

# GALLOYLGLUCOSES OF LOW MOLECULAR WEIGHT AS MORDANT IN ELECTRON MICROSCOPY

## II. The Moiety and Functional Groups Possibly Involved in the Mordanting Effect

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### ABSTRACT

Synthetic pentamonogalloylglucose applied to fixed tissues acts as a mordant, inducing high and diversified contrast similar to that obtained with natural gallo-tannins of low molecular weight (LMGG). By the separate use of each of the two moieties of the galloylglucose molecule, it was found that gallic acid is the mordanting agent. Glucose may contribute, however, to the effect by increasing the solubility and cross-linking potential of the compound, since the mordanting induced by gallic acid alone is weaker than that produced by its hexose esters. As suggested by results obtained with various phenolics and benzoic acid derivatives, the functional groups required for the mordanting effect of such agents are the carboxyl group, and at least one hydroxyl group concomitantly present on the benzene ring. In the case of galloylglucoses, it is assumed that the effect is due to hydrolysis products (gallic, digallic, or trigallic acids) or to the multiple hydroxyl groups of the intact molecule. Esters of gallic acid (propyl- and methylgallate), as well as pyrogallol, produce a "reversed staining" of all membranes, except for those of communicating (gap) junctions.

In the previous paper (21), we showed that natural gallotannins of low molecular weight (LMGG) commercially available as tannic acid AR, usually extracted from Turkish nutgalls, can be used for increasing and diversifying contrast in electron microscopy. The effect was obtained on tissues previously fixed in one step by  $\text{OsO}_4$  or in two steps by aldehydes and  $\text{OsO}_4$ . We demonstrated that the gallotannins act as mordants and ascribed this effect to their major components, namely, the penta- and hexagalloylglucoses, the relative low molecular weight of which may explain their satisfactory penetration into the cells.

To exclude the possibility that other minor components or contaminants occurring in these natural gallotannins might be responsible for the mordanting effect, we have carried out experiments with synthetic pentagalloylglucose (i.e. synthetic ester of gallic acid with glucose [5, 6] which has been found to be chemically identical to one of the main components of natural gallotannins [1, 6, 15, 18]).

In additional experiments we tried to find out which part of the galloylglucose molecule accounts for the mordanting effect and which functional groups are involved in this process.

## MATERIALS AND METHODS

### Reagents

Synthetic  $\beta$ -penta-*o*-mono-D-galloyl glucose and the data on its chemical composition, 90-95% -penta-*o*-mono-D-galloyl glucose<sup>1</sup> (Fig. 1), and 5-10% 2, 3, 4, 6-tetra-*o*-mono-D-galloyl glucose with an overall molecular weight of 940,<sup>2</sup> was provided by T. A. Beasley. This material was prepared at the laboratories of Mallinckrodt, Inc., St. Louis, Mo., by A. D. Bell.

Other reagents used were obtained as follows: gallic acid (purified, "G," crystals, C<sub>7</sub>H<sub>6</sub>O<sub>5</sub>·H<sub>2</sub>O, formula weight (F.W.) 188.14) from Mallinckrodt; catechol (pyrocatechol), crystalline, anhydrous, mol wt 110.1, phloroglucinol (1,3,5-trihydroxybenzene), anhydrous, mol wt 126.1, *n*-propylgallate, anhydrous, mol wt 212.2,  $\alpha$ -3,5-resorcylic acid, anhydrous, mol wt 154.1, resorcinol, *p*-aminobenzoic acid, crystalline, free acid, 3,5-diaminobenzoic acid, and 3,3'-diaminobenzidine, tetrahydrochloride (3,3',4,4'-tetraaminobiphenyl) from Sigma Chemical Co., St. Louis, Mo.; *p*-hydroxybenzaldehyde, mol wt 122.13, *p*-hydroxybenzoic acid, mol wt 138.12, and methylgallate (practical grade), mol wt 184.15, from Eastman Kodak Co., Organic Chemicals Div., Rochester, N. Y.; hydroquinone (purified), F.W. 110.11, and pyrogallol (pyrogallic acid), F.W. 126.11, from Fischer Scientific Co., Fair Lawn, N. J.

### Animals, Tissues

For these experiments, adult rats of the Sprague-Dawley and Wistar-Furth strains (the latter obtained from Microbiological Associates, Inc., Bethesda, Md.) were used. Each experiment was carried out on five to seven animals, and the tissues processed were the pancreas, oviduct, omentum, and skeletal muscle.

### Experimental Procedure and Results

The methodology of the experiments designed to determine the carrier of the mordanting effect is diagrammatically represented in Fig. 2. Solutions of compounds to be tested for mordanting activity were prepared in arsenate or cacodylate buffer (pH 7.0) and used to treat, for a standard period of 30 min at 22°C, tissue fragments fixed by either a two-step or a one-step procedure (21). Subsequently, the blocks were briefly washed in buffer, dehydrated in ethanol, and embedded in Epon. All sections obtained from these blocks are stained for 5 min in lead citrate (17).

<sup>1</sup> Although the exact molecular configuration is not clearly established, it is likely that the glucose backbone is esterified by individual molecules of gallic acid, but the possibility that the latter are chain-like, linked by depsidic bonds to form digallic acid or trigallic acid, cannot be ruled out.

<sup>2</sup> Carried out in Mallinckrodt laboratories (St. Louis, Mo.) by T. H. Beasley.

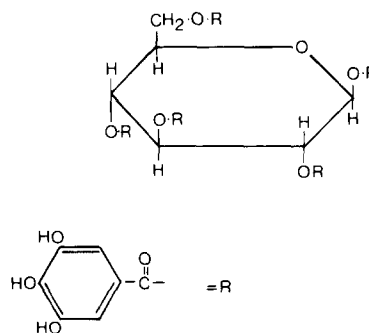


FIGURE 1 The structure of the  $\beta$ -penta-*O*-galloyl-D-glucose molecule (mol wt 940).

### I. Effects of Synthetic Pentamonoalloylglucose

To check whether synthetic pentagalloylglucose has a mordanting effect similar to that observed with natural gallocannin containing pentagalloylglucose, we treated tissue blocks as described above, in a 1% solution of the synthetic product.

The mordanting effect was similar to that obtained with the natural LMGG used in the first part of this work (21). The synthetic pentagalloylglucose satisfactorily penetrated the cells, usually did not produce interstitial precipitates, and induced high contrast of both intra- and extracellular structures (Figs. 3 and 4). Since there was no detectable difference between specimens treated with either the synthetic or the natural product, it can be concluded that the mordanting effect obtained with LMGG is due to the galloylglucoses they contain.

### II. Effects of Gallic Acid or Glucose

To find out which of the two moieties of the galloylglucose molecule is responsible for mordanting, we treated tissue blocks as described above with either 1% gallic acid or 1% glucose.

The gallic acid does not penetrate uniformly into the tissue blocks. In the penetrated regions, its mordanting effect is weaker than that of galloylglucose, but is nonetheless evident as demonstrated by the high contrast and the trilaminar appearance of membranes (plasmalemma and membranous organelles) (Fig. 5). After glucose alone fixed tissues showed a satisfactory preservation of their constituents but no enhanced contrast. These observations indicate that in the galloylglucose molecule the galloyl moiety is the mordant.

### III. Effects of Phenolic and Gallic Acid Derivatives

To determine the functional groups possibly involved in the mordanting effect, we took advantage of the large variety of commercially available phenolic compounds

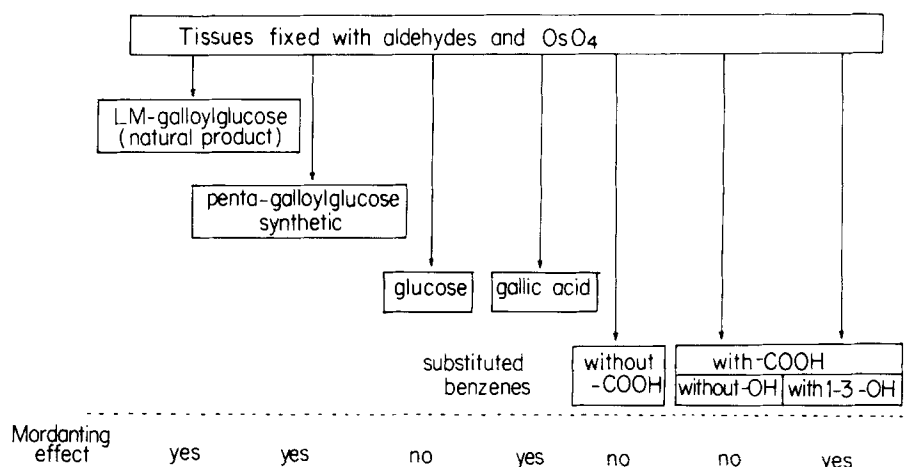
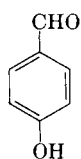


FIGURE 2 Methodology of the experiments designed to determine the carrier of galloylglucose-mordanting effect.

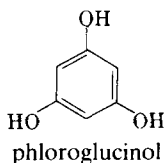
that are similar to, or derivatives of gallic acid. Using such phenolics, we investigated the dependency of the mordanting effect on the functional groups present on the benzene ring. The following compounds were tested at the concentrations given in brackets.

(a) Phenolics devoid of a -COOH group, obtained either by substitution of -COOH by -CHO: *p*-3-hydroxybenzaldehyde (0.5%),

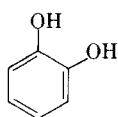


hydroxybenzaldehyde

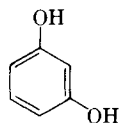
or by elimination of -COOH, with the remaining -OH groups in various numbers and positions: phloroglucinol (1,3,5-trihydroxybenzene) (1%), catechol (1,2-dihydroxybenzene) (1%), resorcinol (1,3-dihydroxybenzene) (1%), and hydroquinone (1,4-dihydroxybenzene) (1%):



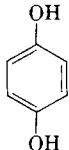
phloroglucinol



catechol



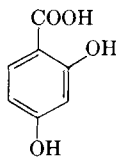
resorcinol



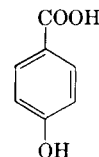
hydroquinone.

When phenolic compounds of this type were used, no mordanting effect or improvement in tissue preservation was detected, irrespective of how many remaining -OH groups were present and in what position. The only exception was catechol which occasionally induced in some areas a moderate enhancement of structure contrast.

(b) Benzoic acid derivatives with fewer -OH groups than gallic acid—resorcylic acid (2,4-dihydroxybenzoic acid) (1%), and *p*-3-hydroxybenzoic acid (0.5%):



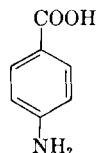
resorcylic acid



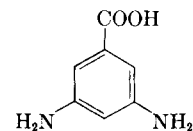
hydroxybenzoic acid.

These compounds showed a weak but rather constant mordanting effect, sufficient to reveal the trilaminar configuration of membranes after lead staining. Even one -OH group was sufficient, provided the carboxyl group was simultaneously present on the benzenic ring. This is the case with the hydroxybenzoic acid as illustrated in Fig. 6. In general, the mordanting effect obtained with such simple phenolic acids is weaker and even less uniform than that of gallic acid.

(c) Benzoic acid derivatives without -OH groups, containing one or more amino groups—*p*-aminobenzoic acid (0.5%), and 3,5-diaminobenzoic acid (1%):



aminobenzoic acid



diaminobenzoic acid.



FIGURE 3 Acinar cell of rat pancreas fixed with aldehydes and  $\text{OsO}_4$  and subsequently treated with synthetic pentamonogalloylglucose. Note the trilaminar pattern and sharp delineation of membranes.  $\times 88,000$ .

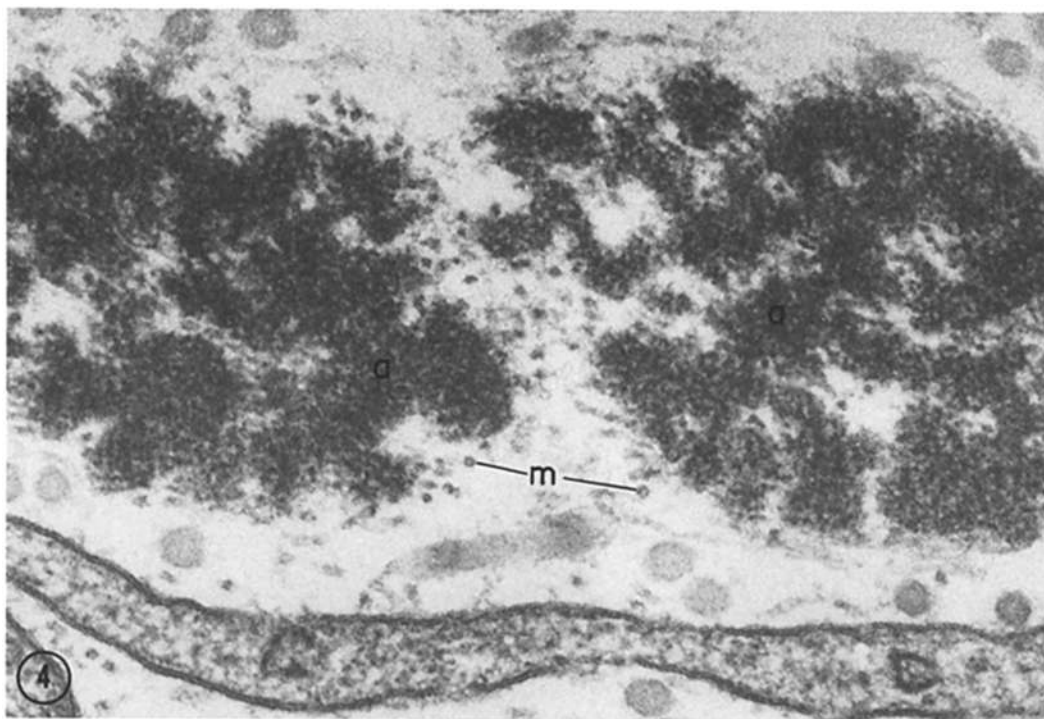
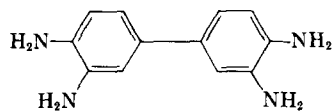


FIGURE 4 Rat pancreas: tissue fixed with aldehydes and  $\text{OsO}_4$ , and treated with synthetic pentamonoalloylglucose: high contrast of both microfibrils (*m*), and amorphous part (*a*) of the elastic fibers, with the revealing of a substructure in the latter.  $\times 10,000$ .

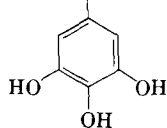
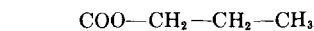
In addition, a biphenol such as diaminobenzidine (3,3', 4,4'-tetraaminobiphenyl) (1%) was tested:



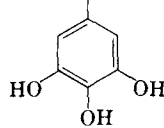
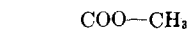
diaminobenzidine.

These compounds showed no mordanting action at the concentration and in the conditions used.

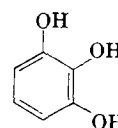
(d) Pyrogallol (1,2,3-trihydroxybenzene) (1%) and gallic acid esters such as methylgallate (1%) and propylgallate (0.2%) were separately tested for their effects on tissue preservation and contrast.



propylgallate



methylgallate



pyrogallol

The pyrogallol effect on subsequent lead staining was checked both on blocks and on thin sections. The latter were obtained from tissues fixed with aldehydes and  $\text{OsO}_4$  and exposed for 5–10 min to a solution of 1%

aqueous or buffered pyrogallol before being stained for 5 min in lead citrate. These compounds induced a striking appearance in tissue exposed to their action which at first approximation gives the general impression of a "reversed staining." The cytoplasmic matrix and, especially, the mitochondrial matrix are highly contrasted. The density of the cisternal content of the endoplasmic reticulum is increased, but the two dense leaflets of all membranes, particularly the outer leaflet of the plasmalemma, show low to absent contrast (Fig. 7). The only membranes stained were those of the communicating junctions<sup>3</sup> in which the contrast appears to be increased primarily in the inner leaflets of the joint membranes (Fig. 8). Comparable results were obtained when sections were treated

<sup>3</sup> For reasons explained in reference 20, we prefer this term to the more frequently used expression "gap junction".

with pyrogallol before their staining with lead (Fig. 9). The dependency of the reversed staining on the duration of specimen exposure to gallates and pyrogallol remains to be investigated. A more detailed inquiry also is necessary to establish the conditions required to produce this appearance and, if possible, to understand its chemical basis. The first impression is that the effect is caused by the general extraction of membrane-bound osmium, the communicating junctions representing an exception. All compounds capable of inducing the "reversed staining" give a fine, dense precipitate and mediocre preservation of cell structures.

## DISCUSSION

### A. Mordanting with Synthetic Pentagalloylglucose

It has been established that the pentagalloylglucose represents the core of natural gallotannins, to

which three to five additional galloyl groups may be attached by depsidic linkages (2, 8-11). By chemical analysis, the tannic acid used in our preceding paper (21), like the Aleppo gallotannin, has as major components penta- and hexagalloylglucoses.<sup>2</sup> The synthetic pentagalloylglucose used in the experiments now reported has been shown to be identical with that isolated from gallotannins (6, 8, 11, 13). Since we have obtained similar effects in both cases, we can ascribe the mordanting activity of the natural products to their galloylglucoses and thereby eliminate the involvement of unidentified impurities. The effects are comparable for both contrast enhancement and improvement of tissue preservation. In both the natural and the synthetic product, the pentagalloylglucose has the advantage of a relatively low molecular weight (~940) over the hepta- to decagalloylglucose

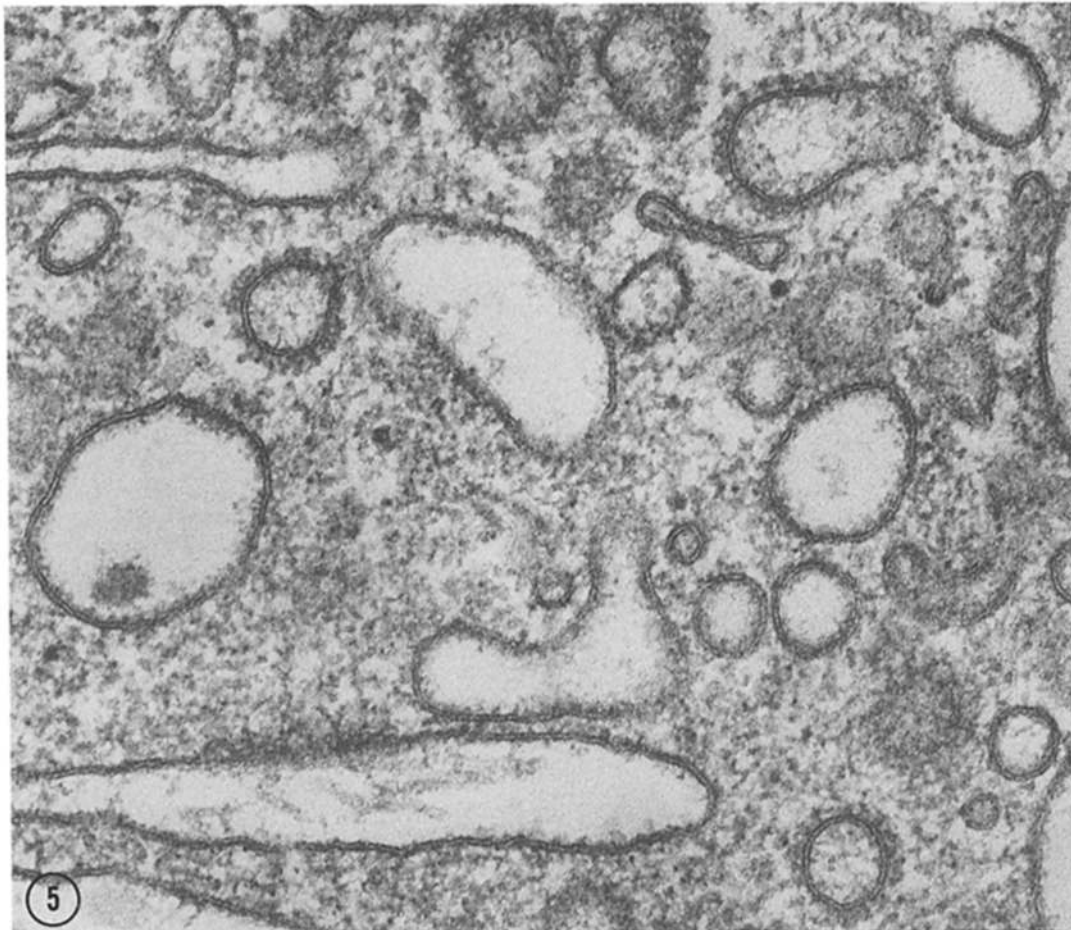


FIGURE 5 Rat pancreas: tissue fixed with aldehydes and  $\text{OsO}_4$ , and exposed to a 1% buffered solution of gallic acid. Note trilaminar membranes, sometimes with uneven delineation.  $\times 120,000$ .

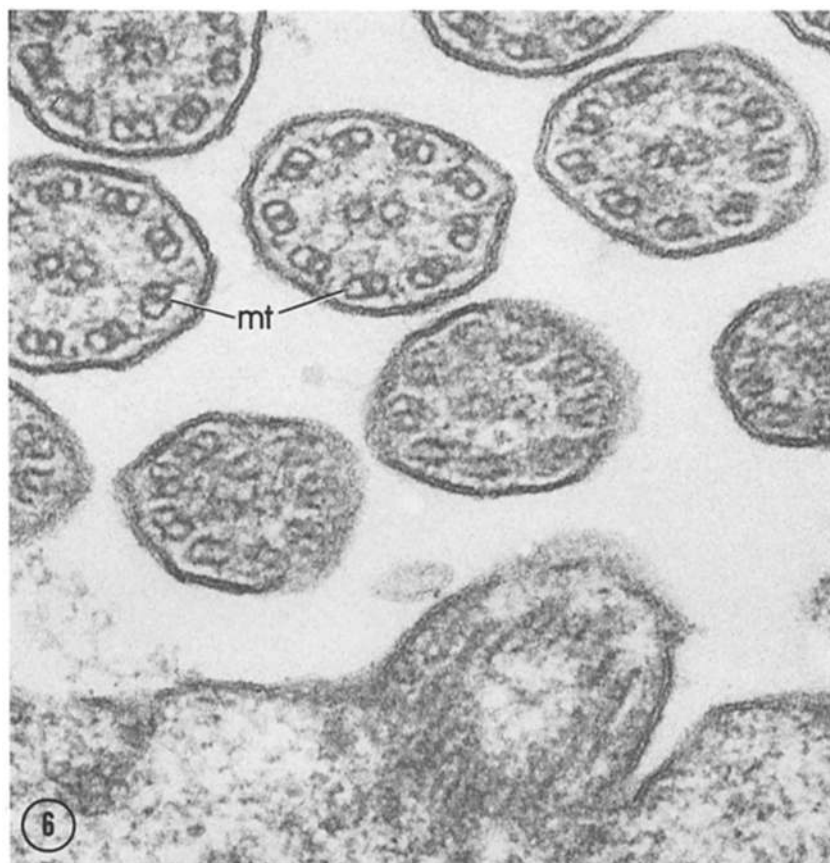


FIGURE 6 Rat oviduct: epithelial cilia: tissues were fixed with aldehydes and  $\text{OsO}_4$  and then exposed to a 0.5% buffered solution of *p*-hydroxybenzoic acid. Uneven but constant appearance of the trilaminar pattern of cell membrane. *mt*, Microtubules.  $\times 125,000$ .

coses which prevail in most commercially available tannic acids ( $\text{C}_{76}\text{H}_{52}\text{O}_{46}$ , mol wt 1,701) used by previous investigators. For work of the type reported, synthetic pentagalloylglucose has the advantage of a homogeneous and chemically well-defined reagent, but it is not yet available commercially.

#### B. Mordanting with Gallic Acid

On hydrolysis with acids, alkali, or enzymes, both the naturally occurring and the synthetic galloylglucoses are converted into glucose and polyhydroxycarboxylic acids: brief hydrolysis produces gallic acid, *m*-digallic acid,<sup>4</sup> and trigallic acid (5, 11,

13), whereas prolonged hydrolysis destroys digallic and trigallic acids, the sole ultimate product being gallic acid. Analytical, chromatographic, and optical identity has been reported between synthetic and naturally occurring gallic acid, digallic acid, and trigallic acid (3, 6, 11, 13, 15). There is no clear evidence on the extent to which, in solution or in contact with certain tissue components, the galloylglucoses yield gallic, digallic, or trigallic acids. In the case of synthetic pentamono-galloylglucose, it is presumed that the galloyl groups are individually attached to the glucose backbone (Fig. 1). Our observations on tissues exposed separately to either gallic acid or glucose showed that only the former acts as mordant. The glucose may contribute indirectly by increasing the solubility of the compound, and thereby facilitating its penetration into the tissue: mordanting is stronger with glucosylated gallic acid than with its simple, nonesterified form. In addition, earlier

<sup>4</sup> Variations in the procedure used for isolating and characterizing gallotannins (tannic acids) from different sources may explain, at least in part the discrepancy in the amount of digallic acid ascribed to different commercial products.

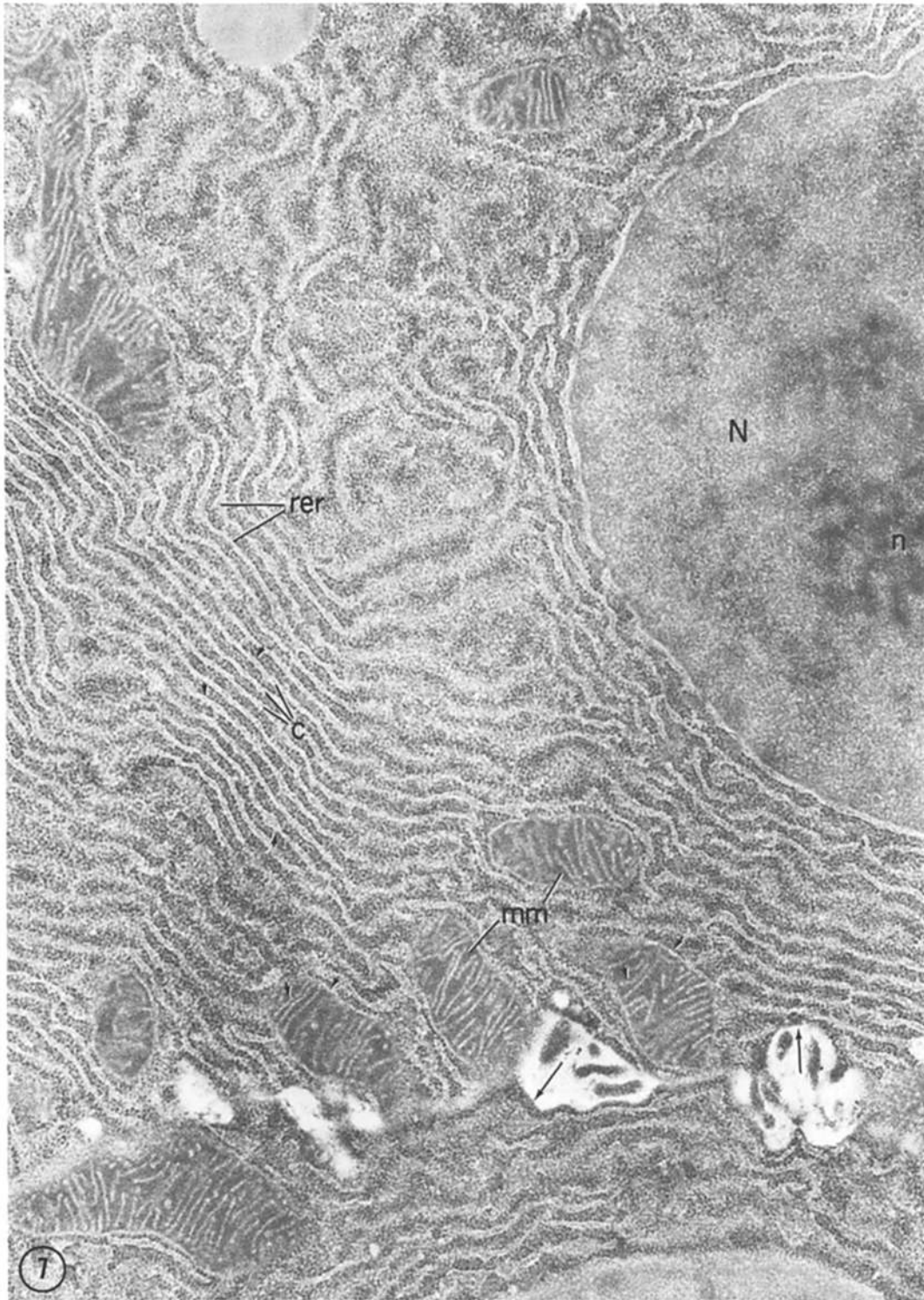


FIGURE 7 Acinar cells of rat pancreas, fixed with aldehydes and  $\text{OsO}_4$  and subsequently treated with 1% buffered solution of methylgallate. Both cell membrane (arrows) and organelle membrane sites (arrowheads) appear unstained against a strongly contrasted mitochondrial matrix (*mm*), ribosomes, and endoplasmic reticulum content (*c*). *n*, Nucleolus; *rer*, rough endoplasmic reticulum; *N*, nucleus.  $\times 31,000$ .



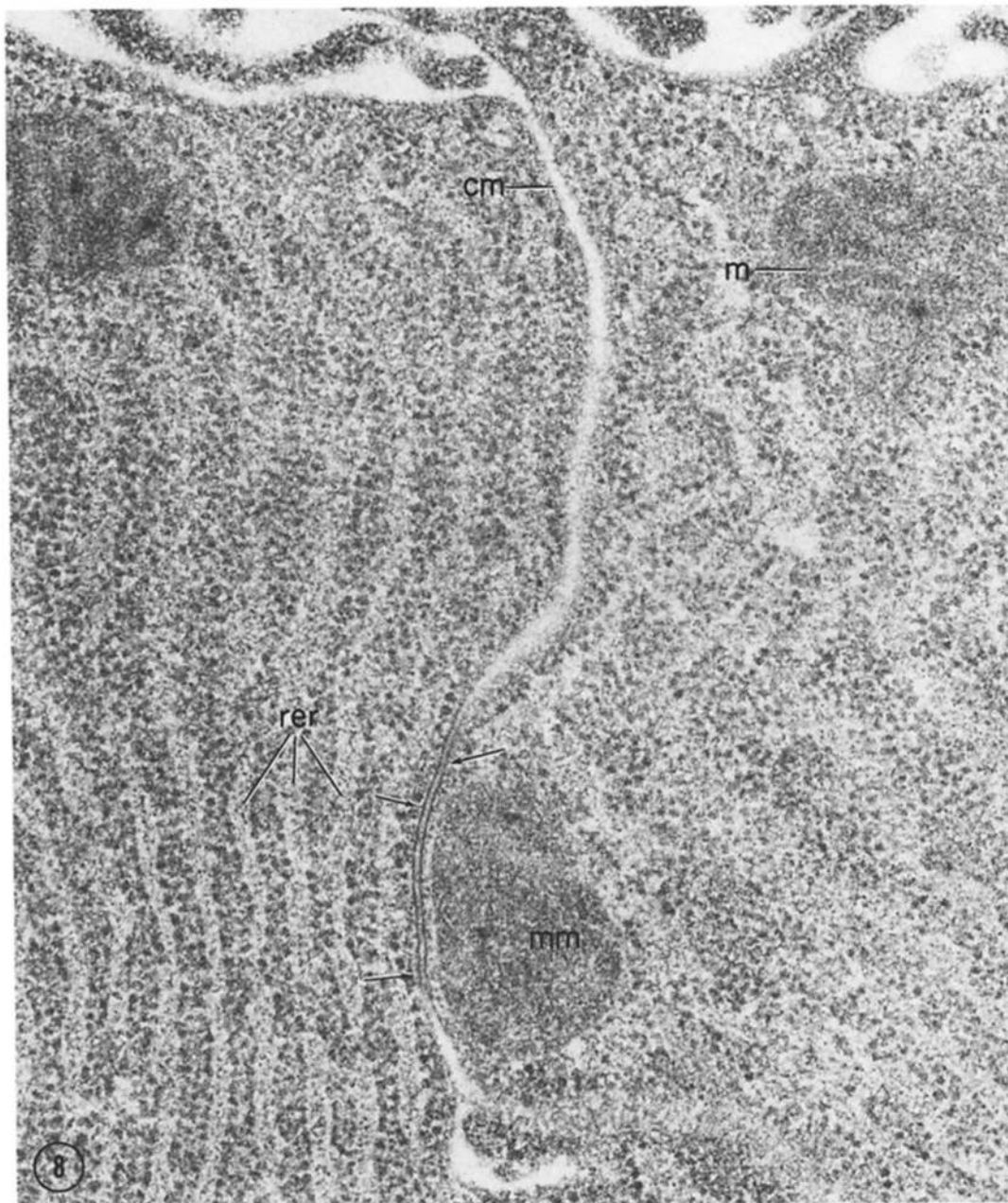


FIGURE 8 Acinar cells of rat pancreas fixed with aldehydes and  $\text{OsO}_4$  and exposed to 1% buffered solution of pyrogallol. Note the lack of staining of organelle membranes (e.g., *rer*, rough endoplasmic reticulum; *m*, mitochondria) and cell membranes (*cm*) with the exception of communicating junction membranes (*arrows*). The mitochondrial matrix is, on the contrary, heavily stained (*mm*). Tissue preservation is generally mediocre, and a fine precipitate frequently occurs.  $\times 65,000$ .

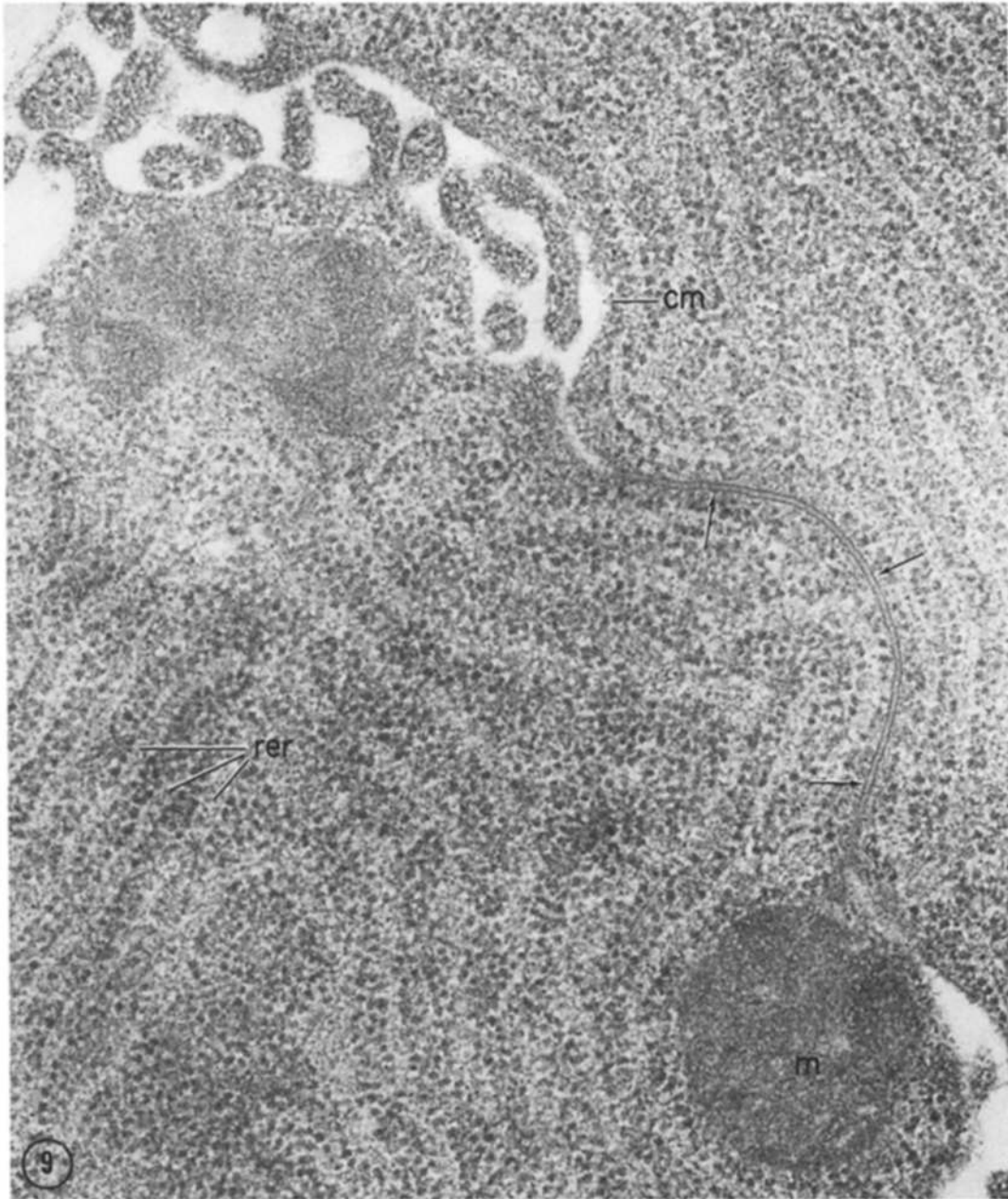


FIGURE 9 Acinar cells of rat pancreas fixed with aldehydes and  $\text{OsO}_4$  and dehydrated without any other previous treatment in block. Thin sections were exposed to a solution of 1% buffered pyrogallol for 10 min then stained in lead citrate for 5 min. Both the organelle membranes (such as those of the rough endoplasmic reticulum, *rer*; mitochondria, *m*) and cell membranes (*cm*) are unstained, except for the communicating junction membranes (*arrows*).  $\times 63,000$ .

studies on the tanning process indicate that polyphenols are in general more reactive with tissues than simple phenols (7, 12, 19).

### C. Functional Groups Possibly Required for Mordanting

The fact that even simple phenols have a modest but detectable mordanting action (Figs. 5, 6) suggests that, for these types of compounds, the functional groups probably involved in the attachment of lead to osmicated structures are -COOH and at least one -OH simultaneously present on the benzene ring. Our observations made on simple phenols may be relevant also for the case of galloylglucoses, provided they yield, in solution or in contact with the tissues, gallic or digallic acids both of which satisfy the requirements indicated above. It should be mentioned, however, that according to the chemistry of tanning (5, 7, 18, 19), galloylglucoses may interact directly by their multiple phenols (5, 7) with available tissue radicals. In our case they may interact with functional groups on osmicated structures as well as with lead.

These attempts at explaining the effects recorded should be considered as strictly tentative since the mordanting mechanism of galloylglucoses and related phenols is still poorly understood even in the simpler cases of leather tanning (5, 7, 18, 19), textile dyeing, and histological-histochemical staining (4, 14, 16). In our case, the chemistry of tissues is much more complex than that of either leather or textile fibers, and in our experiments the mordanting involves heavy metals, not dyes.

In conclusion, our results show that galloylglucose treatment of fixed tissues enhances and diversifies structure contrast, with significantly less extraction and better preservation of cellular components than is obtainable with conventional techniques (e.g., uranyl acetate or ferrocyanide-reduced osmium tetroxide).

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