GALLOYLGLUCOSES OF LOW MOLECULAR WEIGHT AS MORDANT IN ELECTRON MICROSCOPY

I. Procedure, and Evidence for Mordanting Effect

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ABSTRACT

Gallotannin, consisting mainly of low molecular weight esters such as penta- and hexagalloylglucoses (commercially available as tannic acid produced from Turkish nutgall), can be used for increasing and diversifying tissue contrast in electron microscopy. When applied on tissue specimens previously fixed by conventional methods (aldehydes and OsO_4), the low molecular weight galloylglucoses (LMGG) penetrate satisfactorily the cells and induce general high contrast with fine delineation of extra- and intracellular structures, especially membranes. In some features, additional details of their intimate configuration are revealed. Various experimental conditions tested indicate that the LMGG display a complex effect on fixed tissues: they act primarily as a mordant between osmium-treated structures and lead, and concomitantly stabilize some tissue components against extraction incurred during dehydration and subsequent processing. Experiments with aldehyde blocking reagents (sodium borohydride and glycine) suggested that the LMGG mordanting effect is not dependent on residual aldehydes groups in tissues.

Tannic acid (TA)[1] was recently introduced as an additional fixative for biological specimens in electron microscopy (28, 29, 16) and, although the reactions in which it is involved were not clearly understood, its effects were alternatively referred to as "fixation" (28, 29, 34, 1) or "staining" (41, 37, 8). The most notable feature of the TA-treated specimens appeared to be increased contrast and sharp delineation of cell membranes and certain extracellular structures (29, 34). When TA-aldehyde mixtures are used to fix fresh tissues,

however, the preparations are usually affected by: (a) unsatisfactory penetration into cells (34, 15) (which may be explained, at least in part, by the high molecular weight of the TA $[C_{76}H_{52}O_{46}$; mol wt 1,701] so far used); (b) formation of interstitial precipitates[2] (29, 34, 42, 15); and (c) extraction or precipitation of some tissue constituents (29). The

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[1] *Abbreviations used in this paper:* A, alcohol; ALD, aldehydes; DAB, diaminobenzidine (3,3', 4,4'-tetraaminobiphenyl); LMGG, low molecular weight galloylglucoses (mostly penta- and hexamonogalloylglucoses); E, Epon; PTA, phosphotungstic acid; TA, tannic acid (gallotannin).

[2] Precipitates can also appear intracellularly, in which case they facilitate the visualization of microtubule subunits. The latter are not directly stained, but negatively outlined by TA-metal deposition in the grooves which separate the adjacent subunits. It is noteworthy that in such preparations the penetration of TA was frequently achieved by addition of detergent (15). Extracellular TA-metal precipitates were used as an alternative to the lanthanum procedure for the visualization of gap and tight junctions (42).

last effects are expected in view of the rather extensive literature on TA-induced damage in a variety of fresh tissues (cf. references 2, 7, 11, 26, 32).

The experimental results we report in this paper show that TA is not a primary fixative but acts essentially as a mordanting agent. Low molecular weight "tannic acids," i.e. gallotannin or galloyl-glucoses, are much more effective than previously used high molecular weight tannins. They give the best results when applied on fixed tissue before dehydration and staining.

MATERIAL AND METHODS

Reagents

Commercially available tannic acids are natural products consisting of complex mixtures of polyphenolic materials, all having in common a galloylated glucose structure (13, 35). The extent of glucose esterification by phenol-carboxylic acids, such as the gallic, digallic, or trigallic acids, varies from one natural source to another (3). Usually, the phenolic content of gallotannins is expressed in number of galloyl groups per glucose molecule, but this does not describe the exact structure of the molecule, especially the extent of depside linkages (14) between gallic acid residues. The tannic acid prepared from oriental nutgalls (Chinese gallotannin) is composed mostly of hepta- to decagalloylglucoses (~80%) and of only a small fraction of penta- and hexagalloyl esters (~9%). Such tannic acids have a relatively high molecular weight (~1,400). In the tannic acid prepared from Turkish (Aleppo) nutgalls (Turkish gallotannin), the predominant constituents are the penta- and hexagalloylglucoses (~51%), and only ~30% are hepta- and octagalloylglucoses. Accordingly, these gallotannins have a relatively low molecular weight (~1,000) (T. H. Beasley, personal communication).[3]

We have tested various commercially available TA preparations: (a) tannic acid, $C_{76}H_{52}O_{46}$, mol wt 1,701.18 (A-310, lot 733829) purchased from Fisher Scientific Co., Fair Lawn, N.J.; (b) tannic acid (practical) — P422 from Eastman Organic Chemicals, Rochester, N.Y.; (c) tannic acid, AR, code no. 1764, from Mallinckrodt Inc., St. Louis, Mo. This product was referred by the manufacturer as chiefly $C_{14}H_{10}O_9$ (i.e. digallic acid), and this specification we have used in a preliminary communication (39). In the present paper, the compound is designated low molecular weight galloylglucoses (LMGG), since this name describes as precisely as possible, at present, the chemistry of the compound. In addition, we have tested two chemically better-defined experimental preparations obtained from

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[3] We are deeply obliged to T. H. Beasley for kindly providing us with these samples and the information concerning their chemical composition.

Mallinckrodt Inc., St. Louis, Mo.,[3] namely: (d) tannin prepared from Aleppo nutgalls which consists mostly of penta- and hexagalloylglucoses and is similar to the tannic acid we designated as LMGG; and (e) tannin prepared from oriental nutgalls which is composed mostly of hepta- to decagalloylglucoses.

Ferric chloride $(FeCl_3 \cdot 6H_2O)$, phosphotungstic acid (PTA), glycine (anhydrous, mol wt 75.1), and sodium borohydride $(NaBH_4,$ anhydrous, mol wt 37.85) were obtained from Sigma Chemical Co., St. Louis, Mo.

Animals, Tissues

Adult rats of Sprague-Dawley and Wistar-Furth strains (the latter purchased from Microbiological Associates, Inc., Bethesda, Md.) were used throughout this work. 5–12 animals were used per experiment. The following tissues and organs were processed in different combinations in each experiment: pancreas, jejunum, liver, thyroid, adrenal, tracheal epithelium, oviduct, myocardium, skeletal muscle, omentum, mesentery, blood vessels, thymus. Some tissues (e.g., thyroid, adrenal, trachea, oviduct, thymus) were examined a limited number of times (4–5 times), while the others were extensively and repeatedly examined (20–40 times).

PROCEDURES AND RESULTS

In preliminary experiments, we tested which of the commercially available gallotannins (see Reagents) when used under identical conditions gave the best results in terms of cell penetration, high contrast, improved tissue preservation, and minimal interstitial precipitates. The best effects were consistently obtained with LMGG (tannic acid AR, code No. 1764, Mallinckrodt Inc.) and the Aleppo gallotannin. The former compound which is commercially available was used in subsequent experiments designed: (a) to determine the optimal position of LMGG treatment during tissue processing; (b) to establish the physical conditions optimal for the LMGG effects on tissues; (c) to investigate the possible nature of LMGG effects on tissue.

In each case, the results obtained were compared with those usually achieved by processing samples of the same specimens with one or the other of the following control methods: (a) two-step fixation — (I) 2% buffered glutaraldehyde, or a mixture of buffered 5% formaldehyde and 3% glutaraldehyde (modified after reference 20, for 90 min at 22°C, followed by (II) 2% buffered OsO_4, for 90 min at ~4°C; (b) one-step fixation — by treating tissue specimens for 30–60 min with a mixture of 5% formaldehyde and 3% glutaraldehyde (3 vol), 2% OsO_4 (2 vol), and saturated solution of lead citrate (1 vol) (38). All solutions

were prepared in 0.05–0.1 M HCl-Na arsenate or HCl-Na cacodylate buffer, pH 7.2–7.4; (c) treatment in block—with uranyl acetate (12), uranyl acetate oxalate (30), or ferrocyanide-reduced osmium tetroxide (21) of tissues previously fixed as indicated in (a) or (b). In all these cases the fixed specimens were dehydrated in ethanol and embedded in Epon.

Optimal Position of LMGG Treatment during Tissue Processing

EXPERIMENTAL PROCEDURE: The following three experimental conditions were investigated (Fig. 1).

(a) LMGG added to fixative to a final concentration of 1% (wt/vol) in either 2% glutaraldehyde, or in a mixture of 5% formaldehyde and 3% glutaraldehyde, in 0.1 M HCl-Na arsenate or HCl-Na cacodylate buffer, pH 7.2–7.4. Tissues were fixed *in situ* for 10–15 min, immersed in the same fixative for 90 min at 22°C, then osmicated (2% OsO₄ in the same buffer) for 90 min at 4°C, dehydrated in ethanol, and embedded in Epon.

(b) Tissue specimens fixed with aldehydes (as in Control a) were exposed for 60 min at 22°C to a solution of 1% LMGG in 0.1 M buffer (as in a), as

FIGURE 1 Experimental design to determine the optimal position of LMGG treatment during tissue processing.

a separate step before osmication and subsequent processing, as in a.

(c) Tissue samples were treated with LMGG as above but after osmication, then dehydrated in ethanol and embedded in Epon.

(d) Tissue specimens processed as under (c) except that they were incubated for cytochemical peroxidatic reactions before osmication (17); these specimens come from experiments in which horseradish peroxidase, myoglobin, and heme-peptides were injected into the blood circulation.

Sections obtained from all these specimens (a–d) were examined either unstained or stained with lead citrate alone (33), or with uranyl acetate and lead citrate. A Philips-301 electron microscope operated at 80 kV and provided with apertures in the condensor (300 μm) and objective (50 μm) was used for microscopy.

RESULTS: Of the sequences tested (Fig. 1), those in which the LMGG treatment was intercalated between osmication and dehydration (procedures c and d) gave the best results. In their case there were satisfactory cell penetration, and, after staining (see below), enhanced contrast and sharp delineation of both intra- and extracellular structures. The layered appearance of cellular membranes was particularly clearly demonstrated in all specimens. The overall preservation of the tissue was satisfactory and interstitial precipitates were absent or minimal.

In procedures a and b the tissue preservation was considerably less satisfactory; contrast was high, but cell penetration was uneven. In addition, precipitates were frequently found in the interstitia. In some respects, the appearance of these specimens was reminiscent of that reported in the literature for the high molecular weight tannins.

Physical Conditions Required for LMGG Optimal Effects

EXPERIMENTAL PROCEDURE: Specimens fixed by the two-step or one-step method (see Controls), or processed through a peroxidatic reaction (17), were treated after osmication with LMGG in experiments in which the following variables were tested: LMGG concentration—0.25%, 0.5%, 1.0%, 2.0%, and 4.0%; pH—5.0, 6.0, 6.4, 6.8, 7.0, 7.2, 7.4; buffers—50–200 mM of either HCl-Tris, Na acetate-Na veronal, HCl-Na-arsenate, HCl-Na-cacodylate, NaOH-NaH₂PO₄, or NaH₂PO₄-Na₂HPO₄[4]; time—5, 15, 30, 45, 60,

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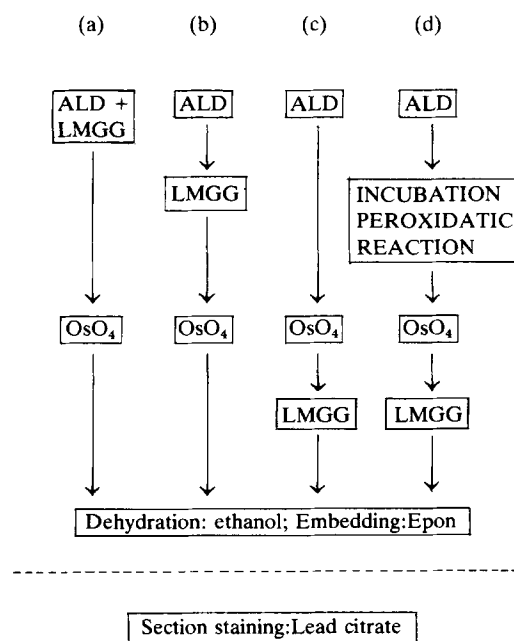[4] LMGG does not dissolve in K phosphate buffer.

120 min; temperature—0-4°, 22-24°, and 36-40°C.

RESULTS: The best results were obtained at a concentration of 1% LMGG in either Na-arsenate or Na-cacodylate buffer, pH 7, applied for 30 min at room temperature (20-22°C). These conditions, however, are not critical: good results were obtained over a relatively wide range of concentrations (0.25-1%) and times of exposure (15-60 min), depending on the size and nature of the specimens.

Possible Nature of LMGG Effects on Tissues

The following experiments were carried out to gain some general insight into the nature of LMGG effects on tissues.

LMGG Used as a Fixative

EXPERIMENTAL PROCEDURE: Fresh tissue blocks were immersed in a solution of 1% LMGG in 0.1 M HCl-Na arsenate or HCl-Na cacodylate buffer, pH 7.0, for 90 min at 22°C, then dehydrated in ethanol, and embedded in Epon; sections cut from these blocks were stained in lead citrate.

RESULTS: The preservation of such specimens is extremely poor: there are massive disor-

ganization and extraction of the tissue, indicating that LMGG does not act as a primary fixative.

LMGG Used as a Mordant

EXPERIMENTAL PROCEDURE: Tissue specimens were processed as indicated in Fig. 2.

RESULTS: The general appearance of the tissues was that expected for each type of fixation used, but high contrast was obtained only in procedures (c) and (d), in which LMGG treatment followed osmium tetroxide fixation or postfixation and was followed in turn by lead staining of the sections (Figs. 3-10). There was no contrast enhancement without OsO₄ used as a fixative or postfixative or with lead staining intercalated in block between OsO₄ fixation and LMGG treatment. Taken together, the results indicate that LMGGs act as a mordant for lead staining of OsO₄-fixed or postfixed specimens.

It should be noted, however, that LMGG treatment of already fixed tissues (with aldehydes and OsO₄) results in a better preservation of membranes in general and fibrillar structure in particular inside and outside the cells, as well as in the retention of more material (presumably soluble proteins) in the cytoplasmic matrix. As a result, LMGG-treated specimens generally have a higher

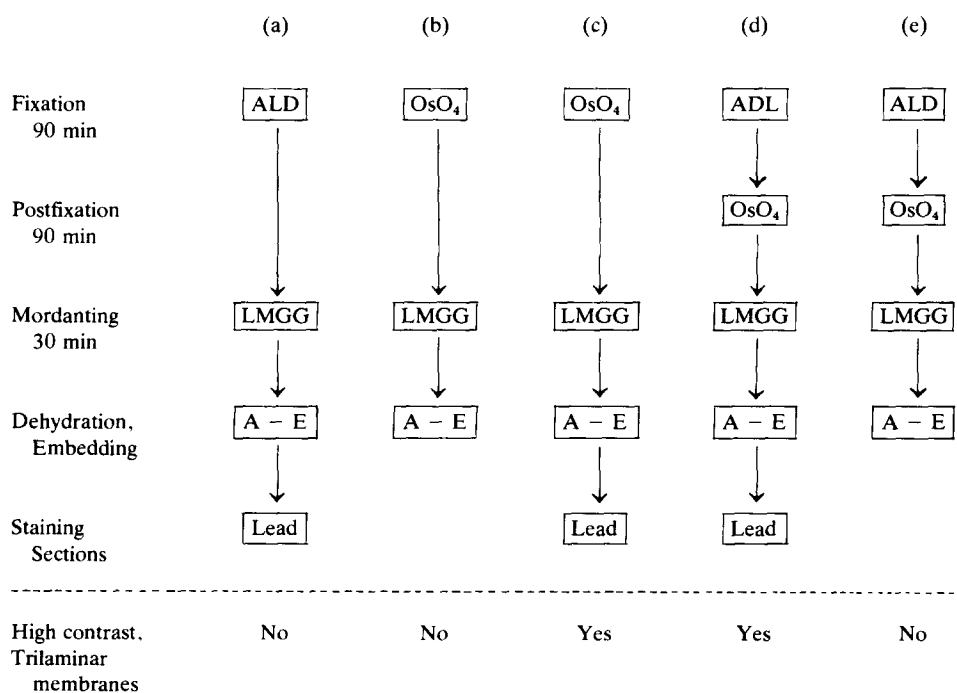|	(a)	(b)	(c)	(d)	(e)
Fixation 90 min	ALD	OsO₄	OsO₄	ADL	ALD
Postfixation 90 min				OsO₄	OsO₄
Mordanting 30 min	LMGG	LMGG	LMGG	LMGG	LMGG
Dehydration, Embedding	A – E	A – E	A – E	A – E	A – E
Staining Sections	Lead		Lead	Lead	
High contrast, Trilaminar membranes	No	No	Yes	Yes	No

FIGURE 2   Experimental design to determine the mordanting effect of LMGG.

background density than specimens processed by the usual procedures.

Experiments in which aldehydes were omitted from the tissue preparation procedure (Fig. 2, columns *b* and *c*) and experiments in which aldehyde-blocking agents were used after fixation showed that the mordanting action of LMGG does not depend noticeably on free aldehyde groups in the tissue. Aldehyde blocking was obtained by treating specimens fixed in aldehydes and OsO_4 with an aqueous mixture of 0.1 M $NaBH_4$ and 0.1 M glycine (in cacodylate buffer, pH 7.0, 30 min at 22°C) before LMGG treatment.

Tannic acids are frequently referred to in the literature as stains for electron microscope specimens, notwithstanding the absence of heavy atoms in their molecules. If the sequence of preparation steps in Fig. 2 column (*d*) is repeated, but lead staining of the sections is omitted or replaced by LMGG treatment, there is no contrast in the preparation. This demonstrates that, as expected, LMGG (and by implication all reasonably pure tannins) do not act as stains.

To find out which metal is preferentially attached to osmicated structures by LMGG treatment, several heavy metal salts were tested (Table I). The results obtained indicated that lead binding to structures is particularly enhanced by LMGG.

The satisfactory preservation of membranes (continuity and trilaminar configuration), microtubules, and filamentous assemblies, as well as the higher density of the cytoplasmic matrix, suggests that when applied to fixed tissues LMGGs also make all these components more resistant to extraction and related damage incurred during dehydration and subsequent processing. Therefore, the mechanism for LMGG effects seems to be complex: LMGGs act primarily as a mordant between osmicated structures and lead and, in addition, stabilize certain tissue components. The chemical reactions involved in such processes are still unknown.

Procedure for Tissue Treatment with Low Molecular Weight Galloylglucoses (LMGG)

The results obtained in the experiments reported were used to work out a final procedure which involves the following steps: I — fixation in aldehydes and OsO_4 by either the two-step or the one-step method; II — block washing in 0.1 M buffer pH 7.2 (three times for 5 min, at 22°C);

TABLE I

LMGG-Treated Tissues. Results Obtained by Staining Thin Sections with Various Heavy Metal Salts

Staining method	High contrast obtained
Lead citrate (5′)	Yes
Uranyl acetate (5′)	No
Uranyl acetate (5′), lead citrate (5′)	Yes
$FeCl_3$ (4.5%, 10′)	No
Phosphotungstic acid (5%, 5′)	No

Tissues were fixed with aldehydes and OsO_4 before being treated with LMGG. LMGG treatment of sections (5%, 30 min) without prior treatment in block results in no contrast; LMGG treatment in block and on sections does not increase contrast over treatment in block only.

III — treatment with 1% LMGG (tannic acid A.R. code no. 1764, Mallinckrodt Inc.) in 0.05 M buffer (preferably Na arsenate or Na cacodylate) pH 7.0 for ~30 min (15–45 min) at 22°C; IV — block washing in the same buffer containing 1% sodium sulfate[5] for 5 min; V — dehydration in ethanol, impregnation overnight at room temperature (in tissue rotor) in 1:1 propylene oxide and Epon, then in Epon for 5–6 h, and embedding; VI — staining of sections, 3–5 min in lead citrate.

The concentration of LMGG and the duration of treatment in step III can be varied to lower the contrast if excessive.[6] As outlined, the technique is generally successful; uneven penetration occasionally encountered generally coincides with poor preservation of the tissue (especially unsatisfactory osmication).

The applicability of the procedure has not been fully explored. The few examples shown in Figs. 3–12 are intended to illustrate in general and in detail the appearance of LMGG-treated specimens (subsequently, the sections were stained with lead). Fine detail or characteristic contrast is demonstrated in such structures as basal lamina (Fig. 8), collagen fibers (Fig. 10), amorphous part and the microfibrils of the elastic fibers (Fig. 9),

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[5] Sodium sulfate is used in the dyeing and printing of textiles to facilitate the washing of unbound mordants or dyes.

[6] Excessive contrast can also be controlled by extending dehydration, in 70°C ethanol or by caffein (19) treatment (anhydrous, mol wt 194, Sigma Chemical Co., St. Louis, Mo.) for 5 min, of the sections before staining in lead.

FIGURE 3   Acinar cells of rat pancreas: fixation with aldehydes and OsO$_4$ and subsequent treatment with LMGG. Note the high contrast of extra- and intracellular structures, and absence of interstitial precipitates. c, Collagen; is, interstitial space; n, nucleus; zg, zymogen granule. × 21,000.
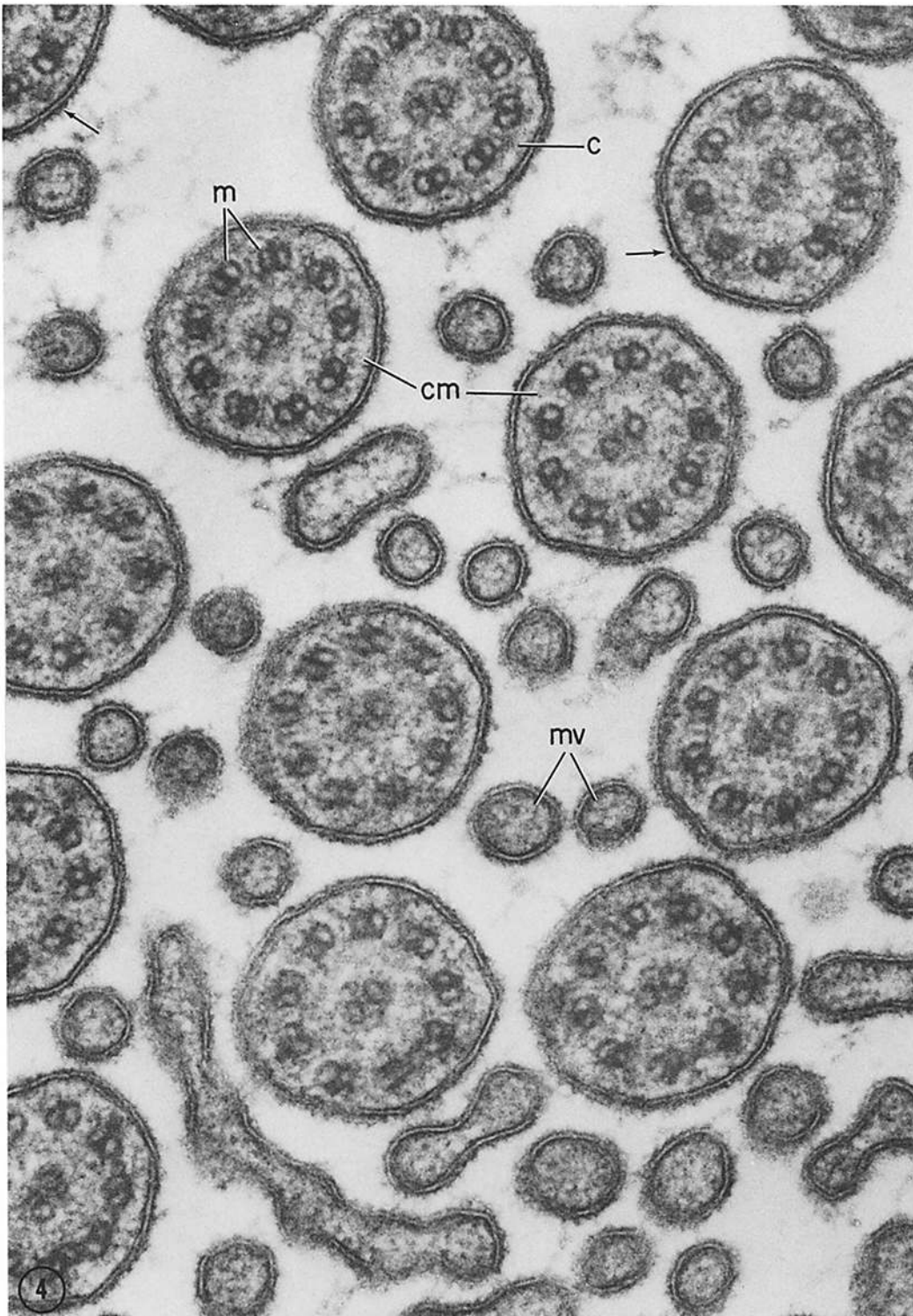
FIGURE 4   Epithelial cilia (c) and microvilli (mv) of rat trachea fixed with aldehydes and OsO₄, and then exposed to LMGG. Note the trilaminar pattern of cell membranes, and the satisfactory preservation of cilium matrix (cm), microtubules (m), and the fine fibrillar structures associated with the outer aspect of the plasmalemma (arrows). × 135,000.

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Figure 5 Rat omentum: endothelial junction in a postcapillary pericytic venule. Note the sharp delineation of the trilaminar plasmalemma (*pl*), and lack of interstitial precipitate. *c*, Chylomicron; *e*, endothelial cell; *l*, lumen; *p*, pericyte; *ps*, pericapillary space. × 125,000.

and crystalline lattice of insulin in the secretory granules of a β cell (Fig. 7).[7]

By comparison with the often used uranyl staining in block, the LMGG procedure outlined above (*a*) induces comparable or higher contrast and sharper delineation of membranes, (*b*) is followed

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[7] A stable combination of tannins with insulin in vitro has been reported (27).

by considerably less extraction of cytoplasmic and mitochondrial matrix components, (*c*) induces contrast and better definition of the fine structure of the fibrillar components in muscle filaments, basal lamina, and collagen, and strong contrast of both components of the elastic fibers.

The ferrocyanide-reduced osmium tetroxide technique gives satisfactory definition of membranes but does not stain nuclei, ribosomes, and
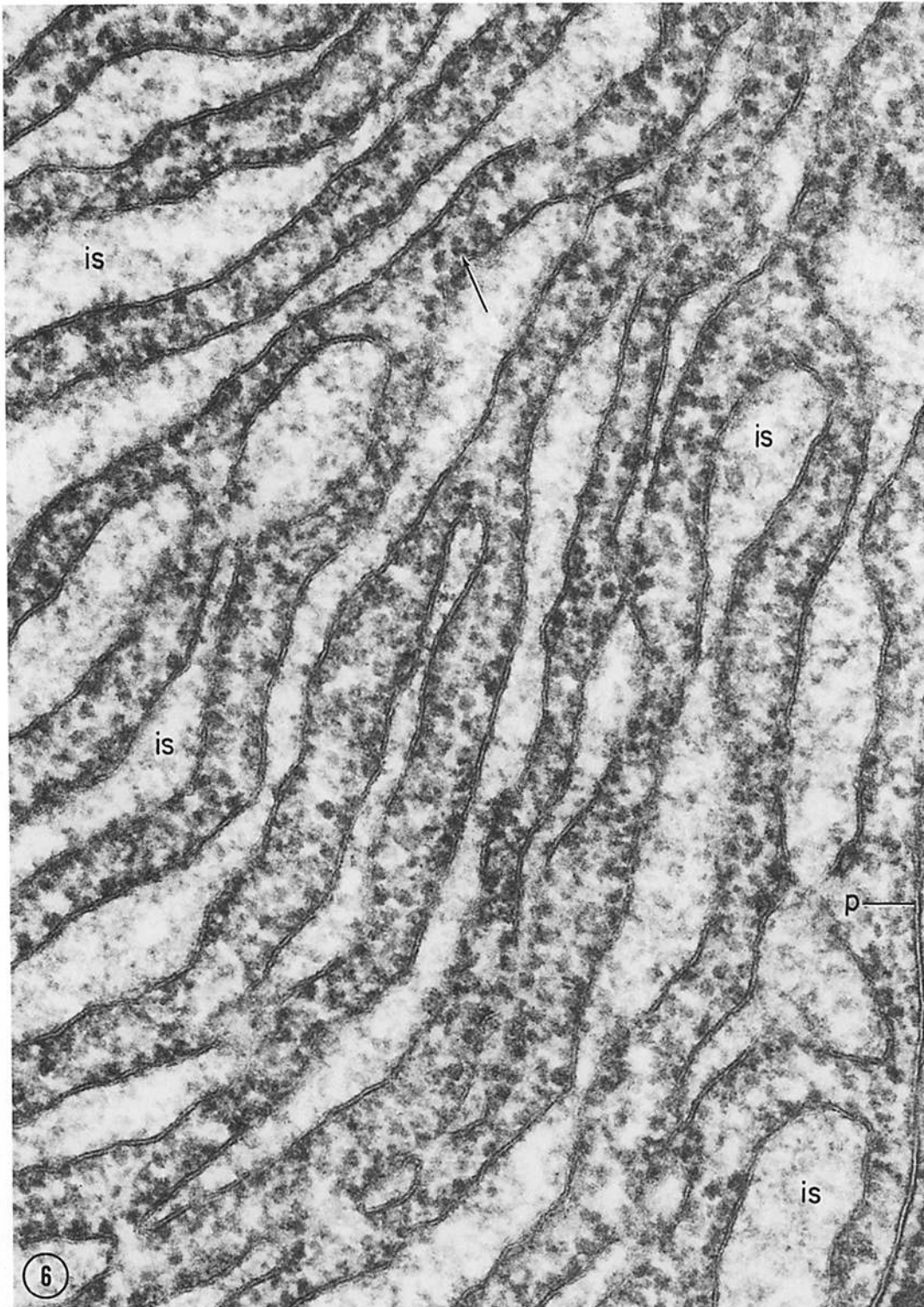
Figure 6 Endoplasmic reticulum of an acinar cell (rat pancreas): fixation with aldehydes and OsO₄ followed by LMGG treatment. Some ribosomes display their subunits (*arrow*). *is*, Intracisternal space; *p*, plasmalemma. × 121,000.
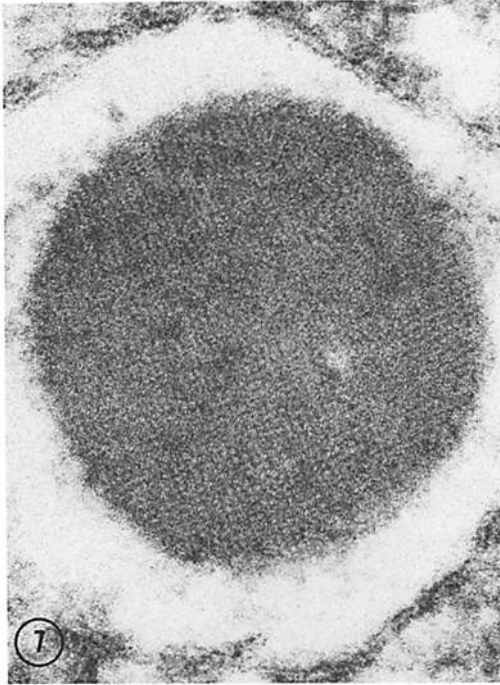
FIGURE 7 Rat pancreas, islet of Langerhans: crystalline pattern of secretory granule in a β cell; tissue sequentially treated with aldehydes, OsO_4, and LMGG. × 250,000.
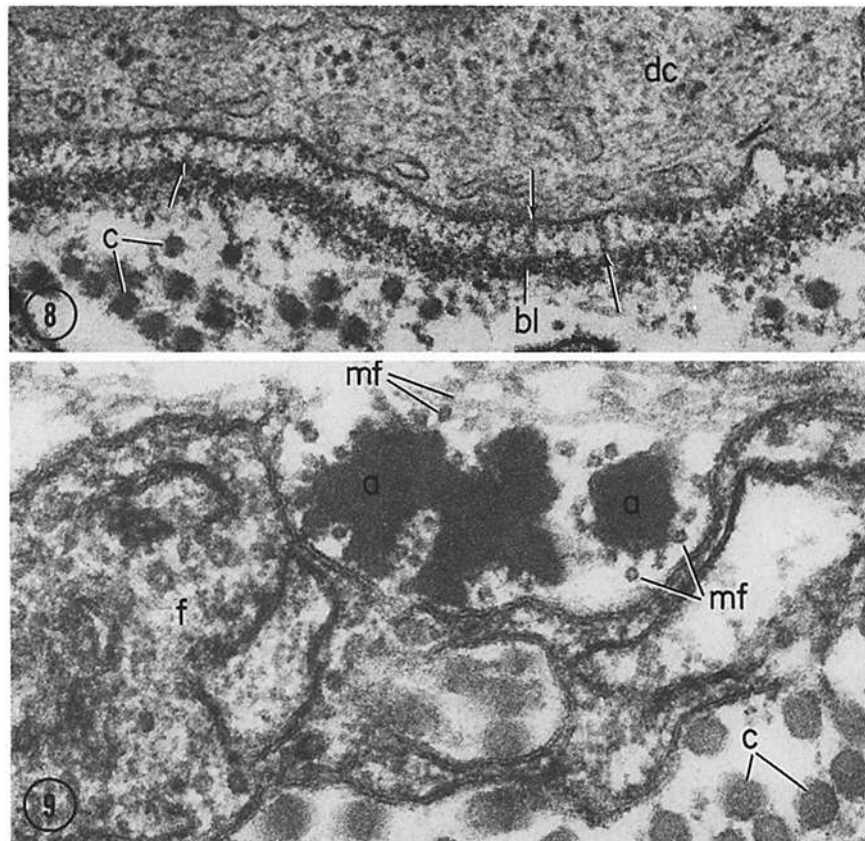

FIGURE 8 Duct cell (*dc*) in the rat pancreas fixed with aldehydes and OsO_4 and exposed to LMGG. Note the appearance of basal lamina (*bl*) and anchoring microfibrils (*arrows*). *c*, Collagen. × 68,000.

FIGURE 9 Connective tissue components in a rat pancreas fixed with aldehydes and OsO_4, and treated with LMGG; both the amorphous part (*a*) and microfibrils (*mf*) of the elastic fibers are strongly contrasted; *c*, collagen; *f*, fibroblast. × 100,000.
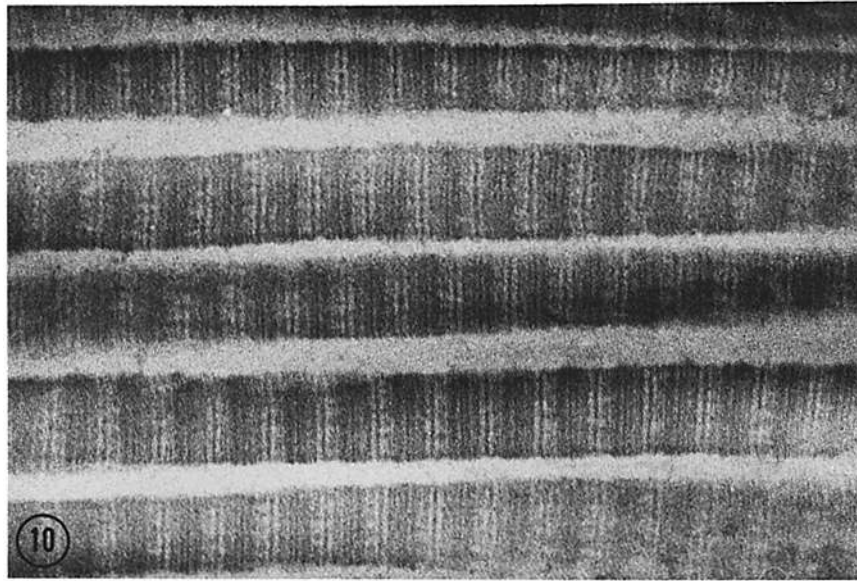
FIGURE 10 Banding pattern of collagen fibers (lamina propria of rat jejunum sequentially treated with aldehydes, OsO₄, and LMGG). × 210,000.
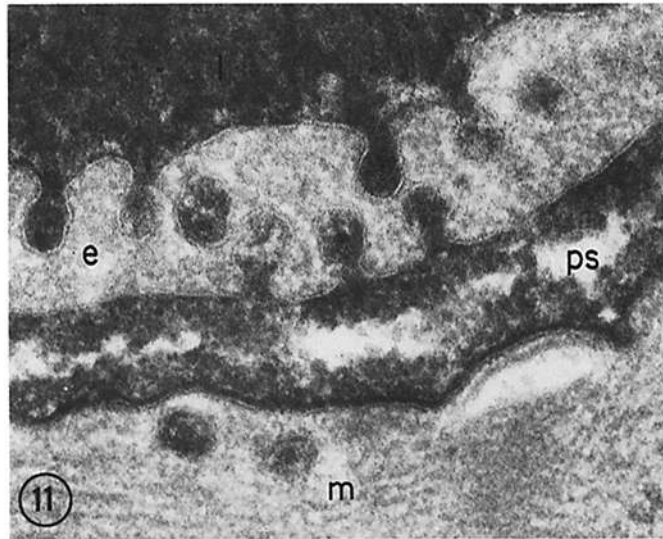

FIGURE 11 Blood capillary in a rat diaphragm, after i.v. injection of a tracer solution of hemoctapeptide. The tissue was fixed with glutaraldehyde and formaldehyde, incubated in DAB-H₂O₂ medium, postfixed in OsO₄ (18) and then treated with LMGG. Note the trilaminar pattern of cell membranes. *l*, Lumen; *e*, endothelium; *ps*, pericapillary space; *m*, muscle. × 110,000.

certain fibrillar structures; their demonstration requires additional treatment with uranyl acetate.

DISCUSSION

The use of tannins or other phenols as mordants is a rather old practice in histological technique; it is

found in ferric-,[8] hematoxylin-, fuchsin-, and cresyl violet staining. In histochemistry, tannins were

[8] It is considered that Link's iron reaction for tannin and gallic acid (1807) was the first histochemical reaction ever reported (24).
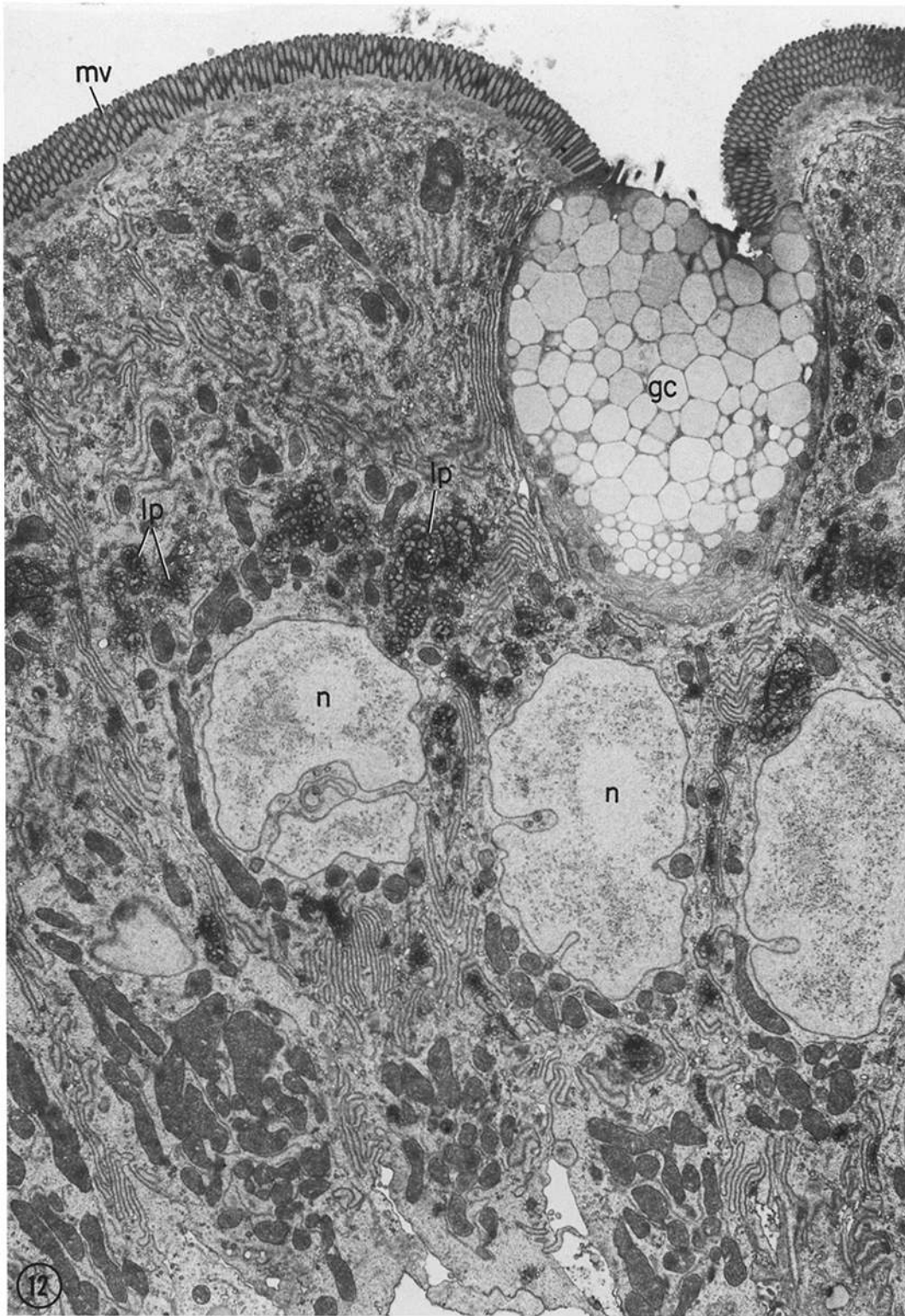
FIGURE 12   Epithelium of rat jejunal mucosa fixed with OsO_4 only and then treated with LMGG. Structures display an overall high contrast, with a particular enhancement of lipoprotein globules (lp). Note the absence of interstitial precipitate. mv, Microvilli; gc, goblet cell; n, nucleus. × 60,000.

successfully employed for the localization of "tannophilic proteins" (10), mucins (31), and arginine-rich sties (22). As indicated by our observations, gallotannin of low molecular weight (LMGG) can be used in processing tissues for electron microscopy for increasing and diversifying the contrast of cellular and extracellular structures.

By their chemical nature, LMGGs are unable to impart by themselves contrast to tissue components in contradistinction to the heavy metals (i.e. uranyl acetate, PTA, $FeCl_3$, OsO_4) customarily used for staining biological specimens in block. As demonstrated by our experiments, LMGG acts indirectly as a mordant primarily between OsO_4 and lead. The physics and chemistry of the mordanting process are still unclear. The experience acquired in the textile and leather industry (19, 35) and in histological practice (4–6, 10) indicates that a mordant is a chemical capable of modifying tissue components so that they can be stained or better stained with compounds which otherwise impart limited staining or contrast. To achieve high contrast, OsO_4 treatment seems to be a prerequisite, irrespective of the sequence, i.e. before or after exposure to LMGG. The former sequence gives better results in terms of cell structure preservation. Our observations show that the LMGG effects are not dependent on the presence of free aldehydes in the tissue. The broad spectrum of reducing capabilities of $NaBH_4$ suggests that, at least in part, the LMGG action does not depend on the presence within tissues of active groups such as ketones, Schiff bases, nitriles, disulfides, acid chlorides, inorganic anions, etc. (9, 23, 25). We do not so far have an explanation for the special affinity that LMGGs show for lead salts. As already indicated, LMGG treatment renders tissues more resistant to extraction and deterioration of structural detail incurred during the rest of the preparation procedures (dehydration, embedding). In this respect, it compares favorably with staining in block with uranyl acetate (40) or uranyl acetate-oxalate (30), for which similar effects have been claimed. Contrast enhancement of some structures was reported in osmium-fixed tissue subsequently treated with ligands such as thiocarbohydrazide (TCH) (18, 36).

At present, there is no satisfactory explanation for the chemistry of LMGG effects on tissues. An attempt to gain some insight into the reactions in which LMGGs are involved is presented in a companion paper.

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