THE ULTRASTRUCTURE OF ISOLATED AND *IN SITU* HUMAN CARDIAC AGE PIGMENT

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The occurrence of fluorescent, pigmented inclusion bodies in muscle, nerve, and other nondividing cell lines has been known since the turn of the century. These pigments, variously known as age pigments, lipofuscin, or abnutzungspigmente (wear and tear pigments), have been subjected to numerous histochemical and histological studies. Little has been firmly established, however, regarding the relationship of these structures to the normal aging process even though the pigment in large amounts is a normal concomitant of aging in a number of different cell lines. A report from this laboratory (1) established the first quantitative relationship between chronological age and the amounts of age pigments in human cardiac muscle. It was shown that the concentration of this substance increased at the rate of about 0.3 per cent of a total heart volume per decade or 0.6 per cent of the intracellular volume per decade. Thus in a centenarian the expected average pigment concentration would be approximately 6 per cent of the total intracellular volume. The concentration of pigment in this study, in contrast to that of Bachmann's study (2) on human liver lipofuscin, was not shown to be significantly altered by the clinical history of the individuals autopsied.

During the past four years we have developed methods for obtaining purified preparations of age pigment utilizing homogenization, sonication, KCl extraction, and centrifugation techniques, and have described a number of the physical and chemical properties of this purified material (3, 4). Similar studies have been conducted by Heidenreich and Siebert (5). Chemical and physical analyses of this purified pigment (3) indicate that it consists of autooxidized lipids in association with a protein moiety.

The relationship of lipofuscin granules to preexisting or known cell organelles has been the subject of some discussion. It has been suggested (6), on the one hand, that they arise from the deterioration of mitochondria, primarily because bodies which have been identified as lipofuscin granules occasionally can be conjugant with partially disorganized mitochondria and may occasionally have double membranes surrounding them. On the other hand, the work of Essner and Novikoff (7) and of Gedigk and Bontke (8) has strongly suggested a relationship or identity between lipofuscin and lysosomes, since the pigmented areas in liver, cardiac muscle, brain, and elsewhere were shown by histochemical tests to be the loci of acid phosphatase and catheptic activity. The lipofuscin we have isolated from human cardiac muscle was, contrary to expectation, found not to be enriched in its content of these two enzymes characteristic of lysosomes. This specific absence of high enzyme activity may be due to damage or disruption of the lipofuscin particles by our particular isolation technique, with subsequent inactivation or liberation of the enzyme into the surrounding medium. Alternatively, lipofuscin may indeed be relatively free of acid phosphatase and catheptic activity and therefore distinctly different in nature from lysosomes.

The present study was undertaken in order to clarify three general questions. Firstly, we wished to examine the preparations of purified lipofuscin in order to describe the ultrastructure of this material. Secondly, we wished to compare this material with that occurring *in situ*. Finally, we hoped that a comparison would shed some light on the possibility that the discrepancy between the chemical studies and the histochemical results

might be resolved by such differences in appearance of the isolated and *in situ* granules as might possibly be observed.

MATERIALS AND METHODS

The human cardiac muscle used in these studies was obtained at autopsy from the Pathology Department, Baltimore City Hospitals, not more than 6 hours post mortem. Material used for the isolation of age pigment had been frozen for a week prior to use. The isolation procedure was essentially as described by Hendley *et al.* (4) and consisted of the following steps: (*a*) The tissue was minced and homogenized at 4°C in a 0.25 M sucrose solution using a Waring Blendor. (*b*) After a preliminary centrifugation to remove the intact cells and other large particles (600 *g*, 10 minutes), the "mitochondrial fraction" was isolated by centrifuging at 12,000 *g* for 10 minutes. (*c*) This material was resuspended in sucrose medium and sonicated in a Raytheon 9 kc sonic oscillator, model no. DF101, for 4 to 8 minutes. (*d*) The sonicated mixture was centrifuged and the residue washed twice with centrifugation with a 0.5 M KCl solution which served to dissolve much of the muscle protein. (*e*) The centrifugates thus obtained were pooled and subjected to a second sonication for 4 to 8 minutes, centrifuged, and washed with 0.5 M KCl.

Suspensions of age pigments were centrifuged in the final medium above and resuspended in 1 per cent buffered osmium tetroxide (pH 7.5 at 4°C) for 1½ hours. The samples were then dehydrated by passing them through alcohols, and embedded in methacrylate.

The procedure used for the myocardial tissue was identical except for the absence of the centrifugation steps. Cubes of tissue approximately 2 mm on the side were used. Sectioning was performed on an LKB ultramicrotome model 4800A, and sections were viewed under an RCA EMU-3F electron microscope.

RESULTS

In Figs. 1 and 2 are shown typical isolated lipofuscin particles.

Figs. 3 to 6 show the dispositions of *in situ* particles in relation to the muscle structure. Note the essential identity between the particles illustrated in Figs. 1 and 2 and those in Figs. 3 and 4.

Throughout the entire preparative procedure, we have observed consistently the same particles measuring approximately 0.5 μ by 0.5 to 2.5 μ. These structures generally assume an ovoid shape and vary in electron opacity. Membranes are not associated with the isolated particles although they may occasionally be seen *in situ*. This membrane (see Fig. 5) is apparently a single one of thickness approximately 10.0 to 20.0 A. The ovoid particle consists of many hundreds of smaller spheroidal homogeneous granules of variable osmophilia, ranging in size from 50.0 A to 0.2 μ (see Fig. 6).

The lipofuscin particles, *in situ*, are located among the mitochondria. Occasionally the particles are seen to be concentrated at the poles of a

FIGURE 1

Electron micrograph of a lipofuscin particle obtained after a single sonication. × 124,000.

FIGURE 2

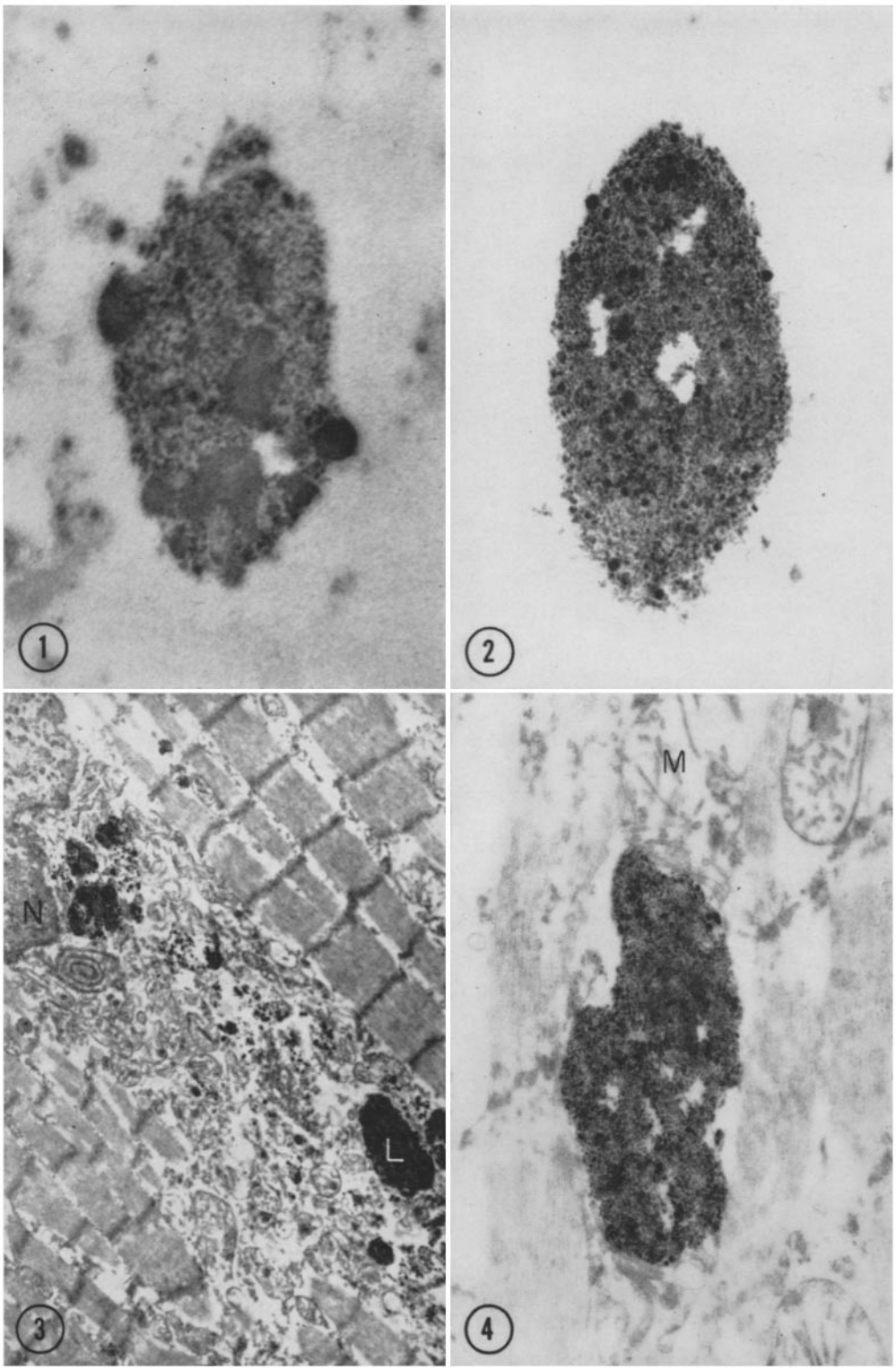
Electron micrograph of a lipofuscin particle obtained from the purified preparation. × 62,000.

FIGURE 3

Electron micrograph of human cardiac muscle obtained at autopsy. Age 75 years. Many small granules are seen near the fully formed lipofuscin particles. These granules are possible precursors of the mature particles. *L*, lipofuscin particle; *N*, nucleus. × 10,500.

FIGURE 4

Electron micrograph of human cardiac muscle. Age 75 years. Note the intimate contact of the lipofuscin particle with adjacent mitochondria. *M*, mitochondrion. × 62,000.



muscle nucleus, but ordinarily this is not the case.

DISCUSSION

It is apparent from the present study that the isolation procedure employed earlier in the preparation of age pigment has not substantially altered the ultrastructure of the isolated particles. Membranes are never seen associated with the isolated particles; however, one would expect such membranes to be disrupted by the sonication procedures, as indeed the membranes of the mitochondria are. There is, of course, always the alternate possibility that polymerization damage to the membranes may have occurred as a result of the use of methacrylate as an embedding material. Finally, it is possible that the single membranes seen enveloping occasional *in situ* particles are, in reality, condensation membranes as opposed to structures intrinsically a part of the pigment particle. Regardless of which is the case, certainly the isolation procedure is not generally sufficiently vigorous to disrupt the aggregation of smaller granules which compose the lipofuscin particles, and which remain morphologically intact. Morphologic integrity nevertheless offers no guarantee that enzymatic activity has not been lost, although it certainly affords circumstantial evidence which should be more strongly pursued by further chemical and histochemical techniques upon *in situ* material.

The proximity of the age pigment particles and the sarcoplasmic mitochondria seems to be coincidental. There is no morphologic evidence in our study to support the theory of a mitochondrial origin of lipofuscin particles; nor are there structures within the pigment particles that would suggest assimilation or degeneration of cytoplasmic components into structures which eventually form a "mature" lipofuscin particle, as is reported by Ashford and Porter (9) in connection with liver lysosomes. There is, however, a marked similarity—if not identity—in ultrastructure between many of the myocardial lipofuscin particles and the lysosomes described by other authors (7, 10–12).

The present studies do not, of course, settle the question of the origin, composition, and fate of these bodies. Resolution of this issue must await a further study of the enzymatic and chemical nature of the pigment. If the absence of a clear relationship to mitochondria is maintained by future studies, then the possibility that these structures represent insoluble waste products formed in the normal course of cellular function would still appear to be one of the most attractive alternatives. Such material might well represent the degraded remnants of previously functional lysosomes, as theorized by Essner and Novikoff (7). Alternatively, one might conjecture the formation of lipofuscin by the aggregation of smaller, particles which, as a result of similar lipophilia, are sequestered in areas where they will interfere minimally with the normal functions of the tissues and cells.

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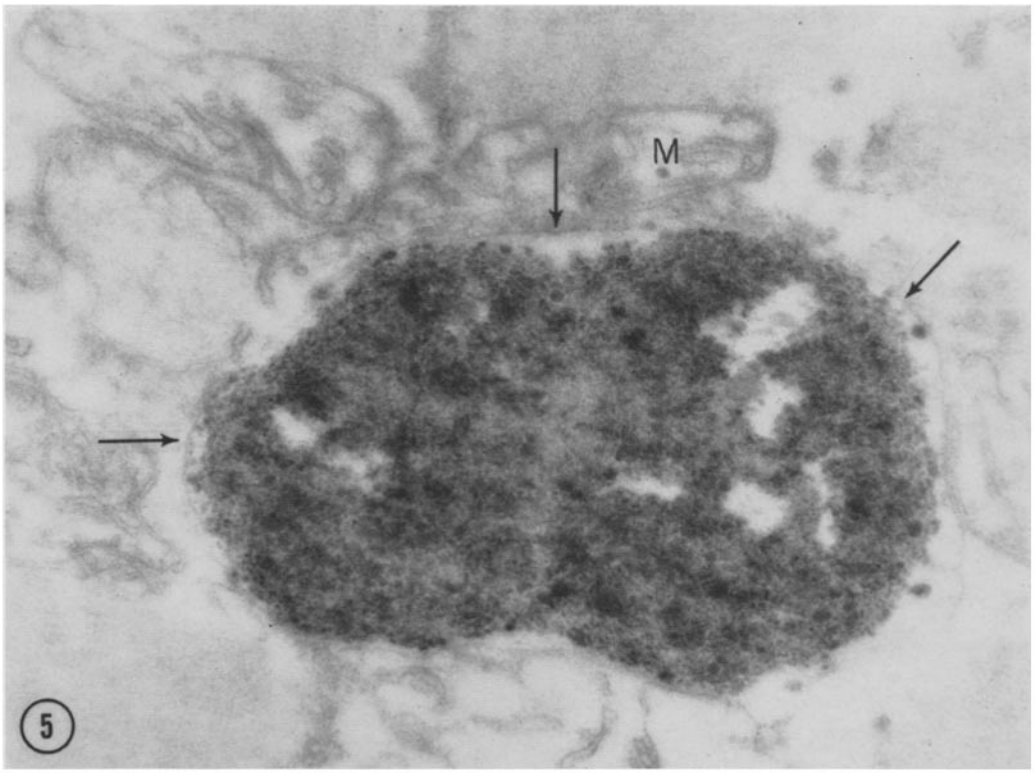
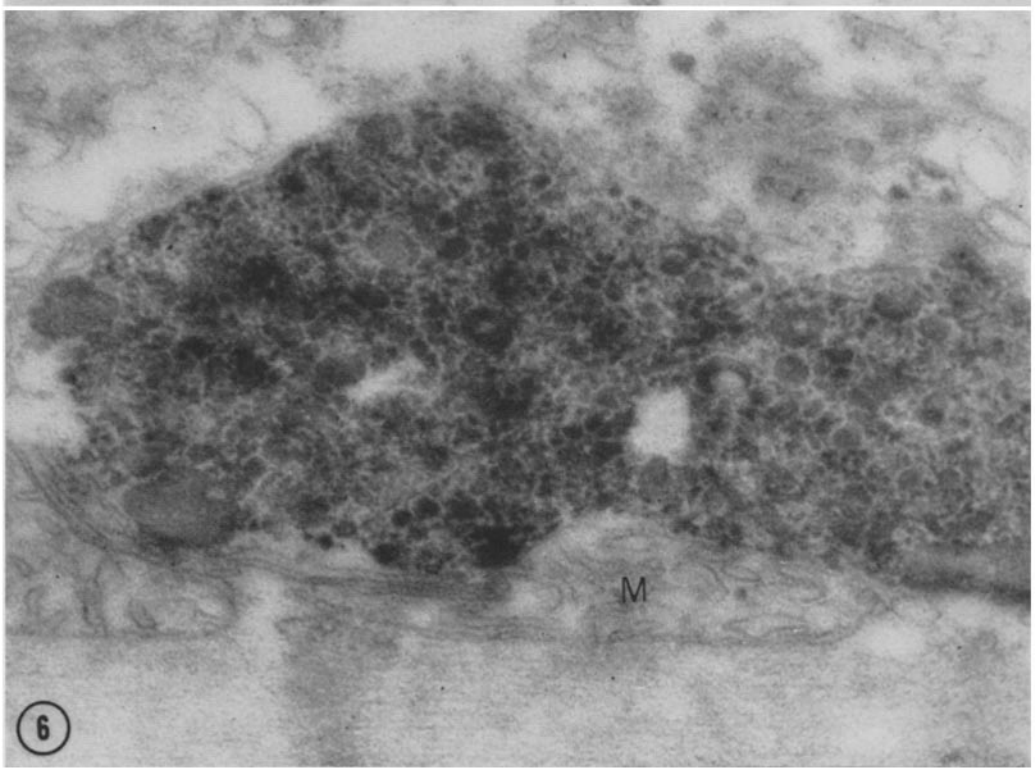
FIGURE 5

Electron micrograph of human cardiac muscle. Age 75 years. The lipofuscin particle is surrounded by a single membrane (arrows). *M*, mitochondrion. × 78,000.

FIGURE 6

Electron micrograph of human cardiac muscle. Age 75 years. High magnification of the lipofuscin particle reveals the marked differences in size, shape, and electron opacity among the granules. *M*, mitochondrion. × 108,000.





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