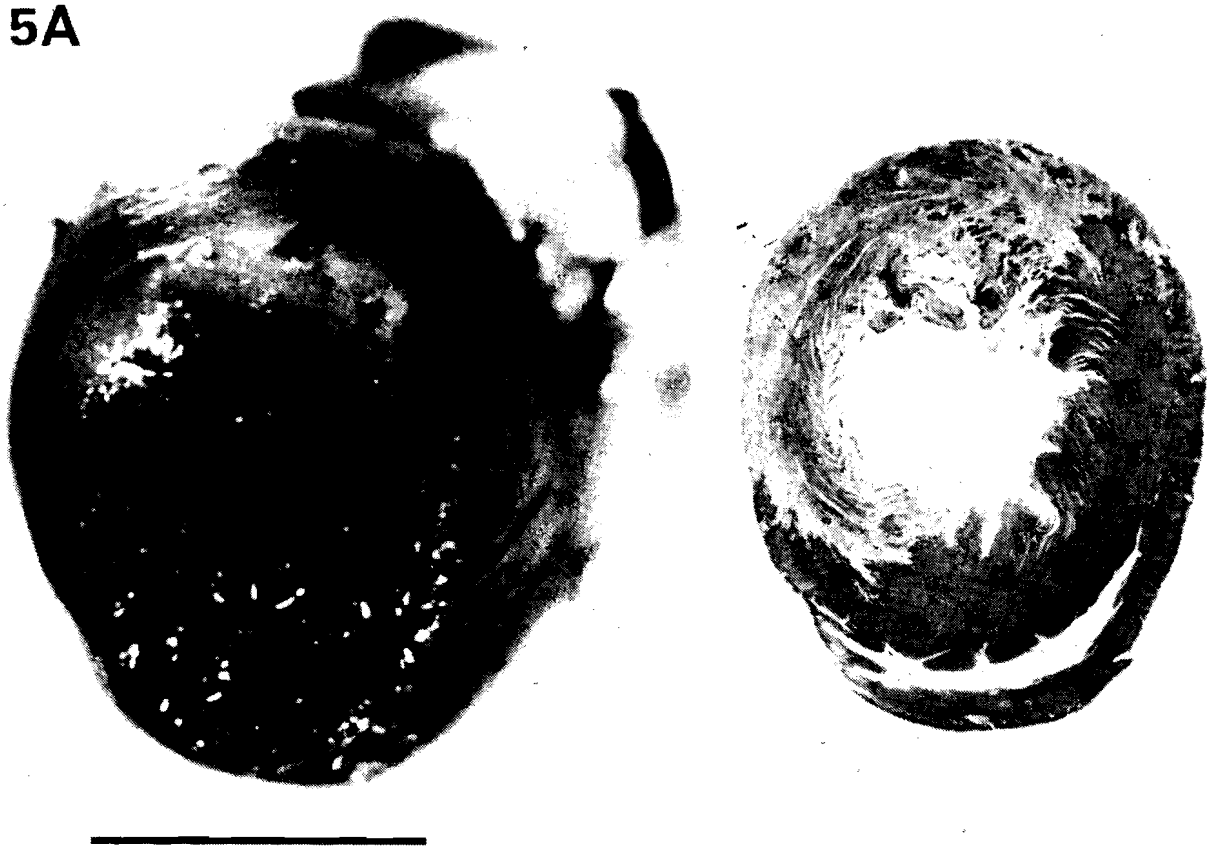
3A

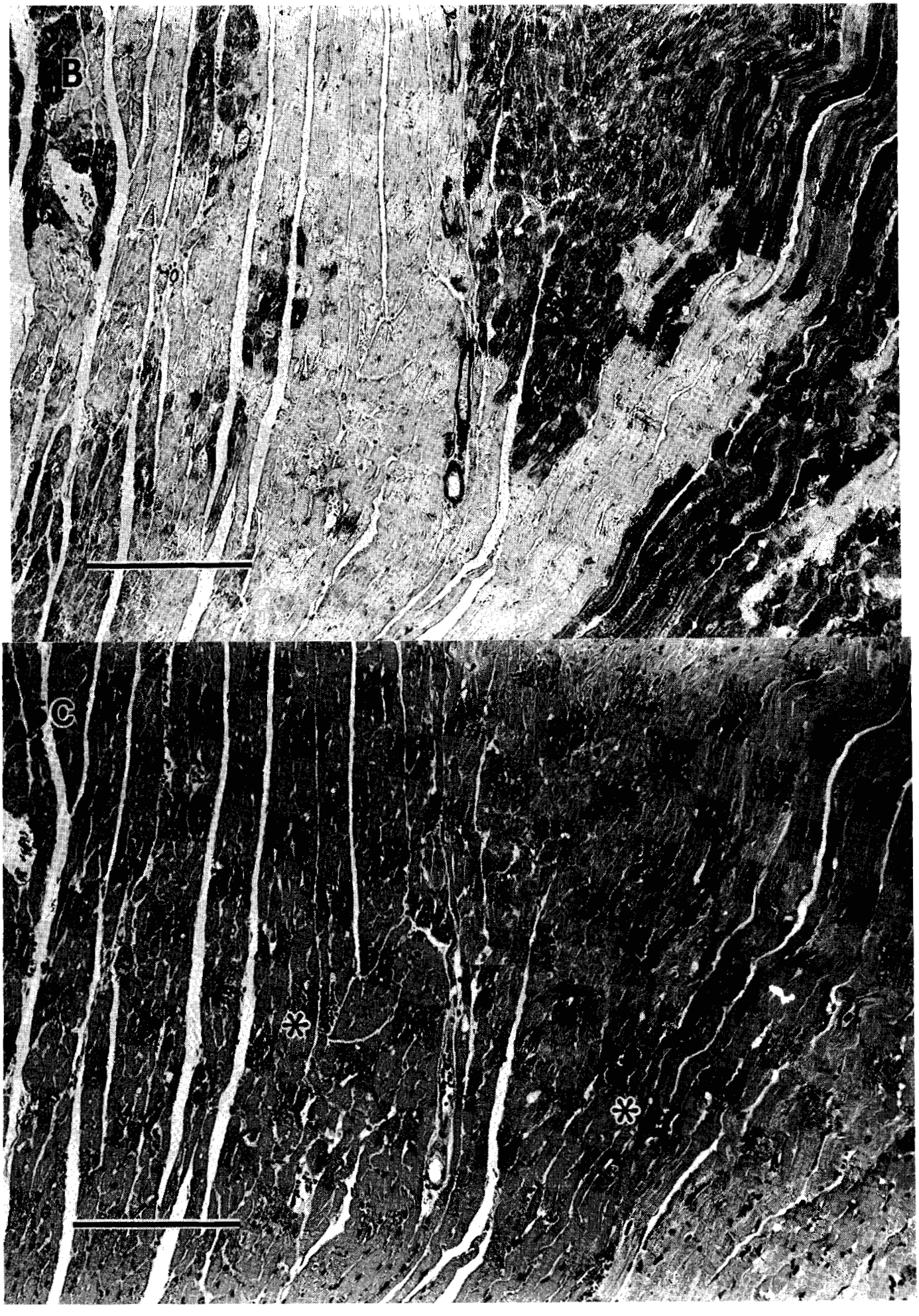



3B
3C



5A

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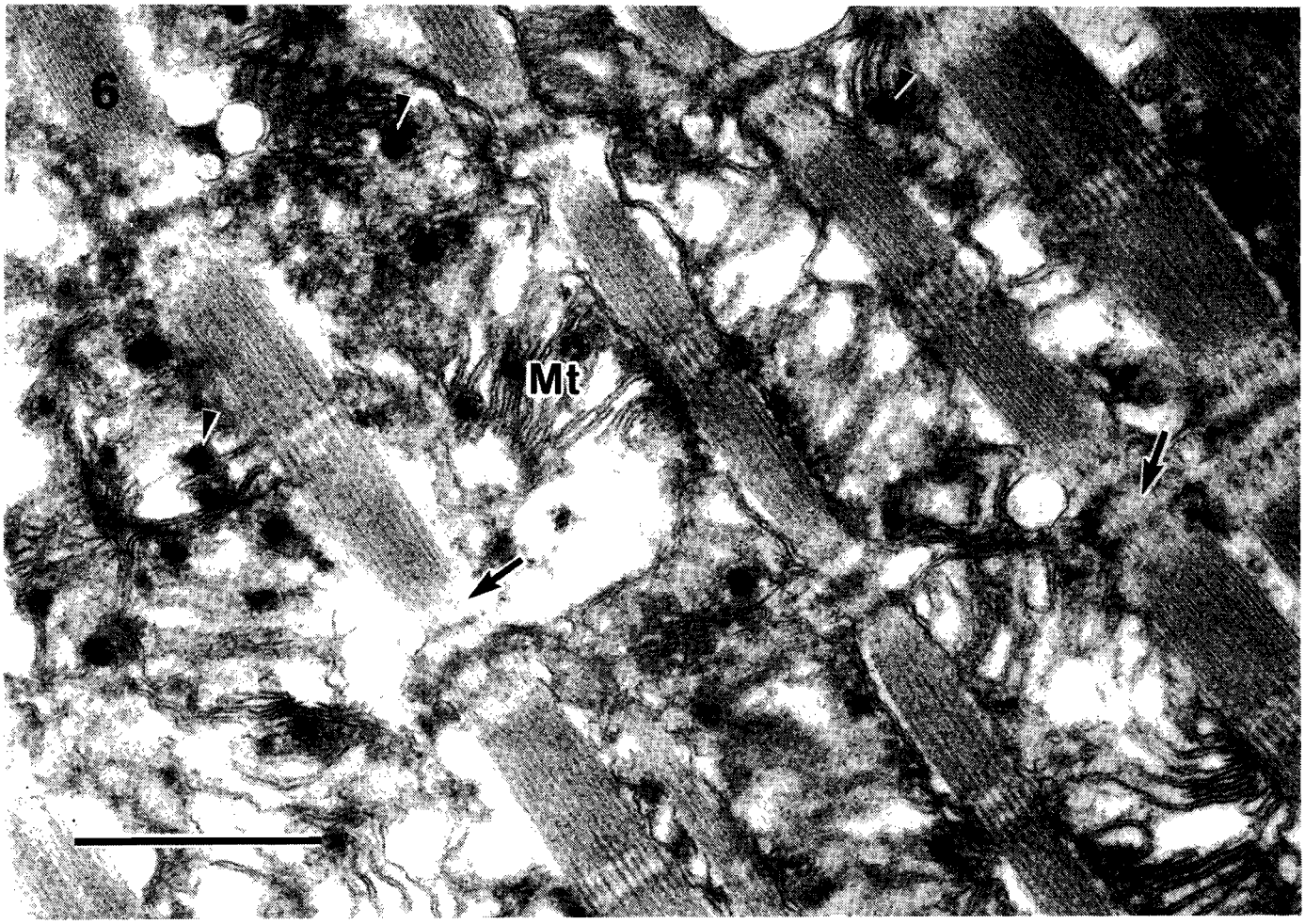

Results

A section of myocardial tissue with 5-minute ischemia that has undergone TTC reaction is shown in Figure 2A. At this stage, the dehydrogenases were still present. However, the microscopic findings of the tissue from the same area as that stained with the TTC reaction, which was reacted with the anti-actin antibody, showed a sporadic presence of fibers wich were negative. In this area, anti-actin antibody reaction was absent in more than half of the cell body (Fig. 2B), even in fibers with clear cross-striations, and there was an absence of eosinophilia by HE staining in most of the fibers at the ischemic site (Fig. 2C).

Even in 30-minute ischemia groups, dehydrogenase was also detected by the TTC reaction in the myocardium (Fig. 3A). However, the anti-actin antibody reaction had extensively disappeared in a wide area corresponding to the ischemic site (Fig. 3A).

Microscopic findings showed complete disappearance of the anti-actin antibody reaction in most of the fibers in the ischemic area (Fig. 3B). HE staining revealed no remarkable nuclear changes and slightly eosinophilic cytoplasm in fibers within the ischemic area (Fig. 3C).

By electron microscopic examination (Fig. 4) of the tissue from 30-minute ischemia, actin fibers are clearly revealed despite the disappearance of the anti-actin antibody reaction. Ischemic myocardial changes such as expansion of the I-band and swelling of the mitochondria were observed, but no animal showed any amorphous structures in the mitochondria at this stage.

As described above, absence of the anti-actin antibody reaction without the disappearance of the TTC reaction was found in all the 35 animals in the acute stage; i.e., 7 animals each with ischemia lasting for 5, 15 and 30 minutes and for 1 and 3 hours.

The TTC findings of the myocardium 6 hours after coronary occlusion indicated the absence of any activity in the ischemic site (Fig. 5A). The macroscopic findings of the same tissue undergoing the anti-actin antibody reaction showed almost complete disappearance of the reaction in the area corresponding to the ischemic region (Fig. 5A). Microscopically, there were many fibers with significant eosinophilic degeneration by HE staining (Fig. 5C), which were diffusely negative for the actin antibody reaction (Fig. 5B). In addition, fibers with

pyknosis were sporadically present. Actin fibers in almost all of the muscle fibers were observed by electron microscopic examination of the myocardial fibers in the area corresponding to the site of disappearance of the TTC reaction and anti-actin antibody reaction (Fig. 6).

The myocardial cells of all rats with sham operations were positive for the anti-actin antibody reaction, but were completely negative in the immunohistochemical test using NRS as a control.

Discussion

Actin was originally extracted by Bruno and Straub, and numerous subsequent studies show that it is widely distributed in cells. Previous studies (Elizinga et al., 1973; Lu and Elginga, 1977; Vandekerckhove and Weber, 1978a; Vandekerckhove and Weber, 1979b) show, on the basis of its primary structure, that there are at least six types of actin in mammals. These actins are present in the skeletal muscle, myocardium, smooth muscle (two types) and non-muscular cells (two types) (Whaleu et al., 1976; Vandekerckhove and Weber, 1978b). The primary structure of actin in rabbit skeletal muscle is completely identical to that of actins in bovine and chicken skeletal muscle (Vandekerckhove and Weber, 1978a; Vandekerckhove and Weber, 1979). These findings indicate that the structure of actin is specific for a particular organ, rather than a species. In the present study, we used actin derived from chicken gizzard, i.e., smooth muscle, as the antigen for the production of anti-actin antibodies. However, in the primary structure of smooth-muscle actin and myocardial actin, only 4 out of the 375 amino acid residues are different, supporting the fact that the antibodies showed a more favorable reaction to myocardial fibers in the present study. We also evaluated the nature of changes exhibited by actin in an ischemic myocardium.

The numerous data available on the myocardial biochemical changes occurring in response to ischemia indicate that such changes appear several seconds after the onset of ischemia. Specifically, a change from aerobic to anaerobic metabolism occurs within cells, resulting in various metabolic disorders, such as inhibition of glycogen metabolism and ATP production (Cho et al., 1963; Kohn et al., 1977) and liberation of intracellular potassium (Jennings et al., 1957, 1964; Harris et al., 1958) and enzymes (Schneider and Urbaszek, 1974) liberated from the myocardium. Although attempts have been made to detect these processes histologically, it is difficult to detect subtle changes.

Our present data show that very early in the acute stage, i.e., 5 and 15 minutes after coronary occlusion, some fibers already show a negative reaction to anti-actin antibodies, and that these fibers increase in number in parallel with the duration of occlusion, with extensive disappearance of the reaction 30 minutes after occlusion. However, in animals with early infarction, i.e., 5, 15 and 30 minutes and 1 and 3 hours after occlusion, in which enzyme-histochemical detection of ischemia was impossible, despite the fact that the anti-actin antibody reaction disappears at the ischemic site, electron microscopic examination reveals the existence of actin fibers. What is more interesting is that we found that the anti-actin antibody reaction was decreased or absent in clearly striated fibers showing no eosinophilia by HE staining.

These facts suggest that ischemia induces some biochemical rather than morphological degeneration, at the molecular level of the actin. This hypothesis is supported by the following two facts.

Firstly, changed actin was extracted from the myocardium in acute and chronic heart failure, and this actin polymerizability was sharply reduced (Karsanov et al., 1986).

Secondly, doxorubicin, an anthracycline antineoplastic drug which has a side effect of irreversible cardiotoxicity, induced polymerization of cardiac actin in vitro, but this polymerization had ultrastructural and biochemical characteristics which were different from actin polymerization induced by salt which is the usual method (Mariono et al., 1986).

The above published facts allow a hypothesis that changes of actin polymerization take place prior to the proteolytic change of actin filaments in the early stage of myocardial ischemia.

The present data indicate that the technique using anti-actin antibodies described here is capable of detecting very early degenerative ischemic changes and is a better method for determining the range and degree of early infarction. In addition, it is a great advantage that routine paraffin sections can be utilized.

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