LYSOSOMES OF THE ARTERIAL WALL

I. Isolation and Subcellular Fractionation of Cells from Normal Rabbit Aorta*

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There is considerable circumstantial evidence that the lysosomes of the arterial wall smooth muscle cells are altered in atherosclerosis. Morphological studies have demonstrated increased numbers of cytoplasmic structures believed to be lysosomes in both human and experimental atherosclerosis (1–5). Similarly, assays of acid hydrolases have shown significantly increased activities in diseased as compared to normal arterial tissue (6, 7).

Because of the dense connective tissue matrix surrounding the smooth muscle cells of the arterial wall, prolonged vigorous homogenization is necessary for disrupting the cells and releasing their contents. Such conditions do not permit the satisfactory isolation of subcellular organelles, particularly the fragile lysosomes. Another difficulty in the study of lysosomes from arterial wall is the very small quantity of cellular material available.

These difficulties were overcome in the present work by the use of: (*a*) an enzymic digestion procedure for disrupting the arterial matrix before isolation and homogenization of the cells; (*b*) the Beaufay automatic rotor for single-step fractionation of disrupted cell preparations by isopycnic centrifugation; and (*c*) microassays for the measurement of up to 10 enzymes, protein, and DNA in the separated fractions. Cells recovered from single rabbit aortae and representing between 0.5 and 2.0 mg of total protein could be processed in this manner and their lysosomes characterized.

Subsequent studies will report sex- and age-related changes, the effect of various treatments (including cholesterol feeding) known to affect the arterial wall, and the application of this technique to human vascular tissue.

Materials and Methods

Isolation of Aortic Cells.—Virgin female chinchilla rabbits weighing 2–3 kg and aged 5–8 months were purchased from Carver's Rabbitry (Summerville, N. J.). They were maintained

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for at least 1 month on Purina Rabbit Chow (Ralston Purina Co., St. Louis, Mo.) and any unhealthy rabbits were discarded. The animals were fasted overnight and killed with 2–4 ml intravenous sodium pentobarbitone, 60 mg/ml. The thorax and abdomen were quickly opened and the entire aorta from the heart to the iliac bifurcation was removed. The adherent fibrous and fatty tissue was removed and the aorta was cut into segments 2–3 cm long which were immersed in ice-cold complete Hanks' medium. The adventitia was carefully dissected from the segments, which were then opened longitudinally to remove any adherent blood clot. The pieces of tissue were washed in three changes of ice-cold Hanks' solution, blotted with absorbent paper, and weighed on a torsion balance. The strips of aorta were cut into slices approximately 0.5 mm thick; initially this was done with fine surgical scissors, but subsequently a specially constructed electro-pneumatically operated guillotine was used.

The tissue slices were placed in a jacketed vessel maintained at 30°C containing Hanks' solution (1 ml/50 mg tissue). The following enzymes (purchased from the Sigma Chemical Co., St. Louis, Mo.) were added to the tissue suspension: elastase, 40 units/ml (type I: 600 units/mg); collagenase 300 units/ml (type I: 200 units/mg); hyaluronidase 800 units/ml (type III: 600 units/mg). The elastase was added to the reaction vessel 5–10 min before the other enzymes. The digestion mixture was gently agitated with a magnetic stirrer and was aerated by a small air pump. Dissolution of the connective tissue matrix was complete in 2–2½ hr and the suspension was filtered through coarse surgical gauze into an ice-cold centrifuge tube. The cells were collected by centrifugation at 800 *g* for 10 min at 4°C; the supernatant was discarded and the pellet gently resuspended in 10 ml of ice-cold Hanks' solution with a Pasteur pipette. This was repeated twice and the final cell pellet was resuspended in 5 ml of SVE[1] medium. All glassware was siliconized (Siliclad; Clay-Adams, Inc., Parsippany, N. J.).

Assessment of Cell Integrity.—Samples of each cell preparation were studied by phase-contrast microscopy before and after homogenization. The ability of the cells to exclude trypan blue was studied by mixing one drop of filtered 1% trypan blue in 0.15 M NaCl with 1 drop of the cell suspension on a microscope slide. The percentage of cells that took up the dye was determined.

Microscopy.—The cell suspension was fixed for 2 hr in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, centrifuged at 10,000 *g* for 15 min, and postfixed for 15 hr in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4. The pellet was stained in block with 0.5% uranyl acetate in 0.05 M acetate-Veronal buffer, dehydrated, and then embedded in Epon. For light microscopy 1-μm-thick sections were cut with a glass knife and stained with azure II-methylene blue (8). For electron microscopy ultrathin sections were cut with a diamond knife, doubly stained with uranyl acetate and lead citrate, and examined in a Philips E.M. 300 microscope (Philips Electronic Instruments, Mount Vernon, N. Y.).

Disruption and Subcellular Fractionation of Aortic Cells.—The pellet of washed cells was suspended in 7 ml of SVE medium and was disrupted with 25 strokes of a type B pestle in a small Dounce homogenizer (Kontes Glass Co., Vineland, N. J.). Examination of the homogenate by phase-contrast microscopy indicated that nearly all the cells had been disrupted, but that many nuclei were still intact. The homogenate was quantitatively transferred to a conical tube and centrifuged at 600 *g* for 10 min. The postnuclear supernatant (PNS) was removed with a Pasteur pipette and kept at 4°C. The sediment of nuclei and debris was suspended in 1 ml of SVE medium (N fraction).

7.5 ml of PNS were layered onto a 24-ml sucrose gradient extending, linearly with respect to volume, from a density of 1.05 to one of 1.28, and resting on a 6 ml sucrose cushion of density 1.32, in a Beaufay automatic rotor (9). The rotor was run at 35,000 rpm for 35 min at 0°C. The design and use of the rotor and associated equipment have been described previously (10).

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[1] *Abbreviations used in this paper:* PNS, postnuclear supernatant; SVE medium, medium containing 0.25 M sucrose, 1 mM Versene (EDTA), and 0.1% ethanol.

Some 16 fractions were collected into tared tubes, and after weighing the density of each fraction was determined in a gradient of organic solvents (11). More recently density has been determined indirectly with an Abbé refractometer (Bausch & Lomb Incorporated, Rochester, N. Y.).

Preparation of Extracts From Rabbit Liver.—Rabbits were fasted overnight and killed with intravenous sodium pentobarbitone. A 2 g portion of the liver was cut into small pieces with scissors and homogenized in 20 ml of ice-cold SVE medium with a small Waring Blendor operated at maximum speed for 1 min. The homogenates were stored in aliquots at −16°C.

Cytochrome Oxidase.—The enzyme was assayed by a modification of the technique of Cooperstein and Lazarow (12). 0.1 ml of suitably diluted tissue fraction was added to 1 ml of buffered substrate previously warmed to 37°C. The final concentrations in the reaction mixture were: cytochrome *c* (type IV; Sigma) 0.50 mg/ml; Triton X-100 (Sigma) 0.01%; Emasol (Kao Atlas, Tokyo, Japan) 0.1%; 1 mM EDTA; 0.1 M sodium phosphate buffer pH 7.0. The cytochrome *c* was 90% reduced with sodium dithionite and passed through a Millipore filter immediately before use. The decrease in absorbance at 550 nm was measured at 37°C in 1 ml glass cells in a Cary 14 Recording Spectrophotometer (Cary Instruments, Monrovia, Calif.) equipped with a 0.1 slide wire. The first-order velocity constant of the reaction was computed from the observed change in absorbance with the cytochrome *c* concentration being taken as 0.034 mM and the millimolar extinction coefficient of cytochrome *c* equal to 19.2. The results were then converted to the units of Cooperstein and Lazarow (12).

Catalase.—A micromodification of the method of Baudhuin et al. (13) was used. 0.1 ml of suitably diluted tissue fraction was incubated for 30 min at 25°C with 0.1 ml of freshly prepared buffered substrate. This was prepared by dissolving 50 mg of bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill.) in 5 ml of 0.2 M imidazole buffer pH 7.0; 5 ml of 2% (w/v) Triton X-100 and 0.1 ml of 30% hydrogen peroxide (Superoxol; Merck and Co., Inc., Rahway, N. J.) were added, and the solution was made up to 50 ml with distilled water. The reaction was stopped and the remaining hydrogen peroxide estimated by the addition of 2 ml titanium peroxysulfate, prepared as described by Leighton et al. (10) but diluted 1:1 (v/v) with distillled water. The absorbance of the solution was measured at 405 nm. Suitable enzyme and substrate blanks were used in all assays. Activities are given in terms of the first-order velocity constant of the reaction, in units defined by Baudhuin et al. (13).

Phosphatases and Glycosidases.—These enzymes were assayed with 4-methyl umbelliferyl derivatives (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England). The substrates were shown to be homogeneous and free from 4-methyl umbelliferone by thin-layer chromatography (14). Stock solutions of the substrates (10 mM) were prepared in moisture-free methoxyethanol and stored at 4°C for up to 4 wk. 0.2 mM solutions of these substrates were prepared immediately before use in the appropriate buffer containing 0.1% Triton X-100. Buffers and pH optimum for each substrate are shown in Table I.

A 0.1 ml aliquot of suitably diluted tissue fraction was incubated at 37°C for 10–60 min with 0.1 ml of the buffered substrate. The reaction was stopped by the addition of 2 ml 50 mM NaOH-glycine buffer pH 10.5 containing 5 mM EDTA. The liberated 4-methyl umbelliferone was assayed in a Perkin-Elmer fluorescence spectrophotometer (Model 204 with xenon lamp; Perkin-Elmer Corp., Norwalk, Conn.) with a 0.5 ml flow-through cuvette connected to a semi-automatic sampling device. The exciting wavelength was 365 nm and the emission wavelength setting was 460 nm. Suitable enzyme and substrate blank assays were performed. Standardization was carried out by mixing 4-methyl umbelliferone in the appropriate buffer with 2 ml NaOH-glycine buffer and measuring the amount of fluorescence as described above. For all enzymes, 1 unit of activity corresponds to the hydrolysis of 1 μmole of substrate/min at 37°C.

Latency of N-Acetyl-β-Glucosaminidase.—0.1 ml of suitably diluted PNS was incubated for 15 min at 37°C with 0.1 ml of 0.2 mM 4-methyl umbelliferyl *N*-acetyl-β-glucosaminide in 0.25 M sucrose containing 0.1 M acetate buffer pH 5.7, with (total activity) or without (free activity)

0.1% Triton X-100. Latency is the difference between total and free activity expressed as per cent of total activity. A small number of latency experiments were performed with other 4-methyl umbelliferyl substrates under identical conditions, except for α-glucosidase, which was determined in 0.1 M phosphate buffer pH 6.9.

Leucyl-β-Naphthylamidase.—This enzyme was assayed fluorometrically by a modification of the method of Panvelliwalla and Moss (15). 0.1 ml of suitably diluted tissue fraction was incubated for 30 min at 37°C with 0.1 ml of 0.2 mM leucyl-β-naphthylamide (Sigma) in 0.1 M phosphate buffer pH 7.3 containing 0.1% Triton X-100. The reaction was stopped by the addition of 2 ml ice-cold 50 mM NaOH-glycine buffer pH 10.4 containing 5 mM EDTA, and the liberated β-naphthylamine was estimated fluorometrically in a Model 204 Perkin-Elmer fluorescence spectrophotometer. The exciting wavelength was 340 nm and the emission wavelength setting was 410 nm. Suitable enzyme and substrate blank assays were performed. Standardization was performed by mixing β-naphthylamine in 0.1 M phosphate buffer pH 7.3 with 2 ml of NaOH-glycine buffer and measuring the fluorescence as described above.

Cathepsin A.—0.1 ml of aortic cell homogenate was incubated for 60 min at 37°C with 0.1 ml of 10 mM *N*-benzyloxycarbonyl-α-L-glutamyl-L-tyrosine (Mann Research Labs, New York) in 0.1 M acetate buffer pH 5.0 containing 0.1% Triton X-100 (16). The liberated tyrosine was assayed with the L-amino acid oxidase-peroxidase-dianisidine technique of Auricchio, Pierro, and Orsatti (17). Suitable substrate, enzyme, and reagent blanks were used.

Cathepsin B.—0.1 ml of aortic cell homogenate was incubated with 0.1 ml of freshly prepared substrate. This contained 0.2 mM benzoyl-L-arginyl-β-naphthylamide (Cyclo Chemical Co., Los Angeles, Calif.) in 0.1 M acetate buffer pH 5.0 containing 0.1% Triton X-100, 2 mM EDTA, and 1 mM dithiothreitol (18). After 60 min at 37°C, 2 ml of the NaOH-glycine buffer were added and the liberated β-naphthylamine was assayed fluorometrically as in the assay of leucyl-β-naphthylamidase.

Cathepsin C.—0.1 ml of suitably diluted tissue fraction was incubated for 30 min at 37°C with 0.1 ml of 0.2 mM glycyl-L-phenylalanyl-β-naphthylamide (Cyclo Chemical Co.) in 0.1 M acetate buffer pH 5.0 containing 0.1% Triton X-100, 1 mM EDTA, and 1 mM dithiothreitol (19). The liberated β-naphthylamine was estimated fluorometrically as described above. Suitable enzyme and substrate blanks were used for each assay.

Cathepsin D.—0.1 ml of suitably diluted tissue fraction was incubated for 3 hr at 37°C with 0.1 ml of ^{14}CNO-labeled hemoglobin (20) in 0.2 M lactate buffer pH 3.6. The final hemoglobin concentration was 0.5 mg/ml and toluene was used to inhibit bacterial growth. The reaction was stopped by the addition of 0.5 ml 1% (w/v) casein (Hammersten quality; Nutritional Biochemicals Corporation, Cleveland, Ohio) and 1.0 ml 10% (w/v) trichloroacetic acid. After standing in an ice bath for 30 min, the tubes were centrifuged at 3500 *g* for 30 min. 0.5 ml of the supernatant was added to 10 ml of Triton-toluene scintillation fluid (21) and counted for 10 min in a Packard Model 2002 scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Enzyme activities are expressed in arbitrary units of net acid-soluble counts per minute released per minute.

5′-Nucleotidase.—This enzyme was assayed at pH 9.0 as described by Avruch and Wallach (22), using adenosine-2-^{3}H-5′-monophosphate as substrate.

Monoamine Oxidase.—This enzyme was assayed as described by Wurtman and Axelrod (23), using tryptamine-2-^{14}C as substrate.

Protein.—The fluorometric technique of Hiraoka and Glick (24) was used, with some modifications. 0.25 ml of each subcellular fraction or suitably diluted homogenate was mixed with 1 ml of 0.1 M sodium citrate buffer pH 3.1; 1 ml of 33 μM eosin Y (C.I. No. 45380; Harleco, Philadelphia, Pa.) in 0.05 M sodium citrate buffer pH 3.1 was added, and after careful mixing the fluorescence was determined in a Perkin-Elmer fluorescence spectrophotometer. The excitation wavelength was 519 nm and the emission wavelength was adjusted to 540 nm. A Corning 0-52 filter (F. J. Gray & Co., Inc., New York) was placed in the path of the exciting beam. A standard curve was constructed for each assay with bovine serum albumin (Armour)

in the range of 0–100 μg/ml. Under these conditions the degree of quenching of the eosin Y fluorescence was almost proportional to the protein concentration. This technique was found to be five times more sensitive than the Lowry technique and to show no interference by sucrose concentrations up to 60% (w/w). This assay, however, gives a 20% lower protein concentration for an aortic cell homogenate than the automated Lowry technique (11) with the same bovine albumin standard.

Deoxyribonucleic acid.—DNA was assayed by the fluorometric technique of Kissane and Robins (25). Calf thymus DNA (type I; Sigma) was used as standard. Recovery of added calf thymus DNA was 85%. The method was also shown to be highly specific, showing no interference by sucrose, protein, or RNA.

Presentation of Enzyme Distribution Results.—All results are presented in the form of frequency distribution histograms as functions of the mean density of the individual fraction. The results of the top fractions, up to a density of 1.09, which relate mostly to soluble enzyme activities remaining in the starting layer, together with any particles of density lower than 1.09, were pooled and plotted over a density interval of 1.034–1.090. The corresponding block is shaded in the histograms to indicate that it is not part of the density distribution proper. Note however, that the limit of 1.09 is itself somewhat arbitrary and is passed by sedimenting proteins of relatively large size, such as catalase. For these, the histograms are distorted on the low density side by the presence of soluble activity.

The calculations and plots were done by computer, according to methods previously described (10, 11). All histograms are normalized and the per cent recoveries (sum of gradient fractions against original PNS) are given in addition.

Pooling and averaging of several distributions were performed as described by Leighton et al. (10). The method requires conversion of all the histograms to the same preset density intervals and causes some loss in resolution.

RESULTS

Composition of the Isolated Cell Suspensions.—Under the phase-contrast microscope, the washed cell suspension consisted of a large number of elongated or rounded cells among debris of varying nature. Approximately 60% of the cells excluded 1% trypan blue. Surface blebbing of some cells was noted.

In fixed and stained preparations, elongated and rounded cells are clearly seen as illustrated in Fig. 1*a*. Many of the rounded cells have their surface covered with finger-like protuberances, and there is a gradation in the amount of stain taken up by the cells (Fig. 1*b*). The elongated cells are the most lightly stained, the round cells are stained more intensely, and the extensively indented cells are the darkest.

Electron microscope examination of many preparations revealed that practically all cells can be identified as smooth muscle cells, but they range in their degree of damage incurred during the isolation procedure. The most intact cells are elongated and stain lightly (Fig. 2). As they become injured, they tend to round up, bleb, and to become increasingly stainable (Fig. 3). The extreme is represented by the highly indented, strongly stained form, which presumably is the one that no longer excludes trypan blue in fresh preparation (Fig. 4).

In addition to cells, the suspensions contained a fair amount of debris, consisting largely of undigested connective matrix fragments, together with

some vesicles and other cell components released from broken cells. Few isolated nuclei were seen.

Fine Structure of the Isolated Cells.—As illustrated in Fig. 2, the elongated cells were practically indistinguishable from the smooth muscle cells seen in

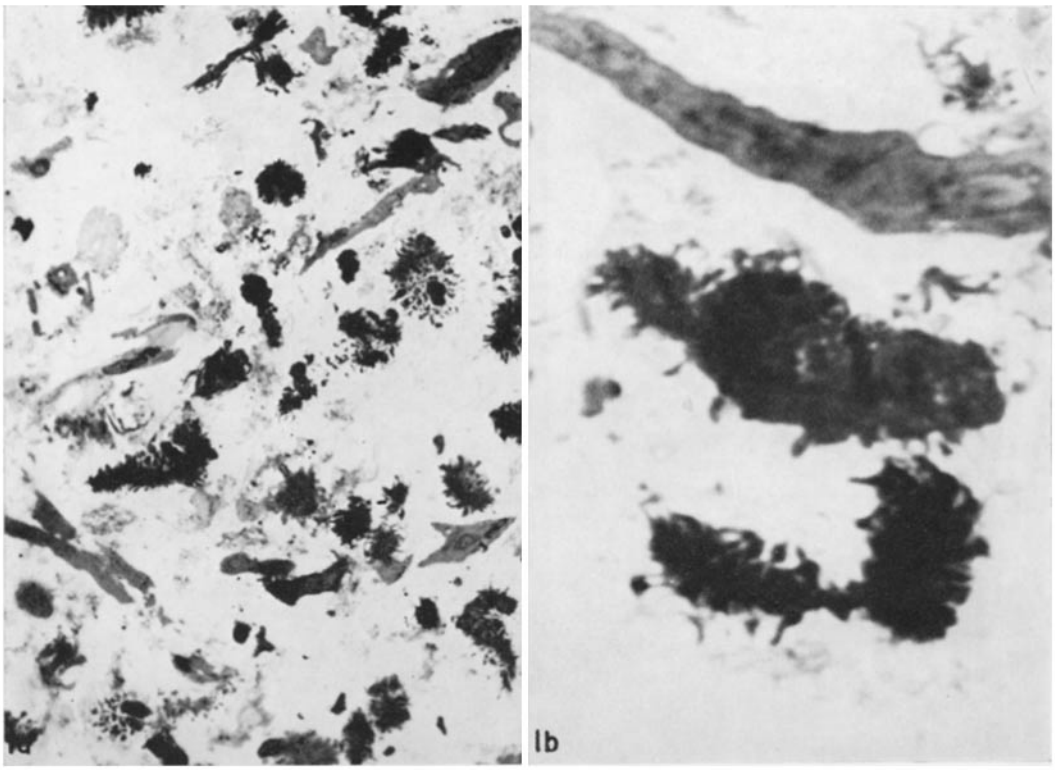

FIG. 1. Cells isolated from rabbit aorta. Stained thin Epon sections. (*a*) A variety of elongated and rounded cells with different staining intensity together with some noncellular debris. × 660. (*b*) Cells in differing states of preservation; top: elongated, lightly stained cell; middle: more intensely stained cell showing some blebbing; bottom: heavily stained rounded cell showing marked blebbing. × 2330.

the intact aorta (3, 5, 26–33). They have a centrally located elongated nucleus with fairly smooth outlines. The cytoplasm, except for an extensive perinuclear area, is filled with myofilaments and contains numerous electron-opaque areas. Groups of small vesicles, possibly of pinocytic nature, are seen connected with the plasma membrane or just beneath it. The perinuclear cytoplasm is rich in organelles. Mitochondria, some rough endoplasmic reticulum, and ribosomes are present together with a large number of membrane-bounded

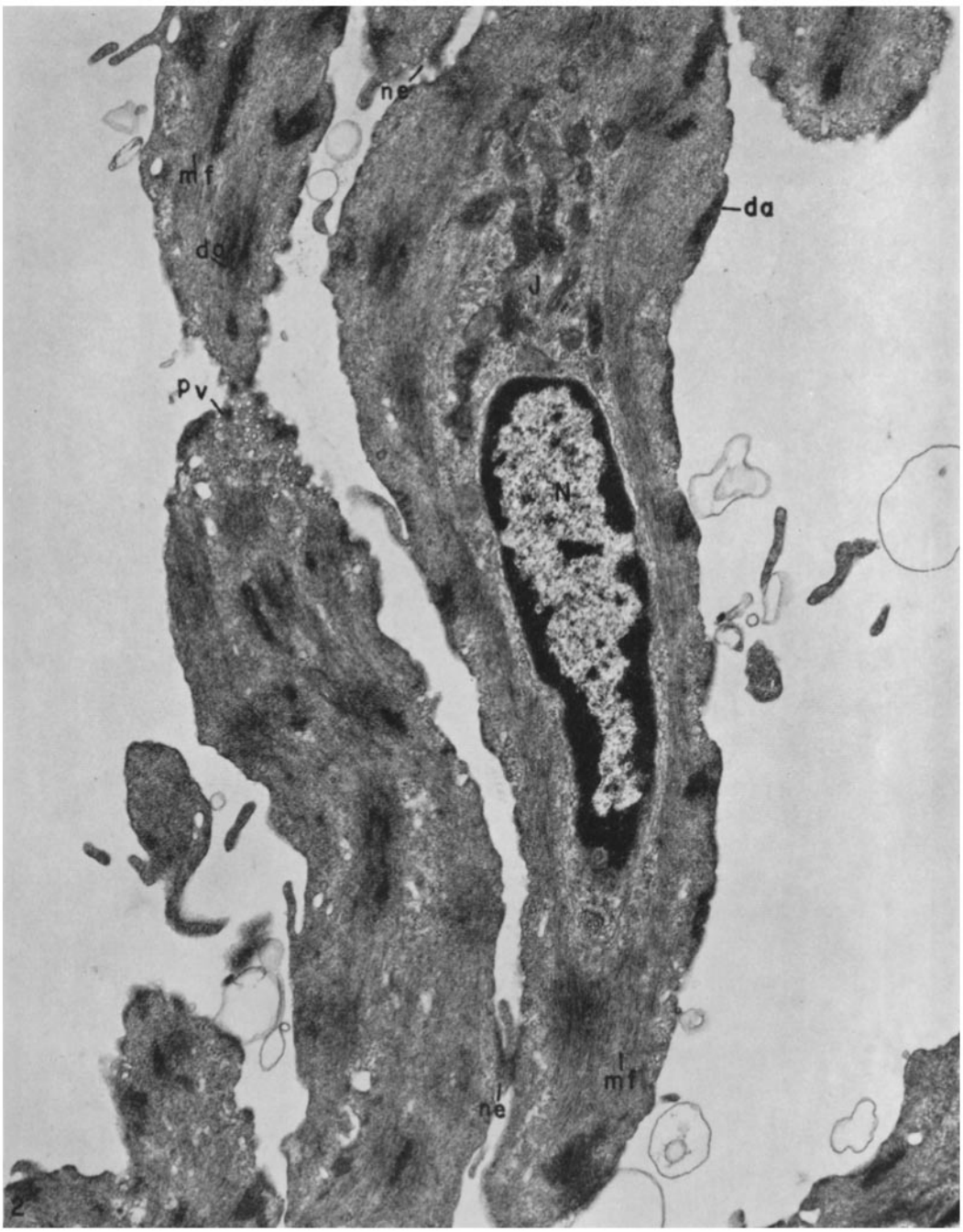

FIG. 2. Isolated smooth muscle cells from rabbit aorta showing normal ultrastructure with characteristic abundant myofilaments (*mf*) and dense areas (*da*). Adjacent to the central nucleus (*N*) is the organelle-rich juxtanuclear area (*J*). Groups of pinocytic vesicles (*pv*) are seen attached to or below the cell membrane. Nexuses (*ne*) still connect the individual cells. × 17,700.

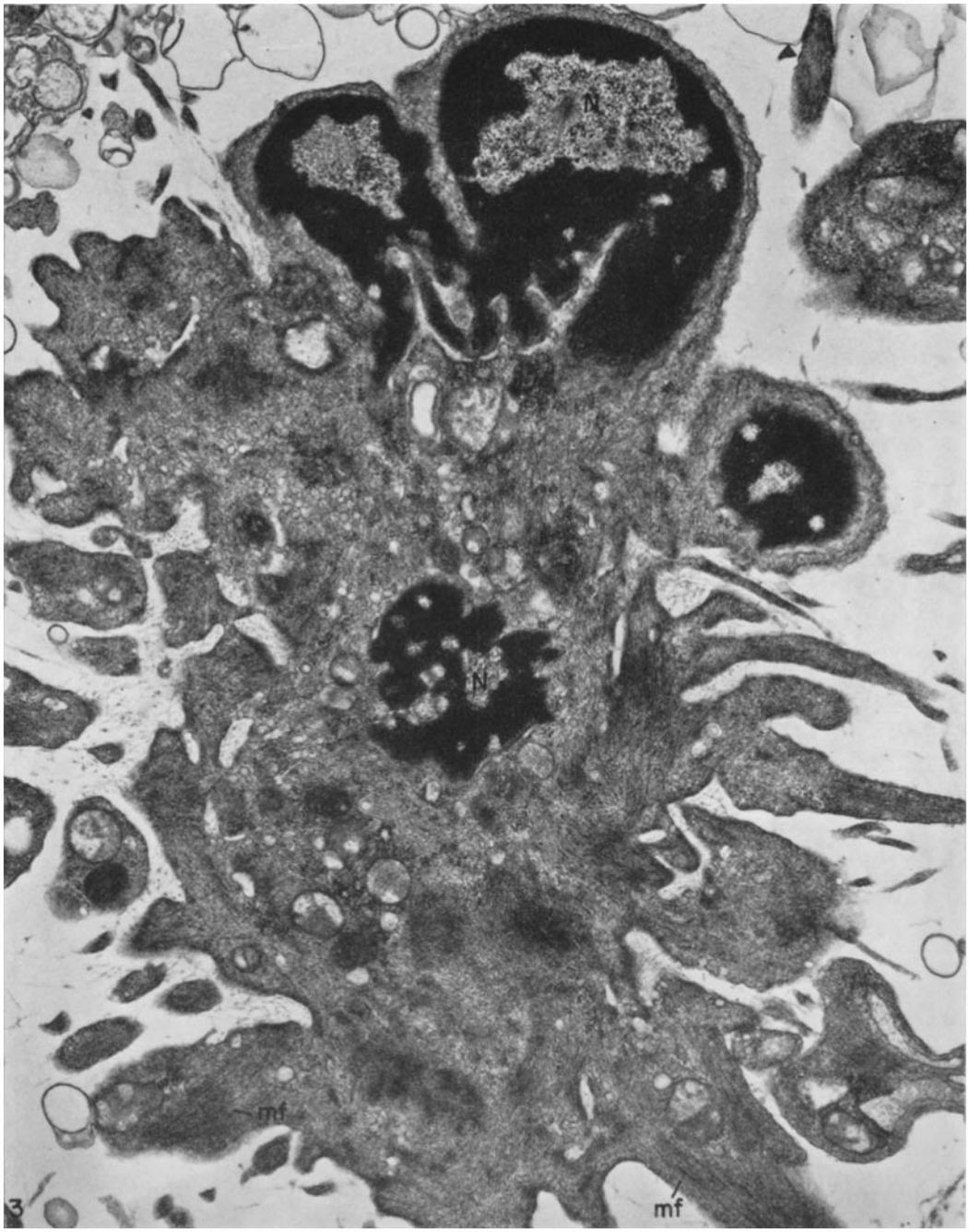

FIG. 3. Isolated cell in highly contracted state. The nucleus (*N*) is displaced to one end of the cell, but otherwise there is no evidence of any serious deterioration. The cytoplasmic processes contain myofilaments (*mf*). × 21,600.

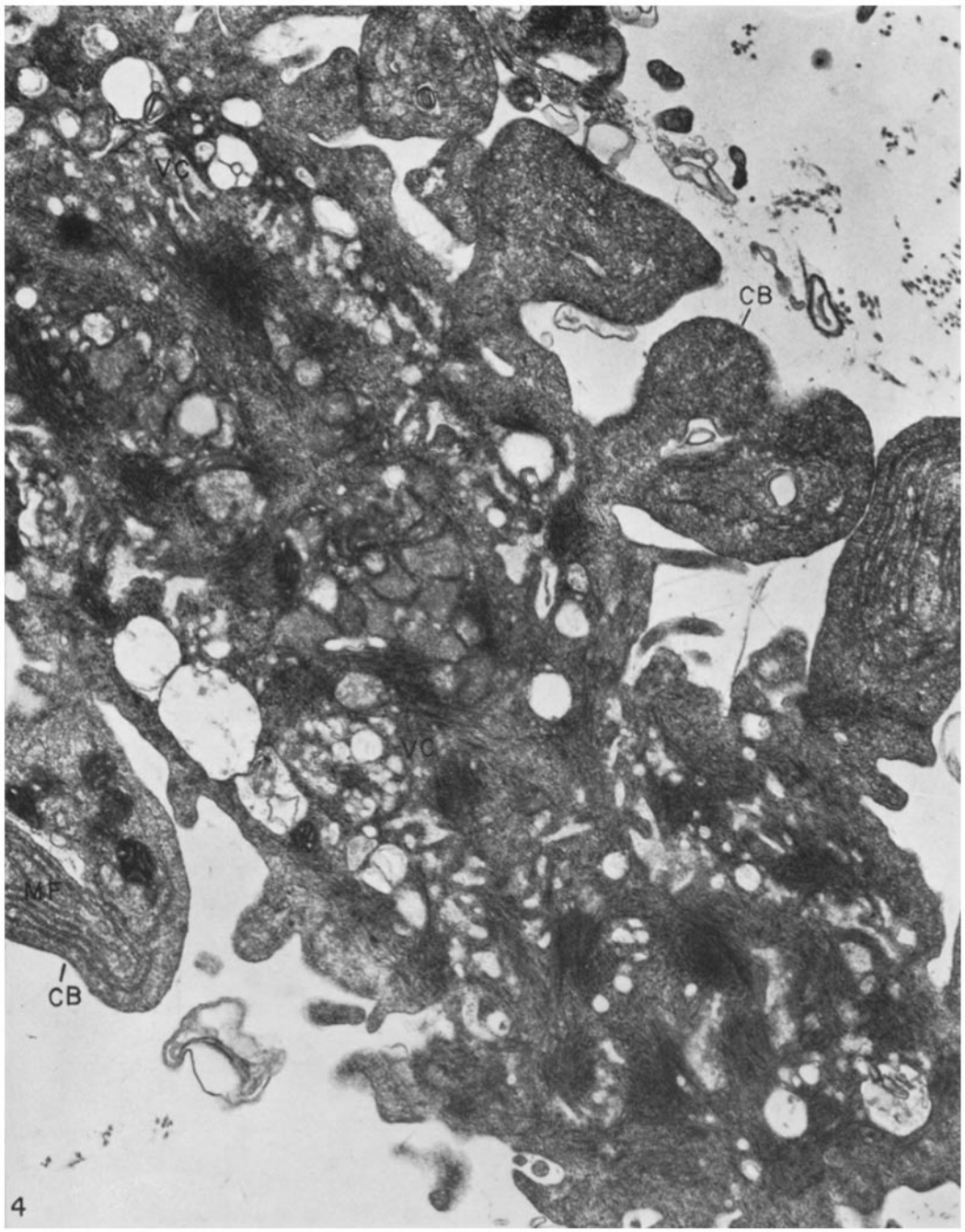

FIG. 4. Isolated cell showing signs of deterioration. The cell is surrounded by cytoplasmic blebs (*CB*) without myofilaments but often containing membraneous formations (*MF*). Inside, the cell vacuolar clusters (*VC*) are visible. × 21,600.

vesicles and granules of different size, some of which may be lysosomes. A well-developed Golgi apparatus (not shown in Fig. 2) is also present in a juxtanuclear position.

The rounding up and blebbing of the cells appears to be accompanied by contraction or hypercontraction of the myofibrils. The nucleus often assumes an irregular shape and is pushed to one side of the cell (Fig. 3). The cytoplasmic protrusions, especially the rounded ones, contain many organelles which appear to be expressed from the cells (Fig. 4). An additional feature of these cells is the increase in the number of round or elongated membrane-limited spaces. The high degree of digitation of the surface makes it probable that many of these are actually cross sections of infoldings of the cell membrane and are not intracytoplasmic structures.

Enzyme Activities of Isolated Cells.—Table I shows the specific activities and pH optima of the various enzymes assayed in the isolated smooth muscle cells. Significant amounts of activity were found for eight hydrolases usually associated with lysosomes in other tissues. All these enzymes, with the exception of α-glucosidase, have a distinctly acid pH optimum. Only trace levels (0.001–0.01 mU/mg protein) of enzyme activity were found with other substrates. These included sulfate, α-arabinoside, β-cellobioside, β-fucoside, α-galactoside, and β-xyloside derivatives of 4-methyl umbelliferone. Significant amounts of cytochrome oxidase, catalase, 5′-nucleotidase, and leucyl-β-naphthylamidase were detected. Only low activity of nonspecific alkaline phosphatase (4-methyl umbelliferyl-phosphate) was found.

Table I also shows the DNA, protein, and enzyme content of the PNS fraction expressed as a per cent of the total activity of the homogenate. With the exception of DNA, between 65 and 70% of each component is found in the PNS fraction. Approximately one-third of the DNA is also found in the PNS, indicating that some nuclei are disrupted during the homogenization procedure.

Latent and Sedimentable Acid Hydrolases.—The mean ±SE latent *N*-acetyl-β-glucosaminidase activity in the PNS preparations from 15 different animals was 58.6 ± 2.4%. A small number of latency determinations performed for α-glucosidase, β-galactosidase, and acid phosphatase gave values of 50–60%. In a comparative experiment carried out with different concentrations of digitonin, latent α-glucosidase and *N*-acetyl-β-glucosaminidase were found to respond identically with this detergent, both showing a 50% decrease in latency in the presence of 0.025 mg/ml of digitonin.

Centrifugation of the aortic cell PNS at 100,000 *g* for 30 min gave a small pellet which contained 50–60% of the *N*-acetyl-β-glucosaminidase, acid phosphatase, and β-galactosidase but 70–75% of the α-glucosidase.

Rate Zonal and Differential Centrifugation Experiments.—Attempts to estimate to the distribution of sedimentation coefficients for particulate β-galactosidase and *N*-acetyl-β-glucosaminidase in the aortic cell PNS, either

by zonal sedimentation or by the moving boundary technique of Deter and de Duve (34), were unsuccessful due to the excessive polydispersity of the particles. It was clear, however, that the particles containing these enzymes are significantly smaller on average in aortic cells than in rat liver.

TABLE I

Enzymic Activities, Protein, and DNA Content of Isolated Aortic Smooth Muscle Cells

Enzyme	Specific activity*	pH optimum	Per cent activity in PNS fraction
	munits/mg protein		
Acid phosphatase	6.08 ± 1.70 (12)‡	5.1a§	66.6 ± 12.0
α-Glucosidase	1.58 ± 0.18 (9)	6.9b	72.8 ± 9.0
N-Acetyl-β-glucosaminidase	0.98 ± 0.44 (15)	5.7a	71.0 ± 8.1
β-Glucuronidase	0.82 ± 0.36 (8)	4.3a	61.6 ± 6.5
β-Galactosidase	0.74 ± 0.33 (9)	4.1a	67.4 ± 7.3
N-Acetyl-β-galactosaminidase	0.41 ± 0.06 (3)	5.3a	68.0 ± 6.1
α-Mannosidase	0.09 ± 0.01 (3)	5.7a	72.3 ± 5.1
β-Glucosidase	0.05 ± 0.02 (5)	5.3a	67.9 ± 6.1
Cathepsin A	Approx. 2 (1)	5.0a, c	—
Cathepsin B	0.07 ± 0.04 (5)	5.0a, c	—
Cathepsin C	1.48 ± 0.31 (4)	5.0a, c	73.0 ± 6.0
Cytochrome oxidase	27.6 ± 8.70 (18)	7.0c	71.4 ± 8.7
Monoamine oxidase	0.56 ± 0.04 (3)	7.4c	70.9 ± 4.1
Catalase	2.81 ± 1.20 (6)	7.0c	67.3 ± 8.8
5′-Nucleotidase	188.0 ± 47.0 (6)	7.0c, d	72.3 ± 7.1
Alkaline phosphatase	0.05 ± 0.01 (3)	8.7d	—
Leucyl-β-naphthylamidase	1.46 ± 0.56 (4)	7.3b	68.5 ± 16.0
DNA	0.121 ± 0.06 (9)e§		33.0 ± 7.4
Cell protein	2.27 ± 1.40 (17)e		66.4 ± 7.5
Cell protein	23.10 ± 0.41 (6)f		—

* Specific activity ± SD.

‡ No. of experiments between parentheses.

§ a, 0.1 M acetate buffer.
b, 0.1 M phosphate buffer.
c, pH of assay: optimum not determined.
d, 0.05 M borate buffer containing 5 mM $MgCl_2$.
e, mg/g whole aorta.
f, μg/μg DNA.

Differential centrifugation experiments were performed on the aortic cell PNS in order to find out whether particulate α-glucosidase, which, as will be seen, differs in several respects from the lysosomal acid hydrolases, would be resolved from particulate *N*-acetyl-β-glucosaminidase on the basis of sedimentation rate. There was however no significant difference between the two enzymes.

Isopycnic Centrifugation Experiments.—Fig. 5 shows the distribution in sucrose density gradients of enzymes likely, from their localization in liver and other tissues, to represent markers for the principal subcellular organelles. Several distinct patterns are recognized. Cytochrome oxidase shows a single peak with a median density of 1.17. Catalase shows a more complex distribution. About half the enzyme is soluble and the remainder is distributed through-

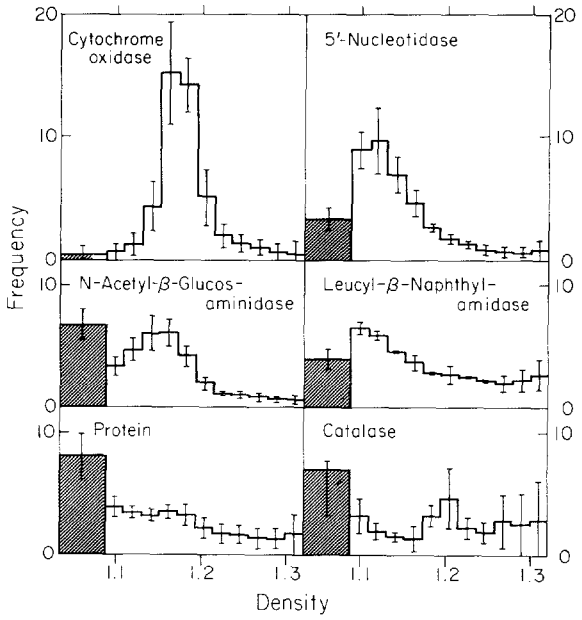

FIG. 5. Isopycnic centrifugation of 6000 *g*-min supernatant from aortic smooth muscle cell homogenate. Graph shows frequency-density distribution (±SD) for various marker enzymes and for protein. The shaded area represents, over an arbitary abscissa interval, the enzyme remaining in the sample layer. The per cent recovered activity (±SD) for each enzyme and the number of experiments (between parentheses) are: cytochrome oxidase, 75.8 ± 12.0 (11); 5′-nucleotidase, 93.5 ± 15.0 (5); *N*-acetyl-β-glucosaminidase, 70.0 ± 10.0 (8); leucyl-β-naphthylamidase, 114.0 ± 20.0 (2); protein, 90.0 ± 7.2 (11); catalase, 170 ± 42.0 (4).

out the gradient with a small peak at a modal density of 1.20. Note however that the apparent recovery of catalase from the gradient is over 100%. 5′-Nucleotidase shows a single skew peak with a modal density of 1.11. Leucyl-β-naphthylamidase has a distribution roughly similar to that of 5′-nucleotidase, although more activity is found at higher densities in the gradient. *N*-Acetyl-β-glucosaminidase is partly soluble, and the remainder forms a single peak of sedimentable activity with a modal density of 1.15. There is a large amount of soluble protein remaining with the original layer, with a smaller amount distributed throughout the gradient. There is a small peak of protein at density of 1.16–1.17.

These averaged distribution plots indicate that there is little resolution between cytochrome oxidase and *N*-acetyl-β-glucosaminidase, which show about the same modal density. In the individual experiments, however, partial dissociation of the two enzyme peaks by at least one fraction was achieved. As mentioned above, the process of averaging the density distributions tends to obscure this resolution.

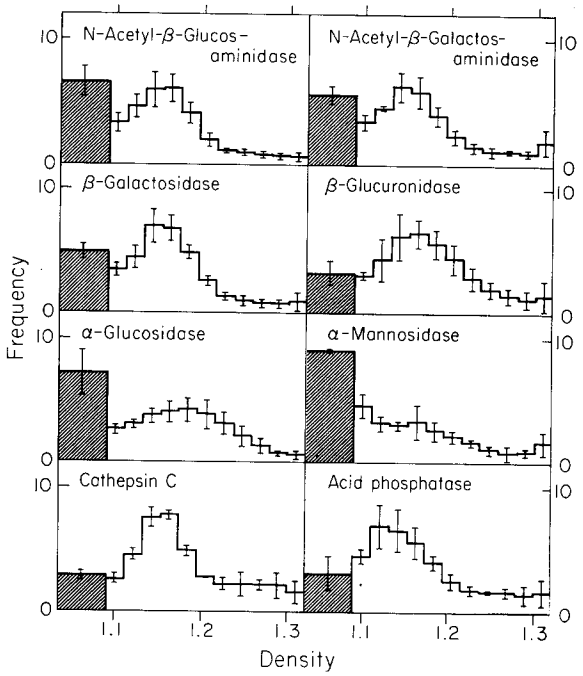

FIG. 6. Isopycnic centrifugation of 6000 *g*-min supernatant from aortic smooth muscle cell homogenate. Graph shows frequency-density distribution (±SD) for eight hydrolases. The shaded area represents, over an arbitrary abscissa interval, the enzyme remaining in the sample layer. The percent recovered activity (±SD) for each enzyme and the number of experiments (between parentheses) are: *N*-acetyl-β-glucosaminidase, 70.0 ± 10.0 (8); *N*-acetyl-β-galactosaminidase, 85.8 ± 12.0 (3); β-galactosidase, 92.2 ± 16.0 (5); β-glucuronidase, 111.0 ± 9.3 (5); α-glucosidase, 72.0 ± 11.0 (4); α-mannosidase, 109.0 ± 12.0 (3); cathepsin C, 114 ± 17.0 (2); acid phosphatase, 113.0 ± 9.8 (5).

Fig. 6 shows the distribution of eight hydrolases in sucrose gradients. All enzymes have significant amounts of soluble activity, varying from 20% for acid phosphatase to over 50% for α-mannosidase. All show a distinct peak of particulate activity around a density of 1.15, but with somewhat different distribution patterns. Acid phosphatase and β-glucuronidase tend to show more "tailing" in the denser fractions, and α-mannosidase gives no more than a shoulder in the region of 1.15. The distribution of α-glucosidase is characterized by a broader and flatter peak extending from a density of 1.13 to 1.22. A

small number of studies have been made on the distribution of β-glucosidase and cathepsin D in the sucrose gradients. They show distribution patterns very similar to that of *N*-acetyl-β-glucosaminidase.

For all enzymes studied, including 5′-nucleotidase, the values quoted are true equilibrium densities, as indicated by experiments in which centrifugation was carried out for up to 3 hr. The density distribution of the particulate enzyme activities was unchanged, but there was some additional sedimentation of the soluble enzyme activities into the gradient, as was to be expected.

The peculiar distribution observed for α-glucosidase, and its pH optimum of 6.9, unusual for a lysosomal hydrolase, prompted us to perform a few additional experiments on this enzyme. Rat liver is known to contain a neutral α-glucosidase localized in the microsomal and soluble fractions, in addition

TABLE II

Enzymic Activities and Protein Content of Rabbit Liver

Enzyme	Specific activity*		pH optimum
	munits/mg protein		
Acid phosphatase	14.20 ± 4.90	(3)‡	5.2
α-Glucosidase	3.80 ± 0.68	(3)	7.2
N-Acetyl-β-glucosaminidase	1.18 ± 0.17	(3)	5.7
β-Glucuronidase	13.10 ± 0.58	(3)	3.5
β-Galactosidase	1.72 ± 0.25	(3)	5.3
Protein	133 ± 10.0a§	(3)	

* Specific activity ± SD.

‡ No. of experiments between parentheses.

§ a, mg/g wet tissue.

to an acid α-glucosidase present in the lysosomes (35). In order to determine whether a similar multilocalization occurs in the smooth muscle cells, the distribution of α-glucosidase, assayed at pH 5.5, 6.9, and 8.5, was studied in the same sucrose density gradient. Although the activity was much lower at pH 5.5 and 8.5 than at 6.9, the relative concentrations throughout the gradient were identical in all three instances. D-Turanose has been shown to be a specific noncompetitive inhibitor of the lysosomal, as distinct from the microsomal, α-glucosidase. It was found that 30 mM and 200 mM turanose caused only 30% and 70% inhibition of the aortic cell α-glucosidase, respectively. In addition, there was no difference in the distribution of α-glucosidase in a sucrose density gradient when assayed in the presence or absence of 200 mM turanose.

Enzymic Activities of Rabbit Liver.—Table II shows the pH optimum and specific enzyme activity of several acid hydrolases in rabbit liver homogenates. The specific activities of the measured enzymes are higher in the liver than in the aortic cells, but markedly so only for β-glucuronidase.

DISCUSSION

The experiments described in this paper represent the first comprehensive fractionation of smooth muscle cells isolated from rabbit aorta. There have been previous attempts to fractionate whole aortae (36–42), but apart from the studies of Miller and his colleagues, no attention has been paid to the characterization of the lysosomes. These latter workers demonstrated the presence of latent and particulate acid hydrolase activity in homogenates of human arteries (41). However, they found that only 15–20% of the lysosomes remained intact. This is in contrast to the present study where nearly 60% of the *N*-acetyl-β-glucosaminidase was latent. No doubt enzymic digestion of the connective tissue matrix helped to reduce homogenization damage.

In this discussion, we will consider first the properties of the cells that served as starting material for the fractionations, then the fractionation techniques, and finally the properties of the subcellular constituents, as deduced from the observed enzyme distribution patterns.

Properties of Aortic Smooth Muscle Cells.—In agreement with the known composition of the rabbit aorta, the cell preparations isolated in this work consisted essentially of smooth muscle cells. As far as we are aware, there have been only three previous attempts to isolate cells from the aorta. In a preliminary study, Day, Newman, and Zilversmit (43) isolated foam cells from the intima of cholesterol-induced atherosclerotic lesions by a technique similar to that used in the present study. They showed that these cells were capable of synthesizing phospholipid in vitro, but have not reported any studies on the subcellular organelles of the cells. Stein et al. (44) attempted to apply a similar technique to the normal aorta but were unable to isolate any smooth muscle cells. Details of their methods are not given, but it appears elastase or collagenase was used separately. No attempts were made to use a combination of these enzymes alone or in conjunction with hyaluronidase. In a brief report, Robertson and Insull (45) described the use of proteolytic enzymes for the isolation of various cell types from both normal and atherosclerotic human arteries. They have, however, not reported any detailed biochemical studies on these cells.

The prolonged exposure of the cells to the powerful hydrolase mixture obviously damages them in that they show certain morphological changes. Nevertheless, there is evidence that similarly prepared cells can synthesize several classes of lipid in vitro (43, 46, 47) and can actively accumulate cholesterol from lipoproteins (48). In the present study, it was found that more than half of the smooth muscle cells still excluded trypan blue at the end of the treatment. In most cells, the subcellular organelles had an essentially normal appearance (Figs. 2 and 3). It is also apparent that at least part of these organelles, in particular the lysosomes, could be isolated intact from such cells.

Compared with rabbit liver, the aortic cells tended to have lower acid hydrolase specific activities, but the difference was marked only for β-glucuroni-

dase. For the other four hydrolases that were measured in the two tissues, the aortic cells had at least 40% of the specific activity found in the liver. Considering the contribution of debris and of myofilaments to the total protein of our preparations, these results may be taken to indicate that the cytoplasm of smooth muscle cells must be well endowed with lysosomes, contrary to inferences made from morphological observations (49, 50).

Taking rat liver as standard of comparison for the other enzymes, we find that the cytochrome oxidase specific activity of the aortic cells amounted to 15–20% of the specific activity in rat liver (51), whereas the catalase reached only 1.5% of the rat liver value (13). Notable activity was observed for 5′-nucleotidase, which in the rabbit aortic cells showed a specific activity twice that of rat liver (52). Other workers (6, 7, 53) have commented on the high 5′-nucleotidase content of arterial tissue. No comparable abundance was found for leucyl-β-naphthylamidase. Alkaline phosphatase was very low, in agreement with previous studies on whole aortae (6, 7).

Fractionation Techniques.—The cells isolated from a single rabbit aorta represented only 0.5–2.0 mg of protein. Such small amounts hardly lend themselves to conventional techniques of differential centrifugation. Even more difficult would have been any attempt at purifying lysosomes or other subcellular organelles for the purpose of characterizing them. These difficulties have been circumvented in the present work by the use of a single-step analytical approach. Even though the resolution achieved was relatively low in terms of actual separation of subcellular organelles, a fair amount of information could nevertheless be obtained simply from an analysis of the density-frequency distribution histograms of the various enzymes measured. The rationale behind such an approach has been discussed elsewhere (54).

Technically, the experiments were considerably facilitated by the Beaufay automatic rotor. There is, however, no reason why a similar fractionation could not be performed in a swinging bucket rotor, though probably with lower resolution and more damage to subcellular organelles. The use of sufficiently sensitive microtechniques, particularly using fluorometric methods and labeled substrates for the biochemical assays, have been invaluable in this study.

In practice, the kind of approach followed in the present work opens interesting possibilities for the future. As will be reported in subsequent papers, it has enabled us to characterize some of the biochemical changes, especially to lysosomes, occurring at the subcellular level during a cholesterol-induced atheromatous transformation. But especially, it can be extended to almost any kind of normal or pathological tissue specimen, including needle biopsies, and could therefore be of general value in the subcellular exploration of disease.

Properties of Subcellular Constituents.—From the distributions observed after density gradient centrifugation, it appears that most of the enzymes

assayed occupy sites in aortic smooth muscle cells comparable to their known intracellular location in liver and other well investigated tissues. Thus, some information on the properties of subcellular organelles in aortic smooth muscle cells can be derived from an analysis of the enzyme distribution profiles.

Plasma membranes: The distribution observed for 5′-nucleotidase suggests that this enzyme is associated with the plasma membrane in the aortic cells, as it is in the liver (55). This appears to be true also for leucyl-β-naphthylamidase (55), except that part of this enzyme seems to occur also in one or more other subcellular sites. The low modal density (1.11) of these enzymes could reflect a relatively high lipid content of the membrane fragments. In rat liver, the modal equilibrium density of 5′-nucleotidase is of the order of 1.17 for plasma membranes separated from the nuclear fraction, but it falls to 1.14 after mechanical comminution of these membranes and is equal to about 1.13 for the plasma membrane fragments present in the microsomal fraction (56). It seems likely from the morphological appearance of the cells that their high 5′-nucleotidase activity reflects the high degree of infolding of the plasma membrane in the aortic smooth muscle cells. According to Verity and Bevan (32), pinocytic vesicles may occupy up to 45% of the cell surface in such cells.

Mitochondria: Cytochrome oxidase shows the expected narrow band characteristic of mitochondria, though with a median density (1.17) lower than the value of 1.18–1.19 observed for mitochondria in rat liver (10). An almost 10-fold increase in specific activity was attained for cytochrome oxidase in the peak fraction, despite the obvious presence of several contaminants. This indicates that the mitochondria of the aortic smooth muscle cells represent only a small fraction of the total cell protein, in agreement with the low specific activity of cytochrome oxidase in the homogenates and with the sparseness of mitochondrial profiles in electron micrographs. A small number of experiments were performed on the distribution of monoamine oxidase in the sucrose density gradients. The distribution was almost identical with that of cytochrome oxidase, indicating the exclusively mitochondrial localization of monoamine oxidase in these cells. If this enzyme is localized to the outer membrane as it is in mitochondria isolated from other tissues (57), these distribution studies indicate that mitochondrial integrity is well preserved during cell isolation and fractionation.

Catalase-containing particles (peroxisomes ?): The finding that about half the catalase activity of the PNS is particle bound, much of it equilibrating at a fairly high density (1.20) in sucrose gradients, suggests the possibility that peroxisomes may be present in the aortic smooth muscle cells. However, the total catalase activity is quite low, and so far we have been unable to detect any of the oxidases characteristic of peroxisomes in our preparations. Even with the highly sensitive fluorometric assay of Guilbault, Brignac, and Zimmer (58), no significant D-amino acid, lactate, glycolate, or urate oxidase activity was found.

The possibility of erythrocyte contamination contributing to the sedimentable catalase was envisaged. However, analysis of the catalase content of washed rabbit erythrocytes showed that they contained 9.30 mU/mg protein. Thus, nearly one-third of the aortic cell preparation would have to consist of red cells to account for the catalase content of our preparations. Kirk (7) has also noted that human aorta contains far more catalase than could be accounted for by red cell contamination.

Lysosomes: The latency and centrifugal behavior of various acid hydrolases indicate clearly that lysosomes are present in aortic smooth muscle cells. We have already commented upon the fact that the level of several of these enzymes is not dramatically lower in the aortic cell preparations than it is in the rabbit liver. It is therefore surprising that few structures that could be clearly identified as lysosomes were detected in these cells morphologically. So far, attempts to identify the lysosomes of the aortic cells at the electron microscope level by cytochemical staining have given inconclusive results. It should, however, be recalled that sedimentation analysis has indicated that the aortic smooth muscle cell lysosomes must be distinctly smaller on an average than rat liver lysosomes, and quite heterogeneous in size. These features may complicate their morphological identification.

All the hydrolases that could be anticipated to be lysosomal, with the exception of α-glucosidase, which will be discussed below, showed modal densities of about 1.15–1.16. This value is low, as compared to the density of lysosomes in tissues such as liver, where these particles take mostly the form of "dense" or "residual" bodies. The scarcity of such bodies in the smooth muscle cells has already been mentioned.

Besides the general agreement of the modal densities, there were significant differences in other features of the distribution diagrams of the various hydrolases. The amounts remaining in the starting layer differed appreciably, and there were also differences in the amounts reaching into the lower parts of the gradients. Artifacts resulting from the rupture of lysosomes and from adsorption of released enzymes to other structures may in part account for these differences. In addition, some of the differences may reflect an intrinsic heterogeneity or multiplicity of aortic cell lysosomes. Before this conclusion is accepted, a careful study of the enzymes themselves and of their intracellular location will have to be made to exclude alternative explanations based on heterogeneity of the enzymes. Many instances are now known of hydrolase activities, especially when measured with nonspecific substrates, involving the contribution of two or more distinct enzyme species occupying different intracellular sites. An additional complication may arise in the present case from the low substrate concentrations used in the fluorometric microassays, which may cause enzyme species with a low K_m to be advantaged over others with a high K_m. Such an effect could explain the high amount of soluble α-mannosidase, in view of the report by Marsh and Gourlay (59) that the soluble α-mannosidase from rat liver has a much lower K_m than the lysosomal enzyme.

The localization of α-glucosidase in the aortic cells is uncertain. This enzyme shows the same sedimentation and latency properties as the typical lysosomal *N*-acetyl-β-glucosaminidase, but differs from this and the other lysosomal hydrolases by its distribution in sucrose gradients. Its neutral pH optimum is not typical of a lysosomal enzyme, but recalls rather the neutral microsomal α-glucosidase identified in rat liver by Lejeune et al. (35). However, efforts to distinguish more than one α-glucosidase on the basis of pH optimum or sensitivity to turanose inhibition were unsuccessful. Thus the question of the subcellular localization of α-glucosidase in the aortic cell must remain open.

Microsomes: It has not yet proved possible to identify a microsomal marker enzyme with sufficient activity so that the distribution of these organelles could be studied in the gradient. In agreement with the negative findings of Kobernick and Hashimoto (60), no glucose-6-phosphatase could be detected, even though the radioassay of Meerov and Ryzhkova (61) was used. Only trace amounts of NADPH cytochrome *c* reductase (62), esterase (63), RNA (64), and galactosyl transferase (with ovalbumin as acceptor [65]) were found in smooth muscle cell homogenates. As mentioned, it is possible, but by no means certain, that the neutral α-glucosidase that we have assayed may be microsomal in the aortic cells, as it is in rat liver.

These investigations have established the principal properties of the lysosomes and other organelles in the smooth muscle cells of rabbit aorta and provide a base line for the study of the changes which occur under the influence of age and various pathological conditions. In addition, these studies illustrate how the technique of analytical subcellular fractionation may be applied to milligram quantities of tissue.

SUMMARY

Smooth muscle cells were dissociated from normal rabbit aorta by incubating the tissue in Hanks' solution containing elastase, collagenase, and hyaluronidase. The isolated cells contained significant amounts of the following acid hydrolases: *N*-acetyl-β-glucosaminidase, *N*-acetyl-β-galactosaminidase, β-galactosidase, β-glucuronidase, α-mannosidase, β-glucosidase, acid phosphatase, and cathepsins C and D. The cells were disrupted and fractionated by isopycnic centrifugation on sucrose density gradients in the Beaufay automatic zonal rotor.

Lysosomes with a modal density of 1.16 were identified by the distribution of these acid hydrolases and by the latency of *N*-acetyl-β-glucosaminidase and β-galactosidase. Other particulate enzymes studied in these sucrose gradients included cytochrome oxidase and monoamine oxidase (mitochondria), 5′-nucleotidase and leucyl-β-naphthylamidase (plasma membrane), and catalase (? peroxisome).

This microanalytical subcellular fractionation technique is applicable to the study of milligram quantities of many other tissues, both normal and pathological.

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