HISTOCHEMICAL AND ELECTRON MICROSCOPIC STUDY IN A CASE OF HURLER'S DISEASE

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The typical clear cells of connective tissues and the lipid-filled cells of the central nervous system were early recognized as key features of Hurler's syndrome. Lately the cellular manifestations of the disease have been obscured by the interest in the biochemically demonstrable polysaccharides.[1-4] These investigations have demonstrated heparitin sulfate and chondroitin sulfate B in excess in tissues and urine of patients with the syndrome but have not yet clarified the pathogenesis of the syndrome. The small number of reported histologic studies, the sometimes conflicting results of histochemical analyses, and the lack of any previous examination with the electron microscope have encouraged us to present our investigation of the cellular alterations, principally in the mitral valve, in a case of Hurler's syndrome.

CASE REPORT

A 3-year-old girl was first admitted to the University of Washington Hospital for the evaluation of increasing hydrocephalus. Hydrocephalus had first been noted at the age of 4½ months. At this time the infant exhibited the characteristic appearance of Hurler's syndrome. She had a typically grotesque facies, clouded corneas, widely spaced pointed teeth, an enlarged liver and spleen, an umbilical hernia, and skeletal changes involving extremities and spine. Her mental development was estimated at the time of her first admission at age 2 to be that of a 1-year-old. Reilly bodies were not found in leukocytes; urinary mucopolysaccharide determinations were not performed.

The parents of the patient were unrelated, and there was no history of Hurler's syndrome or hydrocephalus in either family. The first born sibling was male and died at age 6 months following operation for hydrocephalus resulting from a "colloid cyst" of the third ventricle. The second sibling was a normal girl and the third a girl with suggestive evidence of Hurler's syndrome who died with hydrocephalus and a mass in the third ventricle. (Dr. Norman T. Crane, Plainfield, New Jersey, kindly provided this information.) The fourth and fifth siblings were normal boys. The patient was the sixth sibling.

During the hospitalization period, ventriculograms revealed communicating hydrocephalus; a right ventriculo-atrial shunt was performed to arrest the rapidly increasing hydrocephalus. The patient's subsequent course was complicated by bilateral subdural hematomas, brain stem hemorrhage, cerebrospinal fluid infection and septicemia. The complications, excepting the brain stem hemorrhage, responded to therapy, and the patient was discharged to a nursing home in a decerebrate state, quadriplegic and akinetic. She died one year after her initial admission.

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METHODS

The mitral valve was obtained at necropsy performed 12 hours after death by Dr. D. D. Reichenbach. A portion of the valve was fixed in 4 per cent neutral buffered formaldehyde and embedded in paraffin. Other samples of the mitral valve were frozen-dried and then fixed in formaldehyde vapor for 48 hours at 50° C. before embedding in paraffin.[5] Pieces of aqueous formaldehyde-fixed mitral valve were also washed in gum acacia-sucrose solution,[6] frozen and sectioned in a cryostat at −20° C. For electron microscopy, samples of mitral and tricuspid valves were cut into small pieces, fixed in 2 per cent osmium tetroxide, buffered with s-collidine, dehydrated, and embedded in epoxy resin (Epon 812).[7] Sections were stained with uranyl acetate.

Acidic mucopolysaccharides were stained with 1 per cent toluidine blue in 75 per cent ethanol, adjusted to pH 3.5 with acetic acid. The periodic acid-Schiff (PAS) reaction was employed in conjunction with acetylation and bromination to differentiate unsaturated lipid from polysaccharide.[8] Lipids in paraffin-embedded sections were stained with Luxol fast blue and Sudan black B.[9] Tissue sections were also extracted with chloroform-methanol at room temperature and at 60° C. and then stained for lipids. Diezel's adaptation of Bial's reaction [10] was applied to tissue sections for the tentative identification of substances containing neuraminic acid.

OBSERVATIONS

The mitral valve leaflet on gross examination was pearly white, thickened and beaded (Fig. 1). Similar changes were evident in the chordae tendineae. Microscopic sections demonstrated two contributing elements: collagen and cells. The collagen fibers were arranged in large parallel bands extending out into the valve leaflets. The collagen stained normally with van Gieson's reagent, and on examination with the electron microscope exhibited characteristic 700 Å periodic banding (Fig. 7). There was relatively little intercellular space not occupied by dense collagen; in many sites the collagen fibers were closely applied to the cell membranes (Fig. 7).

The most frequent cell in the valve leaflet had the configuration of the classic "clear" or gargoyle cell in conventional preparations. It was a large oval or polygonal cell, 20 μ in diameter, with a pale central nucleus. The cells were often aligned in parallel rows between the collagen bundles, and the cytoplasm was devoid of material stainable with hematoxylin and eosin or acidic toluidine blue. When frozen-dried, formaldehyde vapor-fixed sections were deparaffined, hydrated to 75 per cent ethanol and stained with toluidine blue, the cells were seen to be filled with intensely metachromatic granular material with no metachromasia in the extracellular sites (Figs. 2 and 3). Intracellular substance was also retained when sections were stained in a 50 per cent acidified ethanol solution of toluidine blue without removing the paraffin. The PAS reaction was very weak in these cells. Sudan black B and Luxol fast blue stained most of the typical clear cells lightly, but in a few cells, densely stained granules were evident (Fig. 5). On electron microscopic exami-

nation, the cytoplasm of the clear cells was seen to be filled with large irregular clear vacuoles varying in size from 0.5 μ to 2 μ (Fig. 6). The membranes of the vacuoles were often broken and the ruptured ends curled. Occasionally mitochondria and cisternas of endoplasmic reticulum were interspersed between the vacuoles. The mitochondria had irregular shapes and short cristas. Ribosomes were often missing from the endoplasmic reticulum. Some of the cells contained vacuoles filled with a dense clumped substance suggesting correspondence with the granules stained with Sudan black B and Luxol fast blue.

A second cell type in the valve leaflets was first noted in the electron micrographs. It was subsequently identified in frozen-dried, formaldehyde vapor-fixed and in aqueous formaldehyde-fixed frozen sections with lipid stains. These cells could not be demonstrated in ordinary formalin-fixed, paraffin-embedded tissues. Characteristically they appeared as elongated aggregations of lipid droplets pressed between collagen bundles (Figs. 4 and 5); it was often difficult to identify the lipid-containing structures as cells. When a nucleus was evident, it was located at one end of the cell but was not obviously different from a clear cell nucleus. These lipid-containing cells were much less frequent and considerably smaller than the clear cells. No acid mucopolysaccharides were demonstrable in them. Luxol fast blue and Sudan black B stained the droplets strongly, and oil red O staining in frozen sections was weak. The intracellular droplets also stained with the PAS procedure; this staining was blocked by acetylation but not by bromination. The cells gave a positive Bial reaction. With electron microscopy the lipid droplets were identified as irregular clumps of extremely dense material, usually surrounded by a membrane (Fig. 6). Although some of the clumps were less dense than others, none resembled the clear vacuoles present in the more frequent gargoyle cells. Only occasional mitochondria and rare endoplasmic reticulum profiles were seen.

In addition to two distinct cell types, one containing almost exclusively metachromatic polysaccharide and the other lipid, there were scattered cells in the valve which contained both lipid and acidic polysaccharide. These were observed in electron micrographs and contained dense aggregates as well as clear vacuoles (Fig. 7).

In other tissues examined (liver, spleen, lymph node and aorta), the fixation of the accumulated substance(s) was not adequate. Large vacuoles in liver cells were devoid of stainable substance. Only in scattered parenchymal and Kupffer cells was metachromatic staining material evident. In spleen and lymph node, clear cells were not apparent, but focal accumulations of cells did contain material which stained metachromatically. Clear cells were evident in frozen-dried aorta, but most

of the metachromatic substance was coarsely precipitated throughout the wall. In only a few spots was intracellular staining convincing. Cell fragments similar to those which stained for lipid in the mitral valve were present in the aorta. There was little stainable lipid in the liver.

Discussion

The first indication that Hurler's syndrome might represent a storage disorder was the abnormal accumulation of lipid in neurons observed by Tuthill [11] in the necropsy of one of Hurler's patients. Subsequent investigators have usually had no difficulty in locating lipid in neurons.[12–21] Histochemical observations [10,20] have indicated that the substance in neurons is possibly a ganglioside, and several investigators [15,22,23] have demonstrated by extraction that gangliosides are considerably increased in the brains of afflicted individuals. These observations have not been uniformly corroborated.[21,24] It should be stressed that it has not been possible to show any qualitative abnormality in the excess ganglioside extracted from brains in patients with Hurler's syndrome and, in particular, that the hexosamine of the extracted ganglioside is the normal galactosamine.[22]

Lipid-filled cells outside the central nervous system were described by Hässler in bone,[25] Schmidt in cartilage,[26] and Brante in heart valves and blood vessel walls [22]; Russell [14] and Magee [27] have found lipid-filled cells in the meninges. More recently Máttyus and Jobst have described their histochemical investigations of extraneural lipid storage cells.[20] In our preparations, the intracellular lipid was well preserved in the formaldehyde vapor-fixed, frozen-dried mitral valve in contrast to the absence of stainable lipid in the aqueous formaldehyde-fixed, paraffin-embedded tissue. The staining properties of the connective tissue lipid cell paralleled those of the lipid in the brain,[20,28] and the likelihood is that the connective tissue lipid is also ganglioside. Extractive studies are, of course, necessary to substantiate this identification. One difference between the lipid in the two sites was the preservation of lipid in the aqueous formaldehyde-fixed, paraffin-embedded neurons and the loss of lipid from connective tissue cells under these conditions. This difference may be a function of the character of the storage sites in the two cells.

In contrast to the ready preservation of the material filling the neurons is the poor fixation of the substance or substances which accumulate in the typical clear cells of the connective tissue, liver and spleen. Extraction of liver and spleen have yielded greatly increased quantities of mixtures of heparitin sulfate and chondroitin sulfate B, with the former usually predominating in liver and the latter in spleen.[29] Hexosamine, present in gangliosides, is also a component of chondroitin sulfate B and

heparitin sulfate; the hexosamine is galactosamine in the former and glucosamine in the latter.

Many methods have been employed with varying success in attempts to stain the soluble polysaccharide component. Haust and Landing[30] have recently re-examined the fixation and staining of this substance and indicated that the use of frozen sections, post-fixed in acetone:tetrahydrofuran is superior to other procedures for preserving the acidic mucopolysaccharide(s).

Two methods of fixation were utilized in our study, aqueous buffered formaldehyde and formaldehyde vapor. The use of the latter fixation combined with staining in 75 per cent alcohol afforded excellent preservation of the mucopolysaccharides in the mitral valve. Frozen-dried tissue was treated with Haust and Landing's fixative and stained in the toluidine blue solution they recommended. The preservation of acidic mucopolysaccharides was not as good as that in the formaldehyde vapor-fixed tissue, but the frozen-dried tissue used may not be suited to this method; a more rigorous comparison of the two procedures, ours and Haust and Landing's, is required.

The preservation of the material in liver and aorta was not adequate in frozen-dried vapor-fixed tissues. Its loss in these sites may indicate greater solubility of the mucopolysaccharide or perhaps increased quantities of hydrolytic enzymes which in the postmortem period may produce breakdown of cell structures with a resultant diffusion of the mucopolysaccharide. Although all the abnormally accumulated mucopolysaccharides may be intracellular, only in the mitral valve is the localization clear in our specimens.

The origins of the two abnormal types of cells in connective tissues are not evident. Since mast cells are normal in distribution and cytologic appearance in this disease, it seems unlikely that they are the cellular precursors. Fibroblasts are the other connective tissue elements known to be capable of synthesizing mucopolysaccharides. The usual assumption that the gargoyle cells are derived from fibroblasts implies that the cells in which the mucopolysaccharide accumulates are the synthesizing cells. Although this assumption is perhaps the simplest, it is conceivable that the gargoyle cells are actually phagocytic and that the accumulated mucopolysaccharide has been synthesized elsewhere.

From the data available in the literature, it is impossible to associate any specific histochemical properties with one or the other of the two genetic forms of the syndrome[31] since, commonly, family histories and occasionally even the propositus' sex are not reported. Analyses of urine in one case of the sex-linked type did not demonstrate any differences between the excreted mucopolysaccharide and that excreted in the auto-

somal recessive type.[29] One speculative but testable hypothesis is that the lipid abnormality is restricted to the autosomal recessive form of the disorder in which mental deficiency is the rule; this is in contrast to the sex-linked form in which mental deficiency may be absent.

In view of the evidence from extraction and histochemical studies that a glycolipid and also two acidic mucopolysaccharides are present in excessive amounts in cells of patients with Hurler's syndrome, the proposed classification of the disease as a mucopolysaccharidosis is a simplification. Although further investigations into the nature of the glycolipid abnormality are necessary to put the knowledge of this pathologic constituent on a par with that of the mucopolysaccharides, any proposed pathogenesis of the syndrome must explain the accumulation of a glycolipid, probably a ganglioside, as well as the two acidic mucopolysaccharides.

The concept that heritable diseases represent a single genic defect which manifests itself as a single protein abnormality has provided considerable insight into the pathogenesis of a number of genetically determined disorders.[32] The usual primary protein abnormality is the deletion of an enzyme. The enzyme involved may be abnormal in character or decreased quantitatively. An abnormal gene may also express itself as a non-enzyme protein abnormality, qualitative or quantitative. It seems worth while, on the basis of available information, to consider the pathogenesis of Hurler's syndrome in terms of the "one gene—one protein" theory.

The group of genetically determined diseases in which excessive cellular accumulation of a normally present compound is a prominent feature includes the glycogen storage diseases and the lipidoses as well as Hurler's syndrome. The actual protein defect responsible for the cellular accumulation has been identified only in von Gierke's disease (glycogen storage disease of the liver and kidney); it is the depression of glucose-6-phosphatase in von Gierke's disease which prevents the utilization of glycogen, and thus leads to its accumulation in liver and kidney.[33]

If a single protein abnormality accounts for the accumulation of a sulfated mucopolysaccharide and an oligosaccharide lipid complex, then some metabolic pathway common to both these substances must be involved. An alternative hypothesis holds that the excess of one of the polysaccharides is the primary abnormality and the other polysaccharide and the ganglioside accumulate secondarily.[34,35] Because so little is known of either the synthetic or degradative metabolism of the mucopolysaccharides or the gangliosides, even speculation becomes difficult. Such speculation as we engage in here can only serve to indicate the unproved, currently tenable assumptions and point out the possibilities for further investigation.

Considering first the metabolic block hypothesis: hexosaminidic bonds are common to gangliosides and sulfated mucopolysaccharides, so that the depression of a common hexosaminidase that acted on heparitin sulfate, chondroitin B sulfate and ganglioside is one conceivable explanation of the pathogenesis of Hurler's syndrome. A serious objection to the postulation of a defective common hexosaminidase is the difference in configuration of the hexosaminidic bond, α in heparitin sulfate and β in chondroitin sulfate B. Cifonelli and Dorfman [35] have suggested the possibility that heparitin sulfate may be a heparin precursor and that the accumulation of heparitin sulfate stems from a block in the transformation of acetamido to sulfamido groups in the polysaccharide, but, as they note, this does not explain the accumulation of chondroitin sulfate B or ganglioside. Another consideration inconsistent with their proposal is that mast cells do not exhibit pathologic accumulations in the face of present evidence that they are prime sites of heparin synthesis.

Mechanisms other than a block in metabolism that could lead to cellular accumulation must be considered even though established models in human disease do not exist. Obviously a substance would accumulate if its synthesis were excessive. Since the mucopolysaccharides not only accumulate in cells but are also excreted in excessive quantities, it has usually been assumed that the production of these compounds is, in fact, excessive. This assumption requires proof in terms of the measurement of the rate in the synthesis of the substances. If such excess production is the case, it could result from either the increased activity of a synthesizing enzyme or from an increase in concentration of a precursor resulting from a block in an alternative metabolic path.

In addition to the possibilities indicated above, a defect in secretion from the synthesizing cell or excretion from the body could also lead to an intracellular accumulation. This defect could be in a protein carrier. Absence of such a carrier would lead to intracellular accumulation by interfering with secretion or excretion; this seems unlikely in view of the well-proven increase in urinary excretion of the two mucopolysaccharides. It is conceivable, though, that an abnormality in a protein carrier could interfere with degradative metabolism producing an accumulation intracellularly as well as extracellularly.

The use of the electron microscope in this case deserves an additional comment. The pathologist with access to an electron microscope has been loath to study necropsy tissues. The preservation of mitochondria and other intracellular membranes and vacuolar structures in the tissues of this case support the recent observations that many intracellular structures are reasonably stable post-mortem [36] and encourages the wider application of electron microscopy to the examination of tissues obtained at necropsy.

Summary

In the mitral valve of a case of Hurler's syndrome, two cell types with intermediate forms have been identified by electron microscopy and histochemical techniques. One type contains glycolipid, the other acid mucopolysaccharide.

Preservation of both substances in connective tissue sites has been accomplished with formaldehyde vapor fixation of frozen-dried tissue.

On the basis of the histologic, histochemical and electron microscopic observations, the syndrome is classified pathologically as an intracellular accumulation. Some possible modes for the development of such an accumulation are discussed.

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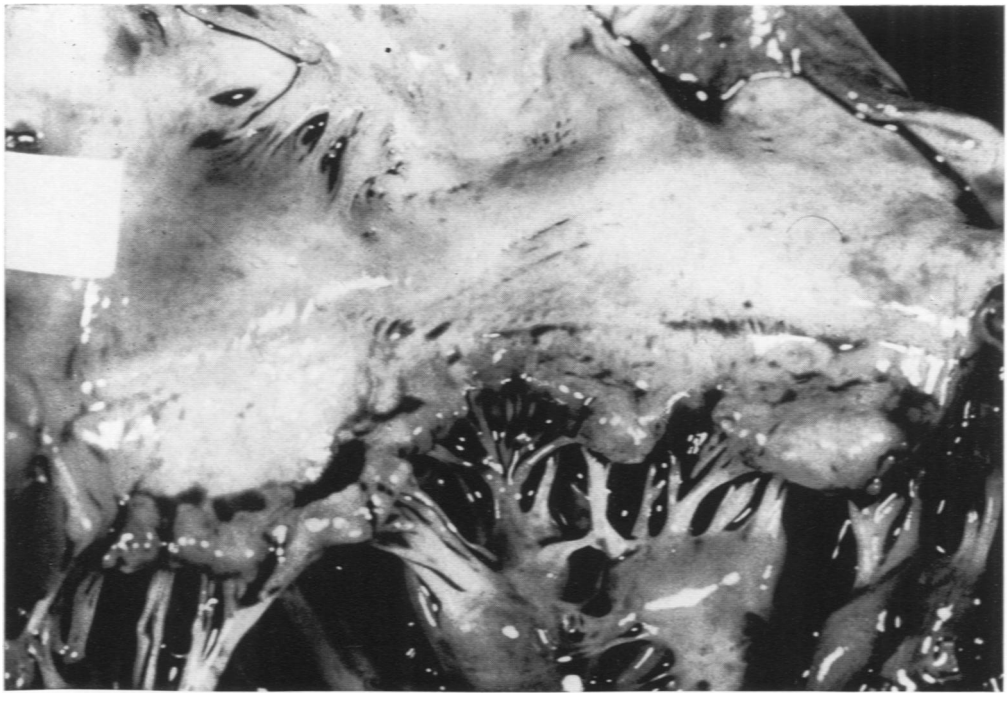
LEGENDS FOR FIGURES

FIG. 1. The mitral valve shows thickening and beading of the leaflet.

FIG. 2. The mitral valve leaflet exhibits gargoyle cells filled with mucopolysaccharide which stains metachromatically with acidic toluidine blue. Frozen-dried, formaldehyde vapor-fixed tissue. × 120.

FIG. 3. Gargoyle cells. Prepared as in Figure 2. × 480.



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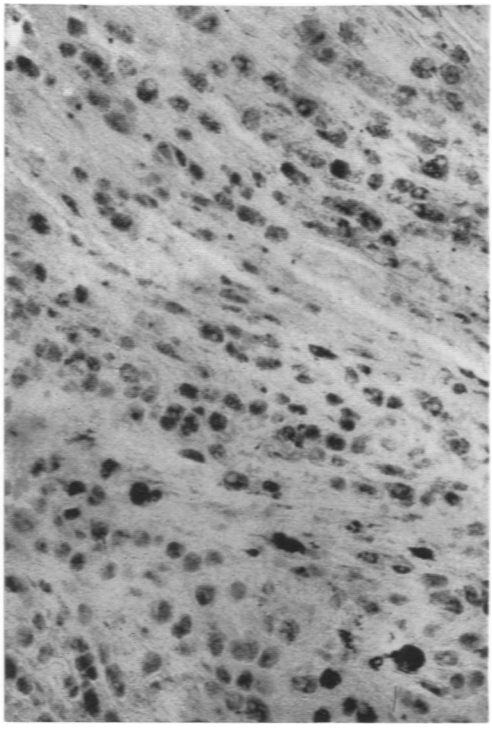

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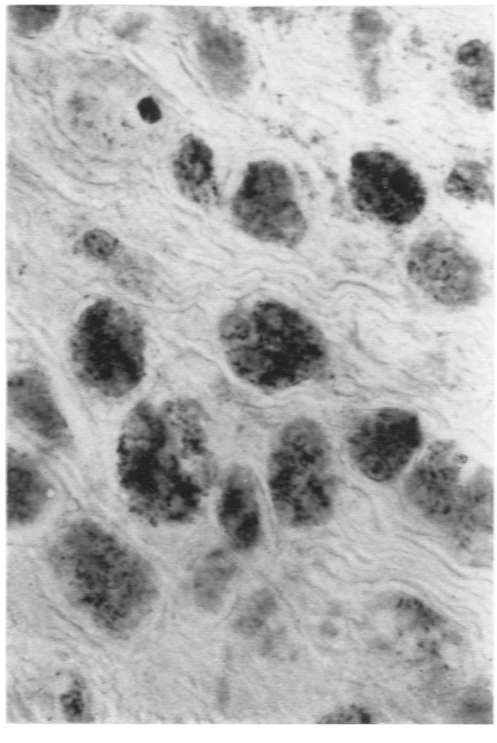

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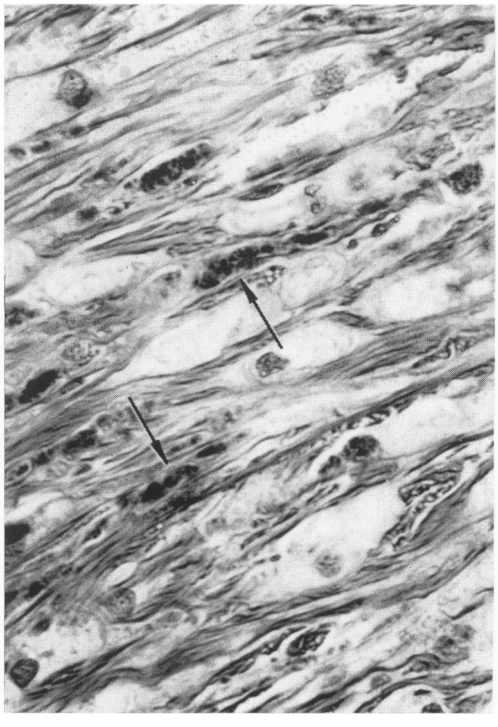

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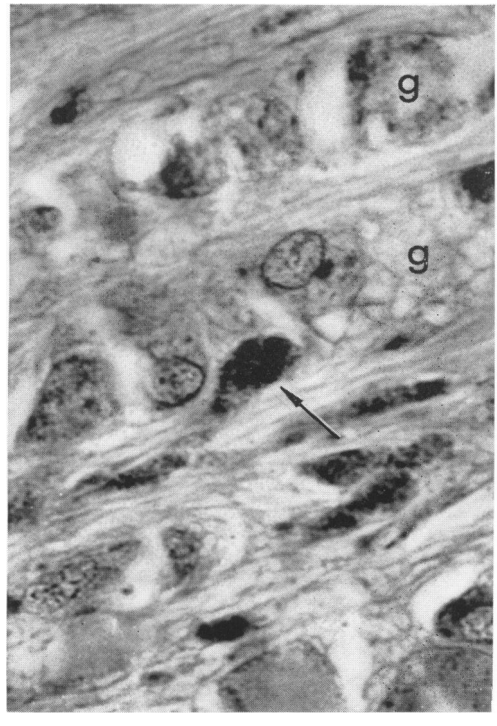

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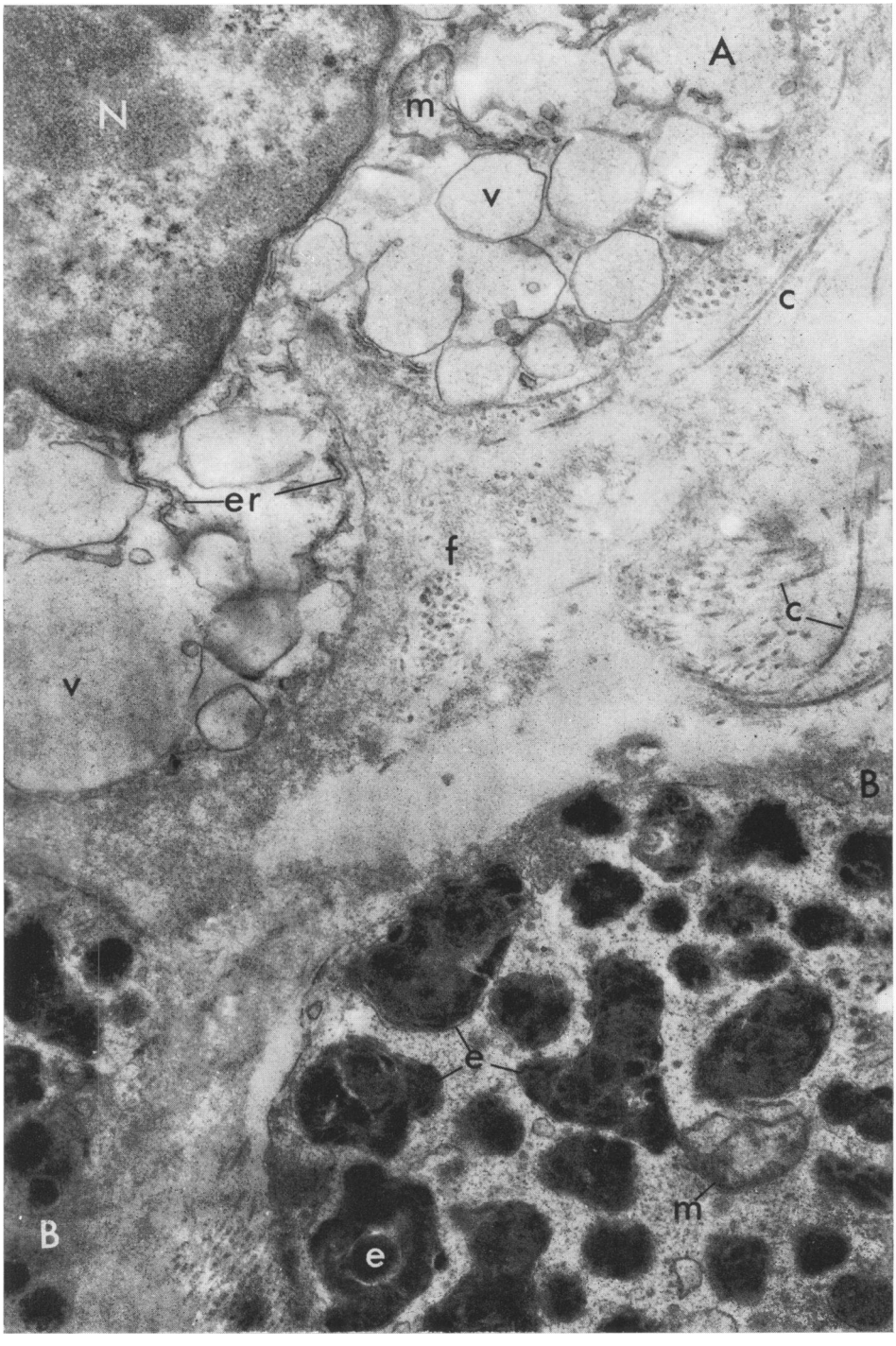
FIG. 4. The mitral valve leaflet shows the collagenous character of the thickening and a population of cells which stain with Luxol fast blue (arrows). Fixed as in Figure 2, stained with Luxol fast blue and van Gieson's reagent. × 480.

FIG. 5. Higher magnification of the lipid-filled cells (arrow) and their relation to the gargoyle cells (g), largely unstained. Fixed as in Figure 2; stained with Sudan black B. × 1,200.

FIG. 6. An electron micrograph of an area from the tricuspid valve in which representatives of the two cell types can be seen. The large clear cell (A) with its numerous clear vacuoles (v), a mitochondrion, an occasional cisterna of the endoplasmic reticulum (er) and the nucleus (N) are evident. Two smaller cells (B) contain dense lipid deposits (e); mitochondria (m) can be seen in one of them. The cytoplasmic matrix is studded with fine particles and occasional profiles of cisternas of the endoplasmic reticulum. The extracellular regions are composed of collagen fibrils (c) cut both transversely and longitudinally, as well as fine filamentous structures (f) containing no clear banding. × 18,000.



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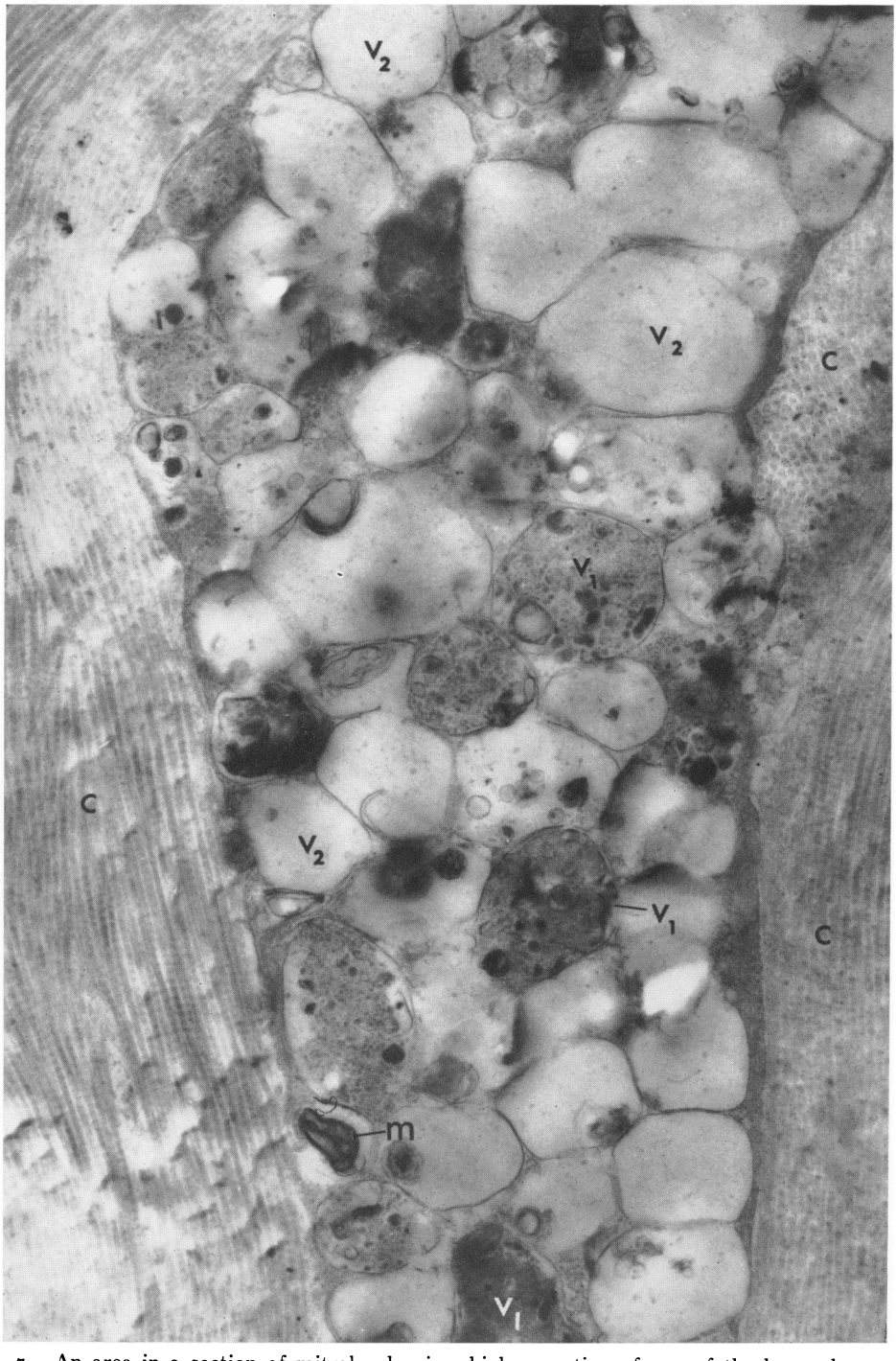

FIG. 7. An area in a section of mitral valve in which a portion of one of the large clear cells is present. Many of the vacuoles contain individual objects of varying density (v_1) while others appear empty (v_2). An occasional mitochondrion (m) can also be observed. A dense matrix of collagen fibrils (c) composes the extracellular substance. × 21,600.