CHANGES OCCURRING WITH AGE IN THE CANINE MYOCARDIUM

by

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INTRODUCTION

The study of changes occurring with age in the myocardium is a study in normal structure and function. Because of the dynamics of growth, differentiation, and decline the term "normal" is nearly meaningless when applied to an organism or any part of an organism unless related to the age or stage of development of that organism.

The animal life span may be divided into two periods: the period of development and the period of decline or senesence. Lansing (1964) contends that the second period begins immediately on completion of the first. In the opinion of some, the period of decline begins even before the completion of the period of development (Sorokin, 1964).

Changes occurring in the animal body during these two periods can be studied at a number of different levels: the molecular level, the cellular level, the tissue level, the organ level, or at a level involving the entire organism. This study is limited to those changes occurring at the cellular and at the tissue levels in the canine heart. The myocardial cell is an excellent subject for the study of cellular aging. It seldom divides after birth, and may, in most cases, be considered to be as old as the individual of which it is a part. The myocardium as a tissue reflects changes occurring, not only in the myocardial cell, but also changes due to the effect of various connective tissue cells.

Extrinsic effects, such as altered blood supply or innervation may also play some part in producing some of the histomorphological changes observed.

Comparative medical research is a major means of advancing the well-being of the human population. The dog is widely used in such comparative studies. It is important that the stage of development of the dog be compared accurately to related stages in human development, and that any normal differences in development and aging in the two species be known. This study is intended to provide additional data which will serve as a further basis for making such comparisons. It is also possible that basic insights into the character and functional mechanisms of the myocardial cell may be applicable across generic lines.

My thesis, then, is that certain morphological changes do take place in the myocardium of the dog during its lifetime which are primarily a consequence of the length of time the individual animal has lived. The major portion of this report will be concerned with the presentation of evidence, and arguments based on this evidence, offered in proof of this thesis. Beyond this, however, statements will be made regarding the effect or functional significance of certain of these changes. These statements will be based almost entirely on morphological evidence and are not a part of the thesis, but are intended to establish new hypotheses, providing a basis for further investigation.

LITERATURE REVIEW

The literature concerning the age-related changes in the myocardium will be reviewed following an outline identical to that which will subsequently be used in reporting the results of this study. This outline is as follows:

Changes in the myocardium as a tissue

- Changes involving collagen fibers
- Changes involving reticular fibers
- Changes involving elastic fibers
- Fatty changes

Changes in the myocardial cell

- Changes in fiber size
- Nuclear changes
- Lipofuscin development and accumulation
- Change in myofibrillar banding

Changes in the myocardium as a tissue

Changes involving collagen fibers Lev (1957) states that in the human there is an increase in collagenous tissue with age, the atria being more markedly affected than the ventricles. He terms the change in the ventricles as "only a slight increase". Also in the human, an increase in collagenous tissue around the fibers of the atrio-ventricular conduction system has been reported (Erickson and Lev, 1952).

Changes involving reticular fibers Reticular fibers are now considered to differ from collagenous fibers chiefly

in their smaller diameter and by their greater tendency to form a network (Copenhaver, 1964). The endomysium around the individual muscle fibers is made up largely of reticular fibers. Lev (1957) found that with advancing age the reticular network in the ventricles becomes very prominent. In the AV conduction system, Erickson and Lev (1952) reported that the reticulum increases in density with advancing age.

Changes involving elastic fibers According to Lev (1957) elastic fibers, by maturity, are scarce in the ventricles while becoming abundant in the atria of man. Miller and Perkins (1927) reported that the heart of the aged human showed an increase of elastic tissue. This increase is, however, very minimal in the ventricles and confined mainly to the endocardium. Karsner (1940) cites Aschoff (1938) as quoting Rondolini as having found an increase of elastica in the nodes of the conducting system. Erickson and Lev's study (1952) of the conducting system also revealed an increase of elastic tissue in that system.

Fatty changes According to Wartman and Hill (1960) two varieties of fatty change occur in the heart: 1. fatty infiltration, manifested by an increase in sub-epicardial fat and replacement and infiltration of the myocardium with fat; 2. fatty degeneration, which affects the sarcoplasm and occasionally the nuclei of the myocardial fibers. Saphir and Corrigan (1933) state that fatty infiltration "affects cells which normally contain fat and represents an alteration simply

of the normal fat depots and transport". Fatty degeneration is, as the term implies, a degenerative change in the myocardial cell caused by the action of an injurious agent.

The right ventricle is a favored site for fatty infiltration to occur. It begins in the sub-epicardium and infiltrates the sub-adjacent myocardium. An excess of fat may cause pressure atrophy of the myocardial fibers. This excess of fat is often noted first in the perivascular areas.

Fatty degeneration is the result of an injury which most often is due to anoxia or to the action of a toxin. The toxin may be bacterial or chemical. When myocardial cells are injured fatty acids are not completely utilized and fat accumulates in the cell.

Cohn (1939) listed, as one of the changes occurring with age in the heart, an increased deposition of sub-epicardial fat.

Changes in the myocardial cell

Changes in fiber size According to Barry and Patten (1960) the fibers of the typical ventricular myocardium of the human average 12 microns in diameter. Copenhaver (1964) gives 14 microns as the normal fiber diameter in the adult human, while stating that in the newborn the diameter averages 6 to 8 microns. Likewise, Roberts and Wearn (1941) found 7 microns to be the average diameter in the human at birth, and 14 microns to be the average size in the adult.

They believed the fiber diameter to be in direct proportion to the heart weight. Angelakas et al. (1964) found the mean diameter in the right ventricle of rats to be 11.5 ± 4.3 microns. In the left ventricle this measurement was 11.8 ± 4.8 microns. In the newborn rabbit 7 microns was found to be the average fiber diameter. These fibers were observed to increase in size by a process of continuous growth, until adult life when they reached a size of 21 microns and remained constant (Shipley et al., 1937). No difference in numbers of fibers per square millimeter between young (4 months) and old (26-27 months) rats was noted by Rakusan and Poupa (1964). They did report a lack of uniformity in the diameters of the older specimens. Harrison et al. (1932) proposed that the diameter of the fiber is directly related to the size of the animal and inversely to the pulse rate.

Different methods have been used to measure length of the myofibers. Linzbach (1956) measured nuclei and internuclear distances along the fibers. The distance between intercalated discs was found to vary from 50 to 120 microns by Walls (1960). He also states that these structures increase in number with age.

Nuclear changes Andrew (1955) has reported on observations of direct nuclear division, most often occurring without accompanying cytokinesis, in aging cells. This

finding has been observed in the Purkinje cells of the cerebellum and in the hepatic parenchymal cell. Andrew describes the process of amitosis as first consisting of a nucleolar division followed by a pinching of the nucleus into an hour-glass shape with subsequent division. Cytoplasmic division may, or may not follow. Each daughter nucleus would receive one of the nucleoli. Clara (1930) describes amitosis leading to binucleate cells in the liver. In cells in which this occurs, Andrew believes that amitosis is an attempt by the cell to reestablish the normal nucleo-cytoplasmic ratio which has been changed due to the growth of the cell. He states, "...amitotic division of nuclei seems to be less often concerned with multiplication of cells than with the reproduction of nuclei within an individual cell to increase the nuclear surface and thus aid in metabolic activity when 'excessive' growth has occurred or when unfavorable conditions threaten the life of the cell". Nuclear invagination or lobulation, which was observed preceding actual division would also result in an increased nuclear surface area presented to the cytoplasm.

In cardiac muscle, abnormally shaped nuclei, binucleate and multinucleate cells have been reported in cardiac hypertrophy in man (Linzbach, 1947; Nieth, 1949; Henschel, 1952). Cellular diameters have been shown to be increased in these conditions. These changes are believed to be indicative of

attempts at amitosis.

In studying aging of the nuclei of cerebellar cells of different animals, Cammermeyer (1963a, 1963b), while critical of many earlier studies on the basis of poorly controlled techniques, is in agreement with most of these in concluding that there is a decrease in the chromatin content of the nuclei with age. In his paper (1963b) he reviews the earlier literature on this subject.

Lipofuscin development and accumulation Two previous studies from this laboratory have been directed toward the relation of lipofuscin to aging (Whiteford, 1964; Whiteford and Getty, 1966; Few, 1966). Various neurons of the dog and swine were the subject of these investigations. The dogs included in these studies made up a sample very similar to that which is the subject of this present study.

Lipofuscin granules have been defined as intracellular entities characterized by pigmentation, autofluoresence, partial insolubility in lipid solvents, positive PAS and Schmorl reactions, acid fastness, and stainability with Sudan black (Bjorkerud, 1964). Bjorkerud gives credit to Hannover (1842) for having first described such granules in dissected nerve cell preparations. Both Bjorkerud and Strehler et al. (1959) credit Borst (1922) with coining the term lipofuscin as applied to these granules. Other specific terms which have been applied to these granules are: fetthaltiges

Abnutzungspigment (Lubarsch, 1902; Sehrt, 1904), hemofuscin (Conner, 1928; Mallory et al., 1921; Sheldon, 1935, cited by Strehler et al., 1949), hemofuchsin (Jayne, 1950). In a recent edition of his histology text Ham (1965) classifies these pigments under the heading "lipochromes" and specifically refers to them as lipofuchsin.

The autofluorescent properties of these granules was first noted in animal hearts in a study by Stubel (1911). A number of investigators have reported on their increasing concentration with age in cardiac muscle (Bohmig, 1935; Jayne, 1950; Strehler et al., 1959; Tcheng et al., 1961 cited by Bjorkerud, 1964). Hamperl (1934) pointed out that they were not present in the myocardium of children under 10 years of age, however, Muller (1935) reports on observing them in cardiac tissue from the first months of life.

Strehler et al. (1959) measured the amount of age pigment in microscopic sections from hearts of 156 humans. No pigment was demonstrated in hearts from individuals under ten years of age. All of the older hearts contained some pigment. These workers showed that the concentration of the pigment increased linearly at 1/3% heart volume per decade. This finding was noted to be independent of sex, race, or cardiac pathology. In their quantification they did not include any data from hearts of individuals in the 10-20 year age group.

Their measurements were taken from blocks of tissue from the apical region of the left ventricle, after studies had shown that there were no significant differences in pigment concentration in blocks taken from different areas of the same heart.

In discussing their findings they defined primary age changes as being time-dependent changes in structure and function contributing to the progressive increase in the probability of death. Their criteria for identifying such a change were: universality, time dependence, intrinsicality, deleteriousness. They concluded that lipofuscin accumulation satisfies these criteria and does indeed reflect a basic biological aging process.

Ulissova (1957) dissents from the view that lipofuscin accumulation is an age change. His study, based on planimetric measurements of scale drawings made from human myocardial sections, indicates that the pigment is present in the most intense concentration up to 40 years of age when it begins to decline. He found no pigment in hearts from children under 12 and did observe it in all hearts studied from individuals who were older than 12. His conclusion was that the accumulation of the pigment represented a physiological phenomenon rather than an age change.

Strehler (1964) summarizes in table form earlier reviews and studies by Gedigk and Fischer (1959), Pearse (1960),

and Tewari and Bourne (1962), and work from his own laboratory concerning the staining properties of these pigments. He states, in summary, that these properties include specific staining reactions for proteins, several types of lipids and acidic groups, and a positive PAS rection. In regard to histoenzymology of lipofuscin, he cites the following enzymes as having been localized in the pigment by one or more studies: acid phosphatase, esterase, B-glucuronidase, alkaline phosphatase, ATPase, and succinic dehydrogenase (Gedigk and Bontke, 1956; Essner and Novikoff, 1960; Gedigk and Fischer, 1959; Tewari and Bourne 1962). There are areas of disagreement among these workers. Strehler has not been able to demonstrate the presence of B-glucuronidase or succinic dehydrogenase in lipofuscin.

Different methods for isolating the pigment for chemical analysis have been applied to cardiac muscle. Brahn and Schmidtmann (1920) used peptic digestion as did Jayne (1950) who combined it with trypsin. Mechanical or sonic disintegration with subsequent centrifugation for separation of the pigment has been the basis for most recent studies (Heidenreich and Siebert, 1955; Hendley et al., 1963; Bjorkerud, 1963, 1964; Lang and Siebert, 1955 cited in Bjorkerud, 1964; Siebert et al., 1962). The main constituents of lipofuscin, isolated by these methods, were shown to be lipids, proteins, and a residue which was insoluble after acid hydrolysis. The

relative amounts of these constituents varied in the different studies. Enzymes have also been assayed from these isolated lipofuscin fractions. Some discrepancies have been found between histochemical findings and findings reached by methods of cell fractionation. These differences have been explained as perhaps being due to fixation effects on the tissue, or resulting from contamination of the separated fractions. A comparison of the findings from the different studies also indicates that lipofuscin from different cells or different species may exhibit different staining properties and enzyme content.

Fine structural studies on both the isolated and in situ lipofuscin of a variety of cell types characterize it as being made up of single-membrane limited granules, exhibiting varying degrees of density (Hess 1955; Duncan et al., 1960; Bondareff, 1957; Novikoff et al., 1956; Essner and Novikoff, 1960; Brandes et al., 1962; Malkoff and Strehler, 1963; Samorajski et al., 1965; Few, 1966). The major portion of the granules is very dense with an internal structure varying from amorphous to granular to a lamellated myelin-whorl like appearance. D'Agostino and Luse (1964) did not find these lamellated structures in what they interpreted to be lipofuscin in the human substantia nigra, and used their absence to identify lipofuscin as opposed to structures identified by them as melanin pigment granules. These myelin-like configurations appear to be more common in

neuronal lipofuscin, and are not a striking feature of cardiac granules.

The pigment granules in cardiac muscle have been found to be concentrated mainly at the nuclear poles. However, Strehler (1964) noted granules developing between the myofibrils with a definite relationship to the banding pattern of the fibrils and he suggests that a migration, probably passive, might occur, explaining their concentration at the nuclear poles.

That some relationship of the lipofuscin granules to the lysosome exists has been rather firmly established. Hydrolytic enzymes, most notably acid phosphatase and non-specific esterase, have been shown to have common sites of localization in these structures (Ashford and Porter, 1962; Gedigk and Bontke, 1956; Essner and Novikoff, 1960; Novikoff, 1961; Strehler, 1964; Samorajski et al., 1965). In these studies certain fine structural characteristics were noted as being shared by lysosomes and lipofuscin granules, and structures resembling intermediate stages between the two have been observed.

The lysosome as a cellular organelle is better understood biochemically than morphologically. Its existence as distinct functional entity was first observed and reported by biochemists (Berthet and deDuve, 1951). The visualization of these organelles by the electron microscopist has been more

or less incidental to the biochemical studies, and, due to the heterogeneity in the morphology of their structure, has contributed to an area of confusion and controversy concerning their identification. Combined efforts using electron microscopy, cell fractionation and chemical analysis, and histochemical methods at the fine structural level have led to our concept of lysosomes as "...membranous vesicles filled with hydrolytic enzymes" (Ham, 1965). Due to the considerable polymorphism exhibited by these organelles in different cell types or even within the same cell, histochemical studies, localizing one or more of the hydrolytic enzymes in a structure believed to be a lysosome, are necessary before identification can be made with any degree of certainty. This polymorphism has been the cause of a profusion of terms which have been applied to this organelle by different workers and reviewers (Ham, 1965; DeRobertis et al., 1965; Ashford and Porter, 1962; Brandes et al., 1962; Brandes, 1965; deDuve, 1961; Gordon et al., 1965; Ericsson et al., 1965).

These authors have pointed out that lysosomes do have different functional roles to play in different cells or even within the same cell, and that the polymorphism is due to this diversified function. Two main lines along which lysosomes may act are evident from a review of these papers. They may act on material extrinsic to the cell which has been brought into the cell by phago- or pinocytosis or they

may act in some way upon other cytoplasmic components of the cell, resulting in their breakdown. Most of the workers in this area agree basically on the mechanisms involved in these relationships but disagree as to terminology.

The simplest morphological form in which lysosomes are found is as a round to ovoid body approximately 0.4 microns in diameter, limited by a single smooth-surfaced membrane. This form is termed an inactive lysosome or a primary lysosome by Ham (1965), a pure lysosome by deDuve (1961), Brandes et al. (1962), Brandes (1965), an original lysosome or storage granule by DeRobertis et al. (1965), a proto-lysosome by Gordon et al. (1965), and it is my interpretation that Ericsson et al. (1965) would refer to it as a cytosome. Linder (1957) used the term "cytosome" in referring to many types of dense bodies in the myocardial cytoplasm. The view is held by most that this simple form represents the inactive or storage form of the lysosome, before it becomes engaged in intracellular lytic processes.

There is general agreement that there are two main types of "active" lysosomes (Ham's term, 1965). Those which fuse in some way with a phagocytic vacuole and act to break down the contained extrinsic material are termed phagosomes by some authors (Novikoff, 1961; Straus, 1958). Ham (1965) and Gordon et al. (1965) reserve the term "phagosome" for the phagocytic vacuole with its contained material. Ham then

terms the body resulting from the fusion of the primary lysosome and the phagosome or phagocytic vacuole a "digestive vacuole". Gordon et al. (1965) and Straus (1964) propose the term "phago-lysosome" as a synonym for this digestive vacuole. The terms "phagosome" and "digestive vacuole" are used synonymously by DeRobertis et al. (1965). Ericsson et al. (1965) use the term "cytosome" as a general name for all types of naturally occurring inclusion bodies, limited by a single membrane, and containing membranous and dense materials of variable appearance in different organs and tissues. Thus the term "cytosome" is purely descriptive and does not indicate the origin of the contained material as to whether it is intrinsic or extrinsic to the cell.

Different terms are suggested for structures resulting from lysosomal action on cellular components. The term "cytolysomes" is used by Ham (1965). DeRobertis et al. (1965) use the term "autophagic vacuole" in making the distinction from the digestive vacuole described above. "Auto-lysosome" is used as a synonym for autophagic vacuole by Gordon et al. (1965). Again Ericsson et al. (1965) suggest a purely descriptive term "cytosegresome" to define inclusion bodies limited by a single, or occasionally a double, membrane and containing recognizable cytoplasmic structures.

The result of the action of the hydrolytic enzymes on the enclosed particles, whether they be of intrinsic or

extrinsic origin, is not usually complete digestion. Certain indigestible residues, usually of a lipoprotein nature, remain. There is some consensus concerning the application of the term "residual body" to the structures formed by these residues. Gordon et al. (1965) believe that small dense bodies, which they term "telo-lysosomes", first form and that these may then fuse with one another to form the residual bodies, or, the telo-lysosomes may fuse with the phago- or auto-lysosomes with the possibility that any hydrolytic enzymes remaining within them could then be re-utilized for further breakdown. Ericsson et al. (1965) apply the term cytosome to these dense residues.

Gordon et al. (1965) contend that multi-vesicular bodies, i.e., membrane-bound vacuole-like structures containing numerous small membranous vesicles, are formed by the penetration of either the phago- or auto-lysosome by numerous small proto-lysosomes.

Maunsbach (1966), in an extensive study of electron dense structures appearing in the cytoplasm of the cells of the proximal tubule of the rat's kidney, suggested that no implication of origin or functional significance be included in the terminology applied to such structures until better evidence concerning such points is forthcoming. He suggests instead the strictly descriptive terms, cytoplasmic dense bodies, types I, II, III, IV. He gives structural and some

histochemical criteria for identifying each type. His type I appears to correspond to the microbodies as described by other workers, type II to either auto- or phago-lysosomes, type III to non-membrane bound lipid droplets, and type IV to autophagic vacuoles or cytosegresomes. He criticizes the use of these terms implying knowledge of origin and/or function of these bodies.

Other organelles of the cell have been implicated in the genesis of lipofuscin. In the neuron, Hess (1955) and later Duncan et al. (1960), noting the proximity of mitochondria to the developing granules, proposed that the degenerating mitochondria play a role in the development of lipofuscin. Tewari and Bourne (1962) and Kumamoto and Bourne (1963) discovered, using histochemical methods, that certain respiratory enzymes, usually considered to be located in mitochondria, were localized in lipofuscin granules. Recognizable mitochondria in different stages of degeneration were seen in liver lipofuscin by Ashford and Porter (1962).

The Golgi apparatus has also been suggested as contributing to the formation of the pigment granules. Gatenby and Moussa (1951) and Bondareff (1957) based this idea largely on the observed proximity of the Golgi to the developing granules. Other workers have since shown a relationship of the Golgi to the development of the lysosome and the production of hydrolytic enzymes (Novikoff, 1963; Brandes,

1965; Gordon et al., 1965). Indirectly this relates the Golgi apparatus to the lipofuscin granule.

The membranes of the autophagic vacuoles or cytosegresomes may have their origin from the smooth surfaced endoplasmic reticulum (ER) (Gordon et al., 1965; Elliott and Bak, 1964). Myelin, whorl-like structures in the hepatic cell cytoplasm have been noted to have their origin from the smooth ER (Stein et al., 1966). These structures may, by fusing with other contributing bodies, take part in the formation of liver lipofuscin. Strehler (1964), noting that the lipofuscin granules of cardiac muscle develop in the interfibrillar space in relation to the banding pattern of the fibril, believes their genesis might be related to the smooth ER (Sarcoplasmic reticulum) which shares this location.

Evidence also indicates that the ribosomes and the rough endoplasmic reticulum have some relationship to the lipofuscin granules in certain cells. Granules have been observed to give a strong Kurnick reaction for RNA (Strehler, 1964). This reaction is abolished by pre-treatment with RNAase. Isolated pigment granules possess a diaphorase (Bjorkerud, 1963) relating them to cytochrome b_5 which is known to be present in the microsome fraction (Chance and Williams, 1954). In the neuron, Hyden and Lindstrom (1950) noted an inverse relationship of pigment to Nissl substance. Histochemical studies of Issidorides and Shanklin (1961) confirmed that a

relationship exists between the developing lipofuscin pigment and the Nissl substance of the neuron.

Change in myofibrillar banding The Z line has been described as a thin band of dense amorphous looking material filling in the spaces between the thin or actin filaments of the I band, halfway along their length (Huxley and Hanson, 1960). These authors state that these actin filaments are slightly thickened in the vicinity of the Z line. Garamvolgyi et al. (1962) isolated Z disc structures from the flight muscle of the bee and from the rabbit psoas muscle by mechanical and chemical disintegration of the cells. On the basis of light and electron microscopic examination of such fractions, they believed the discs to be made up of structures oriented perpendicular to the long axis of the fibril. Conversely, Knappeis and Carlsen (1962) and Carlsen and Knappeis (1963) observed in frog muscle that the Z disc was made up of filaments oriented mainly in the long axis of the fibril, but lying at enough of an angle to form pyramids at whose peaks the I band filaments were attached. These pyramids were arranged in such a way that I filaments on one side of the Z line were attached at a point midway between the attachments of two I filaments on the opposite side of the Z line.

A study of recent reports involving myocardial reaction to different types of stress has revealed no reference to

striking changes in the normal myofibrillar banding pattern (Martin et al., 1964; Lev et al., 1965; Muscatello et al., 1965; Martin and Hackel, 1966; Merkow and Leighton, 1966).

MATERIALS AND METHODS

The hearts included in this study were collected from 99 dogs of both sexes and various ages, ranging from 2 days to 19 years. These animals were raised and maintained in a controlled environment, either in the gerontological beagle colony of the Department of Veterinary Anatomy at Iowa State University, or in the kennels of a large commercial dog food manufacturer[1]. Strict care was taken to keep these animals as disease and parasite free as possible. This care consisted of a careful regimen of sanitation, vaccination, and anthelmenthic therapy. Their diet, after weaning was composed solely of one dry commercial dog food[2]. The majority of the animals in the study were beagles, but many of the older dogs were purebreds of various other breeds. Genetic histories were available for all specimens.

The myocardial tissues collected from these animals were subjected to study by a number of histomorphological methods. Sections of the tissue from each of the animals were studied both qualitatively and quantitatively using bright field and fluorescence microscopy. Tissues from 35 of the dogs were studied by means of the electron microscope.

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[1]Gaines Dog Research Laboratories, Kankakee, Illinois.

[2]Provided by Gaines Dog Food Division, General Foods Company, Kankakee, Illinois.

Method of tissue collection

After weighing, all animals were killed by electrocution and exsanguinated by severing the axillary vessels. The thoracic wall was opened as rapidly as possible after death, the pericardium dissected away, and the heart removed. All chambers of the heart were opened and it was placed in a large jar containing 10% buffered neutral formalin. Later, blocks were trimmed from the lateral wall of the right ventricle and processed further for bright field and fluoresence microscopy. An attempt was made to secure this section of tissue from the same site in all specimens.

In those animals from which tissue was to be processed for electron microscopy, the pericardium was incised immediately after opening the thoracic cavity and a small section of the lateral wall of the right ventricle was taken to be processed as described below. In all cases this piece of tissue was in the fixative used for fine structural studies within five minutes after the death of the animal.

Techniques for bright field microscopy

As noted above, the tissues for bright field studies were fixed in 10% buffered neutral formalin. After washing and dehydration by routine procedures, the blocks were infiltrated and imbedded with Paraplast[1]. Sections were cut from all blocks at 6-8 microns and mounted for staining.

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[1]Scientific Products, Evanston, Illinois.

The staining procedures used in this portion of the study were as follows:

1) Hematoxylin and eosin (AFIP, 1960).
2) Mallory's triple connective tissue stain (as modified by Crossman, 1937).
3) Mallory's phosphotungstic acid hematoxylin (Mallory, 1942).
4) Heidenhain-Van Giesen-Weigert (as modified by Getty, 1949).
5) Orcinol-new fuchsin (Fullmer and Lillie, 1956).
6) The long Ziehl-Nielsen acid-fast stain (Mallory, 1942).
7) Gomori's reticulum stain (Mallory, 1942).

After staining, the slides were then given a second code number, obscuring the kennel number of the animal from which they were taken. This was done to avoid the chance of subjective influences affecting the evaluation of the slides, as the kennel number could give a clue to the age of the animal. Qualitative studies were conducted, noting differences in the myocardium with particular attention to the relative amounts of collagen, reticular, and elastic fibers and adipose cells in the different specimens.

Fiber diameters were measured in all specimens by means of a Filar micrometer eyepiece, which was calibrated with a stage micrometer. In all cases the narrowest diameter of a

nucleated cross-section of the myofiber was measured. The narrowest diameter was chosen for measurement in order to rule out artifacts introduced by slightly oblique sectioning. Ten such cross-sections were measured in each specimen and the mean was recorded.

In order to get information regarding the change in size of the myofibers in a longitudinal direction, nuclear density per unit area was measured. This was done by counting all infocus myocardial cell nuclei lying within the confines of a 5 $mm.^2$ grid which was placed in the ocular in the same focal plane as the section. Five such areas were counted in each specimen, choosing in each case areas of the section in which the fibers were oriented longitudinally.

Nuclear dimensions which, if variable, would affect the above count, were measured in a number of randomly selected animals from different age groups. Longitudinal and transverse axes of the nuclei, as seen in longitudinally oriented sections, were measured using the Filar micrometer eyepiece.

Techniques for fluorescence microscopy

After evaluating the above staining methods it was decided that the technique of fluorescence microscopy was best for quantifying the concentration of the lipofuscin pigment granules in the myocardium. Slides, with mounted paraffin sections, were deparaffinized in xylol and mounted

in low fluorescing immersion oil. These slides were then examined using a Bausch and Lomb Dynoptic microscope using a 43X, .65 N.A. objective and a 10X ocular fitted with a 5 mm.^{2} net reticule, ruled into 0.5 mm. squares. The light source was a Bausch and Lomb 200 watt mercury arc lamp and the filters were a B. & L. 5-58 exciter filter, which transmits mainly to the 400 millimicron range, and a B. & L. Y-8 barrier filter which transmits above the 550 millimicron range.

The method used in quantifying the pigment was based on that used by Strehler et al. (1959) which was a modification of a method for quantification proposed originally by Chalkley (1943). The net reticule was placed in the ocular in such a way that it was in focus in the same plane as the magnified image of the specimen. A green filter that did not transmit the low wave length light necessary to ellicit the autofluorescence of the pigment granules was substituted for the exciter filter and a field was selected in which most of the myofibers were oriented longitudinally. Using the green filter the granules were not visible and so no subjective influences could play a part in the selection of the fields to be evaluated. After the field was thus selected and focused, the exciter filter was replaced and the green filter removed. The reticule, now superimposed on the image of the myocardium, exhibits 121 sites of intersection of its

lines. If any of these intersections was found to be superimposed over the in-focus image of a fluorescing pigment granule this was counted as a "hit". The total number of "hits" per field were counted and the process repeated until a total of ten fields had been counted for each specimen. The total number of "hits" per all ten fields was figured and then divided by 1210, the total possible "hits", to give a percentage.

Techniques for electron microscopy

Tissues from 35 dogs were processed for electron microscopic study. The animals included were of varying ages and are indicated in Table one. As noted above, small sections of the right ventricle were removed as soon as possible after death and placed in the fixative where they were minced to a final size of approximately 1 $mm.^2$. The type of fixative used varied during the course of the study. Those used were as follows:

1) 1% OsO_4 buffered with veronal-acetate to a pH of 7.4 - 7.8 (Palade, 1952).
2) The above fixative with 0.045 gm. sucrose added per cc. (Caulfield, 1957).
3) 3% or 6% glutaraldehyde buffered with Millonig's phosphate buffer to pH 7.4 (Millonig, 1962).
4) 3% glutaraldehyde buffered with 0.1M sodium cacodylate buffer to pH 7.4 (Sabatini et al., 1963).

Fixation in all cases was carried out at 4 degrees centigrade for one to five hours. The osmium-fixed tissues were immediately processed further and embedded after fixation, but the glutaraldehyde-fixed tissue was stored in the buffer at least overnight and, in some cases, for as long as four months before further processing.

After an overnight wash in buffer, frozen sections, at a thickness of 50 microns, were made from certain of the glutaraldehyde-fixed tissue. These sections were then incubated according to Gomori's lead phosphate method for acid phosphatase determination as modified by Sheldon, et al. (1955) for electron microscopy. Control sections were incubated in the same medium with the substrate, sodium glycerophosphate, absent.

Glutaraldehyde-fixed tissue was post-fixed in 1% OsO_4 buffered with the same buffer used in the primary fixative. After rapid dehydration through a series of alcohols, the sections were embedded in one of the following embedment mixtures:

1) A mixture of ethyl methacrylate (3 parts) and butyl methacrylate (4 parts) with 1% benzoyl peroxide.
2) The above methacrylate mixture with 0.8% benzoyl peroxide and 3% divinylbenzene added as a cross-linking agent.
3) Maraglas-D.E.R. 732 mixture (Erlandson, 1964).

4) A mixture of Dow epoxy resins, D.E.R. 732 and 332. (Lockwood and Langston, 1964.)

Polymerization was effected in every case by heat with the time and temperature varying depending on the embedment.

Sectioning was done using glass knives in the Porter-Blum MT-1 or MT-2 microtome. Sections were cut exhibiting interference colors in the silver range. Methacrylate sections were mounted on parloidon coated 200 mesh copper grids and the Maraglas and Dow sections were mounted on uncoated 300 and 400 mesh grids. The sections were stained with either $KMnO_4$ (Lawn, 1960) or lead citrate (Reynolds, 1963; Venable and Coggeshall, 1965) and examined in an Hitachi HU-11A electron microscope.

RESULTS

The results of this study will be reported following an outline identical to that used in the review of the literature.

Changes in the myocardium as a tissue

Changes involving collagen fibers

Collagen, stainable by the methods used, was not noted in the endomysial area of the younger animals, from birth to three years of age. In these animals, it was limited to the epi- and endocardium and to the perivascular regions of the perimysium. Variable amounts of stainable collagen were seen in the endomysium of most animals in the three to eight year age range. It was seen in all animals older than eight years of age and its presence was very definite in most of these aged animals (Figure 1). Even in the most marked cases, however, it did not appear in amounts that could be considered to significantly interfere with the normal function of the myocardium.

Changes involving reticular fibers

The reticulum of the aged animals was noted to be much more pronounced and more distinctly stained than that of the young animals. The change was very gradual and variable among the different individuals in the study. No quantitative statements can be made other than to say that the reticular fibers appeared to increase in number and in density with increasing age (Figures 3 and 4).

Due to the necessarily small areas of sampling with the electron microscope, no comparisons were made with this instrument. The fibers which are considered to correspond to those taking the silver stain in paraffin sections appeared in the electron microscope as very fine collagen fibers. Numerous typical fibroblasts were observed between the myofibers and related to these fine collagenous (reticular) fibers.

Changes involving elastic fibers Elastic fibers were not found in the myocardium proper of the right ventricle in any age group studied. Elastic connective tissue appeared in all cases to be confined to the endocardium. The only exception noted was that in the older dogs it was observed in increased amounts in the sub-endocardial Purkinje network (Figure 2).

Fatty changes In animals from birth to six years of age, any observed adipose tissue was limited to the sub-epicardial region and to the area around the larger of the intra-myocardial vessels. In most animals over six years of age adipose cells were seen in the endomysial spaces. This invasion of the myocardium was most marked in the areas directly sub-epicardial, and in many cases was confined to this area. The groups of adipose cells varied in size, number, and extent. They appeared to be extensions of adipose deposits in the epicardium and the perimysium (Figures 5, 6, and 7).

Changes in the myocardial cell

Changes in fiber size The fiber diameter was measured directly in all animals studied. The results of this series of measurements are recorded in Table 1. The fiber diameter was noted to remain rather constant from birth until six months of age when it was observed to increase to approximately twice its original diameter (Figures 8 and 9) remaining relatively constant at that size (mean diameter, 2 to 100 days: 6.54 ± 1.28 microns; mean diameter 0.5 to 19.0 years: 14.5 ± 0.24 microns).

As an indication of a change in fiber length, the nuclear concentration per unit area was measured in the animals of different ages. A marked decrease in nuclear concentration was noted in animals over six months of age when compared to those younger than six months (Figures 10 and 11). The mean nuclear count per five fields in the 12 animals under six months of age was 351.25 ± 28.14. That of the 11 animals between six and eight months of age was 47.55 ± 16.23 and the mean for all animals in the study over six months of age was 31.03 ± 1.23.

The measurements of nuclear concentration of those dogs older than six months were found to fit a straight line with a slope of -1.54 ± 0.26 per year (Graph 5).

Nuclear change In animals from birth to one year of age the nuclei appeared to be uniform in shape (Figure 10).

From one to seven years a slight lobulation was noted in some of the nuclei, becoming very marked in many nuclei after seven years (Figure 12 a, b). At about this same age, six to seven years, binucleate cells (evidenced by the appearance of two distinct nuclei in the same perinuclear clear area) were noted (Figure 12 c, d and e and Figure 38). The lobulation in the aged animal, ten years plus, became very pronounced, resulting in many "link-sausage" shaped nuclei. This lobulation and the binucleate appearance were seen with both the bright field and electron microscope.

Lipofuscin development and accumulation Pigment granules first appeared and became concentrated at the poles of the nucleus in the myofibril-free area of the sarcoplasm. Some granules, however, were observed to be located between myofibrils near the ends of the myofibers. The pigment did not stain with the H. and E. stain but exhibited its own yellow color and could be readily visualized in the older animals using this stain. With Mallory's Triple Stain the pigment appeared red or scarlet, similar to the color exhibited by the erythrocyte. The pigment stained varying shades of blue or purple using the phosphotungstic acid hematoxylin method. It was very indistinct with the Heidenhain-Weigert-Van Giesen combination stain, as it appeared light yellow-green, very similar to the color of the myofibrils. Lipofuscin appeared red when stained for 12 hours by the Ziehl-Nielson technique.

The best visualization of the pigment was accomplished by means of fluorescence microscopy (Figures 13, 14, 15, 16 and 17). When illuminated by blue-violet light in the 400 millimicron range, the pigment exhibited an orange autofluorescence. This fluorescence was very distinct. Isolated granules which were not clumped at the nuclear poles could be easily resolved with this method.

The accumulation of the pigment was quantified using fluorescence microscopy (Table 1 and Graph 1). No pigment was observed in any dog under six months of age. The amount was very small and variable in animals in the six month to three year age range. It was present in trace or barely measureable amounts in 18 of 26 animals in this group. Some pigment was found to be present in all dogs studied which were over one year old, becoming measureable, by the method employed, in all animals over 3.4 years. From this age on it appeared to increase linearly with age, although showing extreme variability. Statistically it was shown to increase at the rate of $0.36 \pm 0.08\%$ of the myocardial volume per year in animals over 3.5 years. No sex differences were noted in this or the other parameters studied.

The lipofuscin granules appeared to be inert and little affected by any change due to poor fixation as observed by either the light or electron microscope.

The morphology and development of the pigment granules was studied with the electron microscope. In all animals

examined small (400-500 millimicron in diameter) electron dense bodies were noted in the sarcoplasm, appearing to be more highly concentrated in the area of the Golgi apparatus (Figure 18). Structures somewhat larger than these, and showing electronlucent areas of varying density, appearing as "vacuoles", were observed (Figures 18 and 19). Other sections revealed yet larger structures which appeared to be made up almost completely of such "vacuoles" surrounded by a very thin rim of electron dense material (Figures 20 and 21). Areas were observed in which it was noted that groups of such granules were clumped together with some evidence that they were coalescing to form a larger and more complex granule (Figure 22). Other such complex granules in older animals were noted to exhibit fewer and smaller electron-lucent "vacuoles" (Figures 23, 26, 27, 28). The majority of the granules observed in aged animals exhibited mainly an electron dense structure, either granular or lamellar (Figures 24, 25, 26, 27, and 28).

The "mature" granule exhibited a varying morphology. They were all recognized easily by their extreme density and their perinuclear clumping. In optimum sections they could be shown to be limited by a single membrane. As noted above, their internal structure varied. Some areas showed very dense granularity. Others had circular areas of low density which gave the granule a vacuolated appearance. Usually these vacuole-like areas were surrounded by a thin peripheral zone

of electron dense material, giving the false appearance of a membrane delimiting the "vacuole" from the rest of the granule. Other areas of the granules had a myelin-like lamellated appearance (Figure 24). This was not as frequently seen in cardiac lipofuscin as has been observed in neuronal lipofuscin. All of the above mentioned dense structures exhibited some degree of acid phosphatase activity when incubated using the method of Gomori (Figures 32, 33, 34, 35, and 36). The lead phosphate precipitate, indicating the site of such activity, was mostly concentrated around the periphery of the electron-lucent areas in the "vacuolated" pigment granules.

It was noted that smooth surfaced membranes of the myocardial cytoplasm appeared to be related to the surface of the developing granules. The majority of the granules developed in the myofibril free area at the poles of the nuclei in close relationship to the Golgi apparatus. In many cases it appeared that the membranes associated with the granules were derived from the Golgi lamellae (Figure 24). In other sections there was evidence to indicate that they were a part of the smooth surfaced endoplasmic reticulum (sarcoplasmic reticulum) of the myocardial cell (Figure 30).

In those animals tested using the Gomori technique for acid phosphatase localization it appeared that the Golgi area was positive in the very young animals (Figure 37) while, in

the older animals, where the Golgi membranes were related to the pigment, these membranes appeared negative (Figure 36).

In certain sections mitochondria, which were closely related either to lysosomes or developing pigment granules, showed a more dense matrix and some loss of their internal membranous structure. Certain of these altered mitochondria appeared to have actually coalesced with the developing lipofuscin (Figures 27, 28 and 29).

Change in myofibrillar banding No change other than increase in size was observed in either the myofibers or fibrils with age as they appeared under the bright field microscope. With the electron microscope a change was observed in the myofibrillar banding pattern in two older animals. While this change was looked for in all the animals studied, it was found only in limited areas in two animals, one 11.2 years old and one 12.8 years of age.

The change appeared as a widening of the "Z" band density (Figures 40, 41 and 42). In some sarcomeres this was extended until the entire sarcomere was of the same apparent density as the normal "Z" band (Figures 38 and 39). In all cases filaments appearing to be of the same dimension and density as the myosin filaments of the "A" band could be noted in these extended dense areas. In one animal the change appeared to be limited in its morphological effect to single sarcomeres (Figure 38) while in the other affected animal it appeared that the orientation of the entire myofibril was disrupted

(Figure 41). The affected sarcomeres in both animals were so few in number and so widely separated that a cross section through any of the affected areas was not observed or, if observed, was not recognized.

DISCUSSION

This thesis reports on changes occurring with age in the canine myocardium at the cellular and the tissue levels. It is not all inclusive even within these two levels. Due to practical problems relating to the method of tissue collection, histochemical changes and certain morphological changes were not measured.

The changes reported will be discussed in regard to the criteria of Strehler *et al.* (1959) for a primary age change, i.e., a change leading to the increased probability of the death of the individual. These criteria: universality, time dependence, intrinsicality, and deleteriousness are very difficult to evaluate, particularly the last. The different changes will be discussed individually, but it can be said at the outset that universality and time dependence apply to most. The intrinsicality of a change must be based in most cases on the fact that care has been taken to rule out extrinsic effects on this sample. Effects due to abnormal environmental or behavioral stresses, accidents, or pathology have been carefully guarded against. The animals included in the study have a uniform known history. Stein *et al.* (1966) stress the importance of this in their base-line study comparing the structure of the liver of pure-bred beagles of known history to ordinary street dogs.

Deleteriousness as a consequence of a structural change

is, according to Strehler et al. (1959) a necessary criteria if the change is to be regarded as primary. Again, this is difficult to evaluate in all cases, as many of the changes observed during senescence may be compensatory changes occurring in response to another effect, and as such could actually decrease rather than increase the probability of death. Such changes are regarded as secondary as opposed to primary age changes. Actually, then, the changes noted in this study are reported as those accompanying aging and include both primary and secondary effects. Enough information is not available for me to state with any certainty in all cases whether these are deleterious or compensatory. Some conjecture concerning this will be included in the ensuing discussion, but the truth must await further investigation.

Discussion of methods

The hearts included in this study were collected from animals which were a part of an extensive histomorphological study in gerontology which has been continuing in the Department of Veterinary Anatomy at Iowa State University over the past 15 years. Since many other tissues were collected from these dogs at the same time as those included in this study, special techniques such as perfusion fixation or vital staining were precluded. Many of the hearts had been routinely collected, fixed, and stored prior to this investigator's becoming involved in the project and for this

reason many histochemical tests were ruled out. Attention was therefore directed mainly to histomorphology at the light microscope level, and, in a more limited sample, at the electron microscope level.

The area chosen for study was a section of the lateral wall of the right ventricle. Strehler *et al.* (1959) have shown there to be little difference in pigment concentration in sections taken from the walls of the different chambers of the heart, and Angelakas *et al.* (1964) found only slight variation in the diameters of fibers of the right or left ventricular walls. Lev (1957), however, does call attention to significant differences in connective tissue changes among the different heart chamber walls, thus, the changes described in this report should not be considered to apply to any area of the heart other than the right ventricular wall.

The area chosen for the study was decided upon due to the fact that the hearts, as noted above, were fixed *in toto* and the thin-walled right ventricle was noted to exhibit more consistently even fixation throughout than the thicker-walled left ventricle.

Optimum fixation for electron microscopy was found to be achieved by using a slightly hypertonic, rather than an isotonic, fixative. Palade's fixative (veronal-acetate buffered OsO_4), which is very nearly isotonic, appeared to cause a swelling of the membrane-lined spaces of the cell.

A 3% glutaraldehyde solution buffered with either the phosphate or cacodylate buffer (resulting in a solution approximately two times isotonicity) gave the most consistently good results. The cacodylate buffer appeared to be the buffer of choice for use with the acid phosphatase determination. Best results from this determination were achieved by shortening the fixation time to one hour.

Subjective bias on the part of the investigator could play a part in the light microscopic evaluation and measurements of the myocardial components. Through the course of the study, the investigator became acquainted with the kennel numbers by which each slide was identified, and was able to relate these to the age of the animal. To avoid this knowledge of the age biasing the results, a second number was given each slide, obscuring the kennel number. Even so, some clue to the age of the animal is given in the morphology of the tissue, and so, all subjective bias could not be ruled out.

Nucleated cross-sections were chosen for measurement to minimize differences due to the tapering of the cells toward their extremities. Oblique cross-sections would appear oval in shape, but the narrowest diameter in any case should represent the true diameter of the cell. Longitudinal sections were not used for this measurement as cell outlines were difficult to resolve in most such areas.

Several methods were attempted to show change in the length of the cells. Ideally, measurement of the distances

between intercalated discs in the same fiber would be the optimal method. However, the intercalated discs do not stain distinctly in the very young and the differences in the lengths of the branches of the cells would lead to a wide variability in this measurement. Since each myocardial cell has one nucleus, the number of nuclei per unit area will have an inverse relationship to the size of the cells. If the cells are small, the nuclei will be closely packed, and, as the cells grow, the nuclei will be "pushed" further and further apart. Therefore, the higher the nuclear count per unit area the smaller the cells, and a decrease in nuclear concentration would indicate an increase in cell size. In this measurement care had to be taken to differentiate the pale, oval, vesicular nuclei of the myocardial cell from the more flattened, dense nuclei of the fibroblasts and endothelial cells, and include only those of the myocardial cell in the count. In most cases this was not difficult.

The method of quantifying the volume of pigment per volume of the myocardium is well discussed by Strehler et al. (1959). This method, first of all, measures the fraction of a known area of myocardium that is occupied by pigment. Then, if the third dimension is limited by the depth of focus of the microscope to a factor of one (only in focus granules were counted), this percent per unit area may also be expressed as a percentage of the myocardial volume.

Discussion of results

Changes in the myocardium as a tissue The histologic changes in the right ventricular myocardium involve mainly an increase in the stromal or connective tissue elements. This increase is most pronounced in those elements stained by the silver staining method of Gomori. These elements have been shown by the electron microscope to be fine collagenous fibers and have heretofore been referred to as reticular fibers. Their increase with age is probably due to the continued activity of fibroblasts in collagen production. As these fibers reach a certain size due to accretion, they become stainable by the so-called collagen stains and this is seen in the tissue as an increase in stainable collagen with age. The build up of the fibers in the endomysium was so slight that it caused very little displacement of the myofibers. It resulted more in a change in density and character of the endomysial connective tissue rather than a change in mass. These changes are similar to those observed in man (Lev, 1957).

Elastic tissue is seldom associated with striated muscle. It was not noted in the myocardium proper in dogs of any age. The orcinol-new fuchsin stain was used without a counter-stain in order to demonstrate any small fibers in the endomysial tissue which might have been masked by the counter-stain in the Weigert-Heidenhain-Van Giesen method. This demonstrated extremely fine fibers, not shown by the other technique, in

the endocardium, but revealed none in the myocardium proper. The significance of the build up of elastic tissue around the fibers of the conducting tissue is unknown.

The increased amounts of collagenous and reticular tissue were seen in all aged animals, satisfying the criteria of universality. This increase appeared to be time dependent. Whether this build up is a primary change intrinsic to the myocardium, or is a secondary change in response to a primary change such as a decrease in blood supply to the myocardium is not known. Likewise, any deleterious effect it might have is difficult to evaluate.

The fatty infiltration noted in the aged dogs of the sample was probably related to the high level of nutrition of the population from which the sample was taken. These animals were kennel-raised and fed a well balanced, highly nutritious diet, free-choice. All animals 8 years of age and older exhibited some fatty infiltration of the right ventricular myocardium. This change, while occurring universally in this sample, and being time dependent, probably varies according to the level of nutrition of the animal and so may not occur universally in all aged dogs.

Although no data is available to support any conjecture as to the deleterious effect of this change, it appears reasonable to expect that it would exert such an effect. In one animal in particular (M29), adipose cells had largely displaced the myocardial cells, leaving very little functional

tissue in the section observed. This, even occurring to a lesser extent, must serve to weaken the contractile abilities of the right ventricular wall.

Changes in the myocardial cell Change in fiber size

The heart grows by hypertrophy of the cells rather than by hyperplasia. This is reflected in the results of this study concerning fiber diameter and nuclear concentration. The rapid growth of the myocardial cell occurs at the time of the most rapid body growth, 4 to 6 months in the dog. Cohn and Steele (1936) have shown this period to be one of very rapid heart development in the dog. This hypertrophy of the cells is probably somewhat under the control of the somatotrophic hormone of the pituitary, or it may be, in part, a compensatory hypertrophy due to the increased demands placed on the heart by the developing body.

As noted in Table 1 and Graphs 3 and 5, growth of the cell slows after this period of rapid activity but does continue to increase in both length and diameter at a lesser rate throughout life.

The growth in length was assumed from the results of the nuclear count per unit area. Admittedly, the decrease in nuclear concentration with age could be due to some factor or factors other than increase in fiber length. Increased amounts of interstitial connective tissue, if present could cause a decrease in nuclear concentration but, as has been mentioned above, the change noted in the connective tissue

elements involved more of a qualitative change than a quantitative one. The increase in diameter of the cells was not great enough to account for the amount of decrease in nuclear concentration. No change in nuclear size was observed.

It is possible that the nuclear division noted in the older animals affected the results of the nuclear count per unit area in that all myocardial nuclei were counted, even if they were observed to occur within the same cell. This would have the effect of reducing the slope of the line in Graph 5 and, if many binucleate cells were included, would mean that the cell size actually increased at a faster rate than indicated by this measurement.

This continual growth, observed in the myocardial cell, has also been observed in the neuron by Andrew (1955) and probably results from a response of the cell to its continued activity.

No series of measurements was made with the electron microscope, but an apparent increase in both diameter and number of the myofibrils appeared to be involved in the increase in cell size.

Nuclear changes The nuclear changes with age which were observed resulted in an increased surface area presented by the nucleus to the cytoplasm of the myocardial cell. The observed nuclear changes ranged from rather shallow invaginations of the nuclear envelope to cells exhibiting two distinct nuclei, each with its own nucleolus. That the binucleate

cells are a result of amitotic divisions has not been proven by this study. No pinching off or division of the nucleoli have been observed. However, stages in the apparent formation of two nuclei by a continuing invagination of the nuclear membrane have been noted with a nucleolus located in either of the two segments which are believed to eventually become separate daughter nuclei. As no mitotic figures were observed in any of the specimens, it is assumed that the binucleate cells resulted from direct or amitotic nuclear division without accompanying cytokinesis.

This nuclear change begins in maturity and becomes more pronounced in old age. It is possible that it is related to the continuing growth of the cell and represents, as Andrew (1955) suggests in regard to the neuron and the hepatic cell, an attempt by the cell to restore the normal nucleo-cytoplasmic ratio. Probably more important to cellular function is the increase in nuclear surface area rather than any increase in nuclear mass.

Lipofuscin development and accumulation This study has shown the canine cardiac lipofuscin to have the following characteristics: a granular form, concentrated mainly at the nuclear poles but also found to a lesser extent throughout the cell, a yellow-brown color, a yellow-orange fluorescence when stimulated by near ultra-violet light, acid-fastness when stained for 12 hours using the Ziehl-Nielsen technique, a characteristic fine structure recognizable in the electron

microscope, some relationship to the lysosome and its effect on other of the cell organelles, and a definite relationship to the age of the animal. Nothing was noted to indicate that canine cardiac lipofuscin varies significantly from lipofuscins found in other areas or in other species.

The development and accumulation of lipofuscin in nervous tissue has been studied in this same population of dogs. Whiteford (1964) and Whiteford and Getty (1966) describe the characteristics of the pigment in the neurons of the brain stem nuclei and noted its accumulation in relation to age. Few (1966) reported similar findings for the spinal cord, dorsal root ganglia, and autonomic ganglia from the same animals. Cardiac lipofuscin from dogs of this sample exhibited no significant differences in its characteristics or relation to age from that seen in the neuron by these workers.

Because of its importance in certain areas of comparative research and due to the general interest of pet owners, the relative age of dogs compared to man has been an area of discussion. The distinct linear relationship of the accumulation of lipofuscin to age has been pointed out by Strehler et al. (1959) in man. The methods used in this study parallel theirs closely enough that meaningful comparisons may be made. Strehler's group reports no pigment in any of 32 children studied under 10 years of age. No

pigment was observed in the 12 dogs under 6 months of age included in this study. Pigment has been first observed in the myocardium of the human during the second decade. The youngest dog in this study in which fluorescent granules, identified as pigment, have been observed was 6 months old. This is listed in Table 1 as appearing in trace amounts.

The designation, "trace", is intended to indicate that pigment was observed in the section, even though in 10 fields no "hits" on grid intersections were observed. Pigment in these cases was present but could not be quantified by the method used. This presented a problem in statistically evaluating the data in dogs under 3.5 years of age. All dogs over 3.5 years had measureable amounts of pigment and so only these were included in the statistical analysis. This problem did not arise in the human study since the sample did not include hearts from individuals in the 10-20 year age group. Also the statistical data of that study may not be exactly comparable to that of this study since theirs included 32 negative specimens. Such negative results were not included in the statistical analysis in this study.

Strehler's group measured pigment in fields selected entirely at random. Many of these fields contained tissue other than myocardium, possibly blood vessels, adipose tissue, etc. Their direct measurement then was a measurement of the percent pigment per total heart volume. The percentage of

the total heart volume occupied by myocardium was calculated and the direct measurement was adjusted to become the percentage of pigment volume per myocardial volume. In the present study, fields were chosen which contained only myocardium and so the results obtained should be comparable to Strehler's adjusted results for myocardial volume.

In the human, a slope of 0.67% pigment volume per myocardial volume per decade is reported. This would equal 0.067% per year which could be compared to the slope of 0.36% per year found in the dog. These figures indicate then that pigment accumulates in the myocardium of the dog at a rate approximately 5.5 times faster than it does in the human. On the basis of the life expectancies of the two species this would be expected. Man lives on the average approximately five times longer than the dog. The average age in the human sample was 51.8 years and in the canine 10.4 years, a difference of approximately five times. Interpreting from a graph in the report on the human study the mean percent pigment volume per myocardial volume appears to equal approximately 3%. This can be compared to a similar figure of 3.49% in the canine.

Based on the above figures the following rather general statements can be made. Puberty at 6 to 8 months in the dog would compare to the 10 to 12 year period in the human. The 8 month to 3 year period in the dog's life is equivalent to

the 12 to 20 year period in the human. Similarly the 5 to 10 year span in the dog would correspond to the 25 to 50 year range in the human and 10 to 15 year old dogs would be equivalent to 50 to 80 year old humans. It must be stressed that this comparison is based solely on the one parameter of cardiac pigmentation and probably will not apply in comparing other parameters.

The morphologic study of these pigment granules at the fine structural level during different stages of their development leads to the conclusion that this development is dependent upon a number of factors. Many organelles of the cell appear to contribute to this development.

Primary or inactive lysosomes were seen in animals of all ages, but were more numerous in those one year and under. These were round to oval structures, 0.4 to 0.5 microns in diameter, limited by a single membrane, and possessing a very dense granular matrix. They exhibited a strong positive reaction for acid phosphatase. Usually they were found in greatest numbers in the perinuclear area of the cell, often associated with the Golgi apparatus. Not enough evidence was gained from this study to prove their production by the Golgi apparatus. However, in the very young animal, the Golgi membranes were shown to also exhibit a positive reaction for acid phosphatase. This, along with the spatial relationship of the lysosome to the Golgi, is a strong

indication that the Golgi is involved in some way in lysosome formation.

Apparently the first change appearing in the lysosome as it becomes active is the formation of an area of decreased density within its granular matrix. Usually this area of lessened density appears at one pole of the lysosome and gives the lysosome a "vacuolated" appearance. It is postulated that further activity results in the increase in size of these "vacuoles" until forms are seen in which the "vacuolated" areas include the major portion of the structure with the dense material occupying a very narrow area around the periphery. An increase in size of this active lysosome appeared to result as this vacuolization occurred.

What is the nature of the activity of the lysosome resulting in this structural change? One explanation is that the lysosome is acting on substances brought into it by a process similar to pinocytosis and that the vacuoles represent masses of digested residue. Little morphologic evidence can be presented to support this view. Cardiac muscle does show, along its sarcolemma, areas in which pinocytosis is very pronounced, but, except for occasional multivesicular bodies, few vesicles were seen in close proximity to the lysosomes and none have been observed actually fusing with the lysosomal membrane. If such a process did occur, then the lysosomes exhibiting the

vacuolated appearance would be related to the phago-lysosomes described in other tissues. Since evidence has not been noted to support the use of this term, the less specific term, "active lysosome", will be used to describe such structures in the following discussion.

A second possible explanation for the formation of these vacuole-like structures in the lysosome is that, as the lysosome becomes active, substances (hydrolytic enzymes) pass out through the membrane. Koenig (1963) has stated that lysosomal enzymes are bound to anionic sites of the matrix by electrostatic bounds and he has demonstrated that B-glucuronidase and acid phosphatase may be liberated from lysosomes in vitro by cationic molecules without disrupting the membrane. If a similar mechanism could take place in the myocardial cell, hydrolytic enzymes could be released and semi-specifically be attracted to, and attack sites on a cellular organelle which had been altered by its continued functioning. Such a mechanism would explain the changes observed in mitochondria in the vicinity of active lysosomes.

It has been noted that in unfixed tissues, there is a diffuse positivity for the hydrolases (Gedigk and Bontke, 1956; Strehler, 1964). The fact that diffuse acid phosphatase activity is not demonstrated histochemically throughout the cytoplasm of fixed tissue could be explained by a differential effect of the fixative. The enzymes free

in the cytoplasm or in relation to certain organelles would be extracted or denatured while those protected in the glycolipid matrix of the lysosome (Koenig, 1962) would be unaffected.

As the activity of the lysosome increases, the area of the hydrolytic activity appears to be walled off by smooth membranes derived from the Golgi and/or the sarcoplasmic reticulum. This segregation may take place before release of the enzymes. The partially depleted lysosome and the altered mitochondria along with the membranes of the Golgi and the sarcoplasmic reticulum coalesce and fuse to form a structure similar to the cytosegresome as described in the proximal tubule cell of the kidney. Continued hydrolytic activity and condensation within this cytosegresome would result in its becoming more dense and more compact, forming a residual body which could then be referred to as a "mature" lipofuscin pigment granule. The varying degree of density observed in the vacuolated areas of these cytosegresomes may be due to differing stages of lipid digestion and extraction in some cases, and in others the vacuoles may result from alterations in the matrix of the incorporated lysosomes due to enzyme loss. Myelin-like figures observed in the cytosegresomes and residual bodies are believed to result from a layering of the mitochondrial or Golgi membranes as they are incorporated, or from the precipitation of phospholipids resulting from the digestive process.

The location of lysosomes and, later in life, lipofuscin granules in the peripheral portion of the cell, away from the Golgi apparatus, implies that some migration of these structures must take place. There is some evidence to indicate that the primary lysosome may move within the closed membrane system of the sarcoplasmic reticulum. This system in cardiac muscle apparently extends throughout the cell (Porter and Palade, 1957) and the primary lysosome could move within it to the distal extremities of the cell before becoming active. Strehler (1964) suggests that perhaps many of the pigment granules are formed in the distal regions of the cell and a migratory process serves to concentrate them at the nuclear poles in the older animals.

Just when along this line of development from primary or inactive lysosome to active lysosome to cytosegresome and finally to residual body the term lipofuscin pigment granule applies is not apparent. It is probable that some significant collection and condensation of lipids in a partially auto-oxidized state would be necessary for the color associated with the term lipofuscin to be demonstrated. This would mean that development would have to proceed to the cytosegresome stage before these bodies would be recognized as pigment granules. Since lysosomes fluoresce very similarly to the lipofuscin pigment, measurement of the pigment using the fluorescence microscope may have included some large lysosomes.

This process of development and accumulation of the lipofuscin pigment was universal in the sample and was certainly time dependent. Since this study included no diseased animals, it provides no basis on which to agree or disagree with the contention of Strehler et al. (1959) that this accumulation with age is solely an intrinsic phenomenon. The question then of whether or not it does represent a primary age change, i.e., does it lead to an increased probability of death, hinges on the fourth of Strehler's criteria, deleteriousness.

They base their claim for a deleterious effect on the considerable volume occupied by the pigment in the myocardial cell cytoplasm. They do allow that a second alternative is possible, that is, in the light of the little correlation which they observed between the volume of pigment and various forms of heart disease, the pigment may produce no effect at all. Based on numerous observations of the intracellular sites of pigment accumulation in many aged dogs with the electron microscope, this study supports this alternative. The pigment, in most cases, is sequestered in areas where it would appear to have very little mechanical effect on the cell, either as to interfering with the action of the myofibrils, or by blocking the diffusion of materials within the cytoplasm. Also, it should be noted that other body cells, such as the neurons of the human substantia nigra are normally packed with a melanin pigment which appears to have

little if any effect on their function.

The pigment accumulation may be an observed change secondary to the primary age related loss of hydrolytic activity in the cell. Such a loss, if it occurs, would result in the aging cell losing its ability to breakdown "worn out" organelles. Hartmann (1928) reports on keeping an amoeba alive and undividing over an extended period of time by repeated "amputation" of areas of its cytoplasm. Mazia (1961) discusses cell division as a necessity for growth and dilution of the cytoplasm. In commenting on Hartmann's work, Mazia states that if cells normally possessed a means of throwing away cytoplasm and regenerating new, cell division might not be a necessity for biological immortality. It may be that the continued segregated autolysis observed in the cardiac muscle cells and the neuron constitutes such a means, allowing these cells to remain in the post-mitotic state throughout the life of the animal. Lipofuscin, then, would represent a by-product of a process which is advantageous to the cell. If this is the case, a leveling off of the rate of pigment accumulation could be expected in the very old animal. While the four oldest dogs in this study exhibited pigment concentration values which were below the line of linear development, these do not constitute enough of a sample on which to base any conclusion. The truth as to the function and significance of lipofuscin must await further study.

Change in myofibrillar banding The change noted in the myofibrillar banding pattern in certain areas of the canine myocardium is reported here, more as a point of interest than as constituting an age change. The observation of this change in only two of the animals, even though both were aged dogs, certainly does not justify relating this causally to aging.

The widening of the "Z" band density was observed to vary in extent. In some cases the entire sarcomere was involved while in others the change appeared only as a somewhat wider and irregular "Z" band. In most cases the entire width of the fibril was involved, but in others finger-like projections of the density were seen to reach across in only a restricted area of the fibril. The degree of density in all cases was similar to that of the normal "Z" band and of the densities associated with the intercalated discs.

When first observed, it was hoped that this change might consist of an exaggerated "Z" band and as such could serve as a model from which more could be learned concerning the normal morphology of this band. Closer examination, however, showed the major change in the affected sarcomeres to be an increase in the density of the amorphous sarcoplasm between the filaments. The sarcomeres in which this occurred were always observed to be in a contracted state, that is,

no "I" band was noted. Adjacent sarcomeres, in most cases, exhibited an "I" band. The "A" or myosin filaments were observed to extend to the ends of the areas of density in the sarcomeres which were completely affected. The smaller "I" or actin filaments were difficult to see in the affected area. In certain areas of the densities it appeared that the cross-bridges, linking the filaments, were exaggerated in size producing a herring-bone effect. It is doubtful that this herring-bone pattern is related to the "Z" band filaments described by Knappeis and Carlsen (1962) and Carlsen and Knappeis (1963). These "Z" filaments were not demonstrated in the affected areas in this study.

The effect or significance of this change cannot be ascertained on the basis of the observations made thus far. No reference to a similar change has been noted in a review of the literature concerning fine structural study of muscle and muscle lesions (Lev et al., 1965; Martin and Hackel, 1966; Martin et al., 1964; Merkow and Leighton, 1966; Muscatello et al., 1965; Wartman and Hill, 1960).

The affected sarcomeres appeared to be hypercontracted, and it is assumed that they were held in this state before death. If such were the case, they would not have contributed to the contractile ability of the cells involved. However, because they are observed to occur so widely scattered throughout the cell, their overall effect in

diminishing contractility was probably negligible. It appears that they would act as non-contractile fibrous bands which would be passively moved by the contraction of the normal sarcomeres in the fibril. In the 11.2 year old dog, the disorientation of the myofibril apparently resulting from these changes, could be assumed to cause more of a deleterious effect than would be caused in the 12.8 year old in which the change caused no disorientation.

More observations on larger sample areas in a number of animals is necessary in order to determine if this is, or is not, age related. The size of the affected areas, somewhat less than one square micron, does not preclude their recognition using the bright field microscope. This has not yet been accomplished but once they have been identified in this way, their distribution and frequency of occurrence could more easily be determined.

SUMMARY

Sections from the right ventricular myocardium of 99 dogs, ranging in age from 2 days to 19 years have been studied histomorphologically at both the light and electron microscope levels. Changes occurring with age at the tissue and cellular levels are reported. The results may be summarized as follows:

1. A slight increase in the amounts of collagenous elements was noted with increasing age. The most pronounced change was an increase in the endomysium of the finer fibers which were stainable by silver staining methods (reticular fibers). Fibers exhibiting the normal staining properties of collagen appeared in the endomysium of the older animals in amounts which increased with age.

2. No elastic fibers were noted in the myocardium proper of any animal studied. With advancing age, increased amounts of elastic fibers were noted around the sub-endocardial Purkinje fibers.

3. Fatty infiltration of the myocardium was common in animals over 6 years of age. This appeared to be an extension of the fatty depots located sub-epicardially and around the perimysial vessels.

4. Myocardial cell size was observed to increase continually with age. A very rapid increase, approximately doubling the cell size, was observed to occur between the

4th and the 6th month. After 6 months the rate of cellular growth was very slow.

5. Invagination of the nuclear envelope and the appearance of binucleate cells was commonly observed in animals over 7 years of age. The significance of this response and its relation to amitosis is discussed.

6. Lipofuscin was observed by fluorescence microscopy to be present in the myocardium of certain animals at 6 months of age and was present in all dogs over 1 year of age. It was present in amounts measurable by the method used in all animals over 3.4 years of age and was observed to increase linearly at the rate of 0.36% pigment volume per myocardial volume per year. The relationship of these findings to the results of a similar study in man are discussed.

7. The development of the lipofuscin pigment granule in the canine myocardium was studied using the electron microscope. Findings indicate that the lysosomes play an important role in pigment development. The "mature" pigment granule appeared to be a residual body formed by the coalescence of altered lysosomes and other cellular organelles in varying stages of autolysis. It is suggested that lysosomal enzymes were responsible for the autolysis seen in these organelles. The possible significance of these findings is discussed.

8. A change in the myofibrillar banding pattern was noted in scattered areas in the myocardium of two aged dogs. This appeared to be a widened "Z" band density in contracted sarcomeres.

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Lastly, thanks must be expressed to Dr. Homer Porter, a truly good teacher, who planted the seed of scientific curiosity in the mind of a young biology student 17 years ago.

Table 1. Breed, sex, age and quantitative data for animals studied

Dog Number	Breed	Sex	Age < 6 mo. in days > 6 mo. in years	Body wt. in kg.	% pigment/ volume[a]	Av. fiber diameter[b]	Nuclei/ 5 fields[c]
B38	Beagle	F.	2	0.13	neg.	5.4	412
B39	Beagle	M.	3	0.13	neg.	5.3	472
*AI[d]	Beagle	F.	8				
B10	Beagle	M.	9	0.59	neg.	8.7	404
B35	Beagle	F.	14	0.55	neg.	5.6	300
*B50	Beagle	M.	14	0.50	neg.	5.6	404
B45	Beagle	M.	28	1.40	neg.	8.8	432
B46	Beagle	F.	28	1.00	neg.	5.4	418
B29	Beagle	F.	36	1.00	neg.	7.2	390
B28	Beagle	M.	36	.90	neg.	5.6	296
B47	Beagle	M.	57	1.60	neg.	6.8	320
B26	Beagle	M.	97	4.00	neg.	7.5	107
B27	Beagle	M.	100	2.60	neg.	6.6	260
B66	Beagle	F.	0.5	6.8	trace	13.9	47
*B61	Beagle	F.	0.6	4.8	trace	11.3	75
*B60	Beagle	M.	0.6	9.2	neg.	11.6	38
*B59	Beagle	F.	0.6	5.8	trace	15.5	31

[a]Method used in calculating these values described on page 26 of text.

[b]Method used in calculating these values described on page 24 of text.

[c]Method used in calculating these values described on page 25 of text.

[d]* indicates the animals included in the electron microscopic study.

Table 1 (Continued)

Dog Number	Breed	Sex	Age < 6 mo. in days > 6 mo. in years	Body wt. in kg.	% pigment/ volume	Av. fiber diameter	Nuclei/ 5 fields
*A41	Beagle	M.	0.6	7.3	neg.	13.2	72
*A22	Beagle	M.	0.7	8.4	neg.	14.5	39
*A23	Beagle	M.	0.7	7.4	neg.	12.7	48
*A37	Beagle	M.	0.7	8.2	trace	15.9	43
*B48	Beagle	F.	0.7	8.8	trace	11.5	41
*B49	Beagle	F.	0.7	12.7	neg.	11.1	64
*B21	Beagle	M.	0.7	4.4	0.4	16.7	25
*B67	Beagle	F.	0.8	6.1	trace	16.4	51
B22	Beagle	M.	0.8	4.8	trace	8.7	71
B53	Beagle	M.	0.8	11.6	neg.	13.2	56
B55	Beagle	M.	0.9	13.5	neg.	13.8	35
B54	Beagle	M.	1.0	9.3	neg.	12.8	29
B56	Beagle	M.	1.0	11.9	trace	15.3	45
B57	Beagle	M.	1.0	9.9	trace	12.2	40
*B62	Beagle	F.	1.0	7.3	0.6	13.3	36
2BD	Beagle	F.	1.1	5.6	trace	11.7	63
B24	Beagle	F.	2.3	7.9	trace	16.7	21
B25	Beagle	F.	2.3	7.9	0.4	16.1	18
M31	Fox terrier	M.	2.5	10.9	trace	12.9	26
B23	Beagle	M.	2.6	9.3	trace	13.4	38
B16	Beagle	M.	3.3	13.6	0.9	16.0	17
B36	Beagle	M.	3.4	11.5	trace	12.7	34
B20	Beagle	M.	3.4	9.1	1.4	15.5	26
*B65	Beagle	F.	3.6	14.7	0.2	11.7	45
*B51	Beagle	F.	4.2	10.2	0.4	13.3	53
B18	Beagle	F.	6.0	12.7	0.8	15.2	33
D27	Dalmation	F.	6.1	23.1	0.3	16.3	25
*7BA	Beagle	F.	6.4	14.8	1.9	15.0	38

Table 1 (Continued)

Dog Number	Breed	Sex	Age < 6 mo. in days > 6 mo. in years	Body wt. in kg.	% pigment/ volume	Av. fiber diameter	Nuclei/ 5 fields
1F	Beagle	F.	6.5	10.5	3.0	10.4	40
M28	Cocker Spaniel	F.	6.8	7.1	3.4	11.4	16
B30	Beagle	F.	7.5	11.8	1.9	15.2	38
M19	Basinji	M.	7.5	9.1	2.4	14.9	26
M20	Basinji	F.	7.5	10.4	1.7	17.1	25
B44	Beagle	F.	7.8	11.1	4.1	12.9	27
B43	Beagle	F.	7.8	10.7	1.8	15.7	28
*M47	Golden Retriever	F.	8.0	38.1			
B42	Beagle	F.	8.6	10.9	3.6	10.3	42
*M35	Fox terrier	M.	8.6	9.8	2.3	14.6	27
*#9	Beagle	M.	8.7	8.6	3.2	14.1	52
M29	Basinji	F.	9.0	14.5	0.9	18.7	
B33	Beagle	F.	9.1	15.0	2.3	14.5	27
B19	Beagle	M.	9.2	10.8	4.2	7.9	26
*B63	Beagle	M.	9.2	12.1	4.3	13.5	35
M23	Basinji	F.	9.3	14.5	3.2	15.2	20
D29	Dalmation	F.	9.4	19.5	1.4	17.1	31
182	Basinji	M.	9.6	11.3	3.5	13.6	16
*M40	Dachshund	F.	9.7	13.3	3.3	17.2	21
*M46	Irish Setter	F.	9.9	29.5			
M8	Labrador Retriever	F.	10.0	34.5	1.2	19.0	25
B64	Beagle	F.	10.0	11.3	4.6	13.3	24
M26	Dachshund	F.	10.3	11.2	6.2	14.8	20
*M38	Labrador Retriever	F.	10.4	30.7	3.7	12.6	17
M21	Welsh Terrier	F.	10.4	9.5	3.9	14.0	25
*M48	English Cocker	M.	10.5	17.2			
M14	Golden Retriever	F.	10.6	29.5	1.9	16.7	14
M13	German Short-Haired Pointer	M.	10.8	28.1	1.9	23.0	12

Table 1 (Continued)

Dog Number	Breed	Sex	Age < 6 mo. in days > 6 mo. in years	Body wt. in kg.	% pigment/ volume	Av. fiber diameter	Nuclei/ 5 fields
M22	Fox Terrier	F.	11.0	5.2	3.2	13.6	35
M4	English Cocker	F.	11.2	9.0	3.3	14.1	33
M27	German Short-Haired Pointer	F.	11.2	28.8	3.5	15.6	25
*M42	Fox Terrier	F.	11.2	8.2	4.6	15.3	26
*M43	Fox Terrier	F.	11.3	27.5	6.1	16.3	13
M9	Shet. Sheep dog	F.	11.5	10.4	4.9	16.4	27
M30	Fox Terrier	F.	11.5	11.6	4.5	11.2	27
*#5	Beagle	F.	11.9	9.8	2.3	14.2	24
M10			12.0		3.2	16.3	13
M17	Fox Terrier	M.	12.0	7.7	3.9	13.8	16
*M44	Welsh Corgi	F.	12.0	13.0	9.7	16.4	25
*M45	Fox Terrier	F.	12.1	8.3	6.1	14.0	19
B31	Beagle	F.	12.4	12.2	5.4	14.7	9
D30	Dalmation	F.	12.5	25.4	2.2	17.4	13
*M32	Pointer	F.	12.8	19.0	7.1	15.4	28
M33	Golden Retriever	M.	12.8	30.0	2.0	16.8	20
B17	Beagle	F.	12.9	11.8	2.5	15.0	19
M24	Fox Terrier	F.	13.0	2.9	4.0	12.3	37
M34	Welsh Corgi	F.	13.1	8.6	6.5	14.1	30
*M37	Golden Retriever	F.	13.1	39.1	5.2	15.4	23
*M39	Welsh Corgi	M.	13.1	12.7	6.3	20.1	22
B32	Beagle	M.	13.6	13.0	3.3	15.7	20
B14	Beagle	F.	13.6	11.3	6.7	16.3	29
M11	Collie-Shep.	F.	15.0	14.5	3.1	12.7	25
*M36	Cocker Spaniel	F.	16.0	19.3	3.7	22.2	22
M3	Fox Terrier	F.	19.0		4.9	12.9	37

Graph 1. Percent pigment volume per myocardial volume vs. age in years for dogs older than 3.5 years.[1] Linear regression data:

$N = 56$
$Y = 0.36X - 0.254 \pm 1.60\%$ pigment vol./myocardial vol.
$b = 0.36 \pm 0.08\%$ pigment vol./myocardial vol./year, $p < 0.01$.
$\bar{Y} = 3.49 \pm 0.21\%$ pigment vol./myocardial vol., $p < 0.01$.
$r = 0.55$, $p < 0.01$.

Of 38 dogs which were under 3.5 years, 18 of these had trace or barely measureable amounts of pigment. Since the pigment in these animals could not be quantified accurately, their data is not included in this graph or in the statistical analysis.

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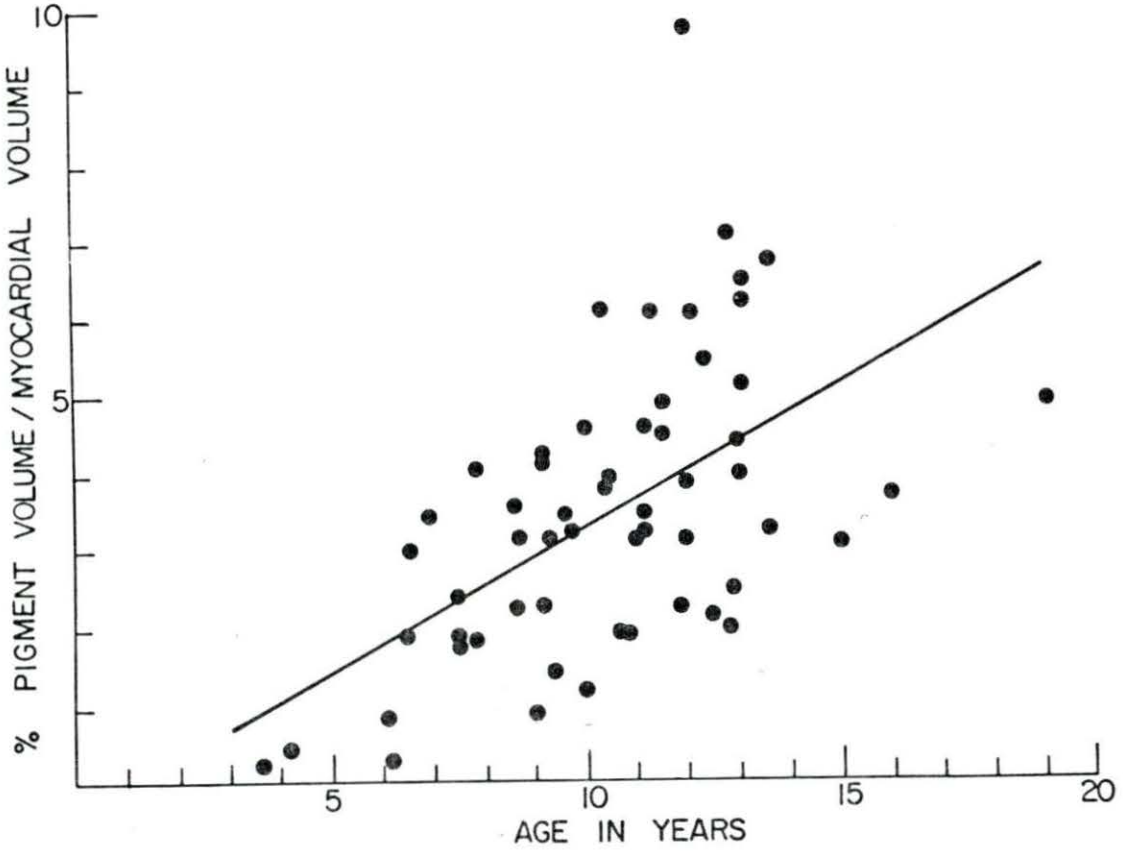
[1] The method involved is described on page 26 of the text.


10
5
% PIGMENT VOLUME / MYOCARDIAL VOLUME
5
10
15
20
AGE IN YEARS

--Graph 2. Fiber diameter in microns vs. age in days for dogs younger than 0.5 years.[1]

Graph 3. Fiber diameter in microns vs. age in years for dogs older than 0.5 years.[1] Linear regression data:

$N = 83$
$Y = 0.16X + 13.34 \pm 2.29$ microns
$b = 0.17 \pm 0.05$ microns/year, $p < 0.01$
$\bar{Y} = 14.53 \pm 0.24$ microns, $p < 0.01$
$r = 0.32$, $p < 0.01$

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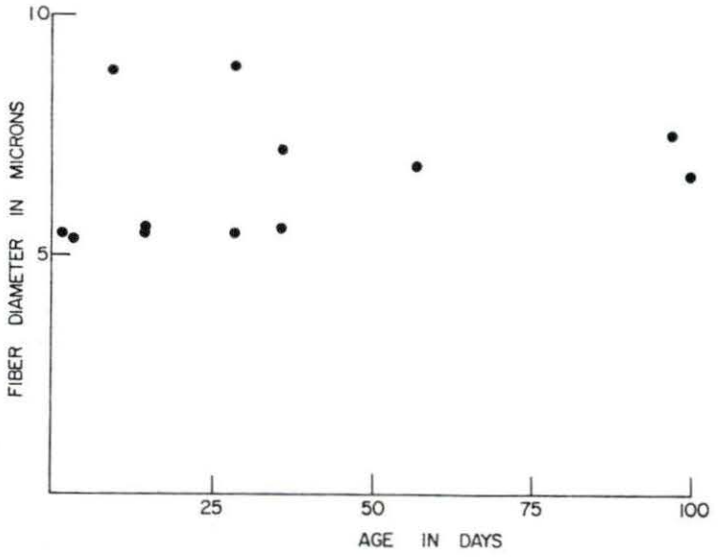
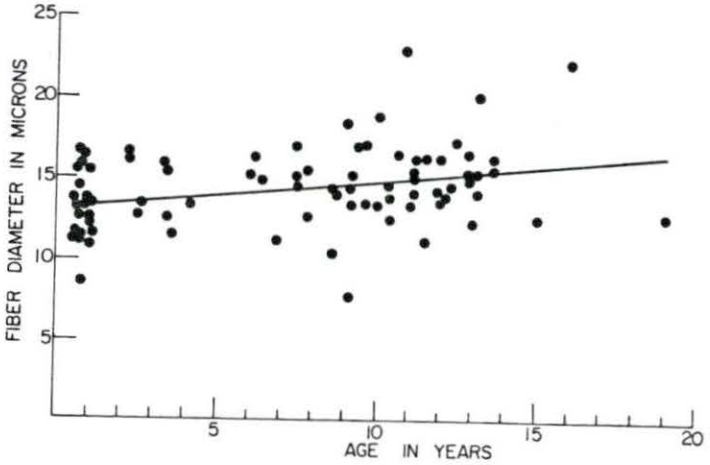
[1]The method involved is described on page 24 of the text.


10
5
FIBER DIAMETER IN MICRONS
25
50
75
100
AGE IN DAYS


25
20
15
10
5
FIBER DIAMETER IN MICRONS
5
10
15
20
AGE IN YEARS

Graph 4. Nuclear count per five fields vs. age in days for dogs younger than 0.5 years.[1]

Graph 5. Nuclear count per five fields vs. age in years for dogs older than 0.5 years.[1] Linear regression data:

$N = 82$
$Y = 42.46 - 1.54X \pm 11.20$ nuclei/five fields
$b = -1.54 \pm 0.26$ nuclei/five fields/year, $p < 0.01$
$\bar{Y} = 31.03 \pm 1.23$ nuclei/five fields, $p < 0.01$
$r = 0.52$, $p < 0.01$

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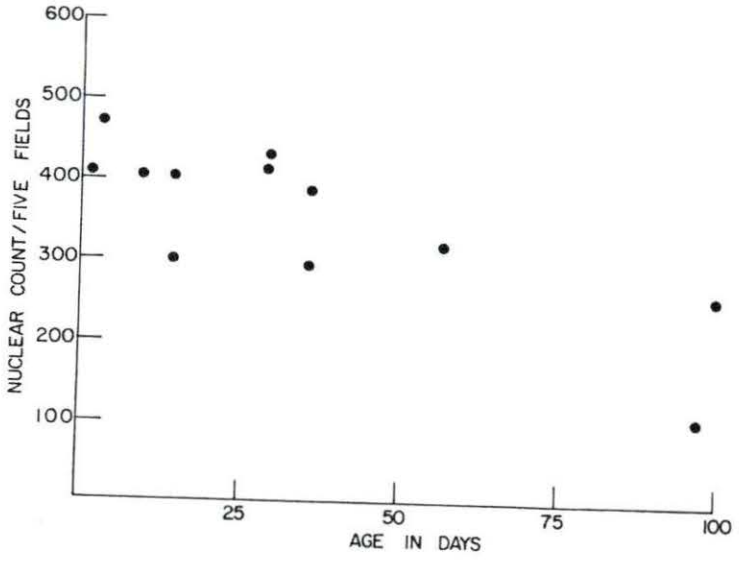
[1]The method involved is described on page 25 of the text.


600
500
400
300
200
100
NUCLEAR COUNT/FIVE FIELDS
25
50
75
100
AGE IN DAYS

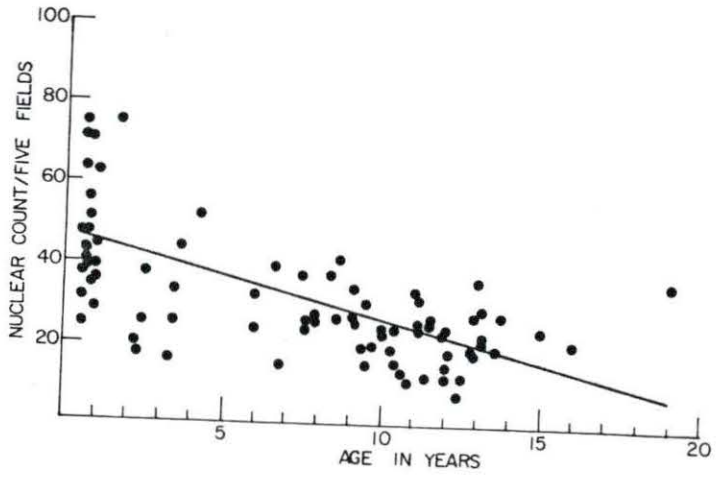
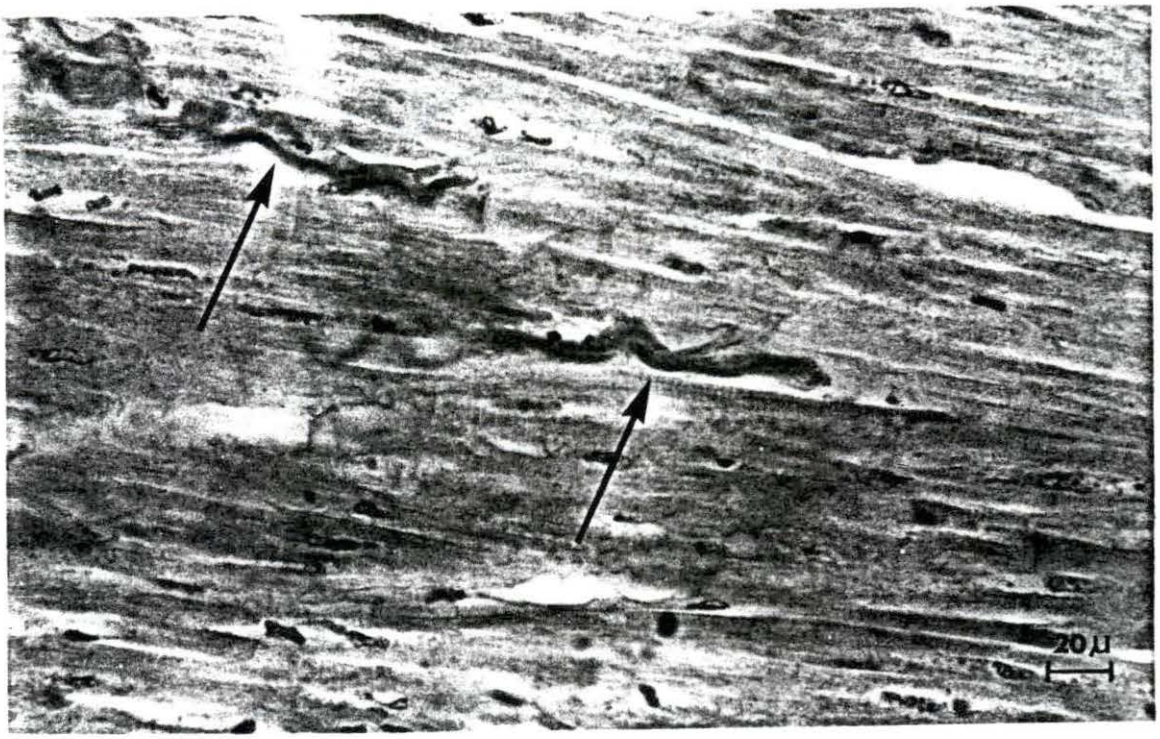

100
80
60
40
20
NUCLEAR COUNT/FIVE FIELDS
5
10
15
20
AGE IN YEARS

Figure 1. Myocardium of right ventricular wall of a 10.0 year old dog (M-8). The arrows point out small bundles of collagen fibers which are widely scattered in the myocardium of older animals. Heidenhain-VanGiesen-Weigert stain.

Figure 2. Purkinje fibers in the right ventricular wall of a 11.2 year old dog (M42). The arrows point out fine elastic fibers which increase in number in this tissue with advancing age. This increase is limited to the Purkinje fiber bundles and is not seen in the myocardium proper. Heidenhain-VanGiesen-Weigert stain.


20μ

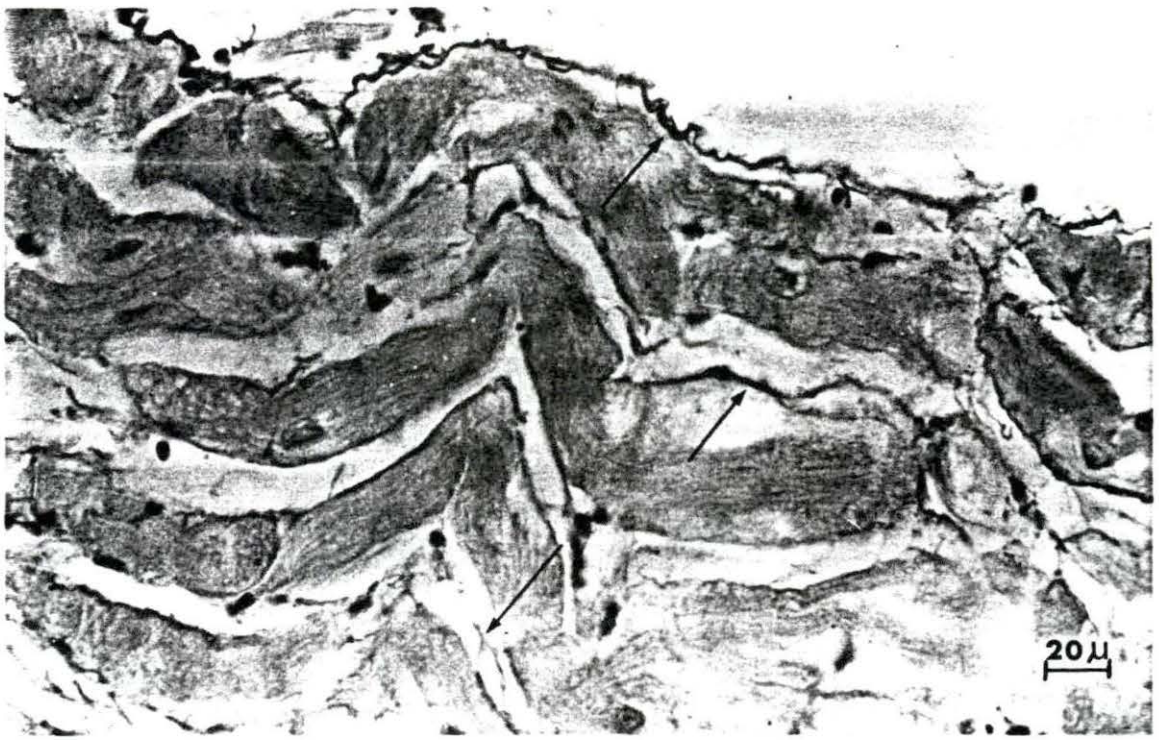
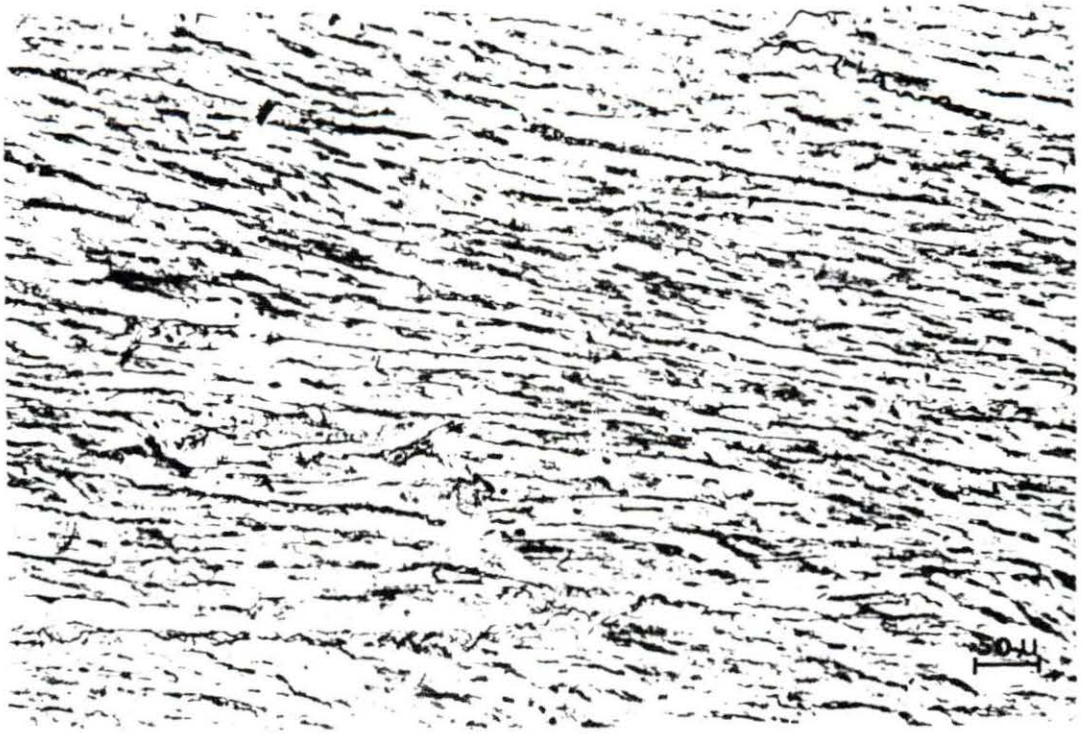

20μ

Figure 3. Myocardium of right ventricular wall of 0.6 year old dog (B-60). The endomysial network of fine reticular fibers is demonstrated and should be compared to that of the older dog seen in Figure 4. Gomori's reticulum stain.

Figure 4. Myocardium of right ventricular wall of 12.0 year old dog (M-44). The density of the reticular network in the endomysium is greater than that seen in younger dogs. Compare to Figure 3. Gomori's reticulum stain.


50 µ


50 µ

Figure 5. Myocardium of right ventricular wall of 13.1 year old dog (M-37). Groups of adipose cells are seen between the fibers of the myocardium. Hematoxylin and eosin stain.

Figure 6. Myocardium of right ventricular wall of 9.0 year old dog (M-29). This was the most advanced fatty infiltration noted in the animals studied. The major portion of the ventricular wall in this section was replaced by fat. Mallory's triple stain.

Figure 7. Myocardium of right ventricular wall of a 13.1 year old dog (M-37). This section demonstrates adipose cells between the fibers of the myocardium proper as well as a large accumulation of such cells around the sub-endocardial Purkinje fibers at the left border of the section. Hematoxylin and eosin stain.


500 µ


500 µ

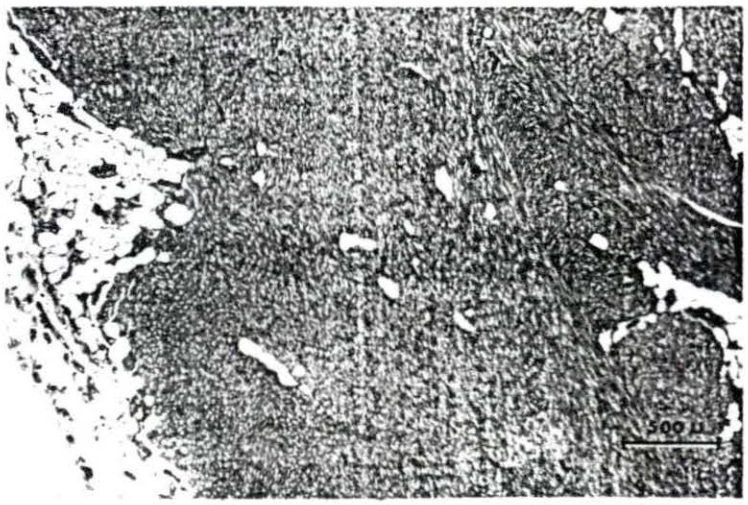
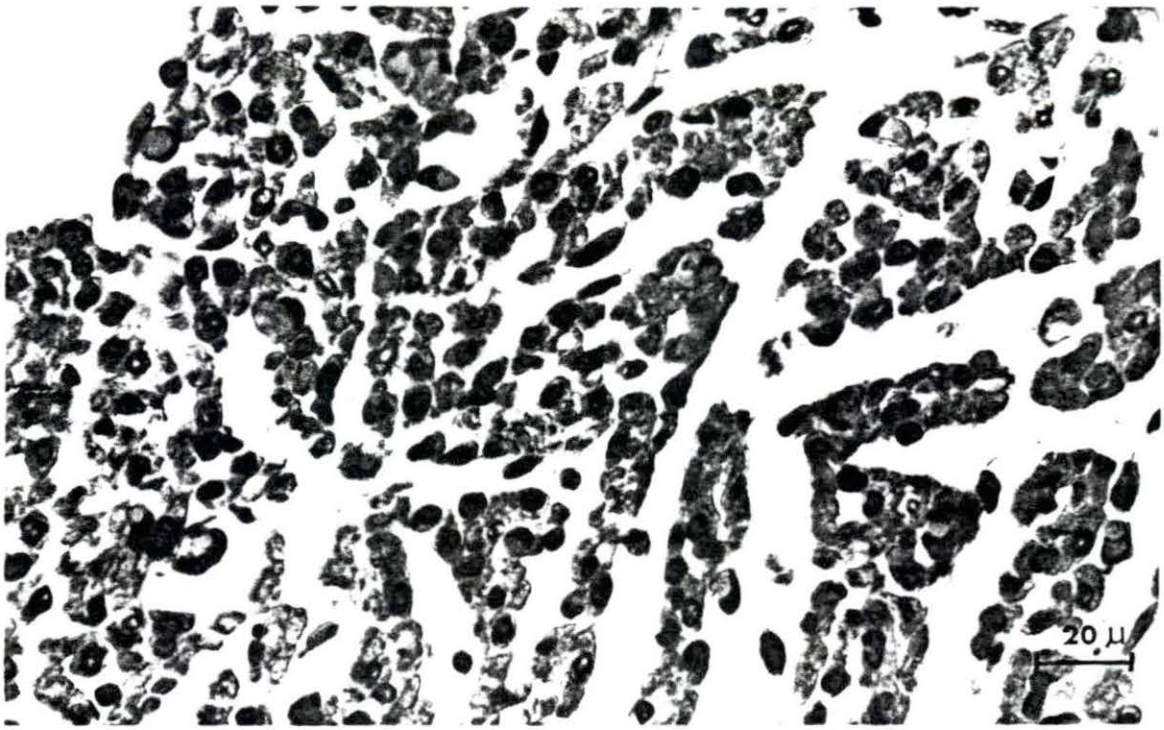

500 µ

Figure 8. Myocardium of right ventricular wall of a 2 day old dog. (B-38). Muscle fibers were oriented so that they were sectioned transversely. Such an orientation was used in measuring cross-sectional diameters. The average diameter of the fibers in the section from which this micrograph was made was 5.4 microns. Hematoxylin and eosin stain.

Figure 9. Myocardium of the right ventricular myocardium of a 0.7 year old dog (A-37). The muscle fibers were oriented similarly to those seen in Figure 8, and were photographed at the same magnification so that size differences could be noted. The arrows indicate the diameters that would have been measured in two of the fibers. The average fiber diameter in the section from which this micrograph was made was 15.9 microns. Hematoxylin and eosin stain.


20 µ

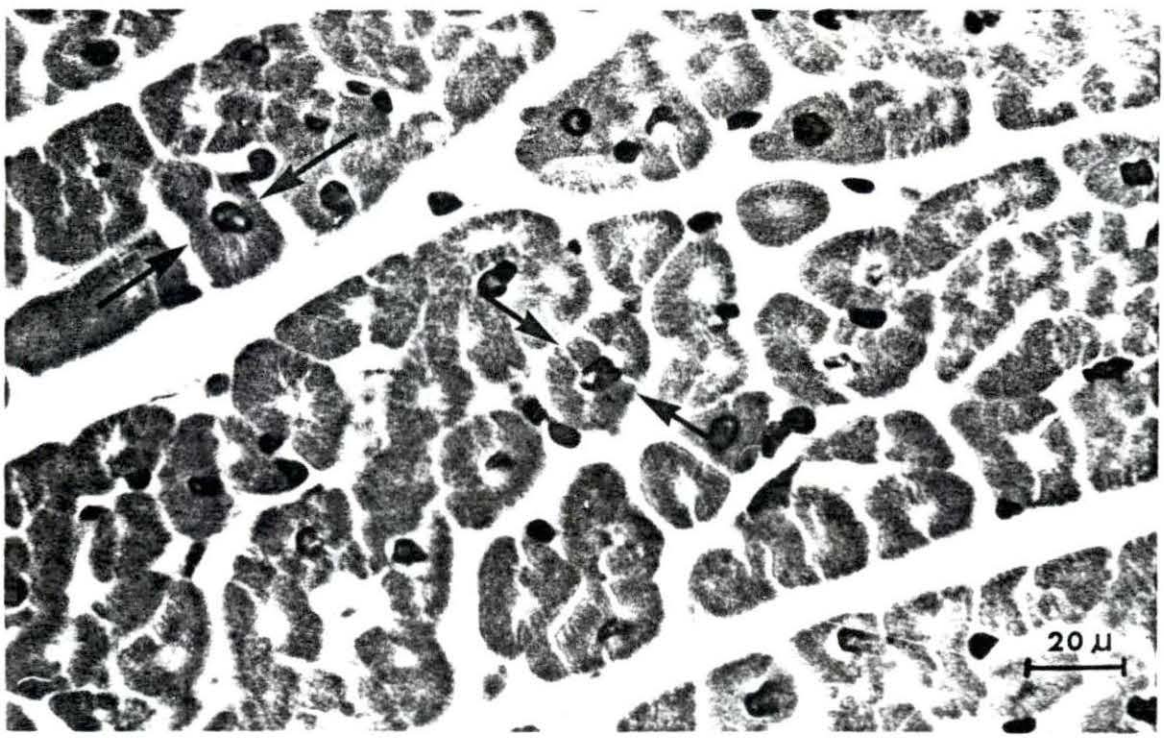

20 µ

Figure 10. Myocardium of right ventricular wall of a 28 day old dog (B-46). The fibers were oriented so that they were sectioned longitudinally. Such an orientation was used to quantify the number of nuclei per unit area. The nuclear concentration demonstrated in this micrograph was typical of animals less than 6 months of age. The actual count in the section from which this micrograph was made was 418 nuclei per five limited fields. Hematoxylin and eosin stain.

Figure 11. Myocardium of the right ventricular wall of an 8.7 year old dog. The fibers were oriented similarly to those seen in Figure 10, and were photographed at the same magnification to show the comparative difference in nuclear concentration. The actual count in the section from which this micrograph was made was 52 nuclei per five limited fields. Hematoxylin and eosin stain.


20

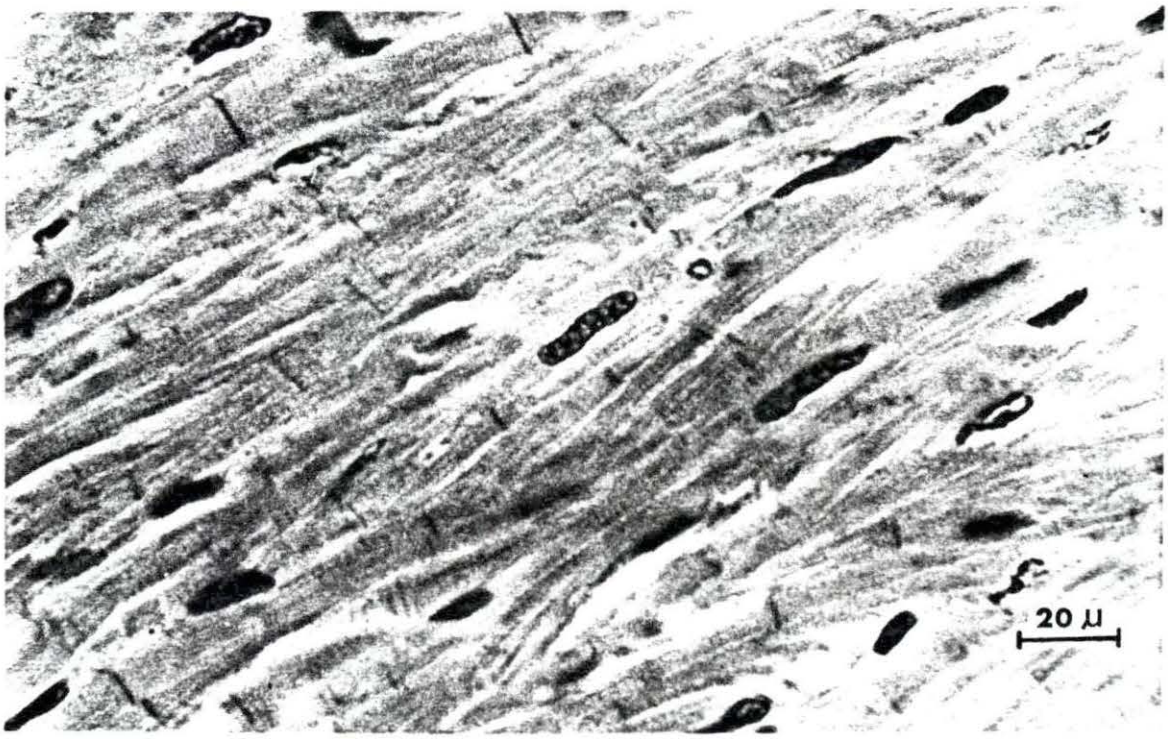

20 µ

Figure 12. Nuclei and peri-nuclear region of myocardial cells from the right ventricular wall of aged dogs.

A. and B. - Lobulated nuclei from 11.3 year old dog (M-43).

C., D., E., and F. - Portions of binucleate cells from 11.5 year old dog (M-9).

Hematoxylin and eosin stain.

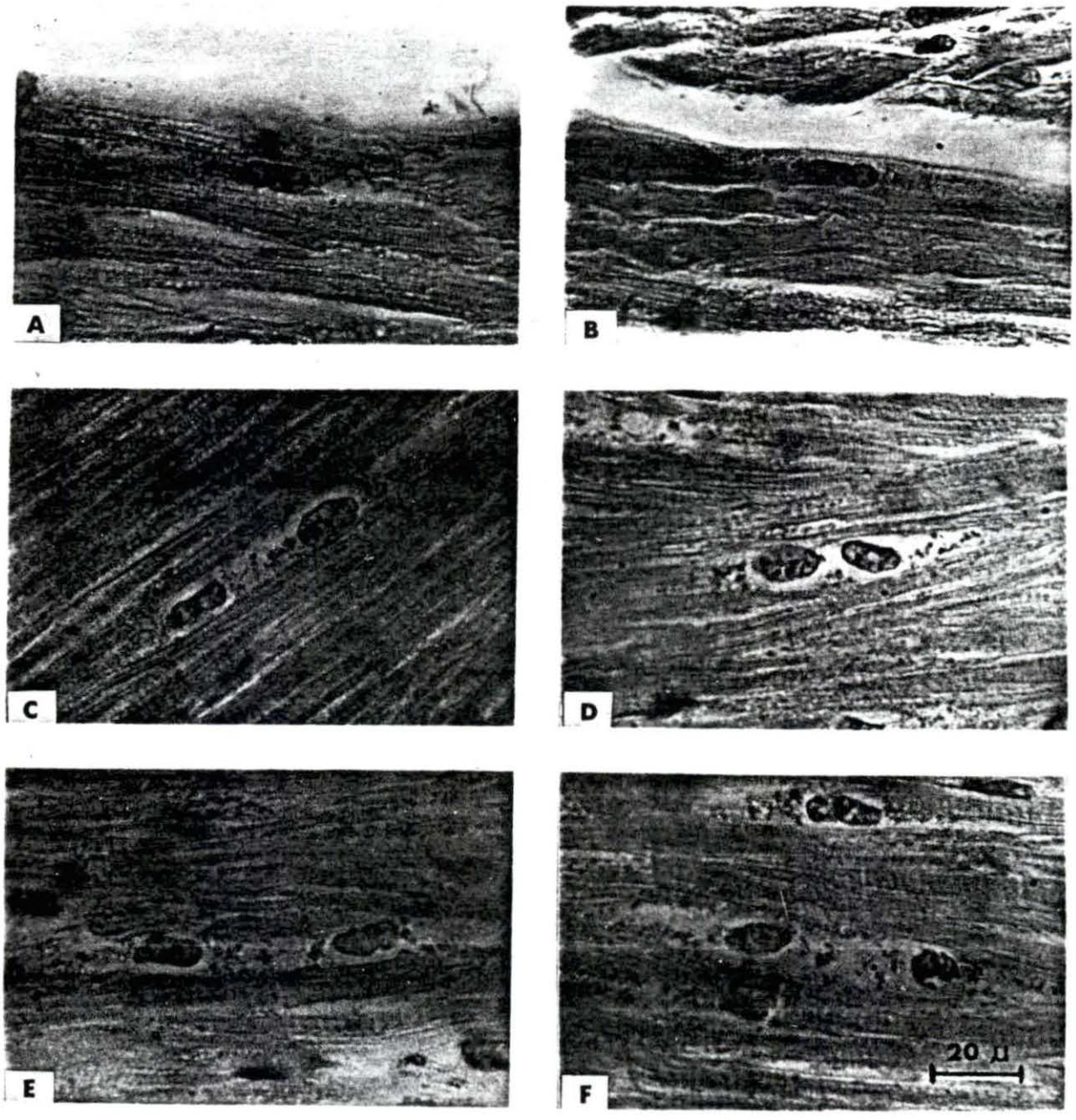
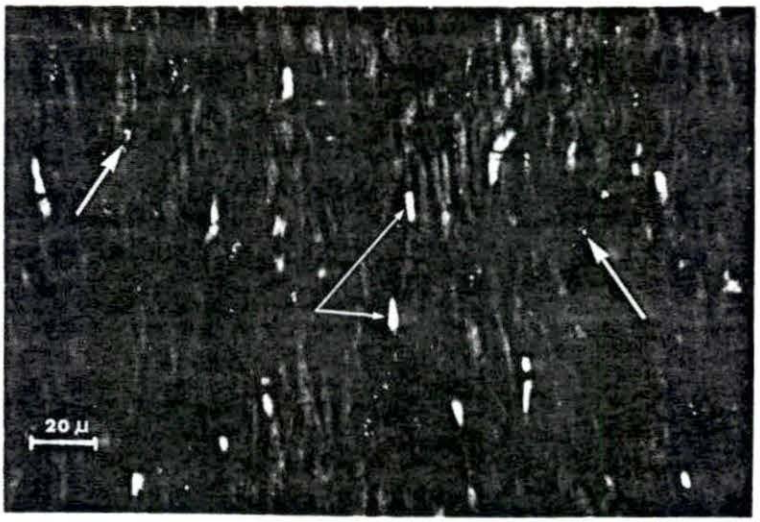
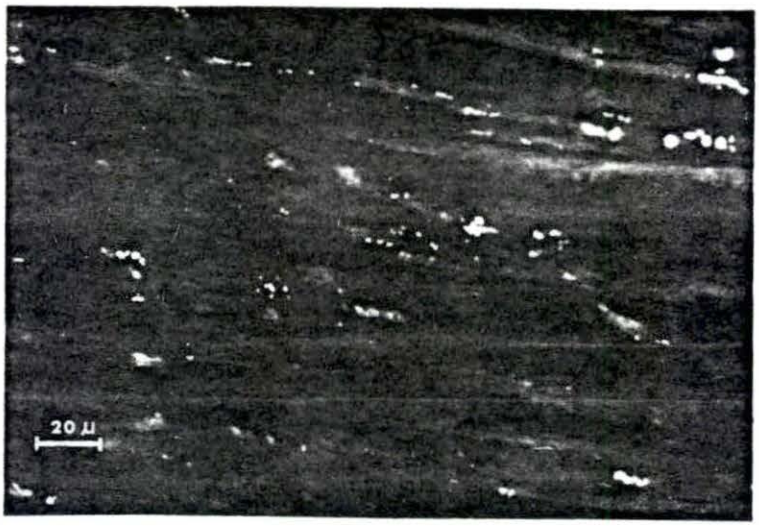

A
B
C
D
E
F
20 µ

Figure 13. Fluorescence micrograph of a section from the right ventricular wall of a 7.5 year old dog (M-20). The pigment appears as small granules, often forming clumps (large arrows). It was distinguished from the RBC's (small arrows) by its color and morphology. The pigment volume in the heart from which this section was taken was calculated to be 1.7% of the myocardial volume.

Figure 14. Fluorescence micrograph of a section from the right ventricular wall of an 11.3 year old dog. The pigment is very evident and appears to form larger clumps than those seen in Figure 13. The pigment volume in this dog was calculated to be 6.1% of the myocardial volume.

Figure 15. Fluorescence micrograph of a section from the right ventricular wall of a 12 year old dog (M-44). A tendency to form still larger clumps is noted with greater pigment accumulation. The pigment volume in this dog was the greatest of any in the dogs studied, equaling 9.7% of the myocardial volume.


20 μ


20 μ

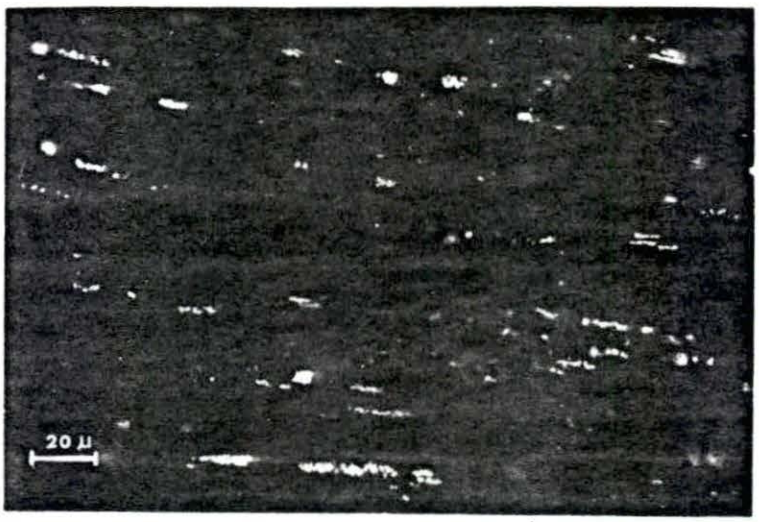

20 μ

Figure 16. A fluorescence micrograph of Purkinje fibers from the right ventricular wall of a 12.0 year old dog (M-44). Some pigment was observed to accumulate in these fibers but to a lesser extent than in the myocardium proper. This can be seen by comparing this micrograph with those in Figures 15 and 17.

Figure 17. A fluorescence micrograph of a section of the myocardium proper from the right ventricular wall of the same animal described in Figures 15 and 16. The distinct clumping noted in this section is typical and occurs at the poles of the nuclei.


20μ


20μ

Figure 18. Electron photomicrograph of the peri-nuclear region of a myocardial cell from a 0.7 year old dog (B48). The ovoid dense bodies are lysosomes in varying stages of activity. The inactive or primary lysosomes (PL) have a granular matrix of a fairly uniform density. Others exhibit circumscribed areas of less density (V) within their matrix which give them a vacuolated appearance. These are considered to be active lysosomes (AL). N, nucleus. M, mitochondrion. Methacrylate embedding, $KMnO_4$ stain.

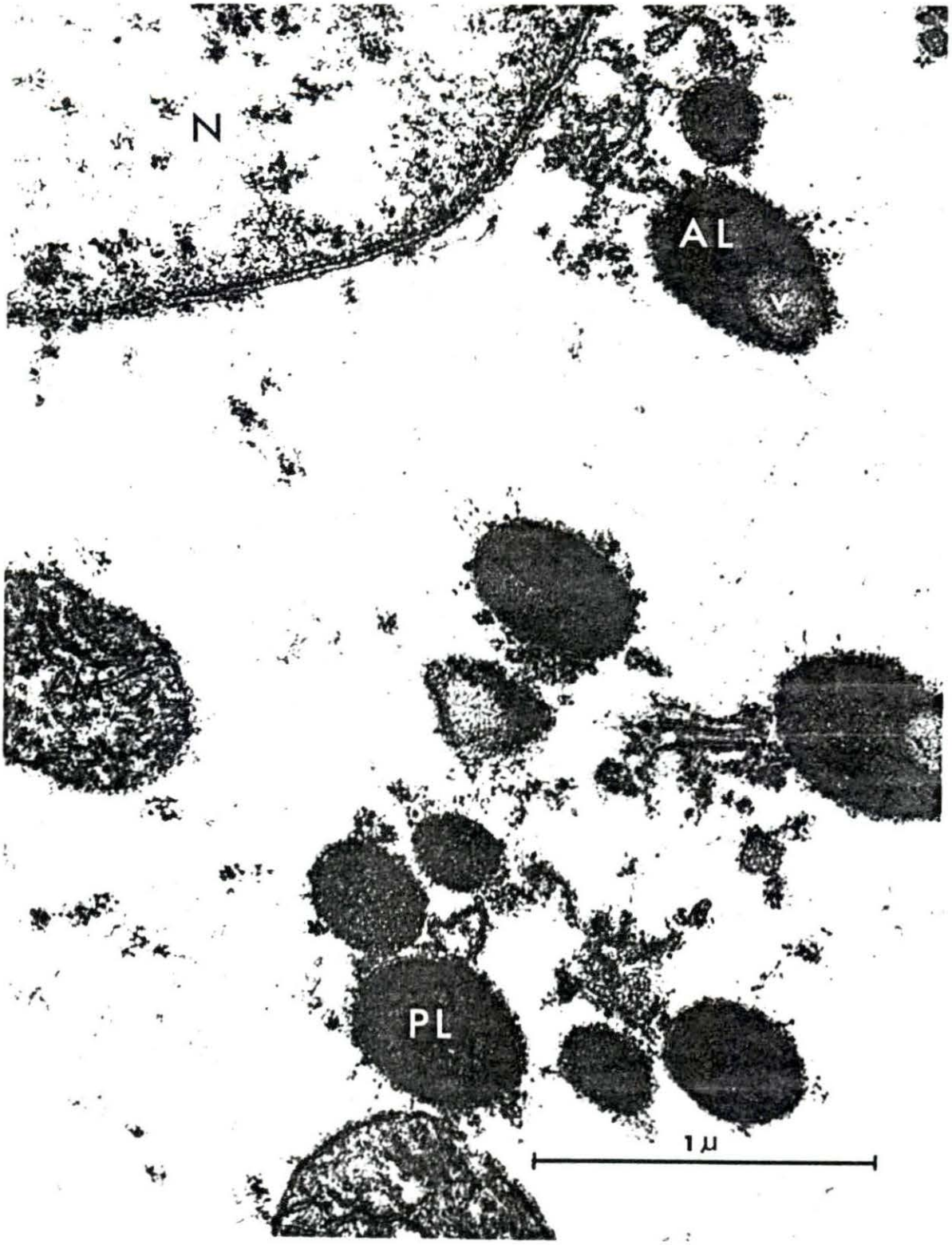

N
AL
V
PL
1μ

Figure 19. Peri-nuclear region of a myocardial cell from a 0.7 year old dog (A37). The "vacuolated area" in the active lysosome is larger than those seen in Figure 18. A thin rim of very dense material around the "vacuole" gives the appearance of a limiting membrane, but examination at higher magnification reveals this not to be the case. D.E.R. embedding, lead citrate stain.

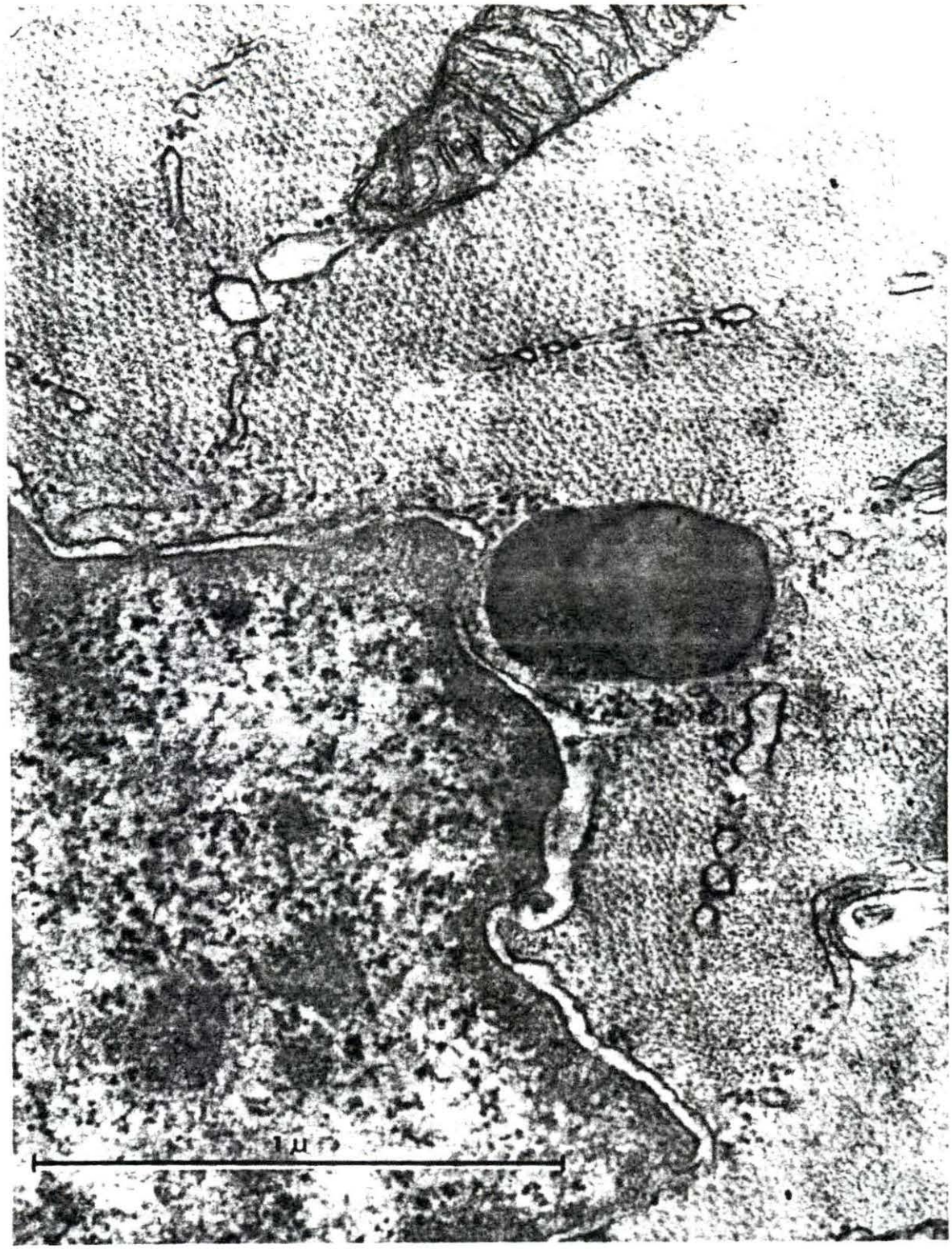

1 μ

Figure 20. A group of active lysosomes in the perinuclear area of a myocardial cell from a 0.7 year old dog (A23). The "vacuolated areas" in two of these structures have enlarged to an extent where the dense matrix of the lysosomes is limited to a narrow rim around the periphery. D.E.R. embedding, lead citrate stain.

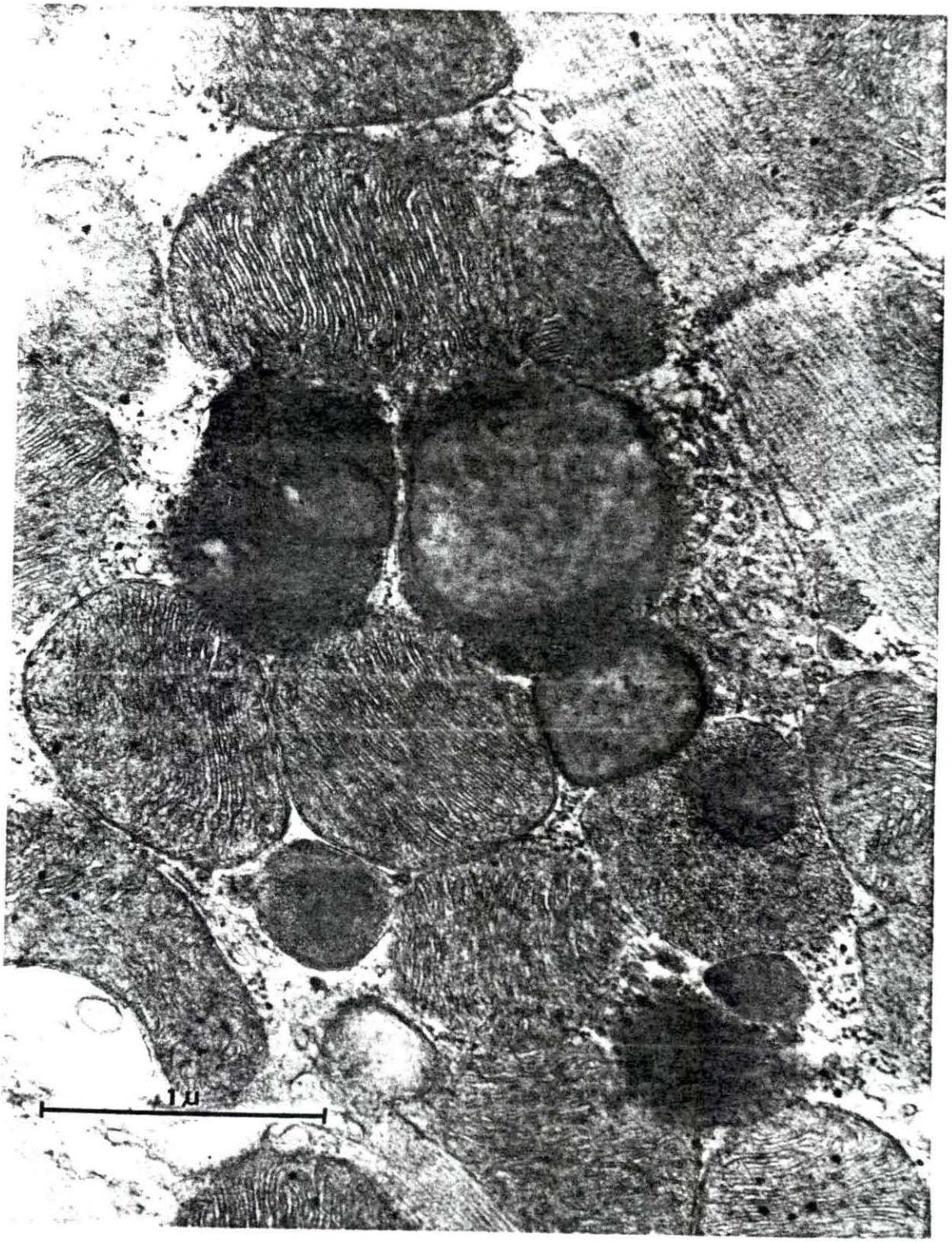

1 μ

Figure 21. Peri-nuclear region of a myocardial cell from a 9.9 year old dog (M46), showing active lysosomes and dense pigment granules (P). Filamentous material (F) is unidentified. D.E.R. embedding, lead citrate stain.

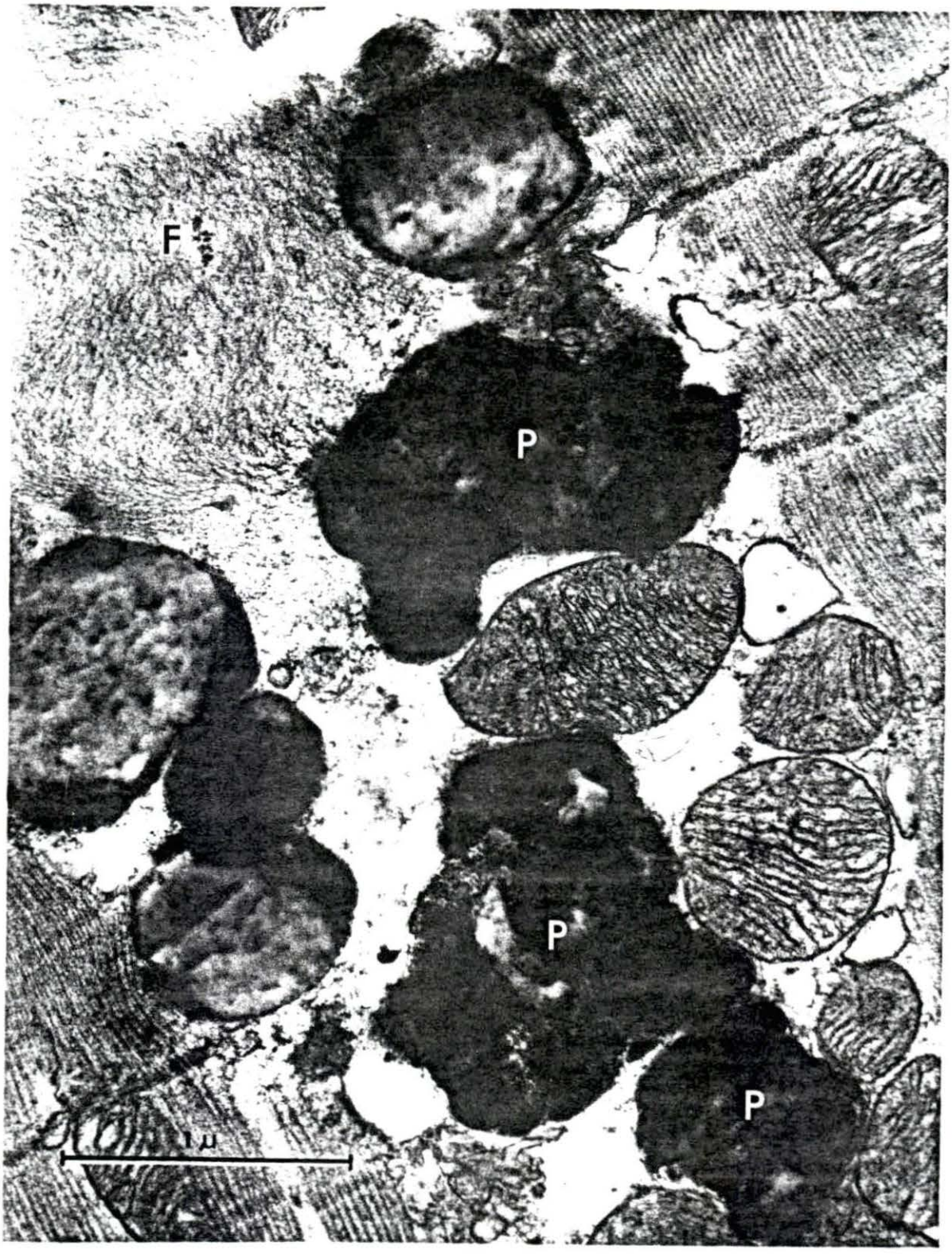

F
P
P
P
1μ

Figure 22. Peri-nuclear region of a myocardial cell from a 12.8 year old dog (M32) showing highly "vacuolated" lysosomes appearing to coalesce, forming larger structures which can then be termed pigment granules. Methacrylate embedding, $KMnO_4$ stain.

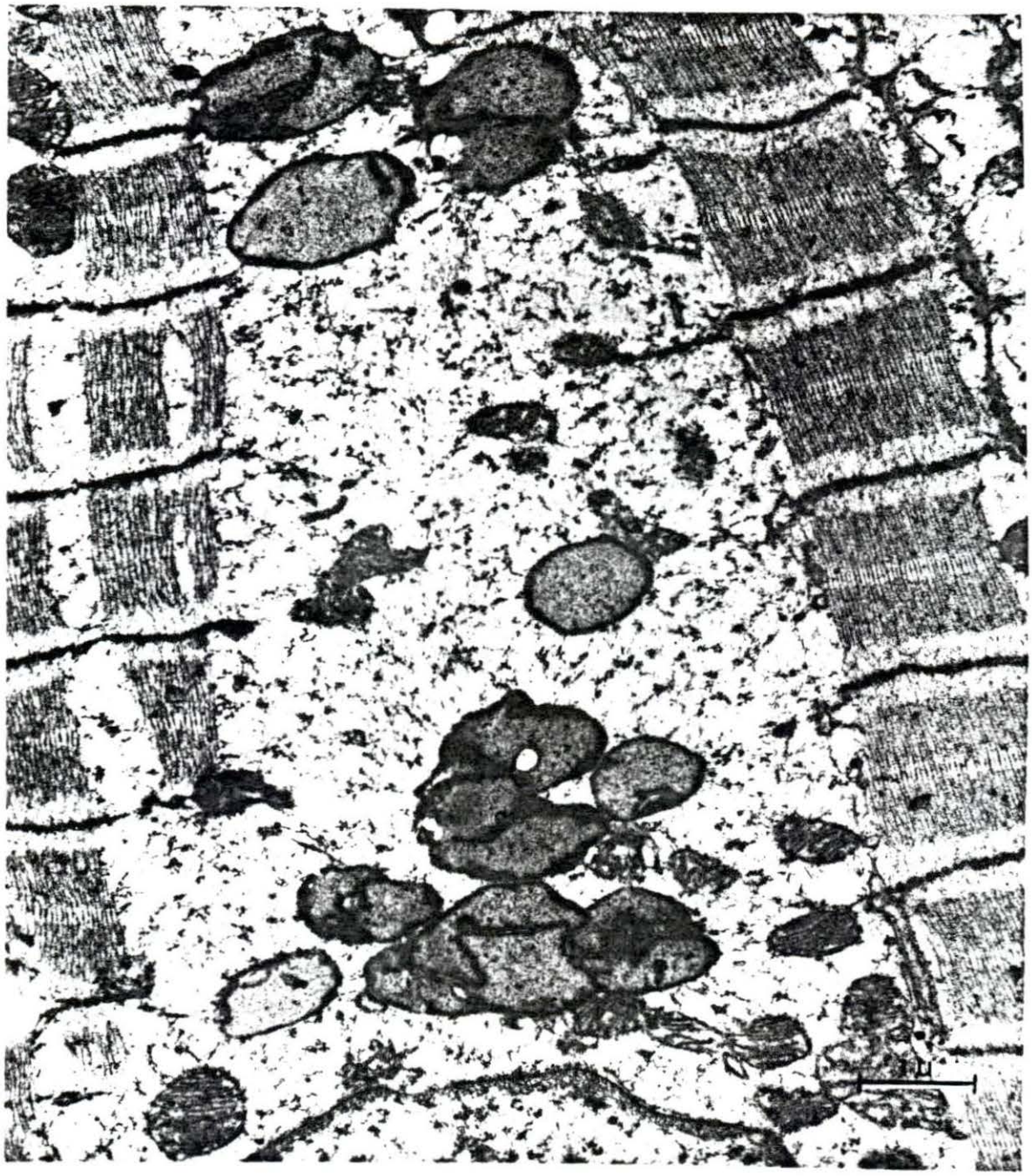


Figure 23. Peri-nuclear region of a myocardial cell from an 11.2 year old dog (M42). This micrograph demonstrates the typical location and morphology of the "mature" pigment granules. Golgi membranes can be noted at the arrow. The fine dense particulate matter distributed rather evenly throughout the sarcoplasm is glycogen. D.E.R. embedding, lead citrate stain.


1μ

Figure 24. Three pigment granules from peri-nuclear region of a myocardial cell from an 11.2 year old dog (M42). The lobulated appearance of these granules indicates the possibility of their being formed by fusion of smaller structures. Layered membranes as seen at the black wedges are associated with the surface of one of the granules and may be continuous with the Golgi membranes (G). Evidence suggestive of this continuity may be seen at the black arrow. Similar lamellated membranes may have been responsible for the myelin-like configurations within the granules, pointed out by the white wedges. A multi-vesicular body (Mv) and a centriole (C) are also seen in this micrograph. D.E.R. embedding, lead citrate stain.

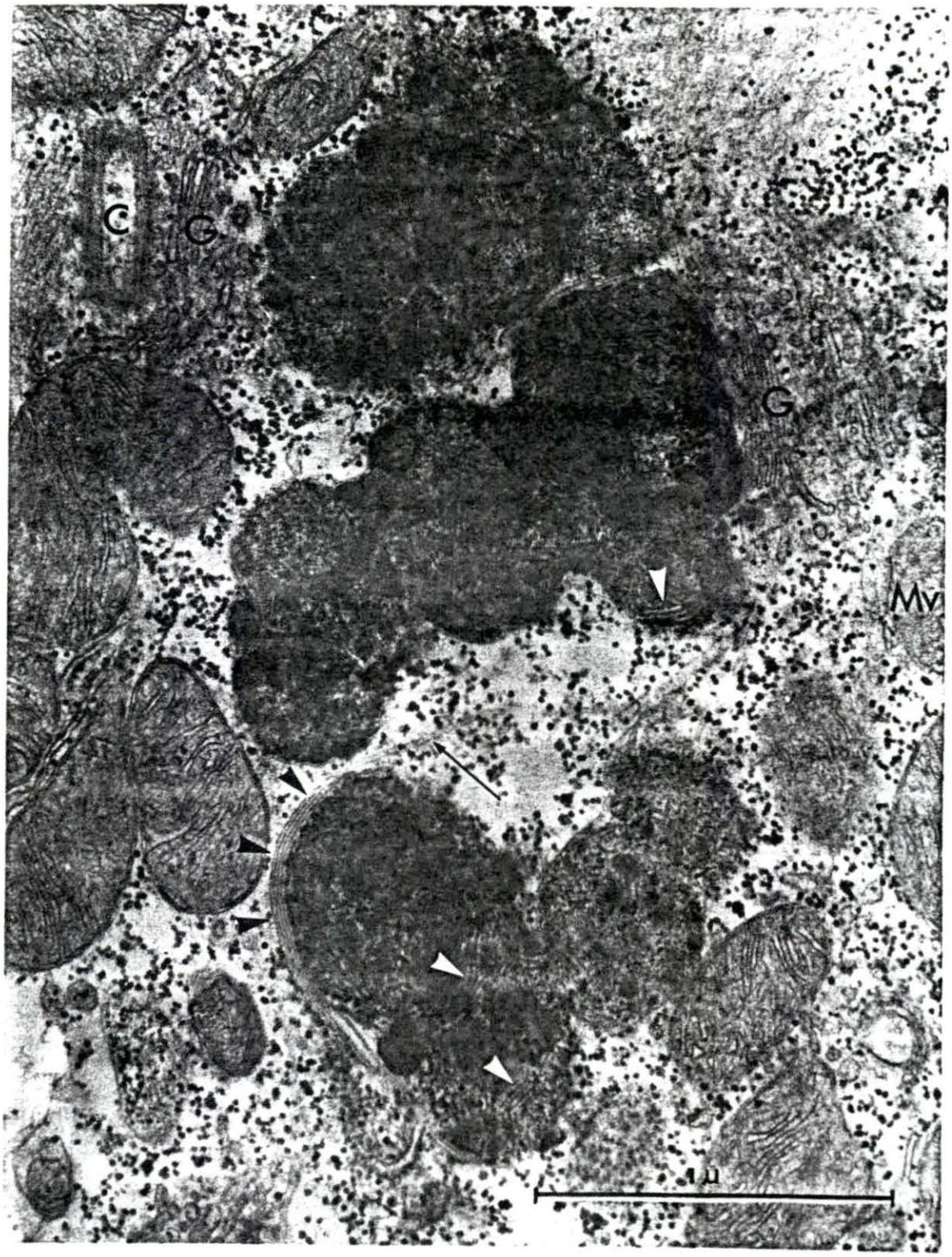

G
G
G
Mv
1 μ

Figure 25. Lobulated pigment granule in the peri-nuclear region from the heart of an 11.2 year old dog (M42). Of interest here is the membrane (arrows) which appears to limit not only the pigment granule but an area of the surrounding cytoplasm as well. This area, plus the pigment granule, is postulated to be the site of a high degree of hydrolytic activity and could reasonably be termed a cytosegrosome. D.E.R. embedding, lead citrate stain.


1 μ

Figure 26. Peri-nuclear region from the myocardium of a 9.7 year old dog (M40). The varying densities and myelin-like configurations typical of lipofuscin can be noted in these granules. The two amorphous dense bodies (DB) are too large to be considered primary lysosomes. They are the size and shape of mitochondria. The fact that a portion of a mitochondrion (M) exhibits an internal structure very similar to that of the dense bodies gives some evidence on which to postulate that these dense bodies may be derived from mitochondria by hydrolytic breakdown of their internal structure. The membrane noted at the black wedges appears to be limiting some areas of the cytoplasm along with the pigment granule. Maraglas embedding, lead citrate stain.

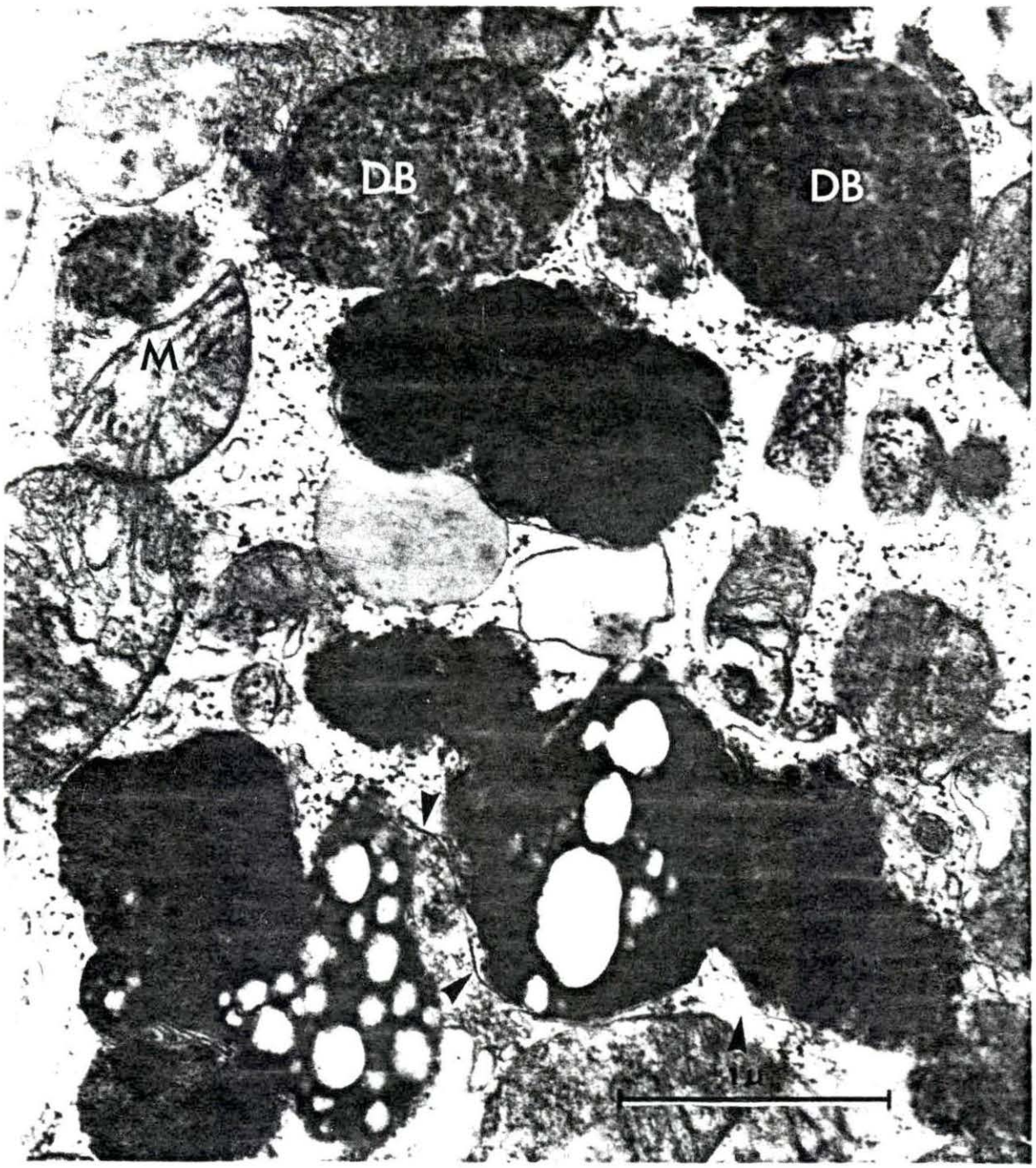
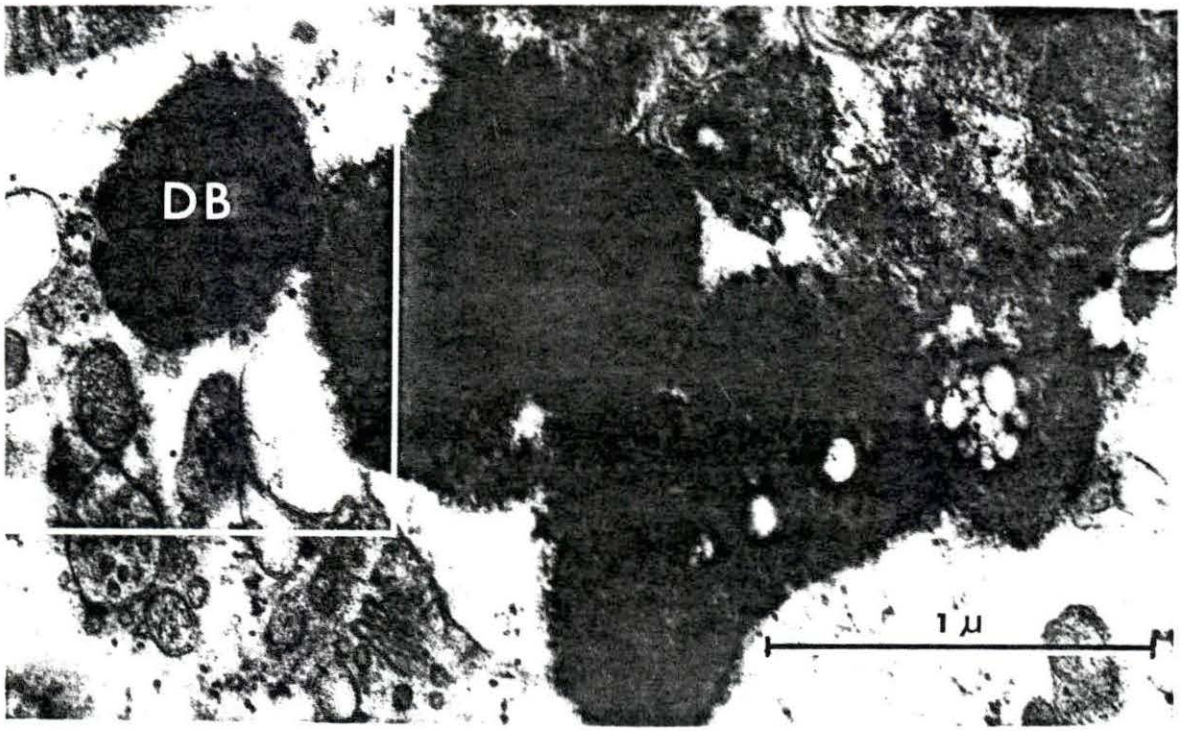

DB
DB
M
1μ

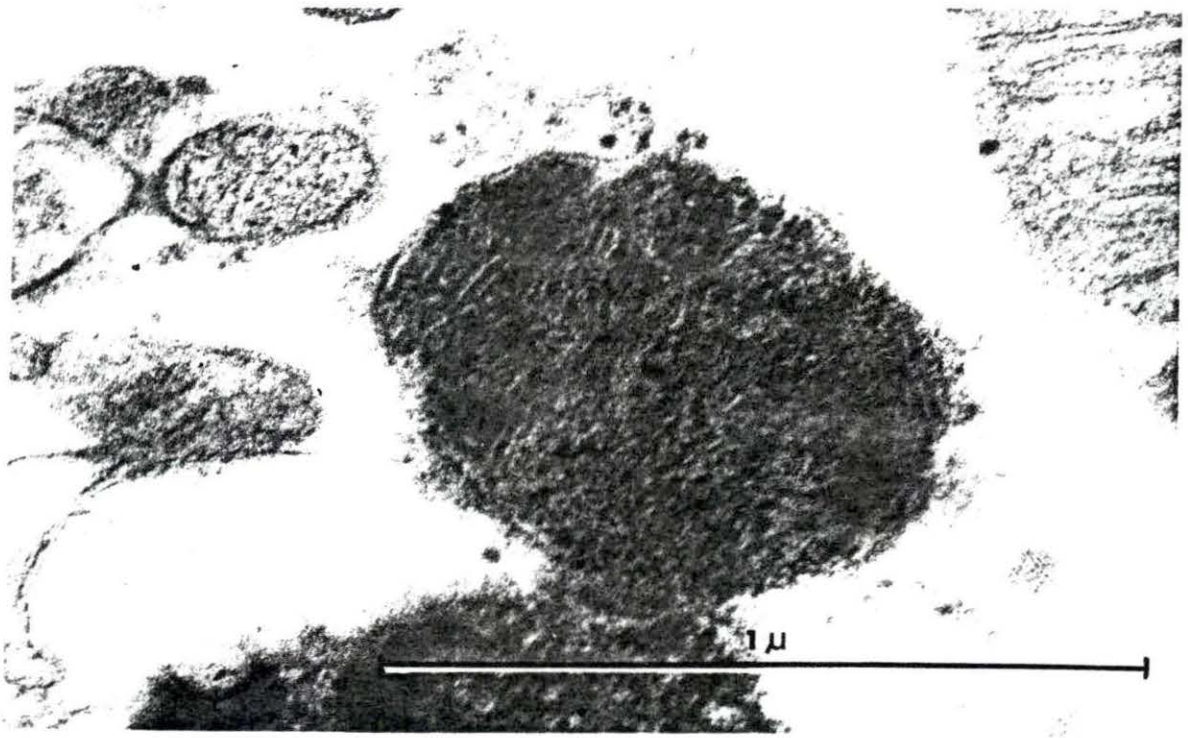
Figure 27. A large pigment granule in the cytoplasm of a myocardial cell from a 10.5 year old dog (M48). Of interest is the disruption of the internal structure of the mitochondria (M_1,M_2,M_3). At the arrow, membranes of what appears to be a disrupted mitochondrion are very closely related to the surface of the pigment granule and may actually be in the process of being incorporated into it. D.E.R. embedding, lead citrate stain.


M2
M3
M1

Figure 28. Lobulated pigment granule from the myocardium of a 9.7 year old dog (M40). The ovoid dense body (DB) exhibits some remnant of an internal membranous structure similar to that of a mitochondrion. It appears to be fusing with and becoming a part of the pigment granule. The portion of the micrograph enclosed by the white lines is enlarged in Figure 29. Methacrylate embedding, lead citrate stain.

Figure 29. An enlargement of the area indicated in Figure 28.


DB
1 μ


1 μ

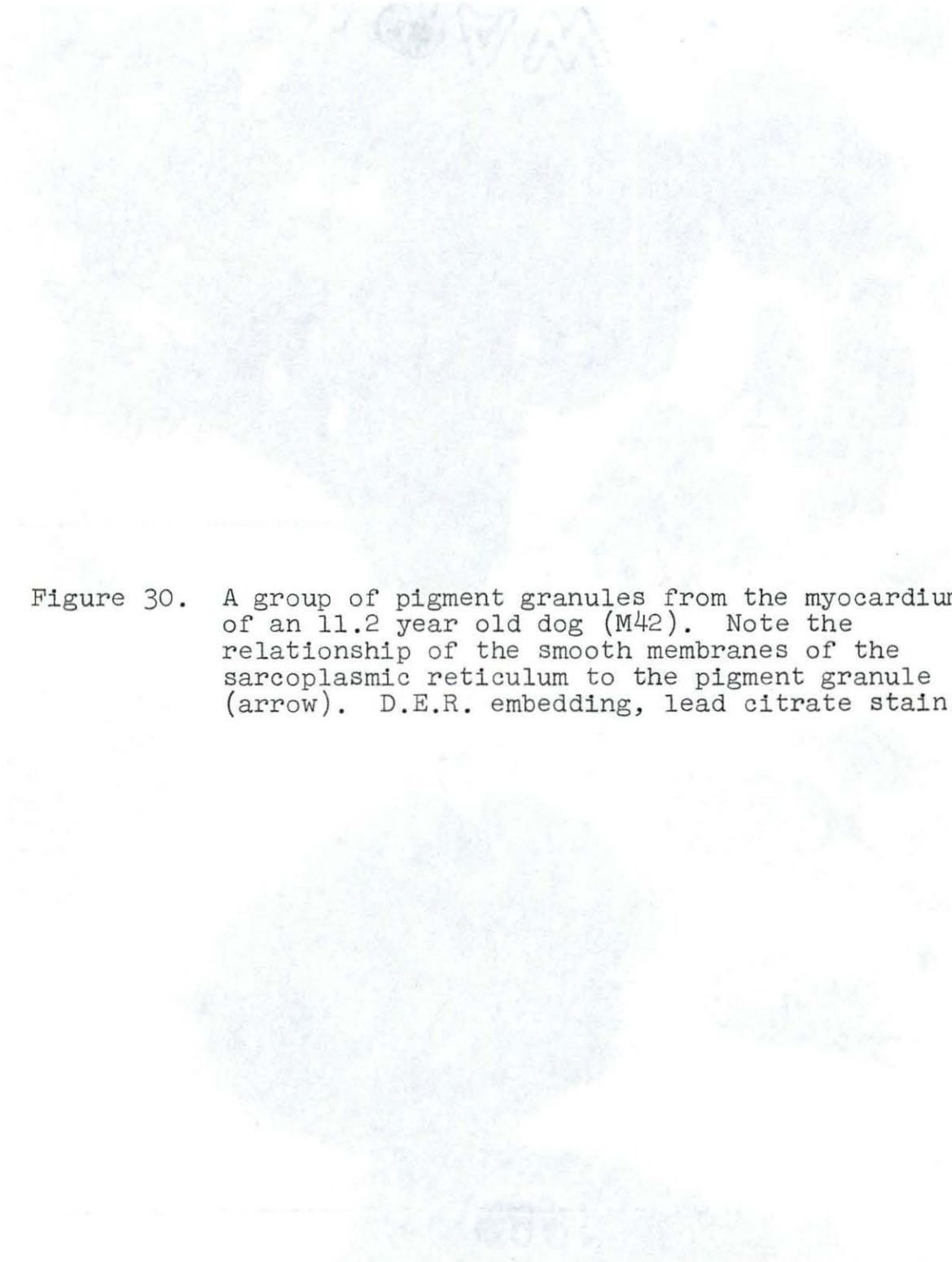


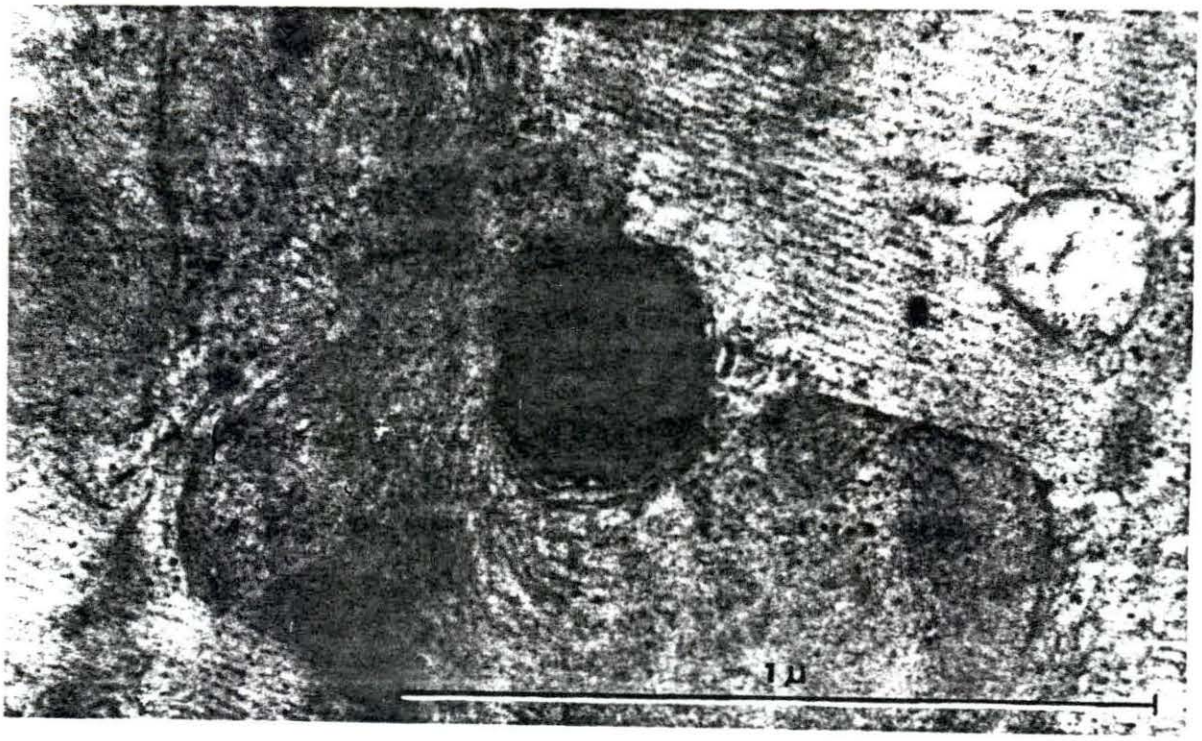
Figure 30. A group of pigment granules from the myocardium of an 11.2 year old dog (M42). Note the relationship of the smooth membranes of the sarcoplasmic reticulum to the pigment granule (arrow). D.E.R. embedding, lead citrate stain.

Figure 31. An active lysosome in the myocardium of a dog 0.6 years old (A41). In this case it appears that the limiting membrane of the lysosome may be continuous with the membranes of the sarcoplasmic reticulum (Arrow). D.E.R. embedding, lead citrate stain.


μ

Figure 32. A primary lysosome from the myocardium of a 10.5 year old dog (M48). This tissue has been incubated 30 minutes in the Gormori medium for acid phosphatase localization. The lysosome is filled with a dense accumulation of the lead phosphate precipitate indicating the site of acid phosphatase activity. D.E.R. embedding, unstained.

Figure 33. Lipofuscin pigment granules from the same tissue, handled in the same manner as that described in Figure 32. Note the concentration of the lead phosphate precipitate in the granules. D.E.R. embedding, unstained.


1μ

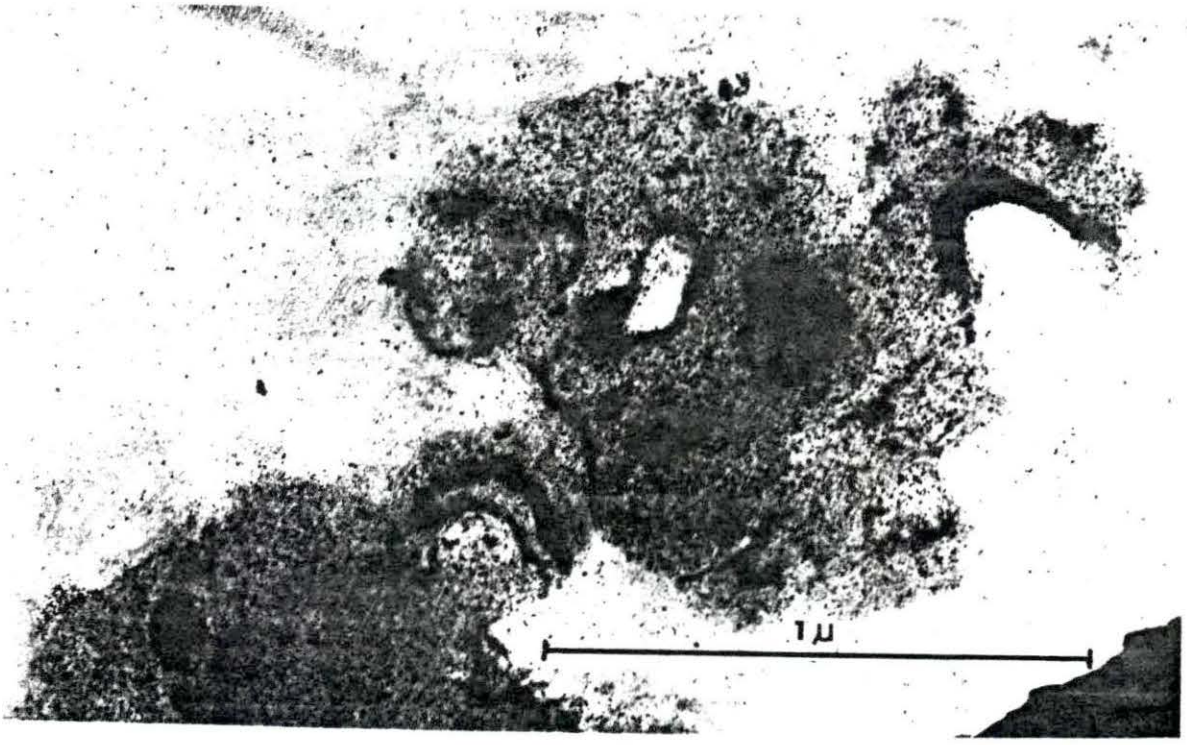
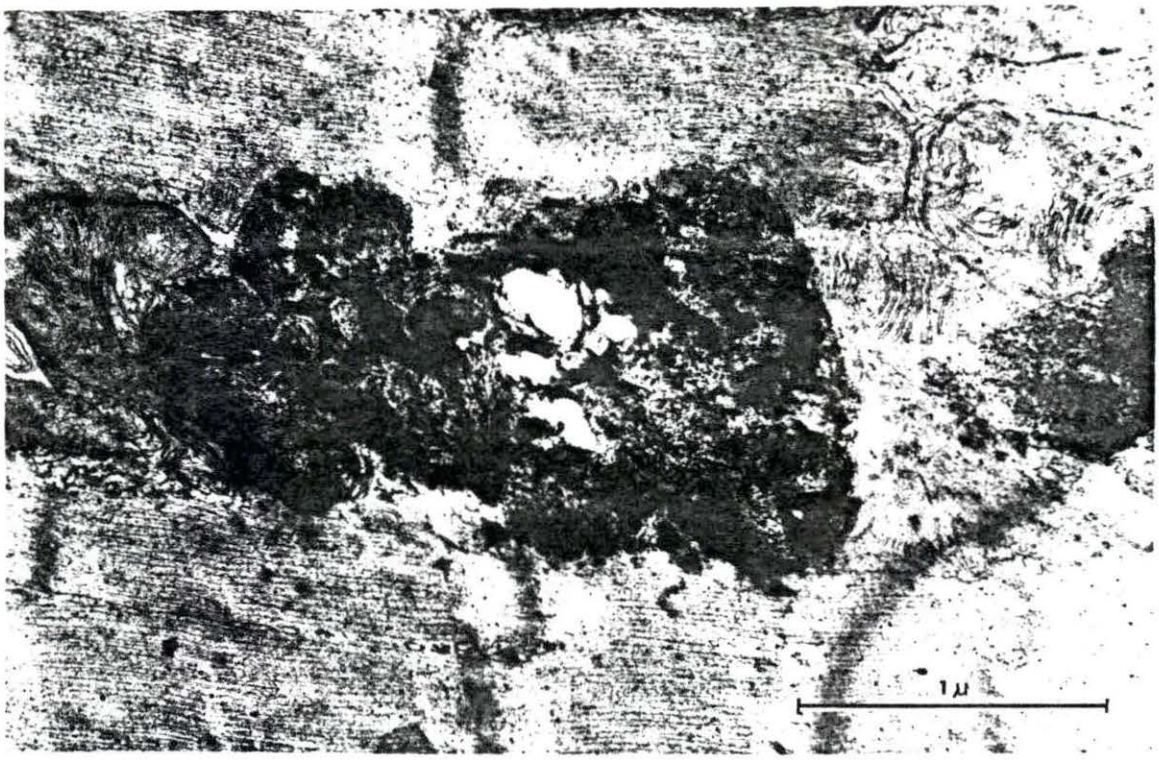

1μ

Figure 34. A lipofuscin pigment granule from the heart of a 10.5 year old dog (M48) showing the localization of the lead phosphate precipitate in the granule following incubation in the Gomori medium for acid phosphatase localization. D.E.R. embedding, light staining with lead citrate.

Figure 35. Same preparation as described for Figure 34. Note the precipitate in the mitochondria adjacent to the pigment granule. Whether or not this is due to artifact has not been established.


1μ

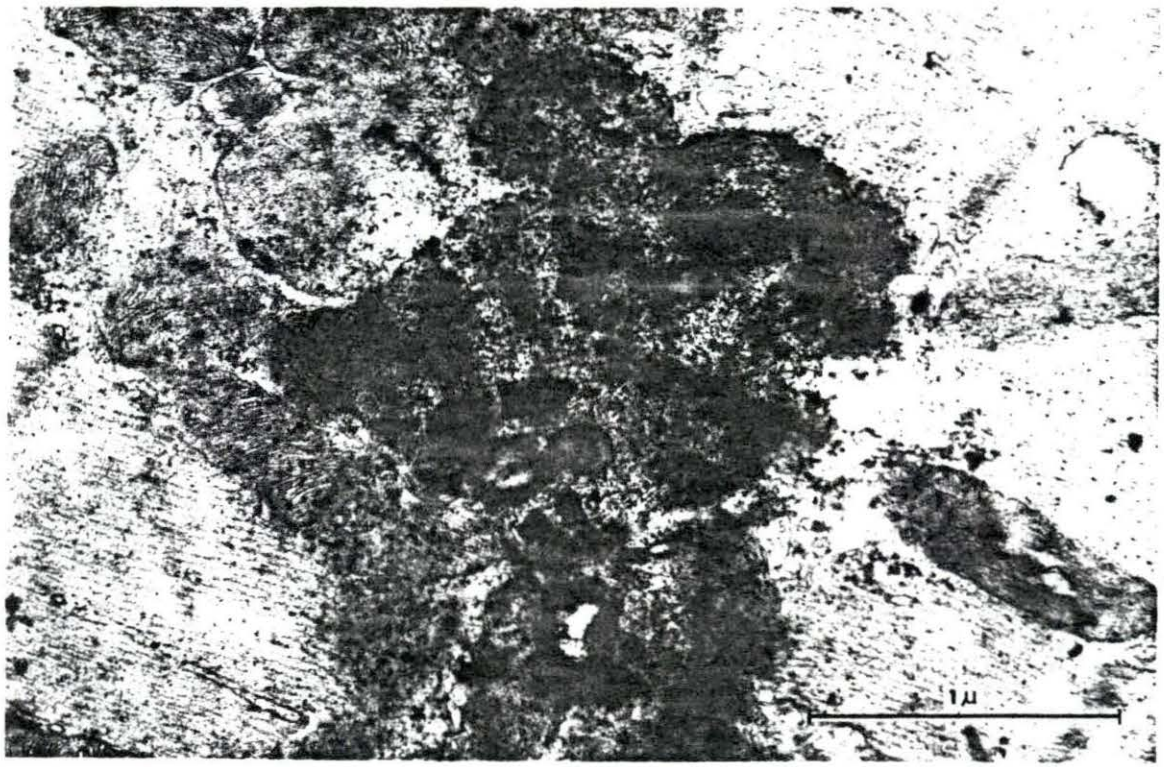
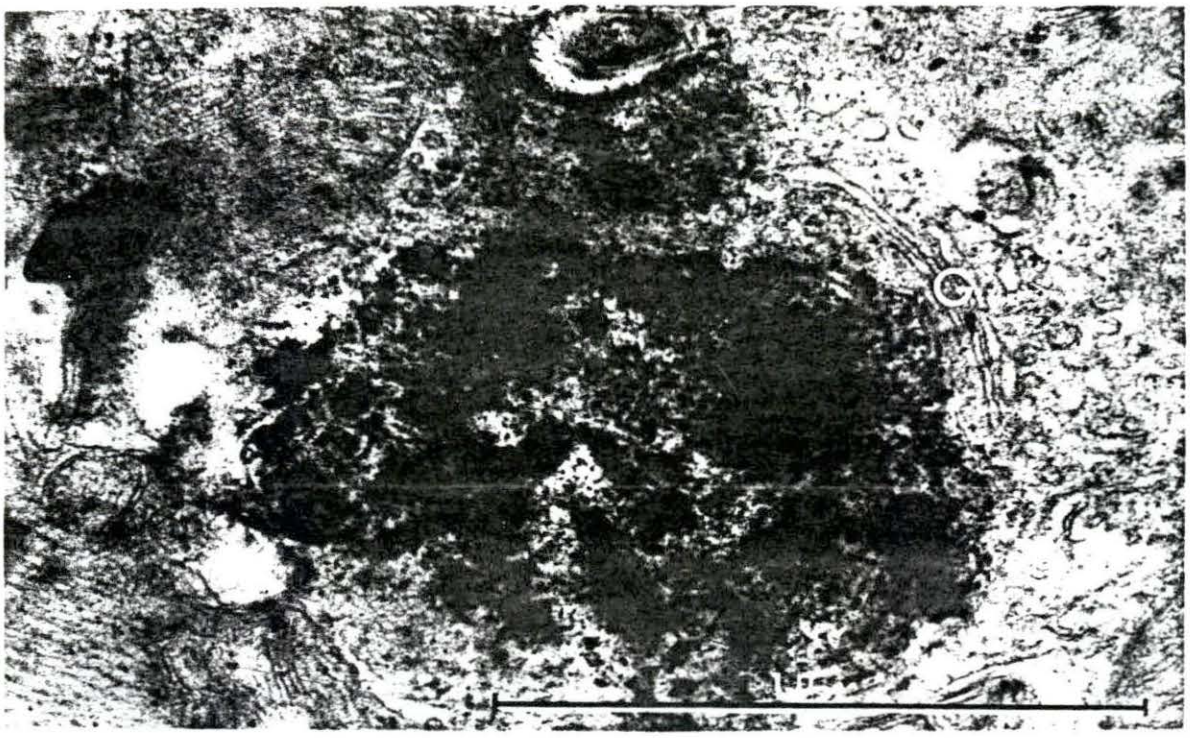

1μ

Figure 36. Lipofuscin pigment granule from the myocardium of a 10.5 year old dog (M48) containing the reaction product of Gomori's method for acid phosphatase localization. Note that the adjacent Golgi area is relatively free of the precipitate. D.E.R. embedding, unstained.

Figure 37. Peri-nuclear region of a myocardial cell from an 8 day old puppy (AI). This tissue has been incubated in Gomori's medium for acid phosphatase localization. The electron dense reaction product was limited to the lysosomes and to areas such as those seen in this micrograph which are interpreted to be the Golgi regions of the cell. D.E.R. embedding, unstained.



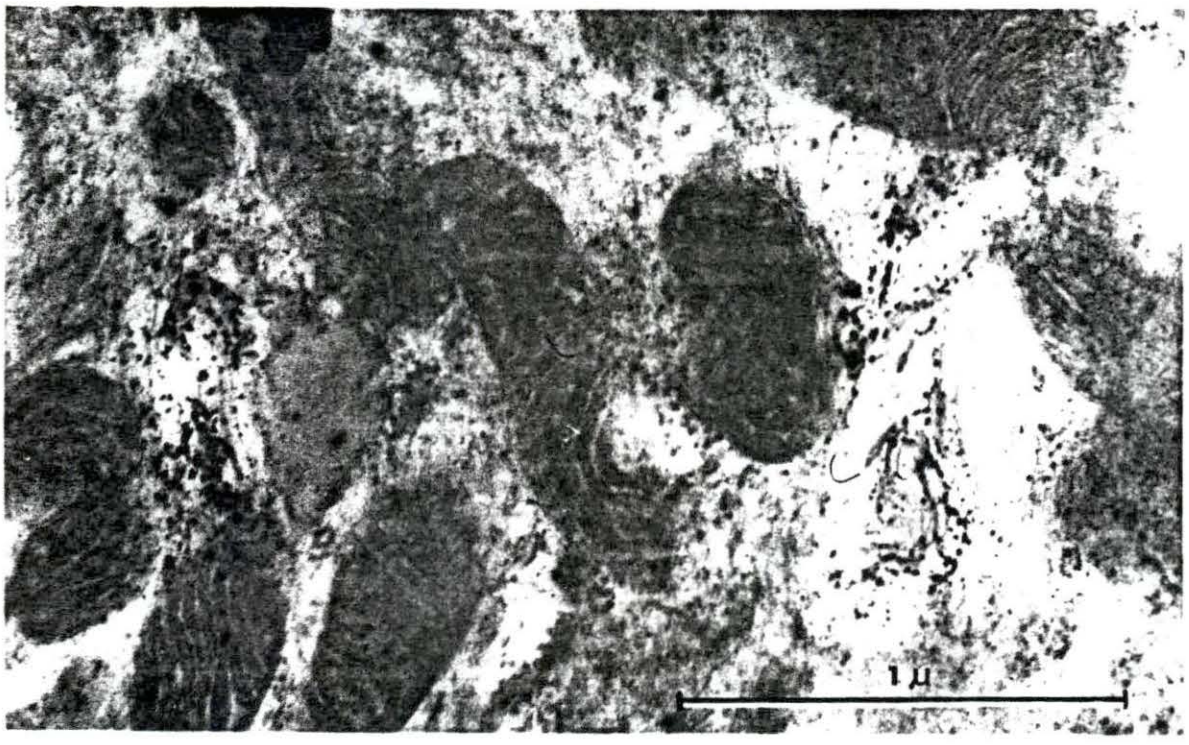

1 μ

Figure 38. Electronmicrograph of the peri-nuclear region of a binucleate myocardial cell from a 12.8 year old dog (M32). Dense lipofuscin pigment granules can be seen at the poles of the nuclei. The arrows point out altered sarcomeres which were scattered through the myocardium in this and one other animal studied. Methacrylate embedding, lead citrate stain.

Figure 39. Highly magnified view of an altered sarcomere similar to those seen in Figure 38. The arrows labeled S span the entire sarcomere. In the adjacent sarcomeres the A bands (A) and the I bands (I) appear normal. Within the affected sarcomere filaments of similar size and density to the myosin filaments of the normal A band are seen, but no banding pattern can be discerned. The density of the affected area is equal to that of the normal Z band. Methacrylate embedding, lead citrate stain.

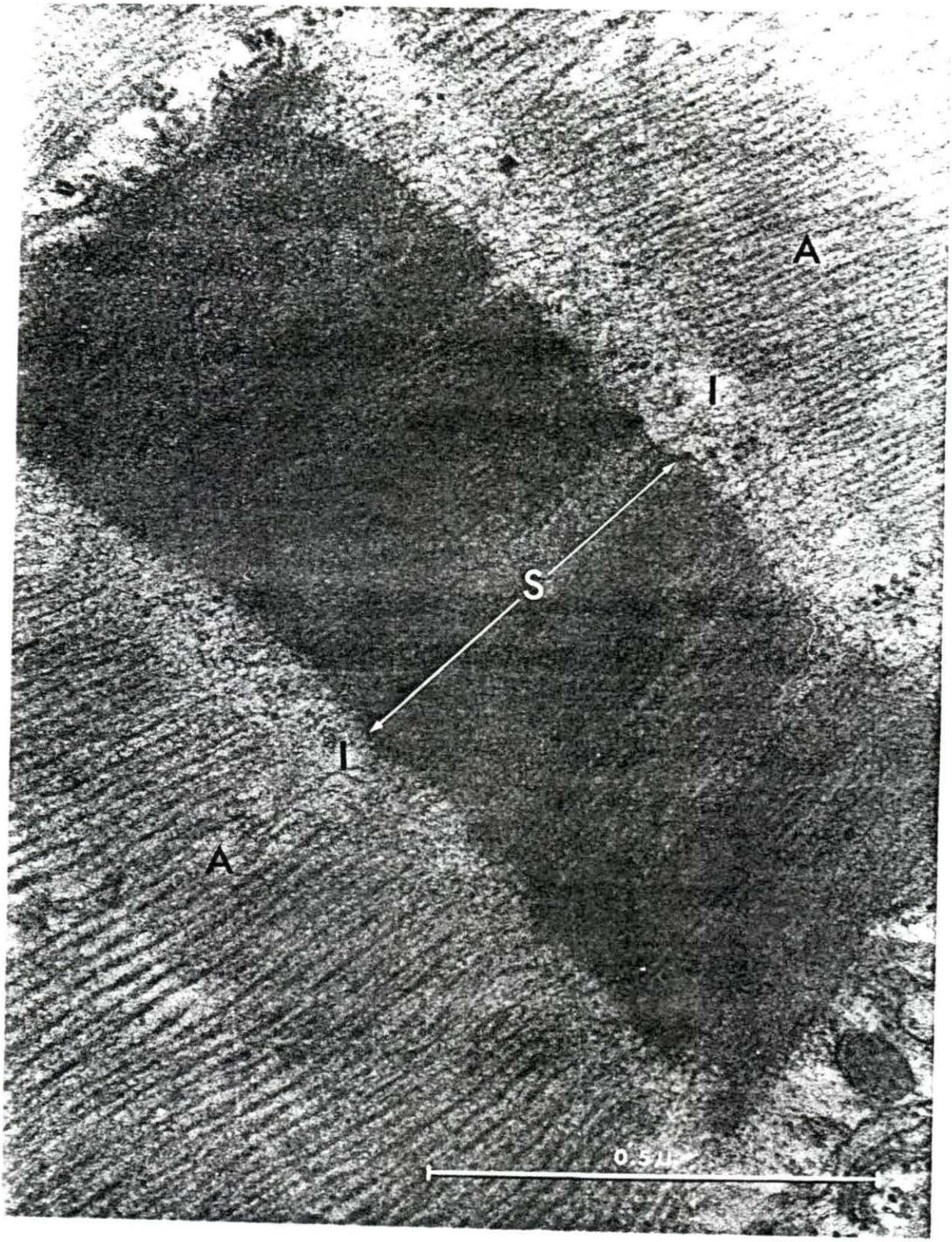

A
I
S
I
A
0.5μ

Figure 40. Alteration in a sarcomere appearing as a widening of the Z band density. A finger-like extension of this dense area extends across the length of the sarcomere. Methacrylate embedding, lead citrate stain.

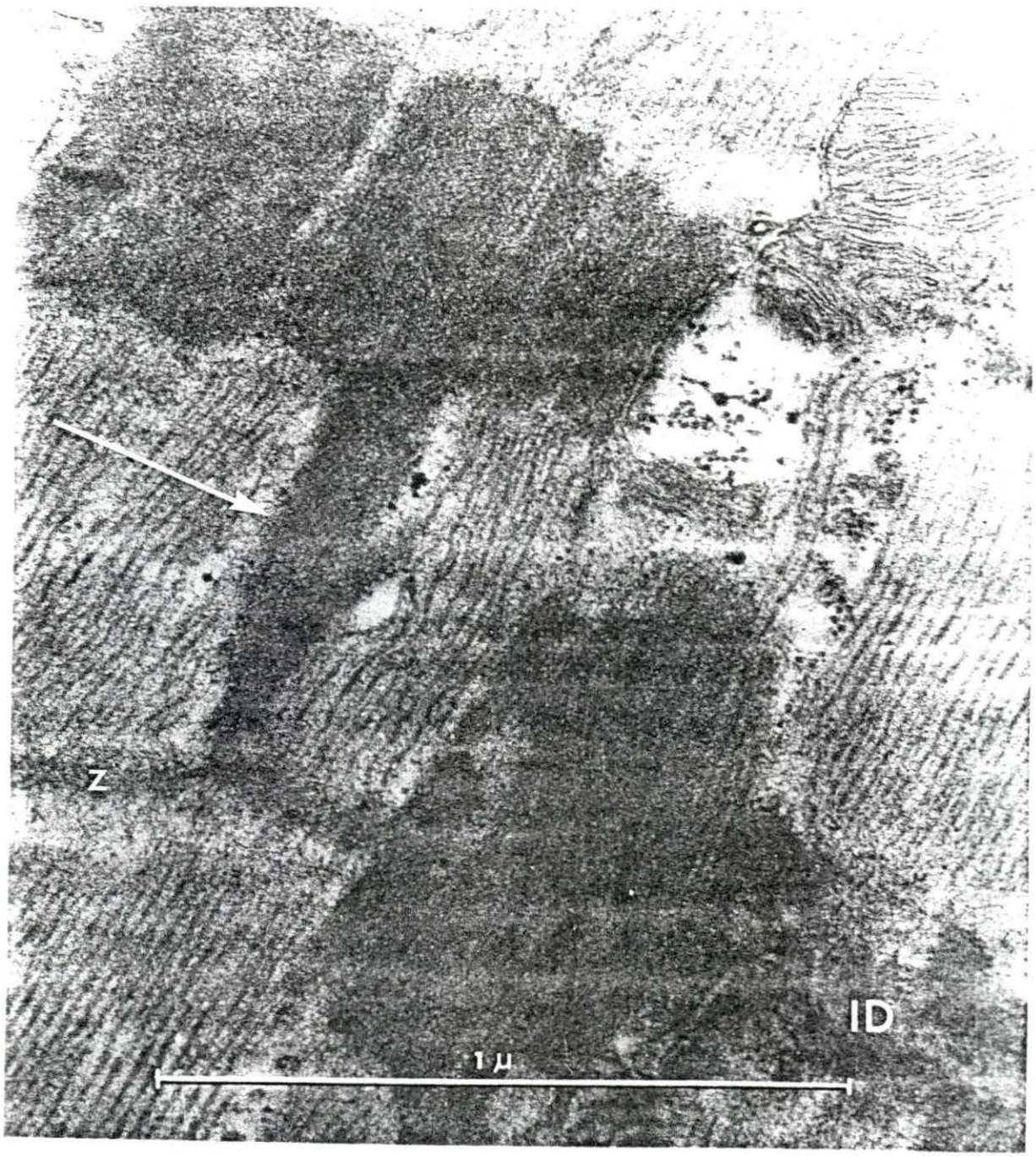
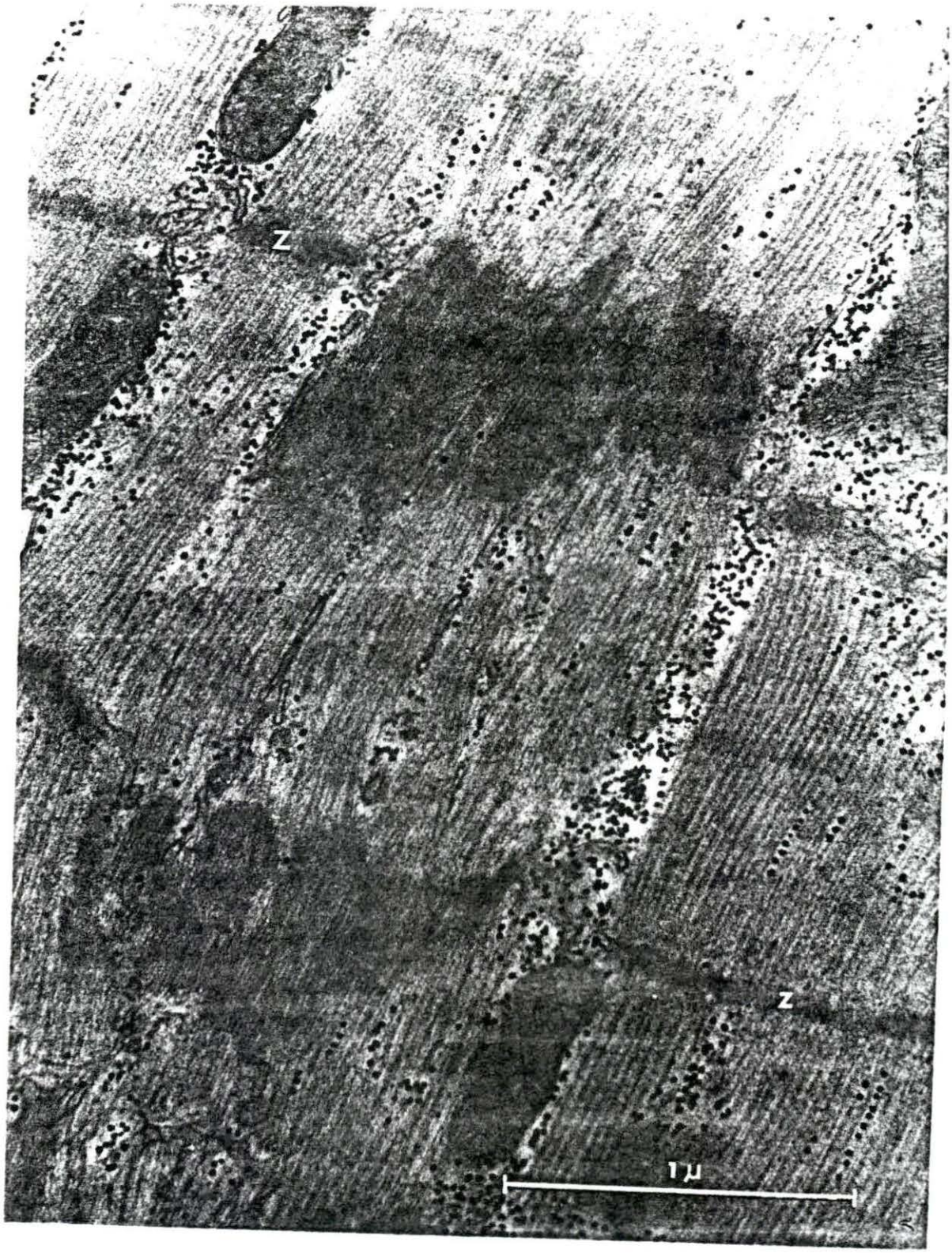

Z
ID
1 μ

Figure 41. Altered sarcomeres in an 11.2 year old dog (M42). The fibrils containing the affected sarcomeres are disoriented in relation to the adjacent fibrils. In this case entire sarcomeres are not affected and the change appears as a widening of the Z band densities. D.E.R. embedding, lead citrate stain.


1 μ

Figure 42. Higher magnification of affected areas from the myocardium of the same animal described in Figure 41. Z, Z band. D.E.R. embedding, lead citrate stain.


Z
Z
1μ