SUBCELLULAR FRACTIONATION AND MORPHOLOGY OF CALF AORTIC SMOOTH MUSCLE CELLS

Studies on Whole Aorta, Aortic Explants, and Subcultures Grown under Different Conditions

STANLEY FOWLER, HELEN SHIO, and HARVEY WOLINSKY

From the Laboratory of Biochemical Cytology, The Rockefeller University, New York 10021, and the Departments of Medicine and Pathology, The Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

A comparative biochemical and morphological study was made of calf aortic smooth muscle cells found *in situ* and grown in vitro under various conditions. Striking alterations in enzyme contents, physical properties, and morphological appearances of lysosomes, endoplasmic reticulum, plasma membranes and, to a lesser extent, mitochondria were observed upon culturing of calf aortic smooth muscle cells. These changes first appeared in cells growing out of tissue explants. They developed further upon subculturing of the cells and depended greatly on the culture conditions used. The alterations included increases in specific activities of some 5- to 25-fold of four acid hydrolases, an average ninefold increase in 5′-nucleotidase, sevenfold increase in cytochrome oxidase, and fourfold increase in neutral α-glucosidase in subcultured smooth muscle cells compared to aortic cells *in situ*. Cell fractionation studies showed significant shifts in the equilibrium densities of plasma membranes, microsomes, and lysosomes, but not of mitochondria, in smooth muscle cells growing out from explants and in subcultured cells, compared to cells isolated from intact aortas. Although the cells grown in vitro exhibited typical phenotypic features of smooth muscle cells such as abundant myofilaments and surface vesicles, alterations in the morphological appearance of the endoplasmic reticulum, Golgi apparatus, and, especially, lysosomes were observed. These results demonstrate significant differences in specific cellular characteristics and functions of aortic smooth muscle cells grown in vitro compared to aortic cells *in situ*.

KEY WORDS aorta · cell fraction · electron microscopy · lysosomes · smooth muscle cells · tissue culture

An important recent focus of attention in atherosclerosis research has been the biology of the arterial smooth muscle cell. Among the techniques being used to study smooth muscle cells is the ex vivo culture of these cells. Popularization of this approach has followed upon the work of Jarmolych et al. (26) and of Ross (39), which convincingly showed that cells phenotypically similar to

those found in the intact vessel could be maintained for many generations in culture. Recently, research efforts have concentrated on elucidating the synthetic and proliferative capabilities of these cells in vitro (40). The effects of hormonal and other environmental manipulations on smooth muscle cell cultures have also been reported (1, 8, 14, 19, 41, 46) and the uptake and degradation of lipoproteins by these cells have been described by several laboratories (1, 6, 22, 44, 48).

Smooth muscle cell culture would appear to be an excellent tool for many pathophysiologic studies because the cells are easily subjected to various treatments under controlled conditions, and the interpretation of results is simplified by the presence of only one cell type. We had hoped to use smooth muscle cell culture to test certain pathologic mechanisms thought to be occurring in vascular disease by following the transformation of normal cultured smooth muscle cells to diseased cells. In the course of our investigations, however, it became apparent that the properties of certain cell functions in culture were already different from those found in normal smooth muscle cells *in situ*. We therefore undertook to determine systematically the extent to which specific cellular characteristics and functions present in vitro differ from those of smooth muscle cells of the intact vessel wall. We have examined the properties of lysosomes, mitochondria, endoplasmic reticulum, and plasma membranes of aortic smooth muscle cells both by cell fractionation and by electron microscopy. These studies were carried out on cells isolated from intact vascular tissue, on cells growing out from primary culture explants, and on cells derived from subcultures. A preliminary communication of these investigations has been given previously (20).

MATERIALS AND METHODS

All aortic smooth muscle cell preparations were derived from aortas of young male calves (~3–6 mo old).

Isolated Aortic Cells

Aortic cells were isolated from thoracic aortic tissue by the method of Peters et al. (34), but incorporating the modifications proposed by Ives et al. (24). Thin strips of inner media (2.5 g) were removed and cut into 0.5-mm squares with a McIlwayne Tissue Chopper (Brinkman Instruments, Inc., Westbury, N. Y.). The tissue chunks were washed in Hanks' medium (23) supplemented with both essential and nonessential amino acids (25) and containing 0.2 mM calcium. The pieces were then incubated in 15 ml of the same medium containing 1,800 U/ml purified collagenase, 230 U/ml purified elastase, and 1 mg/ml soybean trypsin inhibitor, in a shaking water bath at 37°C. The released cells (monitored by phase microscopy) were separated from tissue debris by filtration through nylon gauze (150 μm) and centrifuged at 1,100 rpm for 8 min in a desk-top International Clinical Centrifuge model CL (International Equipment Co., Boston, Mass.). Pooled cells were washed twice in supplemented Hanks' medium and then twice in cold 0.25 M sucrose containing 1 mM EDTA and 0.1% ethanol (SVE medium).[1] This SVE medium has been used in all previous work from this laboratory on fractionation of arterial smooth muscle cells (33–35). The ethanol contained in it serves to prevent conversion of catalase to the inactive Compound II.

Homogenates of calf aorta were used to determine the specific activity of enzymes of cells *in situ*. Strips of the inner media of the thoracic aortas (2.0 g) were cut into 0.1-mm squares with the McIlwayne Tissue Chopper and transferred to a large Duall homogenizer (Kontes Co., Vineland, N. J.). The chopped tissue was homogenized in 10 ml of cold SVE medium and then quantitatively transferred to a graduated cylinder. Half the preparation was used for determination of DNA content and the remainder used for assay of various enzymes.

Aortic Smooth Muscle Cell Cultures

EXPLANT CELLS: Explants of calf aortic smooth muscle cells were grown as described by Coltoff-Schiller et al. (12). In summary, approximately 40 pieces of dissected inner media of the thoracic aorta, about 1 mm^3 in size, were placed in a 28-cm^2 plastic petri dish containing 0.5 ml Dulbecco's modified essential medium (30) supplemented with 1% nonessential amino acids and 10% fetal calf serum (FCS), and buffered with 40 mM Tris-acetate, pH 7.4. A mixture of penicillin (100 IU/ml), streptomycin (100 μg/ml), and Fungizone (0.25 μg/ml) was routinely added to the culture media. The tissue explants were incubated at 37°C in 95% air-5% CO_2; the medium was changed every 2 days. After 3–4 wk, cells had grown to near confluence. These cells will be referred to as "explant cells."

SUBCULTURED CELLS: Subcultures were prepared from explant cells as follows: after removal of adherent aortic tissue pieces, explant cells were harvested with 0.05% trypsin-0.5 mM EDTA in Puck's saline (36). The cells were placed into 75-cm^2 plastic flasks (Falcon Plastics, Oxnard, Calif.) at a density of 2.5×10^6 cells per flask and grown in the supplemented medium described above. Seeding efficiency was >90%

[1] Abbreviations and symbols used in this paper: CS, calf serum; FCS, fetal calf serum; 2 CS, 2% calf serum; 10 CS, 10% calf serum; 50 CS, 50% calf serum; 10 FCS, 10% fetal calf serum; 50 FCS, 50% fetal calf serum; PNS, postnuclear supernate; RER, rough endoplasmic reticulum; SVE medium, 0.25 M sucrose containing 1 mM EDTA and 0.1% ethanol.

and the cells grew to confluence in about 4 days. At this stage the cells are considered to be generation one. For further subculturing, splits of cells in a 1:4 ratio were used. For the experiments on effects of culture conditions, confluent cultures of 1st, 3rd, 5th, or 7th generation were used.[2] Cells brought to confluence in 10% fetal calf serum (10 FCS) were rinsed twice with 15 ml of serum-free Dulbecco's medium supplemented as described above and then incubated for 1 wk in 15 ml of supplemented medium containing 10 FCS, 50% fetal calf serum (50 FCS), 2% calf serum (2 CS), 10% calf serum (10 CS), or 50% calf serum (50 CS). The medium was changed three times during this period. Before use, the calf serum (CS) was passed through a 0.45-μm membrane filter (Nalge Co., Nalge Labware Div., Rochester, N. Y.). These preparations will be referred to as "subcultured cells."

For harvesting of explant cells or subcultured cells, the medium was poured off and the cell layer rinsed three times in SVE medium. The culture was then incubated for 5-10 min at room temperature in 5.0 ml of SVE, during which time the cells began to detach. The cells were then removed with a rubber policeman when necessary, and washed twice in ice-cold SVE medium. The cells harvested in this manner were used in fractionations and for enzyme and DNA determinations. When cell counts were made, the cells were detached by brief incubation with 0.05% trypsin-0.5 mM EDTA in Puck's saline. The cells were uniformly suspended in 50 ml of culture medium, and a small aliquot was placed in a hemocytometer for counting by phase microscopy. The remainder of the cells was concentrated (800 rpm for 10 min) at 4°C, washed three times in Puck's saline, and assayed for DNA content.

Electron Microscopy

AORTIC TISSUE: Morphologic studies were carried out both on aortic cells fixed *in situ* and on cells isolated after enzymic digestion of the tissue. Thin rings of calf thoracic aorta were transported to the laboratory either in ice-cold Karnovsky's paraformaldehyde fixative (27) or in 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4. Diced pieces of inner media were placed in fresh fixative for an additional 3–4 h followed by postfixation for 1½ h in ice-cold 1% OsO_4 in 100 mM cacodylate buffer, pH 7.4. Isolated aortic cells suspended in the supplemented Hanks' solution were mixed with an equal volume of ice-cold 5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, and further diluted with ice-cold 2.5% glutaraldehyde in the same buffer. After fixation for 3 h, the cells were concentrated at low speed on a table-top centrifuge and cell pellets prepared by centrifugation of the cells for 5 min in a Beckman 152 Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The pellets were diced into small pieces with a razor blade and postfixed in 1% OsO_4 as above. All specimens were stained en bloc with uranyl acetate (17), then dehydrated in graded ethanol, treated with propylene oxide, and embedded in Epon 812.

CELL CULTURE: Calf aortic smooth muscle cells cultured in plastic petri dishes were fixed for 5 h *in situ* with ice-cold 2.5% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, treated with ice-cold 1% OsO_4 in 100 mM cacodylate buffer, pH 7.4 for 1 h and stained en bloc with uranyl acetate. The fixed cells, still in the plastic dish, were dehydrated, equilibrated with a mixture of Epon 812 and absolute ethanol (1:1, vol/vol), and subsequently, with pure Epon. Polymerization was done overnight at 60°C with only a thin layer of Epon left in the bottom of the dish. The resulting thin sheet of polymerized Epon, containing embedded cells, was carefully separated from the petri dish and cut into small pieces. These pieces were then re-embedded in Epon in a manner to permit sectioning for perpendicular or tangential views of the cells (16).

Cytochemical staining for acid phosphatase by the Gomori method (2) at pH 5.0 using β-glycerophosphate as substrate was carried out on cultured cells after fixing in the culture dish with ice-cold 1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.4, for 3 h. Specimens incubated in medium without substrate served as controls. Incubation was carried out at 37°C for 1½ h on a shaker bath with constant agitation. After incubation, the cells were washed in cacodylate buffer, and then processed as above.

Silver sections were cut on a Sorvall MT-2B ultramicrotome with a Dupont diamond knife (DuPont Instruments, Sorvall Operations, Newtown, Conn.). Sections were doubly stained with uranyl acetate and lead citrate (37) before viewing in a Philips EM-300 electron microscope operated at 80 kV. For light microscope evaluation, 1-μm thick Epon sections were cut with a glass knife, stained with azure II-methylene blue (38) and photographed with a Zeiss Universal Photomicroscope (Carl Zeiss, Inc., New York) on Kodak Panatomic-X film.

Disruption and Subcellular Fractionation of Smooth Muscle Cells

The washed cells were suspended in 4 ml of cold SVE medium and disrupted with 15 strokes of a type B pestle in a small Dounce homogenizer (Kontes Co.). The homogenate was transferred to a conical tube and centrifuged at 750 rpm for 10 min at 4°C in an International PR-2 refrigerated centrifuge (International Equipment Co.) equipped with a no. 253 rotor. The supernate was removed with a Pasteur pipette and kept at 4°C. The pellet was resuspended in 4 ml of SVE medium and homogenized again as described above. After centrifugation, the postnuclear supernates (PNS) were pooled and brought to a vol of 10 ml. The pellet of nuclei and debris was suspended in 2.5 ml of SVE medium (N

[2] All results are pooled since no consistent difference in any of the observations made was found between generations over this narrow range.

fraction). Examination of these fractions by phase microscopy indicated that nearly all the cells had been disrupted. Assay of latent *N*-acetyl-β-glucosaminidase activity (34), a measure of lysosome integrity, revealed latencies of 70% or more for all PNS preparations except for the explant cell PNS which averaged only 61%.

The PNS was subfractionated by density gradient centrifugation in a Beaufay automatic zonal rotor (3, 28). 7.5 ml of PNS was layered onto a 24-ml sucrose gradient extending linearly with respect to volume from a density of 1.05–1.28, and resting on a 6-ml cushion of density 1.32. All sucrose solutions contained 1 mM EDTA and 0.1% ethanol. The rotor was run at 35,000 rpm for 35 min at 4°C. Under these conditions, all but the smallest subcellular particles are brought to their equilibrium position. At the end of the run, some 17 fractions were collected into tared tubes and weighed. The density of each fraction was determined with an Abbé refractometer (Bausch & Lomb Inc., Analytical Systems Div., Rochester, N. Y.).

Analytical Techniques

Glycosidases and cathepsin C were assayed fluorometrically according to Peters et al. (34), except that the following incubation conditions were used: *N*-acetyl-β-glucosaminidase, 0.25 mM 4-methyl umbelliferyl-2-acetamido-2-deoxy-β-D-glycopyranoside, 50 mM Na-citrate, pH 4.8, and 0.1% Triton X-100; β-galactosidase, 0.25 mM 4-methyl umbelliferyl-β-D-galactopyranoside, 50 mM Na-formate, pH 4.3, 5 mM $MgCl_2$, and 0.1% Triton X-100; neutral α-glucosidase, 1 mM 4-methyl umbelliferyl-α-D-glucopyranoside, 50 mM Na-phosphate buffer, pH 7.1, and 0.1% Triton X-100; cathepsin C, 0.5 mM glycyl-L-phenylalanyl-β-naphthylamide, 50 mM Na-acetate, pH 4.2, 40 mM NaCl, 3 mM dithiothreitol, and 0.1% Triton X-100. Incubations were carried out for 15–60 min at 37°C. Each enzyme assay was checked for linearity with time and enzyme concentration. Assays for cytochrome oxidase and 5'-nucleotidase were performed as described (34). Gradient fractions were diluted with SVE medium before enzyme assay.

Acid cholesteryl esterase was determined by a modification of the radioisotopic methods of Brecher et al. (7) and Takano et al. (47). Cholesteryl oleate, labeled in the fatty acid moiety (about 100 μCi ^3H/μmol), was incorporated into egg lecithin liposomes (7). The final substrate solution, including liposomes, contained 2.54 mM egg lecithin, 25.4 μM cholesteryl oleate, 1.0 mM Na taurocholate, and 50 mM Na-lactate-50 mM Na-acetate, pH 3.9. The reaction was begun by addition of 0.1 ml of this mixture to 0.1 ml of suitably diluted enzyme. Except for the 10 FCS studies in which detergent was omitted, all dilutions of the gradient fractions were made in SVE medium containing Triton X-100 adjusted in amount to give a concentration of 0.2%. The latter detergent was found necessary to prevent adsorption of the enzyme to test tubes, pipettes, etc. After incubation for up to 60 min at 37°C, the reaction was stopped and the radioactive fatty acid released was extracted by the procedure of Belfrage and Vaughan (4). A 0.5-ml aliquot of the upper phase was added to 10 ml of scintillant (8 g of naphthalene, 600 ml of toluene, and 400 ml of 2-ethoxyethanol) and counted. Under these conditions, enzyme activity was observed to be linear with time and enzyme concentration.

Protein was determined fluorometrically by the O-phthalaldehyde reaction (5). DNA was measured by the method of Burton (9).

For all hydrolases, 1 U of activity corresponds to the hydrolysis of 1 μmol substrate/min at 37°C. Cytochrome oxidase activity is expressed in the units of Cooperstein and Lazarow (13).

Presentation of Enzyme Distribution Results

Results of gradient experiments are presented as normalized frequency histograms of the density distribution of the enzymes (15). The results of the top fractions, up to a density of 1.085 which relate mostly to soluble enzyme activities remaining in the starting layer, were pooled and plotted over a density interval of 1.035–1.085. The corresponding block is shaded in the histograms to indicate that it is not part of the density distribution proper. The limit of 1.085 is somewhat arbitrary and is passed by sedimenting proteins of relatively large size. For these, the histograms are distorted on the low density side by the presence of the soluble activity.

The calculations and plots were done by computer, according to methods previously described (15, 28). Pooling and averaging of several distributions were performed as described by Leighton et al. (28). The method requires conversion of all the histograms to the same preset density intervals and causes some loss in resolution. To facilitate interpretation, the distribution for isolated calf cells is depicted on the figures as a dashed line. In addition, the number of experiments performed for each culture condition is given.

Materials

All chemicals were of analytical grade. Sucrose was purchased from Mallinckrodt Inc. (St. Louis, Mo.). The 4-methyl umbelliferyl substrates were purchased from Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England. Glycyl-L-phenylalanyl-β-naphthylamide was prepared by Vega-Fox Biochemicals (Tuscon, Ariz.). Purified elastase, collagenase, and soybean trypsin inhibitor were purchased from Supelco, Inc. (Bellefonte, Pa.), and most other biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Purified O-phthalaldehyde was obtained from Durrum Chemical Corp., Palo Alto, Calif. Osmium tetroxide and 10% glutaraldehyde, sealed in ampules, were purchased from Electron Microscopy Sciences (Fort Washington, Pa.). Dulbecco's modified essential medium, Puck's saline, trypsin, penicillin, streptomycin, Fungizone, and culture

serums (non-heat-inactivated fetal calf and calf [up to 6 mo old]) were obtained from Grand Island Biological Co. (Grand Island, N. Y.).

RESULTS

Growth Characteristics of the Smooth Muscle Cell Cultures

Our investigations were carried out with smooth muscle cells grown under conditions typically used by others (Table I). Out-growths of cells from the explants appeared 10–14 days after initiation of ex vivo culture, and nearly confluent dishes of explant cells were obtained within 3–4 wk. After trypsinization and replating for subcultures, the cells grew rapidly with a population doubling time of ~48 h. Cells reached confluence in about 4 days and cell numbers remained nearly constant for the next 3 days. Exposure of these confluent week-old cultures for 1 additional wk to 10 FCS or to 2 CS, 10 CS, or 50 CS had relatively little effect on cell number (Table II), but the apparent DNA content of the cells was increased 30–40% when FCS was replaced by CS in the medium. The reason for this increase is not evident, but it might have been caused by: (*a*) underestimation of cell counts due to more abundant connective tissue fibers in cultures grown in CS, which tended to interfere with cell dispersion, (*b*) accumulation of DNA derived from phagocytosis of dead cells, or (*c*) occurrence of polyploidy in the cultured cells. The latter was not investigated by us. It is a possibility, however, in view of the results of Martin and Sprague (29) who found 10–14% tetraploid

TABLE I

Representative Examples of Culture Conditions Used for Studies of Smooth Muscle Cells

Cell source	Serum source and concentration	Culture conditions*	Reference
Human aorta	10% Fetal calf serum	Confluent subculture 3–4 wk after seeding	(1)
Human aorta	10% Fetal calf serum	Nonconfluent subculture 6 days after seeding	(22)
Monkey aorta	10% Calf serum	Explant grown 6–12 wk	(19)
Monkey aorta	1% Monkey serum	Nonconfluent subculture maintained 7 days	(41)
Monkey aorta	10% Fetal calf serum	Subculture, 80% confluent, <7 days after seeding	(44)
Swine aorta	1.5% Swine serum	Nonconfluent subculture 4 days after seeding	(8)
Swine aorta	20% Swine serum	Explant grown 4–9 days	(14)
Swine aorta	10% Fetal calf serum	Subculture, 50–75% confluent 4–6 days after seeding	(48)
Rabbit aorta	10% Fetal calf serum	Confluent subculture 7 days after seeding	(32)
Rat aorta	10% Fetal calf serum	Confluent subculture 8–15 days after seeding	(7)
Calf aorta	10% Fetal calf serum	Confluent subculture 1 wk after seeding; culture continued additional week in various media	This study

* Other variables include type of culture medium used, passage number of subculture, use of heat-inactivated serum, and method by which cell cultures were established before use.

TABLE II

Characterization of Subcultures Incubated in Various Media

	Total amount per confluent culture (75 cm²)				
	Initial subculture (10 FCS)	10 FCS*	2 CS*	10 CS*	50 CS*
Cell Count (× 10^6)	12.4 ± 2.3 (9)	16.8 ± 2.9 (6)	10.3 ± 1.4 (6)	13.1 ± 0.8 (6)	16.1 ± 2.5‡ (4)
DNA (μg)	76.7 ± 17 (9)	104.4 ± 18 (6)	91.6 ± 19 (6)	106.9 ± 16 (6)	170.7 ± 64 (6)
pg DNA/cell	6.21 ± 1.1 (9)	6.26 ± 1.0 (6)	9.11 ± 2.4 (6)	8.14 ± 0.9 (6)	8.18 ± 0.8 (4)

Values are given as means ± SD with number of experiments within parentheses.

* Confluent initial cultures of first to seventh generations were incubated for 1 additional wk in the medium indicated.

‡ Probably underestimated due to high cell density.

cells in confluent cultures of monkey aortic smooth muscle cells. Further aspects of cell patterns and morphology in vitro are described below.

Biochemical Studies

ENZYME ACTIVITIES: As shown in Table III, in vitro growth of the calf aortic cells was associated with remarkable increases in the activities of the assayed enzymes. These increases were already manifest in the explanted cells, ranging from less than twofold for cytochrome oxidase and cathepsin C to between three- and fivefold for 5′-nucleotidase and the three other acid hydrolases. Neutral α-glucosidase was the only enzyme measured that did not increase in the explanted cells. The activities recorded for the various enzymes were notably variable from one set of explants to another, but with a high degree of internal correlation, i.e., all enzymes (with the single exception of cathepsin C) were either high or low in a given preparation. Conceivably, this variability is due to cell breakage and loss of cytoplasm (but not of nuclei) upon harvesting of explant cells, which were the most difficult to remove from the culture flask.

Subculturing led to further increases in levels of all enzymes. These increases were similar whether the final subculture was carried out in 10 FCS or in 10 CS, and averaged (with respect to the aortic cells *in situ*) ninefold for 5′-nucleotidase, fourfold for neutral α-glucosidase, sevenfold for cytochrome oxidase, 21-fold for acid cholesteryl esterase, 26-fold for β-galactosidase, 15.5-fold for *N*-acetyl-β-glucosaminidase, but only 4.5-fold for cathepsin C. Protein also increased severalfold. Part of this was extracellular, consisting of matrix proteins (collagen, elastin, etc.) secreted by the cultured cells. Decreasing the CS concentration to 2% (2 CS) for 1 wk caused the protein content and most enzyme levels to fall substantially, although they remained much higher than the values obtained in aortic homogenates. Increasing the serum concentration to 50% (50 CS) had relatively little effect, except for decreasing cytochrome oxidase and increasing cathepsin C.

SUBCELLULAR FRACTIONATION OF SMOOTH MUSCLE CELLS: Figs. 1 and 2 show the results of subfractionation of the PNS of isolated calf aortic cells, explant cells, and subcultured smooth muscle cells. Enzyme recoveries for these and subsequent experiments are given in Table IV. Examining first the results of subfractionations of isolated aortic cells (far left panels, Figs. 1 and 2), we see several distinct enzyme distribution patterns. 5′-nucleotidase shows a single skewed peak with a modal density of 1.11. Neutral α-glucosidase is partly soluble and partly particulate (modal density 1.13). Cytochrome oxidase shows a single peak with a mean equilibrium density of 1.17. The four acid hydrolases are largely sedimentable, with modal densities ranging between 1.15 and 1.17. Much of the protein is soluble and remains with the original layer, although some is distributed throughout the gradient. In general, these results are similar to the gradient distribution patterns obtained for cells isolated from rabbit aorta (34) and hog carotid artery (45). Especially notable is

TABLE III

Enzyme Content of Calf Aortic Smooth Muscle Cells

Enzyme	Calf aorta* (3)	Explant cells‡ (4)	Subcultured cells			
			10 FCS (4)	2 CS (4)	10 CS (4)	50 CS (4)
			specific activity			
5′-Nucleotidase	420 ± 20	1,530 ± 1,500	3,760 ± 1,400	1,870 ± 800	3,910 ± 1,200	5,290 ± 480
Neutral α-glucosidase	25 ± 1	19 ± 7	105 ± 10	77 ± 20	124 ± 20	107 ± 10
Cytochrome oxidase	130 ± 10	190 ± 150	1,020 ± 380	810 ± 290	1,240 ± 310	590 ± 200
Acid cholesteryl esterase	1.3 ± 1	6.3 ± 4	28.2 ± 2	21.5 ± 4	36.6 ± 4	28.2 ± 5
Cathepsin C	38 ± 2	62 ± 30	139 ± 90	152 ± 100	105 ± 10	290 ± 110
β-Galactosidase	22 ± 1	110 ± 70	590 ± 90	450 ± 130	699 ± 150	568 ± 90
N-acetyl-β-glucosaminidase	70 ± 3	230 ± 150	1,210 ± 400	800± 460	1,150 ± 450	1,190 ± 120
Protein	–	20 ± 10	139 ± 10	61 ± 10	122 ± 40	82 ± 40

Enzyme content expressed as mU of activity/mg DNA; protein expressed as mg/mg DNA. Data are presented as means ± SD with number of experiments within parentheses.

* Measured in aortic homogenates; mean DNA content: 0.912 ± 0.04 mg/g aorta.

‡ Each sample consisted of pooled near-confluent cells from eight 28-cm² petri dishes.

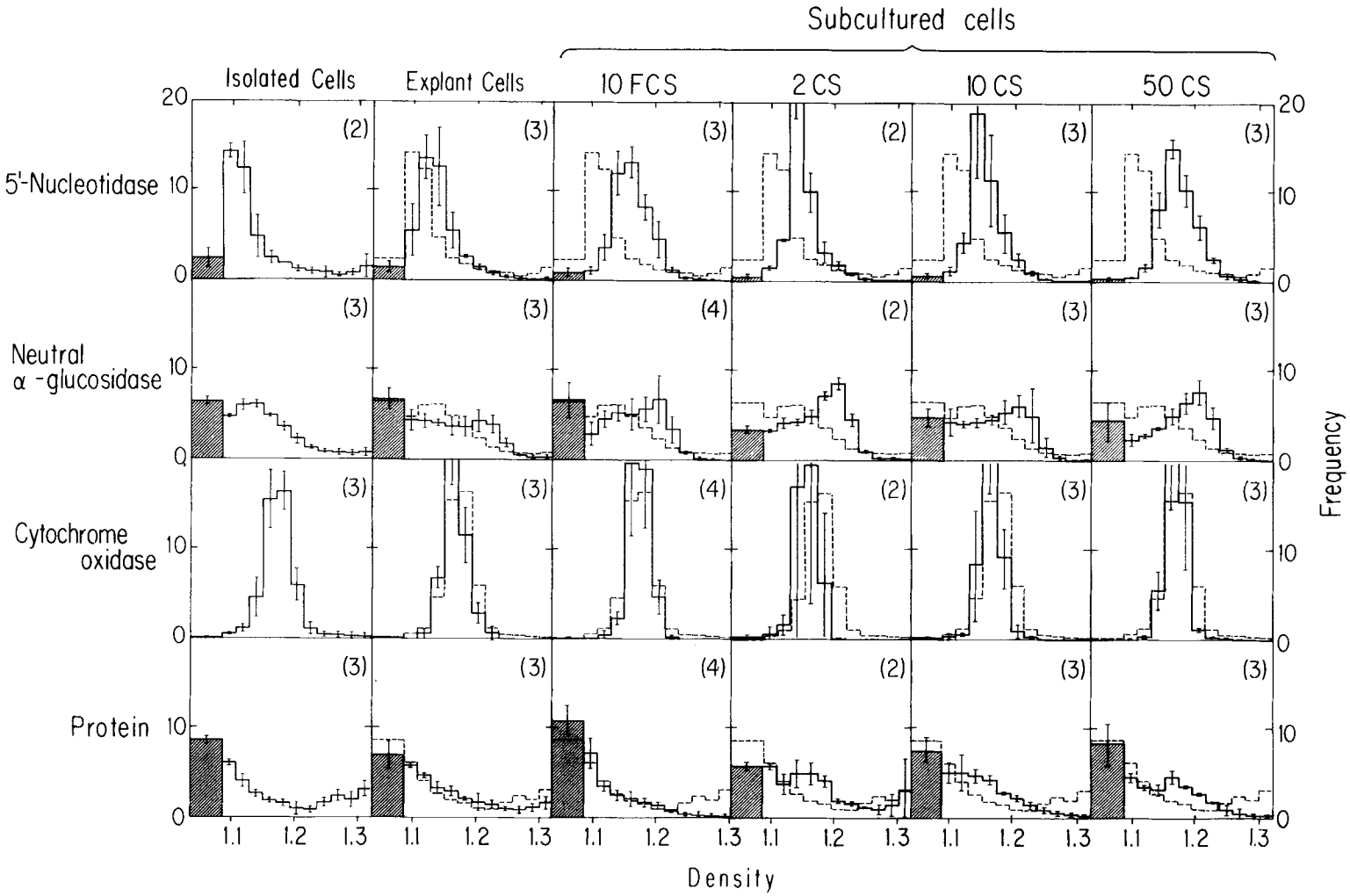

FIGURE 1 Isopycnic centrifugation of PNS of isolated calf aortic cells, aortic explant cells, and subcultured aortic smooth muscle cells cultured under various conditions. Graphs show frequency-density distributions of three marker enzymes and protein. The shaded area represents, over an arbitrary abscissa interval, the amount remaining in the sample layer. For purposes of easier comparison, the enzyme distributions for isolated cells (dotted line) are superimposed on those of other cell preparations. Numbers of experiments are given in parentheses; recoveries are shown in Table IV.

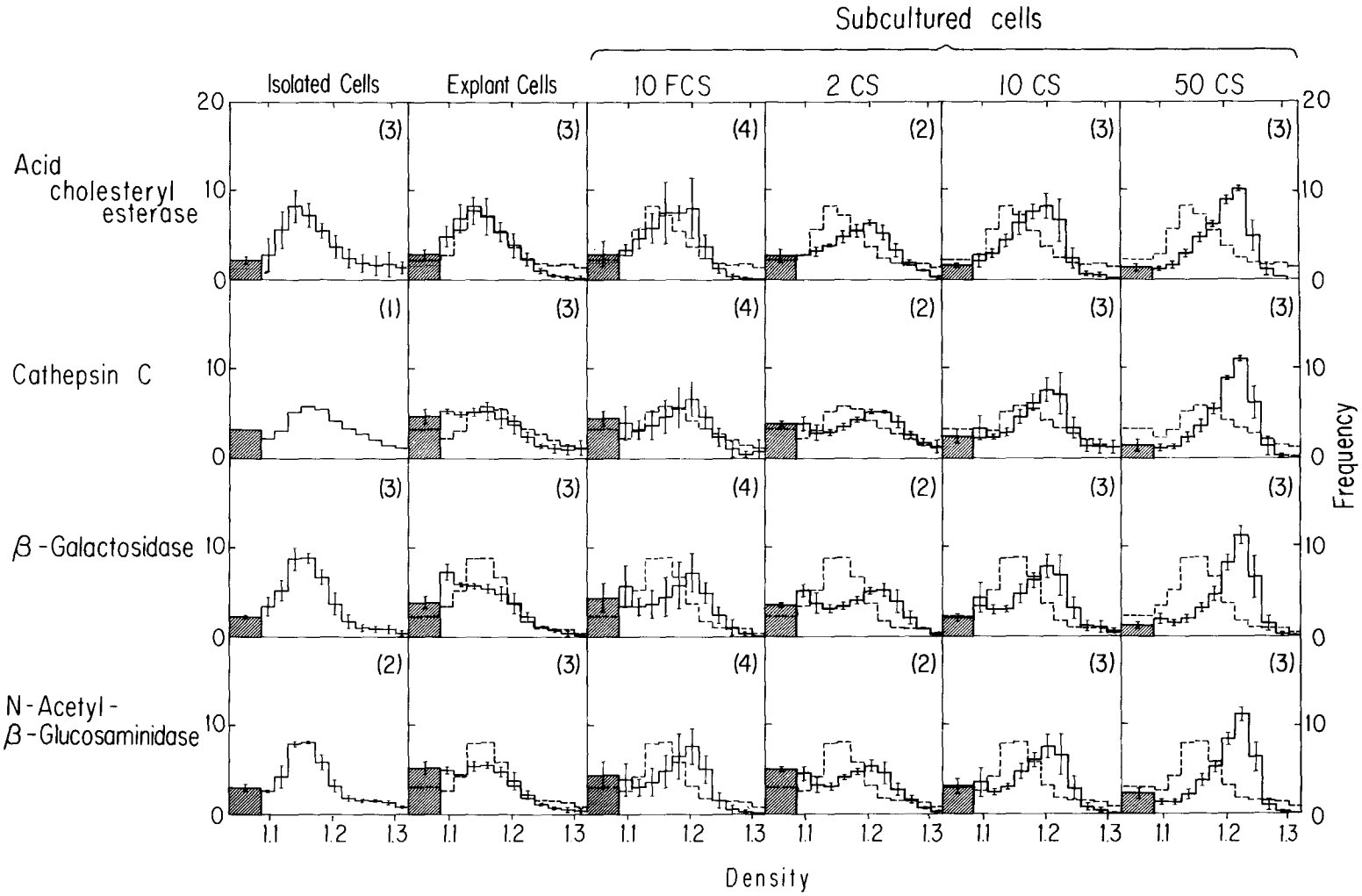

FIGURE 2 Isopycnic centrifugation of PNS of isolated calf aortic cells, aortic explant cells, and subcultured aortic smooth muscle cells cultured under various conditions. Graphs show frequency-density distributions of four acid hydrolases presented as described in Fig. 1. Broken lines repeat enzyme distributions of isolated cell preparations for comparison. Recoveries are shown in Table IV; numbers of experiments are given in parentheses.

TABLE IV

Complementary Data on Cell Fractionation Experiments

Enzyme	Aortic cells	Explant cells*	Subcultured cells					
			10 FCS	50 FCS	2 CS	10 CS	10 CS (3 wk)	50 CS
			Enzyme Recoveries in Gradients‡					
5′-Nucleotidase	94.4 ± 25 (2)	89.4 ± 11 (3)	91.5 ± 3 (3)	89.2 (1)	95.4 ± 3 (2)	82.2 ± 17 (3)	127.8 (1)	117.2 ± 32 (3)
Neutral α-glucosidase	113.5 ± 7 (3)	99.0 ± 9 (3)	110.4 ± 5 (4)	119.6 (1)	98.8 ± 1 (2)	100.5 ± 2 (3)	103.5 (1)	99.8 ± 6 (3)
Cytochrome oxidase	108.3 ± 14 (3)	65.4 ± 6 (3)	78.5 ± 15 (4)	217.3 (1)	77.1 ± 14 (2)	89.5 ± 15 (3)	62.2 (1)	110.8 ± 15 (3)
Acid cholesteryl esterase	87.2 ± 9 (3)	119.6 ± 27 (3)	89.1 ± 21 (4)	85.1 (1)	73.2 ± 2 (2)	70.4 ± 9 (3)	72.2 (1)	80.7 ± 8 (3)
Cathepsin C	132.1 (1)	103.1 ± 13 (3)	98.6 ± 11 (4)	99.2 (1)	93.6 ± 2 (2)	90.2 ± 2 (3)	89.2 (1)	88.1 ± 6 (3)
β-Galactosidase	108.5 ± 7 (3)	82.3 ± 4 (3)	86.1 ± 7 (4)	96.0 (1)	81.8 ± 2 (2)	79.9 ± 6 (3)	81.0 (1)	84.9 ± 4 (3)
N-acetyl-β-glucosaminidase	65.6 ± 12 (2)	57.7 ± 6 (3)	77.3 ± 6 (4)	94.0 (1)	61.4 ± 2 (2)	74.1 ± 3 (3)	89.4 (1)	78.0 ± 3 (3)
Protein	118.5 ± 13 (3)	124.5 ± 17 (3)	93.0 ± 13 (4)	102.1 (1)	103.7 ± 30 (2)	91.4 ± 16 (3)	100.6 (1)	90.0 ± 4 (3)

Values refer to experiments described in Figs. 1 and 2 and Table V. They give means ± SD with number of experiments within parentheses.

* Each experiment consisted of pooled near-confluent cells from eight 28 cm^2-petri dishes.

‡ Sum of activities of gradient fractions expressed as percentage of activity of PNS fraction.

the finding in all three species that acid hydrolases believed to be associated with lysosomes equilibrate around a density of 1.15–1.17.

Comparison of the distribution patterns observed on the cultured cells (remaining panels, Figs. 1 and 2) with those of isolated cells shows clearly that ex vivo growth was associated with marked increases in the equilibrium density of all enzymes except cytochrome oxidase (and acid hydrolases in the explant cells). For easier assessment of these increases, we have calculated for each experiment the median equilibrium density of sedimentable activity, the latter being defined somewhat arbitrarily as the activity recovered at densities higher than 1.085. This limit may truncate some distributions on the low density side to the extent that they contain particles of density lower than 1.085, or it may overload them on the same side if the enzyme measured is of sufficiently high molecular weight to sediment significantly in soluble form. A look at the distribution patterns shows that the resulting errors are likely to be small or negligible in most cases.

The calculated median densities are shown in Table V. In this table, assumptions are made that 5′-nucleotidase is associated with plasma membranes, neutral α-glucosidase with microsomes, cytochrome oxidase with mitochondria, and the four acid hydrolases with lysosomes. Besides conforming to the conclusions derived from previous studies on rabbit aortic cells (33, 34) and to observations made on many other cell types, these assumptions are further supported by the differences observed between the distributions of enzymes believed to be located in different cell sites, as well as by the close similarities in the behavior of the acid hydrolases. The only uncertainty concerns neutral α-glucosidase. We cannot exclude the possibility of the contribution of a lysosomal acid glucosidase to the measured activities in the preparations where lysosomal enzyme activities have risen considerably (Table III). The same problem has been encountered with atheromatous rabbit aortic cells (33).

Looking first at the explants, we observe large increases in the density of plasma membranes and of microsomes, but not of lysosomes (Table V and Figs. 1 and 2). Subculturing leads to further increases in plasma membrane and microsome density, associated now with a marked rise in lysosome density. In all cultures, the density of mitochondria is either the same as, or lower than, the value observed on the freshly isolated cells. In general, density increases tended to be higher in 50% serum than in 10% or 2% serum for all three groups of affected particles. On the other hand, no consistent difference was found between FCS and CS. In 10% serum, lysosome density was higher in CS than in FCS, but the opposite was true in 50% serum. Prolonging the time of culturing in 10 CS from 1–3 wk caused the microsomes to reach a particularly high density, without affecting the density of the other particles.

Morphologic Studies

The intact calf aorta contains typical smooth muscle cells with characteristic abundant myofilaments, surface vesicles, and a prominent base-

TABLE V

Centrifugal Properties of Smooth Muscle Cell Organelles

			Subcultured cells					
	Aortic cells (3)	Explant cells (3)	10 FCS (4)	50 FCS (1)	2 CS (2)	10 CS (3)	10 CS (3 wk) (1)	50 CS (3)
	Median equilibrium density							
Plasma membranes								
5'Nucleotidase	1.114 ± 0.001	1.130 ± 0.007	1.158 ± 0.007	1.162	1.146 ± 0.002	1.148 ± 0.008	1.149	1.168 ± 0.004
Microsomes								
Neutral α-glucosidase	1.138 ± 0.003	1.162 ± 0.014	1.173 ± 0.016	1.173	1.179 ± 0.004	1.176 ± 0.017	1.203	1.184 ± 0.004
Mitochondria								
Cytochrome oxidase	1.172 ± 0.005	1.165 ± 0.003	1.173 ± 0.005	1.166	1.153 ± 0.015	1.161 ± 0.007	1.161	1.169 ± 0.004
Lysosomes								
Acid cholesteryl esterase	1.161 ± 0.021	1.151 ± 0.010	1.172 ± 0.014	1.223	1.189 ± 0.004	1.188 ± 0.013	1.202	1.204 ± 0.006
Cathepsin C	1.169	1.159 ± 0.005	1.180 ± 0.019	1.231	1.196 ± 0.009	1.197 ± 0.004	1.199	1.208 ± 0.002
β-Galactosidase	1.155 ± 0.008	1.155 ± 0.016	1.173 ± 0.021	1.230	1.191 ± 0.004	1.191 ± 0.008	1.192	1.212 ± 0.007
N-acetyl-β-glucosaminidase	1.158 ± 0.002	1.156 ± 0.002	1.173 ± 0.015	1.231	1.180 ± 0.011	1.191 ± 0.009	1.191	1.211 ± 0.007
Mean lysosome density	[1.159]	[1.155]	[1.176]	[1.229]	[1.189]	[1.192]	[1.196]	[1.209]

Population median equilibrium density of sedimentable activity (defined as activity equilibrating at densities >1.085). Values are expressed as means ± SD with number of experiments within parentheses. They are derived from experiments described in Figs. 1 and 2, and from two additional experiments (50 FCS and 10 CS, 3 wk) not reported in detail in the paper.

ment membrane (Fig. 3*A*). Adjacent to the centrally located elongated nucleus are mitochondria, numerous ribosomes, and rough endoplasmic reticulum (RER) sometimes dilated by the presence of intracisternal material. Glycogen deposits are sometimes evident. A well-developed Golgi apparatus is often seen in the juxtanuclear region and membrane-bounded vesicles of varying size and appearance, some of which may be lysosomes, are present. Aortic cells isolated from the intact tissue by enzymatic digestion (Fig. 3*B*) exhibit all of these features except that the cells are found in a highly contracted state, resulting in some disorganization of the cytoplasm, and lack a basement membrane.

Electron microscope studies of explant cells and subcultured cells revealed that they exhibit characteristic features of smooth muscle cells including numerous myofilaments with dense attachment plaques and arrays of surface vesicles; the basement membrane is less prominent (Fig. 4). These cells differ from *in situ* cells in having more lysosomal structures, including multivesicular bodies, vacuoles containing myelin-like figures, and, sometimes, autophagic vacuoles. Another notable feature is the presence of a dilated RER filled with amorphous material. Examination of explant cells in particular shows them to differ little from subcultured cells except for their having fewer lysosomal structures (Fig. 5). Numbers of organelles varied considerably from one explant culture to another.

Under the culture conditions studied here, all subcultures of calf aortic cells show the characteristic features of cultured smooth muscle cells described by others (8, 21, 29, 39, 42). These include, in part, growth in hills and valleys and the appearance of an extracellular matrix with prominent collagen fibers. Subcultured cells grown in 10 FCS (Fig. 4) have the abundant organelles typical of all cells in culture but have increased numbers of lysosomes compared to explant cells. Lysosomal structures containing membranous whorls and other debris are more prominent. These cells also accumulate cytoplasmic glycogen and lipid droplets (not shown).

Cultured aortic smooth muscle cells exposed to CS undergo further changes in appearance compared to cells grown in FCS. Prominent cytoplasmic features of cells cultured in all concentrations of CS studied are an extensive RER and an extremely well developed Golgi apparatus. Accumulations of cytoplasmic glycogen and lipid droplets are also greater in these cells than in cells grown in FCS and seem to increase in relation to the CS concentration. Most notable generally is the more complex appearance of the lysosomes. Specifically, in cells grown in 10 CS (Fig. 6), the matrix within lysosomes is denser than that of cells grown in 10 FCS. The lysosomal

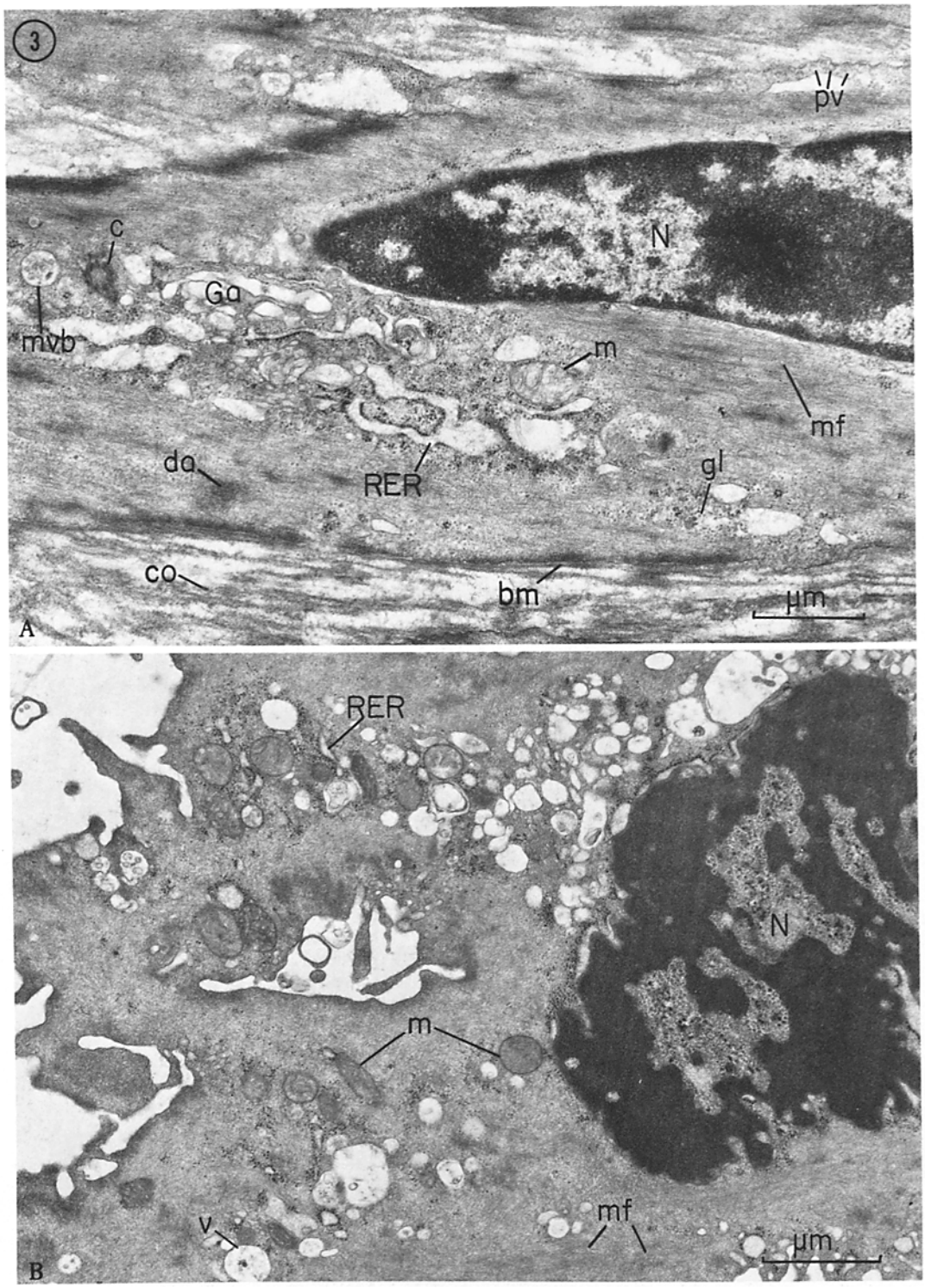

FIGURE 3 Appearances of calf aortic smooth muscle cells *in situ* and in isolated form after enzymic digestion of extracellular matrix. These cells exhibit typical features of smooth muscle cells such as abundant myofilaments (*mf*) with associated attachment plaques or dense areas (*da*), and numerous surface pinocytic vesicles (*pv*). (*A*) Portions of an aortic cell in vessel wall. Located in the juxtanuclear region are Golgi apparatus (*Ga*), rough endoplasmic reticulum (*RER*), mitochondria (*m*), and multivesicular body (*mvb*). A prominent basement membrane (*bm*) is adjacent to the cell periphery and the cells are embedded in an extracellular matrix of collagen (*co*) and elastin fibers. *N*, nucleus; *gl*, glycogen, *c*, centriole. × 17,000. (*B*) Portion of an isolated aortic cell. Structures readily discerned are the nucleus, RER, numerous mitochondria, and membrane-bounded vesicles (*v*) of varying sizes. × 18,000.

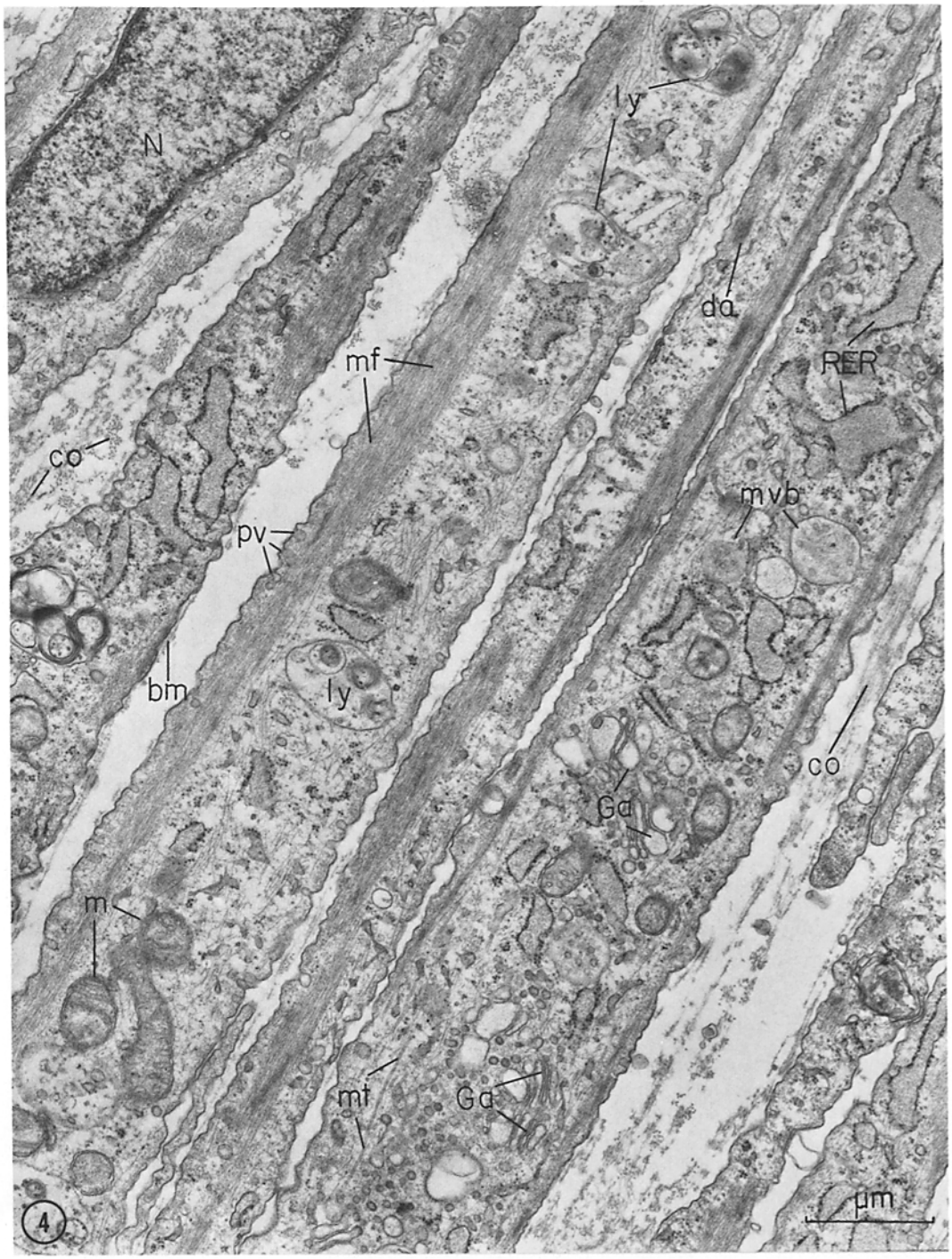

FIGURE 4 View of layers of calf aortic smooth muscle cells subcultured in 10 FCS. Sectioning is perpendicular to the culture dish bottom. Subcultured calf aortic smooth muscle cells have features typical of smooth muscle cells such as myofilaments (*mf*) with associated attachment plaques (*da*), surface pinocytic vesicles (*pv*), and a basement membrane (*bm*). The basement membrane tends to be less evident in culture than those seen *in situ*. The layers of cells are surrounded by extracellular matrix consisting mainly of collagen fibers (*co*) with little recognizable elastin. *N*, nucleus; *m*, mitochondria; *Ga*, Golgi apparatus; *mvb*, multivesicular body; *ly*, lysosomes; *RER*, rough endoplasmic reticulum; *mt*, microtubule. × 19,000.

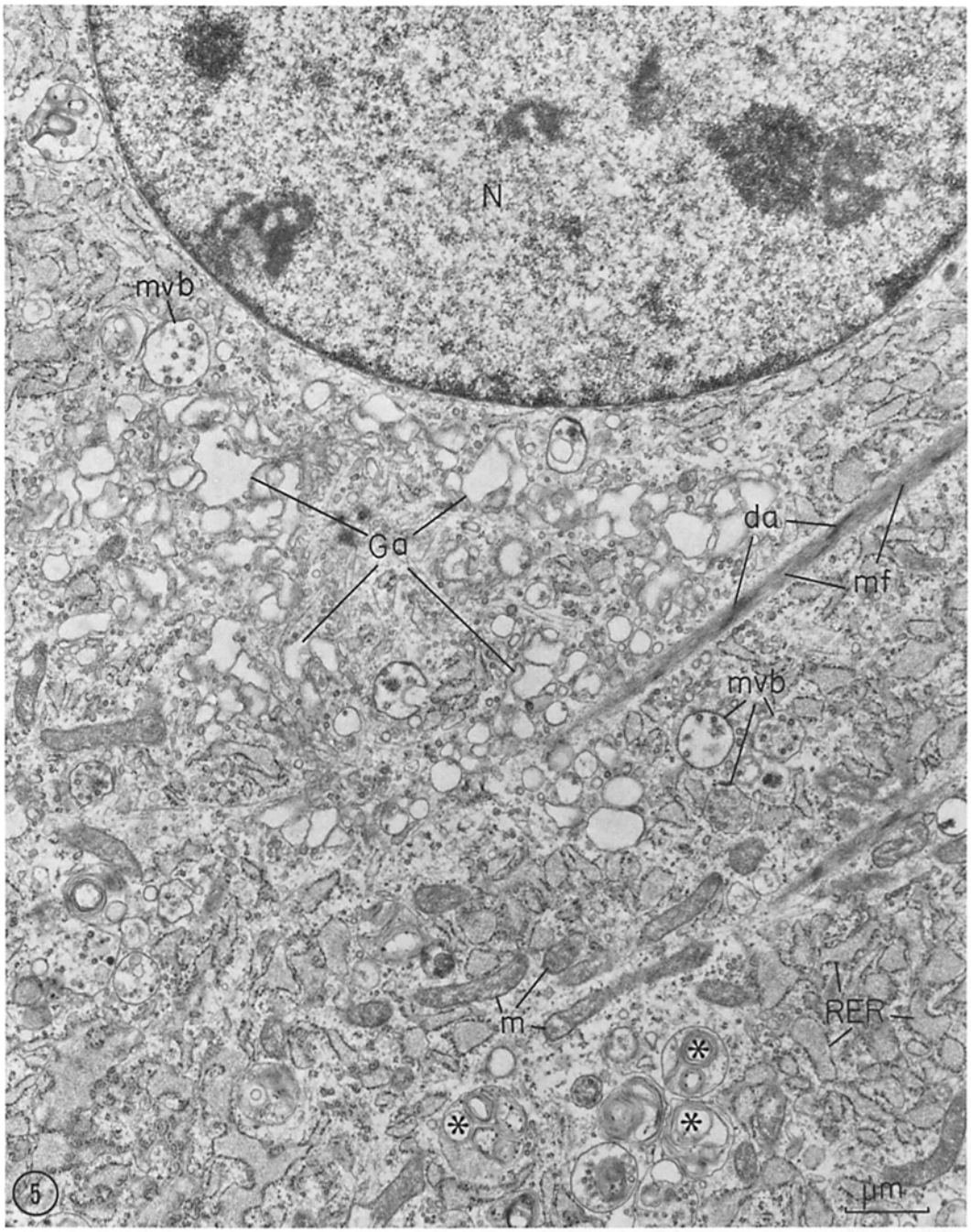

FIGURE 5 Tangential view of portion of a calf aortic explant cell grown for 3 wk in 10 FCS. The appearance of the cytoplasm differs from that of *in situ* and freshly isolated aortic cells. Numerous lysosome-like structures of various sizes are seen. Some of these structures contain membranous whorls (*) and some appear as multivesicular bodies (*mvb*). The rough endoplasmic reticulum (*RER*) is extensive and contains amorphous material. *N*, nucleus; *m*, mitochondria; *Ga*, Golgi apparatus, *mf*, myofilament; *da*, dense area or attachment plaque. × 12,000.

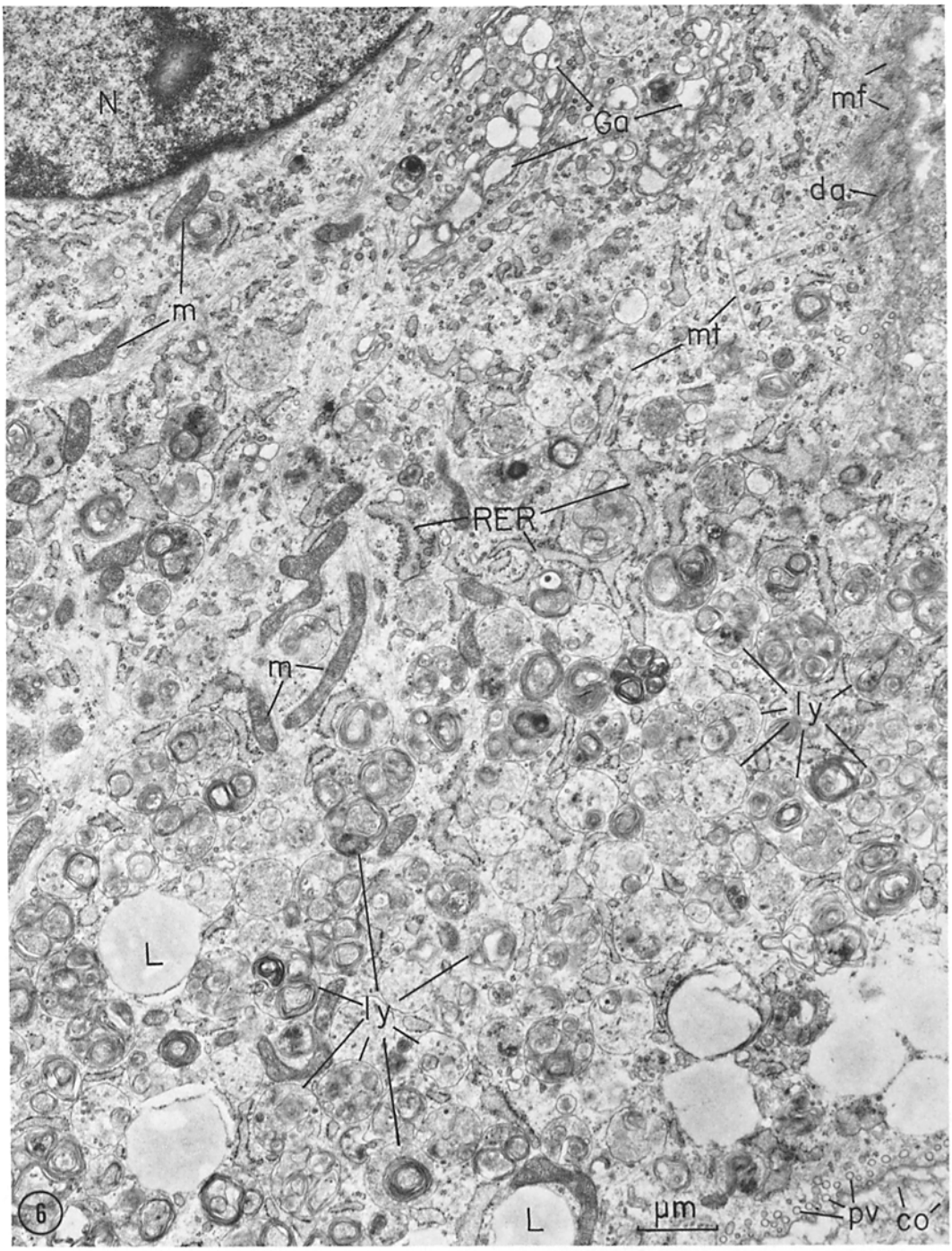

FIGURE 6 Tangential view of portion of a calf aortic smooth muscle cell subcultured for 1 wk in 10 CS. A change in the appearance of the lysosomes is seen. The lysosomes (*ly*) are filled with myelin figures and their matrices contain moderately electron-dense material. Intracytoplasmic lipid droplets (*L*) and glycogen (not shown in this section) are more prevalent. *N*, nucleus; *m*, mitochondria; *Ga*, Golgi apparatus; *RER*, rough endoplasmic reticulum; *mt*, microtubule; *mf*, myofilaments; *da*, attachment plaque, *pv*, pinocytic vesicles; *co*, collagen. × 12,000.

matrix becomes even denser in cells cultured in 50 CS (Fig. 7). The cytochemical reaction for acid phosphatase exhibited by these particles (Fig. 7*C*) supports their identification as lysosomes. These electron-dense lysosomes are ~1 μm in diam, which makes them large enough to be identified at the light microscope level. Many of the black dots seen in the light micrographs (Fig. 8*C* and *D*) may well correspond to the same structures seen at the ultrastructural level.

Considerable variation in the complexity of the cytoplasmic appearances of cells grown under different culture conditions can also be appreciated when seen over larger fields at the light microscope level (Fig. 8).

DISCUSSION

Under the conditions used in this work, calf aortic smooth muscle cells undergo striking biochemical and morphological changes upon culturing in vitro. As they grow out of the original explant in a medium containing 10 FCS, their plasma membrane becomes denser as well as enriched severalfold in 5′-nucleotidase. Neutral α-glucosidase activity, the marker used for microsomes, remains unchanged, but the equilibrium density of these particles in a sucrose gradient increases significantly. This change is associated with a morphologically observed dilation of the endoplasmic reticulum, which appears filled with amorphous material, presumably secretory protein. Cells from explants also show particularly striking changes affecting the lysosomes, which are increased in number, diversified in morphological appearance, and enriched considerably in acid hydrolase activities, without, however, showing any change in equilibrium density. Least affected are the mitochondria which show only a moderate increase in cytochrome oxidase activity and little change in density.

The above modifications are exaggerated, and new ones appear, when the smooth muscle cells are removed from the explant and subcultured. Plasma membrane density and 5′-nucleotidase activity increase even more, while evidence of a high pinocytic activity can be seen morphologically. Further development and dilation of the endoplasmic reticulum is observed, in association with further increases in the density of microsomes. In addition, the microsomal marker enzyme neutral α-glucosidase now rises severalfold in activity. Further marked changes also affect the lysosomes which become even more numerous and polymorphic. Their equilibrium density in a sucrose gradient increases also, sometimes very markedly. The acid hydrolase levels of the subcultured cells are now 5–25 times greater than in aortic cells *in situ*. Even the mitochondria change in the subcultured cells, displaying as much as a sevenfold increase in cytochrome oxidase activity, but their equilibrium density is not altered.

The functional correlates of the observed changes are not easily identified, but seem to center around the main processes of protein import and export. The biochemical indication of plasma membrane proliferation, the morphological signs of active pinocytosis, the high development and enzymatic enrichment of the lysosome system, together with the physical and morphological evidence of overloading of this system with dense material, all point toward enhanced uptake and processing of proteins. Since the changes tend to increase with increasing serum concentration, stimulation of pinocytosis and of lysosome development by a serum factor, such as found by Cohn and Benson (11) for monocytes, could be implicated. There are also signs that that the cells engage in enhanced protein synthesis and secretion, as evidenced by the development and dilation of the endoplasmic reticulum, and by the deposition of collagen. The increased activity and equilibrium density of the microsomal marker neutral α-glucosidase could be a reflection of this change.

Summarizing the observed modifications, one might say that the calf aortic smooth muscle cells tend, upon in vitro cultivation, to become both more macrophage-like and more fibroblast-like. Such a modification is not uncommon. For instance, it is seen in cells undergoing transformation to foam cells in atherosclerotic lesions due to lipid overloading (33). Two points must be stressed. First, the cells involved are the smooth muscle cells themselves. Even in the cultures showing the greatest changes, characteristic myofilaments and other typical features of smooth muscle cells can readily be recognized in all cells (Fig. 7*B*). Second, the altered cells do not seem to be in any special pathological state. They proliferate rapidly on subculture, oxidize glucose very actively (M. M. Daly and H. Wolinsky, unpublished observation), and show an abundance of cytoplasmic organelles.

We do not know the nature of the factors that

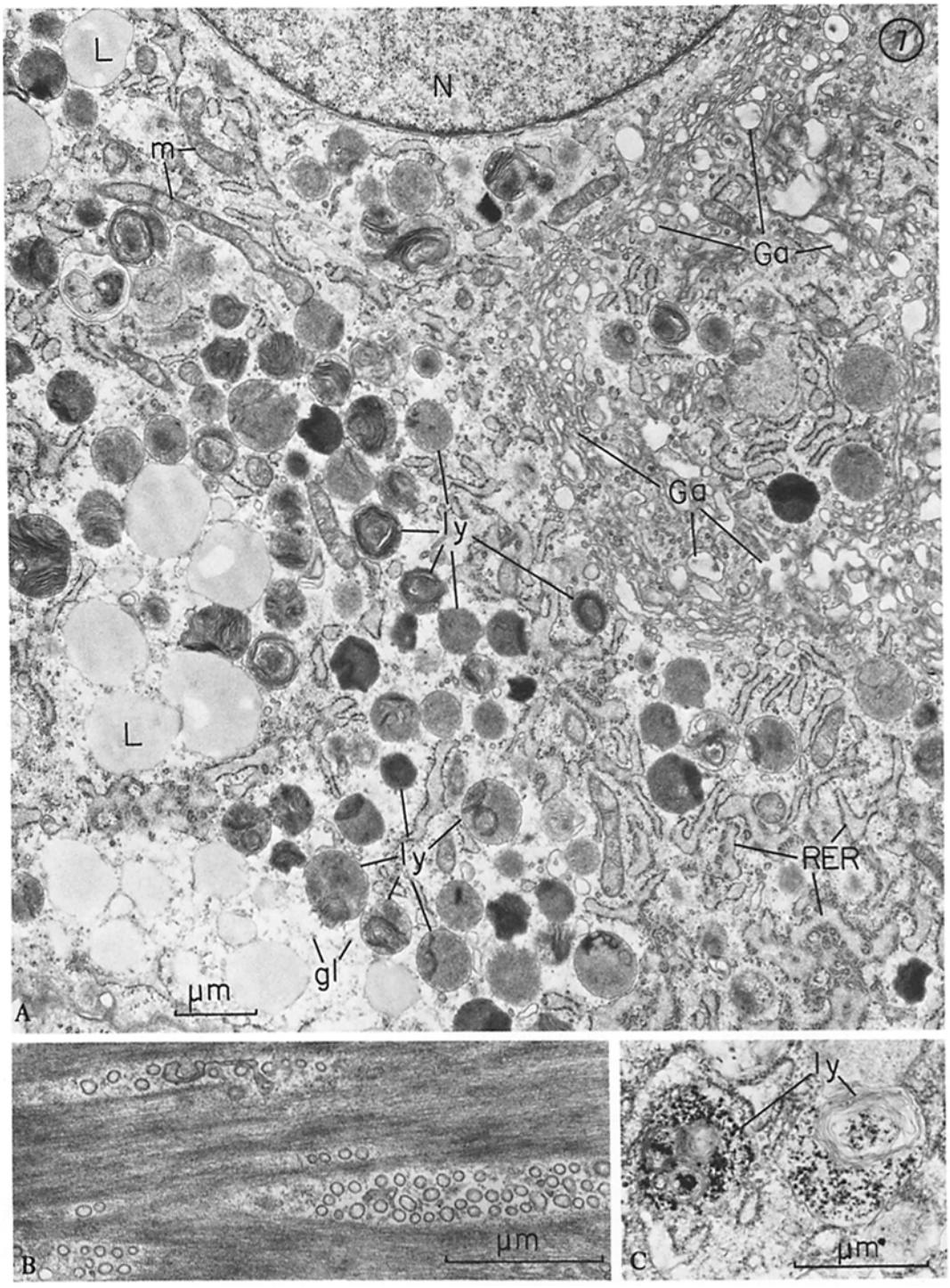

FIGURE 7 Tangential view of portion of a calf aortic smooth muscle cell subcultured for 1 wk in 50 CS. (*A*) The most striking change is the increase in electron density of the lysosomal matrix. Most lysosomes (*ly*) still contain myelin figures. The well-developed Golgi apparatus (*Ga*) is prominent. *N*, nucleus; *m*, mitochondria; *RER*, rough endoplasmic reticulum; *L*, intracytoplasmic lipid droplets; *gl*, glycogen. × 12,000. (*B*) Abundant myofilaments and surface vesicles seen in the periphery of a cell from the same grid as in (*A*). × 23,000. (*C*) Acid phosphatase reaction product in two dense lysosomes. × 20,000. Bars, 1 µm.

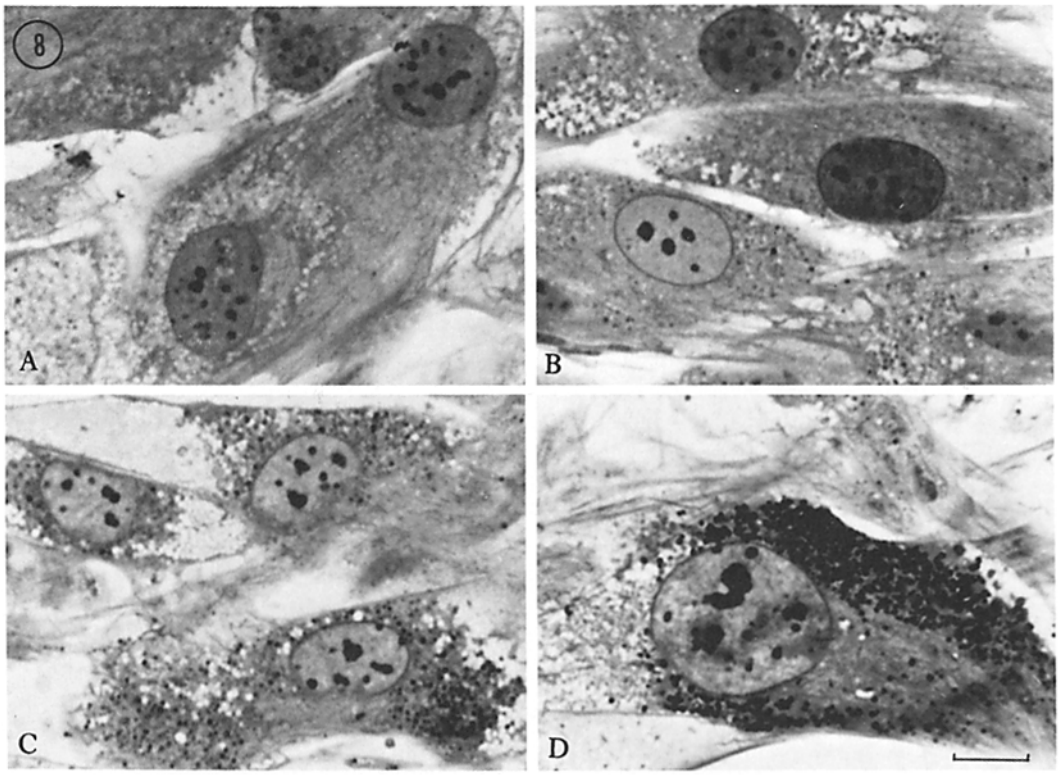

FIGURE 8 Tangential view of smooth muscle cell cultures. 1-μm thick Epon sections were stained with methylene blue-Azure II and viewed by light microscopy. (*A*) explant cells in culture; (*B*) subcultured cells in 10 FCS; (*C*) subcultured cells in the presence of 10 CS; (*D*) subcultured cells in the presence of 50 CS. The appearance of darkly stained granules in (*C* and *D*) may be correlated at the ultrastructural level with lysosomes having electron-dense matrices. Bar, 10 μm. × 1,000.

cause the changes we have observed, nor whether similar alterations occur in the smooth muscle cell cultures developed by others, especially under conditions which appear to return cells to a quiescent state comparable to that prevailing in vivo (43). However, our culture conditions are comparable to those employed by other investigators (Table I) and our morphological findings are not peculiar to the calf aortic smooth muscle cells described in this paper. Similar observations have been made on cultured smooth muscle cells derived from swine (8, 26, 42), guinea pig (39), rat (6), monkey (18), and human (21, 31) arteries. It is not unlikely, therefore, that some of the biochemical changes we have found to be associated with the morphological alterations also occur upon subculturing in these other preparations. That important functional changes may take place upon culturing arterial cells is shown in a recent paper by Chamley et al. (10), who have found that cells isolated from humans, monkeys, and rabbits become unresponsive to angiotensin II and lose their ability to bind antimyosin antibodies after about 9 days in culture. By then, the cells are said to resemble fibroblasts morphologically.

Cultured arterial smooth muscle cells have become a popular system for the testing of various factors suspected of playing a role in the development of arterial disease. Such a system clearly has powerful potentialities, provided its relevance to arterial pathophysiology can be established. The results described in the present paper indicate that a great deal of caution should be exercised in transposing from the in vitro to the in vivo situation, especially since some of the changes induced by culturing resemble alterations found in vascular disease.

The authors wish to express their gratitude to Dr. C. de Duve for his continued interest in this work and for his valuable suggestions in the preparation of the manu-

script. The authors also express their gratitude to Dr. A. M. Adamany (Albert Einstein College of Medicine) and Dr. N. Haley (The Rockefeller University) for helpful advice and cooperation. The excellent technical assistance of Ms. A. Yee for tissue culture and Ms. B. Koss for biochemical studies is also gratefully acknowledged. We also express our thanks to Mr. A. Pelaschier for conscientious maintenance of equipment used.

This work has been supported by Public Health Service grants AG-00367 from the National Institute of Aging, and HL-18157, HL-12766, and HL-13979 from the National Heart, Lung, and Blood Institute. Dr. Wolinsky is the recipient of a Research Career Development Award from the National Heart, Lung, and Blood Institute.

Received for publication 21 January 1977, and in revised form 10 June 1977.

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