MORPHOLOGIC CHANGES ACCOMPANYING SENESCENCE OF CULTURED HUMAN DIPLOID CELLS*

By E. ROBBINS,‡ M.D., E. M. LEVINE,‡ Ph.D., and H. EAGLE, M.D.

(From the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461)

(Received for publication 19 January 1970)

Most diploid human fibroblasts in early culture passage have a cell doubling time of approximately 24 hr. After a variable but finite number of transfers, the average generation time begins to increase progressively, bizarre forms appear, the cells eventually cease to divide, and finally degenerate (1, 2). In vivo also, normal cells, transplanted serially, proliferate for only a finite number of generations (3). This gradual "senescence" at the cellular level has been compared to senescence of the whole animal (1–3), and contrasts with the behavior of human aneuploid cancer cells or transformed cells, most of which can be subcultured indefinitely.

We have followed the ultrastructural changes which have developed in nine human fibroblast strains (seven diploid, one trisomic 21, and one Turner's syndrome) in the course of their serial propagation, and have also examined human skin biopsies from four donors of different ages. It has become clear that in terms of morphologic characteristics, senescence in tissue culture and aging in the intact organism are not strictly analogous. Progressive morphologic changes begin to develop in diploid cultures shortly after their in vitro isolation, regardless of the age of the donor; and the most prominent changes are not characteristic of cells in tissues of aging subjects.

Materials and Methods

The human cell strains used in these studies are listed in Table I, and were derived from embryonic lung or skin. The general procedures have been described elsewhere (4, 5). Six of the culture strains listed in Table I were initiated in this laboratory from 2 mm punch biopsies of the right shoulder. Fragments of four of these (EL, HE, HM, KT) were fixed immediately in 2.5% buffered glutaraldehyde and processed for electron microscopy as described below. Three of these six strains (EL, Penny, MS-2) and the six other strains listed in Table I were serially propagated until the cultures were no longer viable. The cultures were divided two- to four-fold at each (weekly) passage, were maintained in antibiotic-free media to mini-

* This work was supported by grants from the National Institutes of Health (AI 4153, GM 14582), the American Cancer Society, and the National Science Foundation.

‡ Recipients of Public Health Service Career Development Awards.

TABLE I

Source of Cultures Used in Present Experiments

Type of cells	Strain	Donor		Tissue	Special characterization	Earliest culture passage studied*
		Age	Sex			
Human fibroblasts	Detroit‡ 510	Newborn	F	Skin	Galactosemia	13
	Detroit 525	3½	F	Skin	Turner's	22
	Detroit 532	Infant	M	Foreskin	Trisomic 21	19
	Detroit 550	Newborn	M	Foreskin	Normal	13
	Detroit 551	Embryo	F	Skin	Normal	6
	EL§, ‖	31	M	Skin	Normal	0
	HE§, ¶	62	M	Skin	Normal	0
	HM§, ¶	64	F	Skin	Normal	0
	KT§, ¶	22	F	Skin	Normal	0
	MS-2A§, ‖	6	M	Skin	Hypoxanthine-guanine phosphoribosyl transferase deficiency (15)	10
	Penny§	8	F	Skin	Homocystinuric patient	8
	Wi 38	Embryo	F	Lung	Normal (1)	21
Human cancer	HeLa		F	Cervix		>400
	KB	54	M	Palate		>400
SV 40-transformed human cells	Wi 26-Va	Embryo	M	Lung		>100
	W 98-E	33	M	Skin		>100
Animal cells	Mouse (3T3)	Whole embryo			(11)	>100
	Rabbit lens epithelium	—		Lens	(12)	>100

* See footnote, page 1213.

‡ The courtesy of Dr. C. S. Stulberg, Director of the Children's Research Center, Detroit, Michigan, in supplying these five Detroit culture strains is greatly appreciated.

§ Isolated in this laboratory.

‖ Examined as biopsy specimen, and in subsequent serial culture as well.

¶ Examined as biopsy specimen only.

mize possible contamination with *Mycoplasma* (6), and were monitored periodically for the presence of *Mycoplasma* by agar plate cultivation. At varying intervals in the course of its serial propagation, each strain was trypsinized and plated on carbon-coated cover slips for electron microscopic study, as previously described (7), and allowed to grow for 6–12 hr. They were then fixed in 2.5% glutaraldehyde, followed by a modified solution of OsO_4 (8), dehydrated in a graded ethanol series, rapidly embedded in Epon 812 (9), sectioned with a du Pont diamond knife, stained with aqueous uranyl acetate followed by lead citrate, and observed in the Siemens Elmiskop 1A electron microscope.

RESULTS AND DISCUSSION

The morphologic appearance of diploid cells from freshly isolated skin tissue has often been described. The nucleus contains mainly fibrillar chromatin, and one or more nucleoli with no distinguishing characteristics. The cytoplasm includes the usual distribution of organelles, with numerous desmosomes. The specimen illustrated in Fig. 1, from a 62 year old male, contained some cytoplasmic glycogen which was not seen in the 21 and 31 year old donors. Significantly, however, the lysosomes (multivesicular bodies) were not visibly abnormal in these biopsy specimens or in early cultures, regardless of the donor's age. Within 3–6 wk after initiation of an in vitro culture, and whatever the donor's age, all cell strains examined began to show intralysosomal alterations.

Figs. 2–5 illustrate the progressive changes in a single cell strain (Detroit 551). Although some of the multivesicular bodies in the sixth culture passage[1] appeared normal (Fig. 2 a), others showed peripheral accumulation of unidentified electron-dense material, and a significant increase in size (Fig. 2 b). No obvious changes were seen at this time in the other cytoplasmic organelles, including endoplasmic reticulum, polysomes, mitochondria, and nucleus.

By the 15th passage (approximately 30–40 cell doublings; see footnote 1), the Detroit 551 cells contained a markedly increased number of lysosomes, which had now expropriated much of the cytoplasmic space (Fig. 3). While many of these lysosomes had transformed into residual bodies, one could usually still find occasional unaltered multivesicular bodies (arrows), suggesting that although turnover of lysosomes and their contents appear impaired, nascent lysosomes continue to be formed. The result is a gradual and eventually overwhelming accumulation of these organelles. Even at the 15th passage, other cytoplasmic organelles still showed relatively little degenerative change. Some

[1] The age of the culture cannot be expressed as the number of cell doublings, primarily because of the indeterminate and variable number of cell generations represented in the population of the primary culture. At the time of the first culture division, some cells will have only recently moved out of the explant (0 cell generations in culture) while others may represent e.g. the 8th or even 16th cell generation, depending on the time between explanation and first subculture. Each of the subsequent subcultures was divided two- to four-fold, and thus represents an additional 1–2 cell generations; but a further complication in assessing the number of cell generations was introduced by the occasional necessity of reconstituting a strain from a frozen stock at intermediate passage.

cells contained more than the occasional, scattered glycogen particle seen in early passage cells. In about half the cells, there was a 30–60% decrease in the number of free polysomes compared to early passage cells, while membrane-bound polysomes remained unaffected.

In this 15th passage, the cells were growing normally, even if at a somewhat slower rate than in early culture passage. By the 22nd passage, however, most cells of the Detroit 551 strain resembled that depicted in Fig. 4, with the cytoplasm largely occupied by degenerating residual bodies. Although some cell images still showed relatively unaffected mitochondria, membrane-bound polysomes, and Golgi saccules, others showed degenerative signs. Large aggregates of glycogen were a common characteristic of cells at this terminal stage, but not as universal as the lysosomal changes (Fig. 5). Cytoplasmic polyribosomes had largely disappeared. This strain ceased to divide and was discarded at the following (23rd) passage.

Although Figs. 1–5 relate to a single cell strain, similar patterns of lysosomal transformation have been noted on serial passage of all human diploid strains here studied. These progressive changes have not been seen in cells which grow indefinitely in culture, whether human cancer cells (HeLa, KB), SV 40-transformed human cells (strains Wi 26-Va and W 98-E) (10), or serially propagated animal cells [mouse fibroblast strain 3T3 (11) and an epithelial rabbit lens cell (12)].

It thus appears that the serial propagation of cells which ultimately become senescent in culture is accompanied by major changes in the lysosomes, which increase progressively in both number and size, and show profound degenerative changes. Less consistently, there is an accompanying decrease in free cytoplasmic polysomes and an accumulation of glycogen.

It is tempting to invoke a causal relationship between these changes in the cellular organelles, and the progressive slowing of the rate of cellular growth and division, with eventual degeneration. It must, however, be noted that the morphologic changes had already become quite prominent at a time when there was no significant change either in the appearance of the cell in the light microscope, in the rate of growth, or in plating efficiency after trypsinization. Further, these degenerative changes occur only in cells propagated in vitro, in contrast to the trivial morphologic changes seen in fibroblasts of aging subjects. Supplementation of the usual growth medium (13) with trace elements (Fe, Cu, Zn), sodium pyruvate, vitamin B_{12}, biotin, lipoic and linoleic acids, insulin, hydrocortisone, cyclic adenosine monophosphate (AMP), ascorbic acid, and a Sephadex-separated fraction of chick embryo extract (14) failed to prevent the progressive degenerative changes here described. Biochemical correlates of these structural changes are under study.

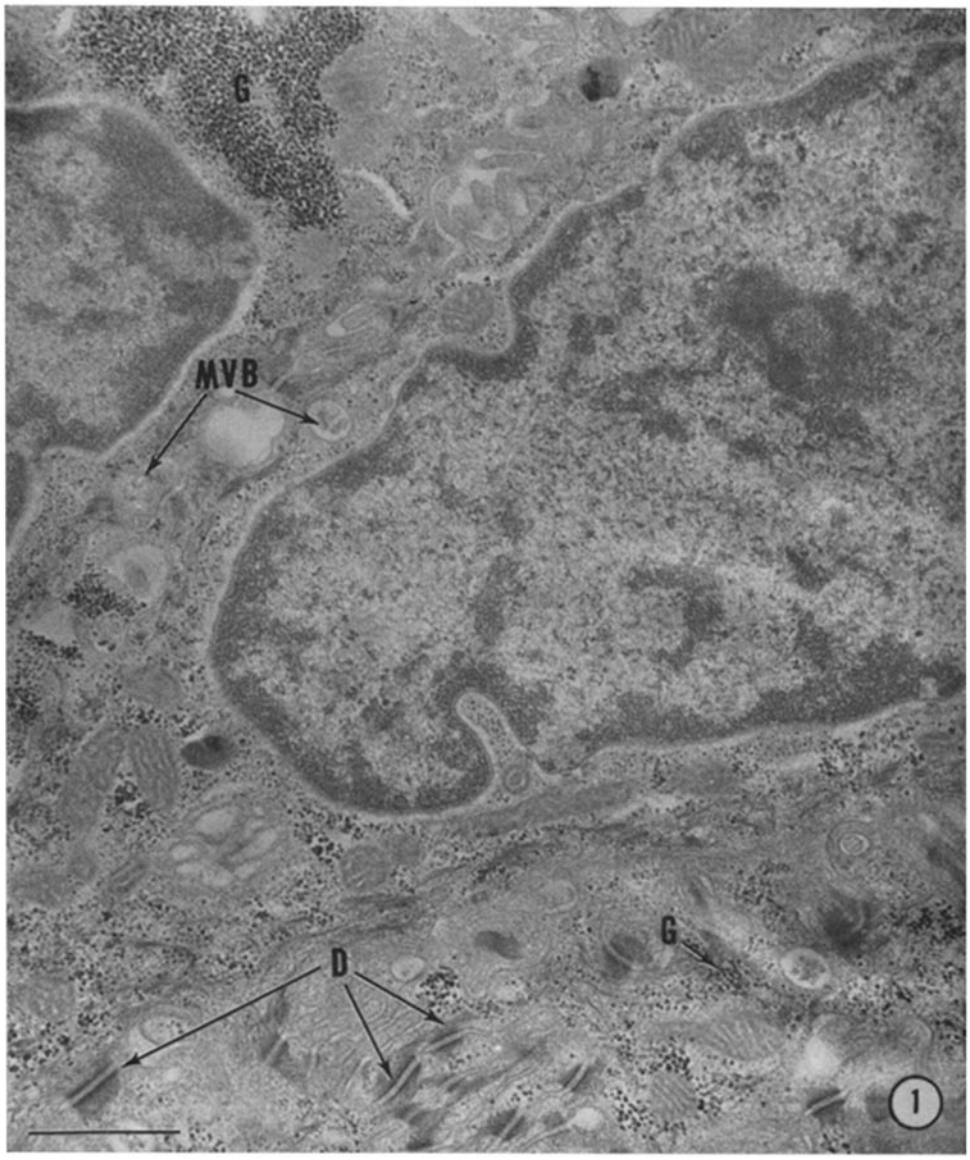

FIG. 1. Section from epidermal punch biopsy showing normal morphology of human skin cell (male aged 62 years). Several multivesicular bodies (MVB) are indicated as well as desmosomes (D) and glycogen particles (G). Scale marker, 1μ.

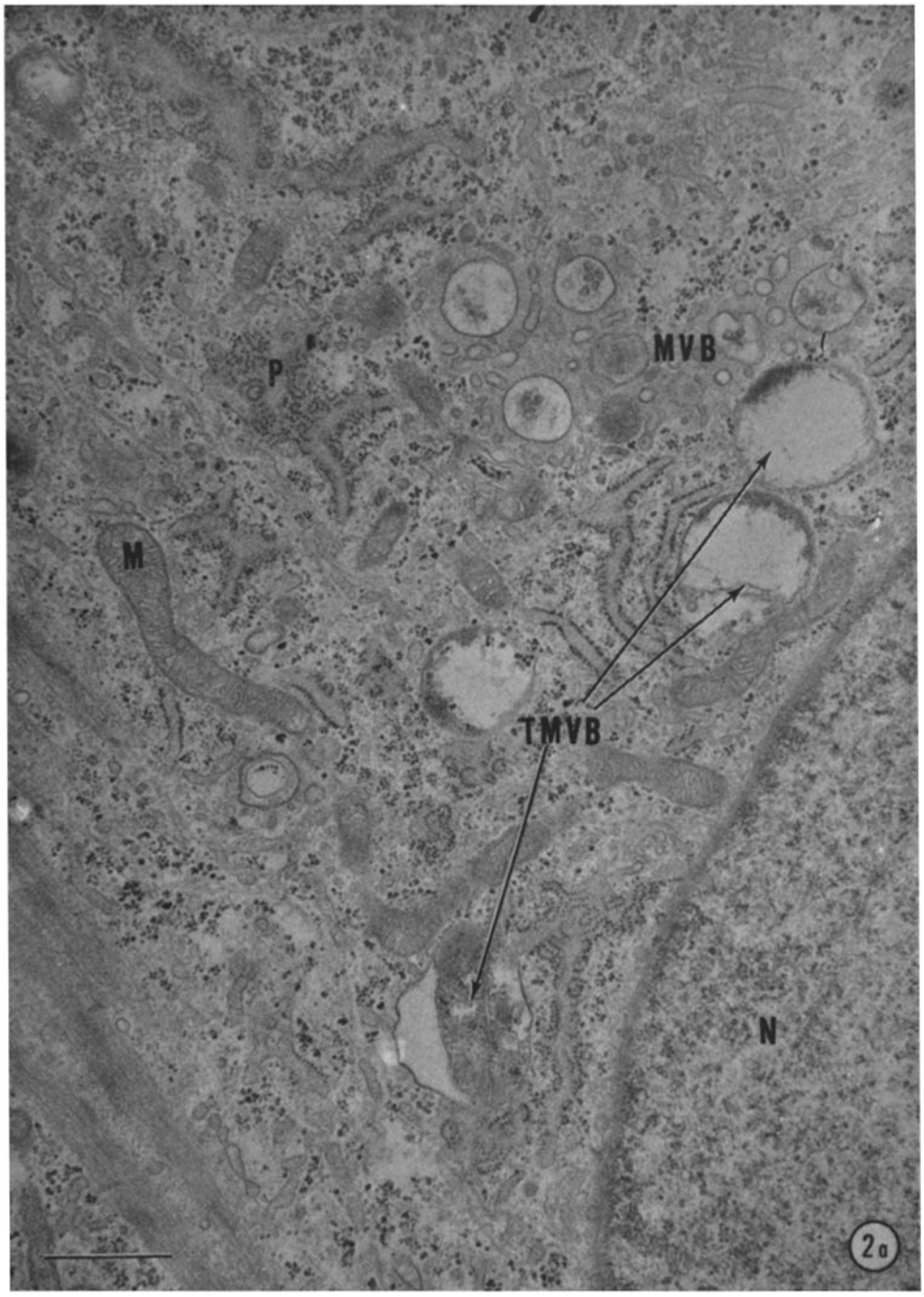

FIG. 2 a. Cell from sixth passage of strain Detroit 551; lysosomal changes are already evident. (TMVB, transformed multivesicular body; N, nucleus; M, mitochondrion; P, polyribosomes). Scale marker, 1μ.

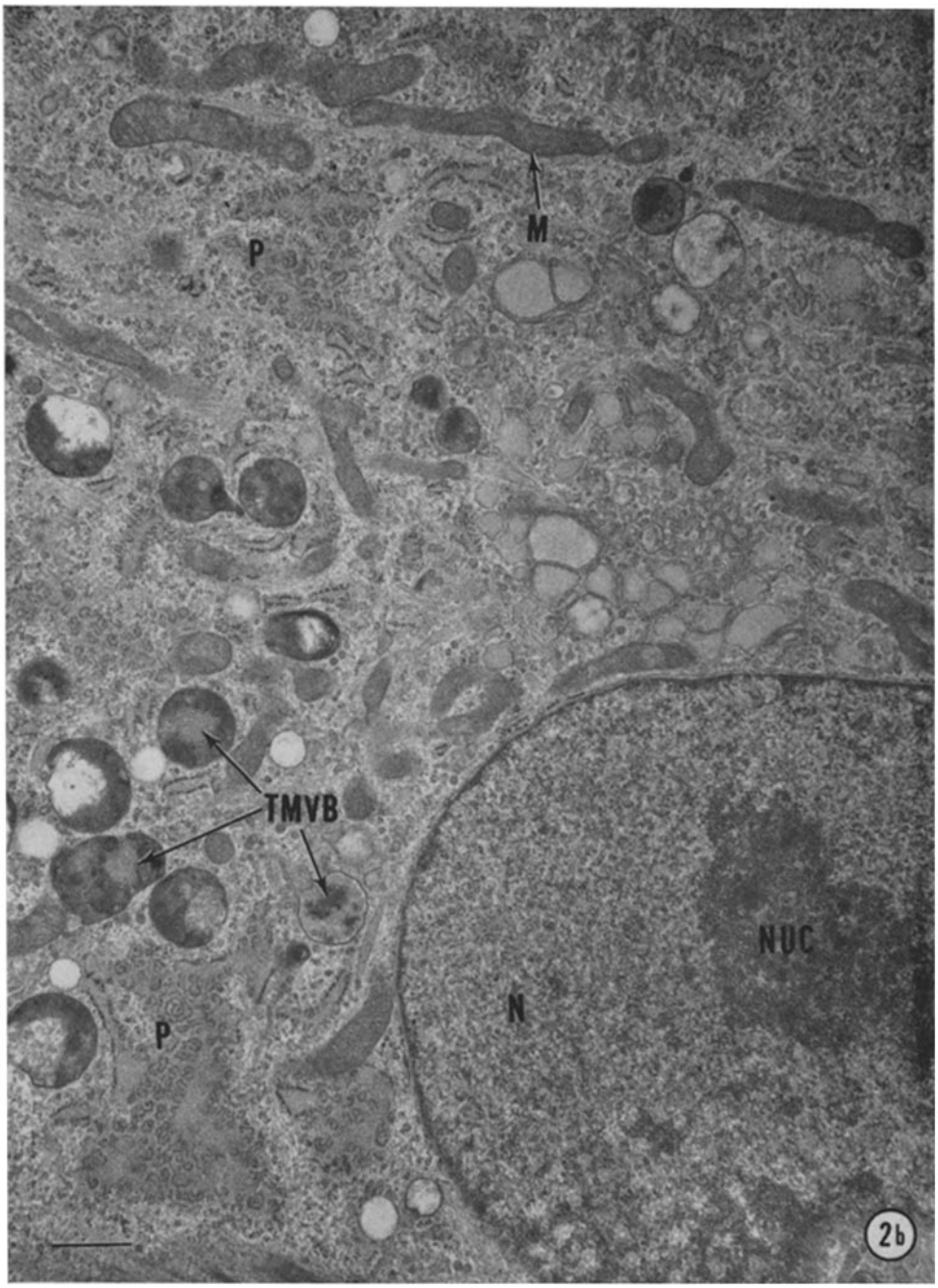

FIG. 2 b. Cell from same preparation as that shown in 2 a; many altered lysosomes are visible (Nuc, nucleolus). Scale marker, 1μ.

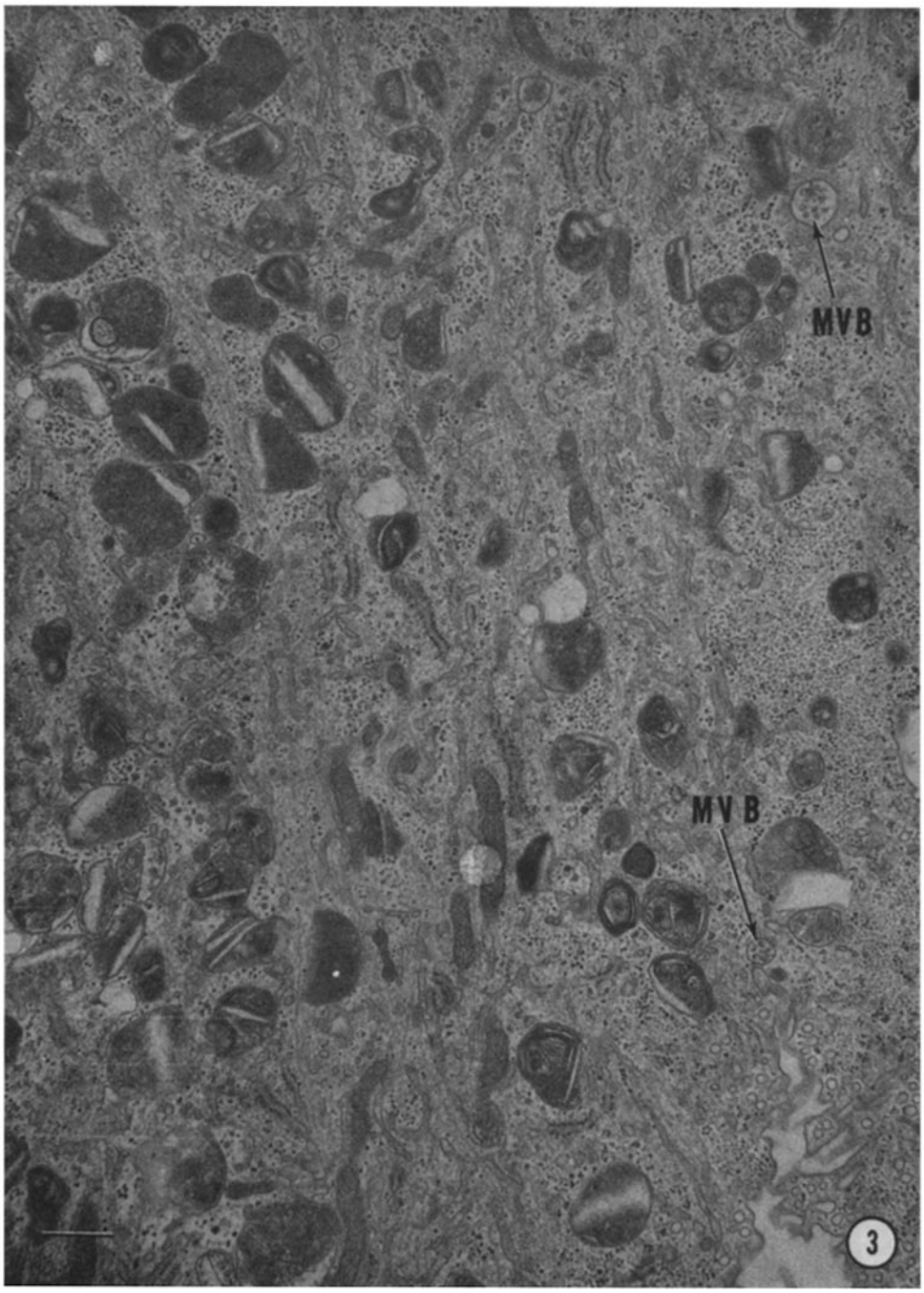

FIG. 3. Cell from 15th passage of strain Detroit 551; most of the lysosomes have transformed into residual bodies, although an occasional normal multivesicular body is seen. Scale marker, 1μ.

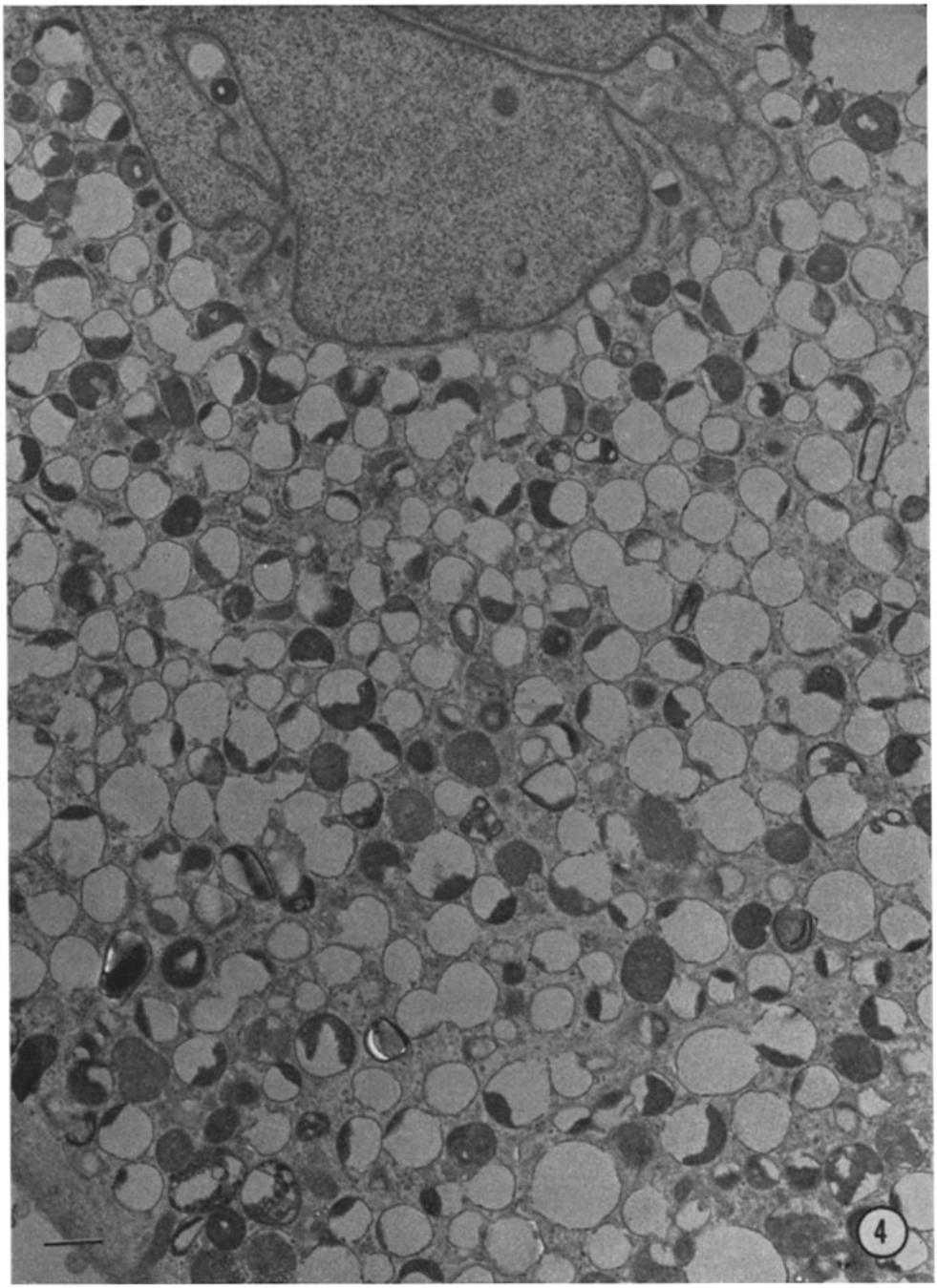

FIG. 4. Cell from 22nd passage of strain Detroit 551; cytoplasmic space is predominantly filled with partially degenerated residual bodies. Scale marker, 1μ.

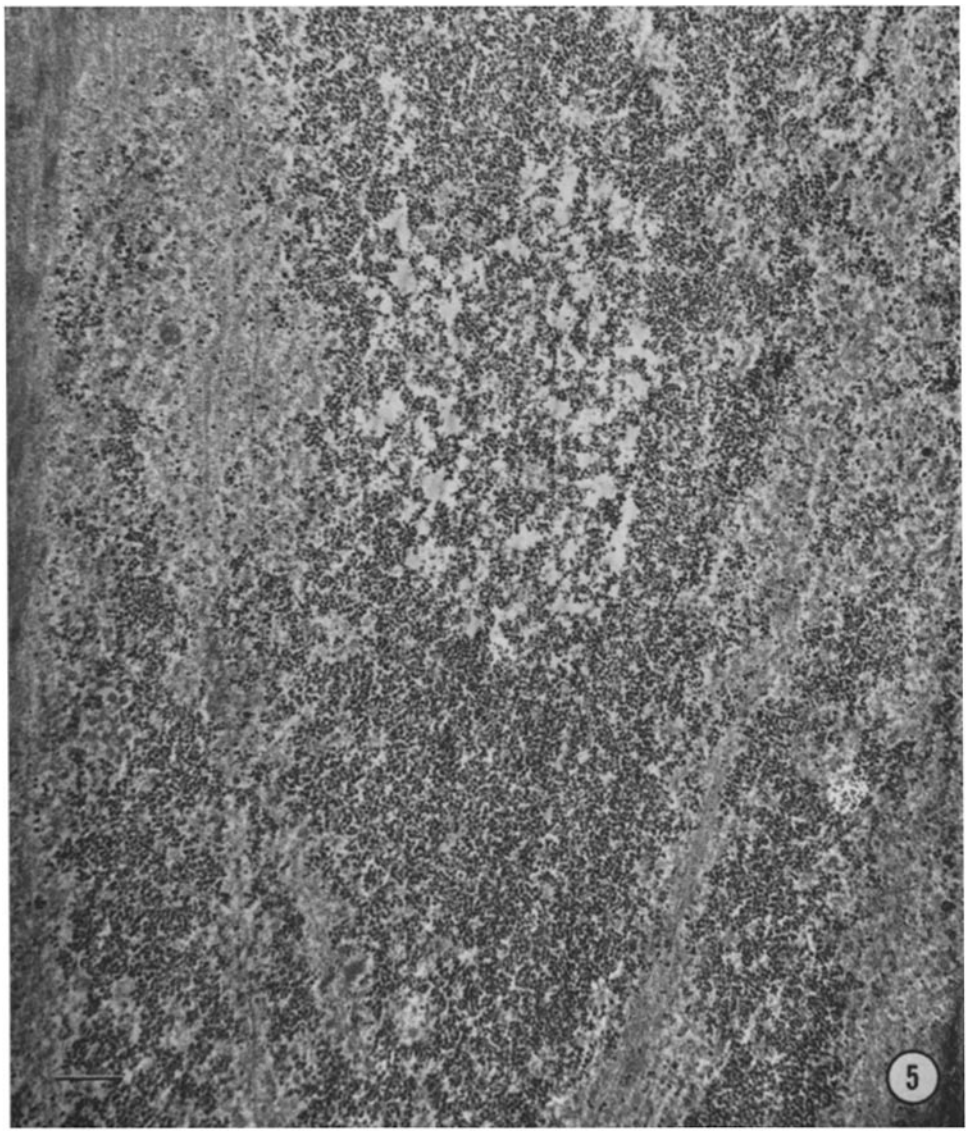

FIG. 5. Area of cytoplasm from Detroit 551 strain cells in 22nd passage; note massive accumulation of glycogen. Scale marker, 1μ.

SUMMARY

The lysosomes of serially propagated human fibroblasts gradually transform to residual bodies which increase in number and size, and show progressive de-

generative changes. There is an accompanying, and less regular, decrease in the number of cytoplasmic polyribosomes and an increased number of glycogen particles. The onset of these morphologic alterations occurs shortly after culture initiation and precedes any marked decrease in the rate of cellular growth; however, in their extreme form these changes may be related to the ultimate cessation of cellular multiplication ("senescence"). The lysosomal changes were seen only in those cell strains which eventually showed senescence, and were absent or minimal either in cell lines which can be propagated indefinitely ("spontaneous" and viral transformants, cancer cells), or in skin sections from aging subjects.

BIBLIOGRAPHY

1. Hayflick, L. 1965. The limited *in vitro* lifetime of human diploid cells. *Exp. Cell Res.* **37**:614.
2. Hayflick, L., and P. S. Moorehead. 1961. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**:585.
3. Daniel, C. W., K. B. DeOme, J. T. Young, P. B. Blair, and L. P. Faulkin. 1968. The *in vivo* life span of normal and preneoplastic mouse mammary glands: a serial transplantation study. *Proc. Nat. Acad. Sci. U.S.A.* **61**:52.
4. Levine, E. M., Y. Becker, C. W. Boone, and H. Eagle. 1965. Contact inhibition, macromolecular synthesis, and polyribosomes in cultured human diploid fibroblasts. *Proc. Nat. Acad. Sci. U.S.A.* **53**:350.
5. Eagle, H., E. M. Levine, and H. Koprowski. 1968. Species specificity in growth regulatory effects of cellular interaction. *Nature (London).* **220**:266.
6. Levine, E. M., L. Thomas, D. McGregor, L. Hayflick, and H. Eagle. 1968. Altered nucleic acid metabolism in human cell cultures infected with *Mycoplasma*. *Proc. Nat. Acad. Sci. U.S.A.* **60**:583.
7. Robbins, E., and N. K. Gonatas. 1964. *In vitro* selection of the mitotic cell for subsequent electron microscopy. *J. Cell Biol.* **20**:356.
8. Robbins, E., and N. K. Gonatas. 1964. The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* **21**:429.
9. Robbins, E., and G. Jentzsch. 1967. Rapid embedding of cell culture monolayers and suspension for electron microscopy. *J. Histochem. Cytochem.* **15**:185.
10. Eagle, H., G. E. Foley, H. Koprowski, E. M. Levine, R. A. Adams, and H. Lazarus. 1970. Growth characteristics of virus-transformed cells. Maximum population density, inhibition by normal cells, serum requirement, growth in soft agar and xenogeneic transplantability *J. Exp. Med.*, **131**:863.
11. Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* **17**:299.
12. Shapiro, A. L., I. M. Siegel, M. D. Scharff, and E. Robbins, 1969. Characteristics of cultured lens epithelium. *Invest. Ophthalmol.* **8**:393.
13. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Washington).* **130**:436.

14. Coon, H. G., and R. D. Cahn. 1966. Differentiation *in vitro*: effects of Sephadex fraction of chick embryo extract. *Science (Washington)*. **153:**1116.

15. Siniscalco, M., H. P. Klinger, H. Eagle, H. Koprowski, W. Fujimoto, and J. Seegmiller. 1969. Evidence for intergenic complementation in hybrid cells derived from two human diploid strains each carrying an X-linked mutation. *Proc. Nat. Acad. Sci. U.S.A.* **62:**793.