Studies on the Mechanisms of Glutaraldehyde Stabilization of Cytomembranes[1]

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The widespread use of glutaraldehyde for routine fixation of biological materials (1, 9, 14, 16) and as an aid to organelle isolation (11, 12, 13) stimulated considerable interest in the nature of its interactions with protoplasmic constituents (2, 3, 6). Using a combined biochemical and histochemical approach, Hopwood (6) demonstrated qualitatively that glutaraldehyde fixation introduced blockable carbonyl groups into tissue protein and that glutaraldehyde and formaldehyde preserved glycogen to a similar degree. However, quantitative studies of the reactions of glutaraldehyde with protoplasmic constituents are limited. Evidence that glutaraldehyde cross-links polyamines (including proteins) and polyhydroxy compounds (such as polysaccharides) has come largely from studies conducted by the tanning industry (2, 6).

This report presents evidence that cross-linking of free amino groups of proteins is critical to glutaraldehyde fixation of cytomembranes, (extractability of membrane lipids is unaffected by glutaraldehyde) and that glutaraldehyde reacts with small molecules such as free amino acids and primary amines. Most of the glutaraldehyde bound by tissue appears to result from its binding to small molecules and to large molecules other than protein.

Materials and Methods

Glutaraldehyde. Excised onion stem and isolated cell fractions from bean leaves, onion stem and mouse liver were treated with 2.5% glutaraldehyde (prepared from a 50% stock solution, Fischer Scientific, Biological Grade, to which 0.16 g/ml of activated coconut charcoal was added to remove impurities) at 0 to 4°. Unless stated otherwise, glutaraldehyde solutions were prepared in 0.01 M sodium phosphate buffer, pH 7.0 to 7.3.

Determination of protein and nitrogen. Total protein was determined from lyophilized fractions which were quick frozen in liquid nitrogen and dried to constant weight over calcium chloride. Tissue was pulverized to a fine powder using a mortar and pestle and a weighed aliquot was extracted exhaustively with hot (90°) 0.1 N sodium hydroxide. Insoluble material was removed by centrifugation, the supernatant was neutralized with trichloroacetic acid, and the protein was precipitated by addition of an equal volume of 1 M trichloroacetic acid (0 to 4°; 2 to 3 hr). Protein

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was estimated by the biuret method (5). Nitrogen was determined by direct Nesslerization (7).

Extraction and determination of lipids. For lipid extraction, about 4 g fresh weight of tissue (either untreated or glutaraldehyde-fixed) was frozen in liquid nitrogen, lyophilized and dried as above, powdered with a mortar and pestle and transferred to microsoxhlet extraction thimbles. The chloroform extractable lipids were removed by a 3 hr extraction with 15 ml of chloroform. Chloroform was evaporated and the lipid residue was brought to constant weight over calcium chloride, redissolved in chloroform, filtered to remove insoluble materials, recovered by evaporation of solvent and brought to constant weight. Final weights were corrected for losses during filtration. To obtain the residual lipid fraction, the thimbles from the chloroform extraction were placed in acetylization flasks with 15 ml of 95% methanol and refluxed for 1 hr over a 90° water bath. Methanol was evaporated over a steam bath and water was removed by transferring the samples to a 90 to 95° oven. After drying to constant weight over calcium chloride, 15 ml diethyl ether were added to the residue followed by exhaustive soxhlet extraction (12 hr). The chloroform-soluble residue after decantation and evaporation of the ether was taken as residual lipid.

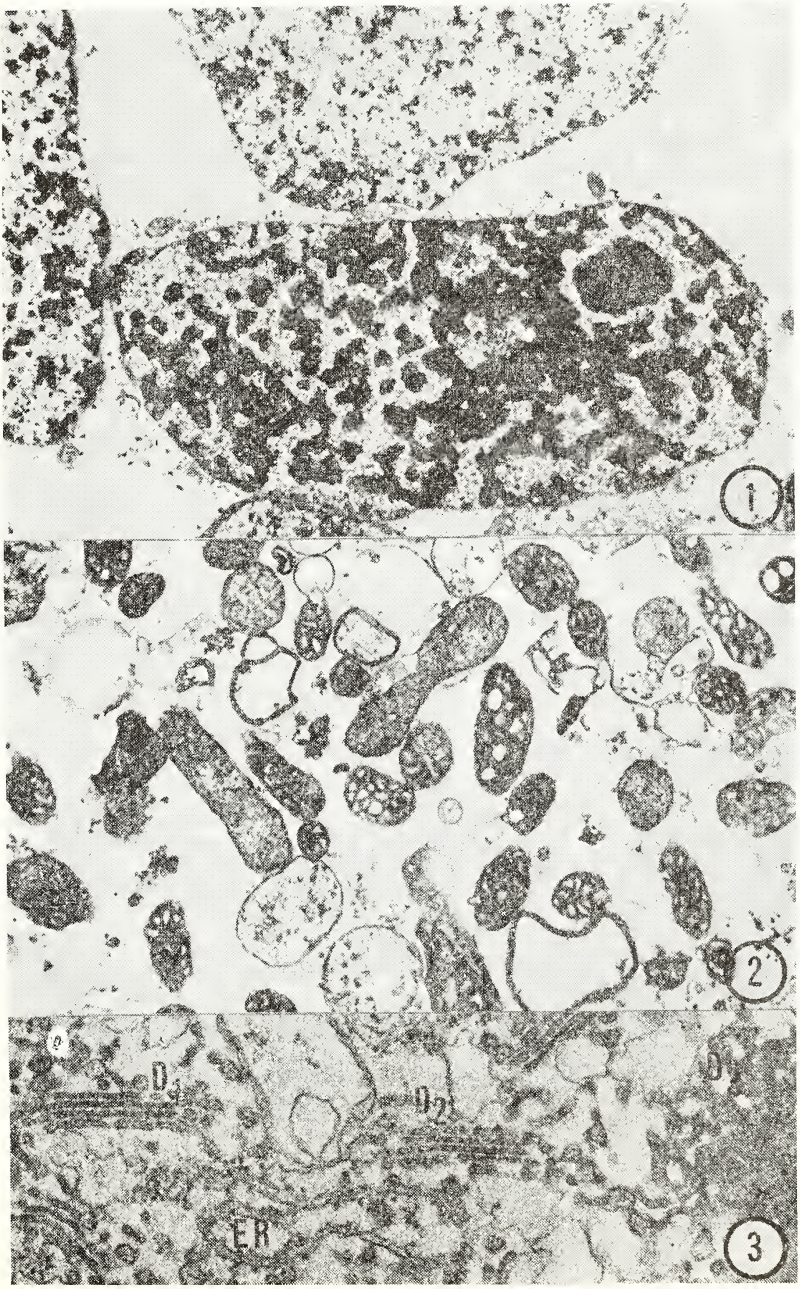
Thin layer chromatography of phospholipids. Approximately 5 mg of lipid in chloroform were streaked on each 20 X 20 cm plate coated with Silica Gel G. Development was in chloroform:methanol:water (65:35:4 v/v). Regions containing lipids were detected by exposing the plates to iodine vapors, scraped from the plates and digested with sulfuric acid and hydrogen peroxide. Phosphorous content of the digests was determined by the method of Fiske and Subbarow (4).

Electron microscopy. Portions of isolated pellets were post-fixed for 1 to 24 hours in 1 per cent buffered osmium tetroxide (0.1 M sodium phosphate, pH 7.2) or exposed for several days to osmium tetroxide vapors. Subsequent dehydration and embedding was as described previously (10). Sections were viewed with a Siemens Elmiskop I or Philips EM 200 electron microscope. Magnifications are approximate.

Results

Glutaraldehyde stabilization of organelles during isolation. Loss of ultrastructural detail in organelles during their isolation was prevented by adding 10^{-1} to 10^{-3} M glutaraldehyde (2.5 to 0.0025%) to the homoge-

Figure 1. Nuclei fraction from onion stem stabilized by addition of glutaraldehyde to the homogenization medium. Osmium post-fixation. X 7,000.
Figure 2. Mitochondrial fraction of onion stem stabilized by addition of glutaraldehyde to the homogenization medium. Osmium post fixation. X 13,200.
Figure 3. Golgi apparatus fraction from onion stem containing sheets of rough endoplasmic reticulum (ER) stabilized by addition of glutaraldehyde to the homogenization medium. Dictyosomes of the Golgi apparatus are shown in cross section (D_1 and D_2) and sectioned tangentially (D_3). Osmium post-fixation. X 40,000.


1
2
3
D_1
D_2
ER

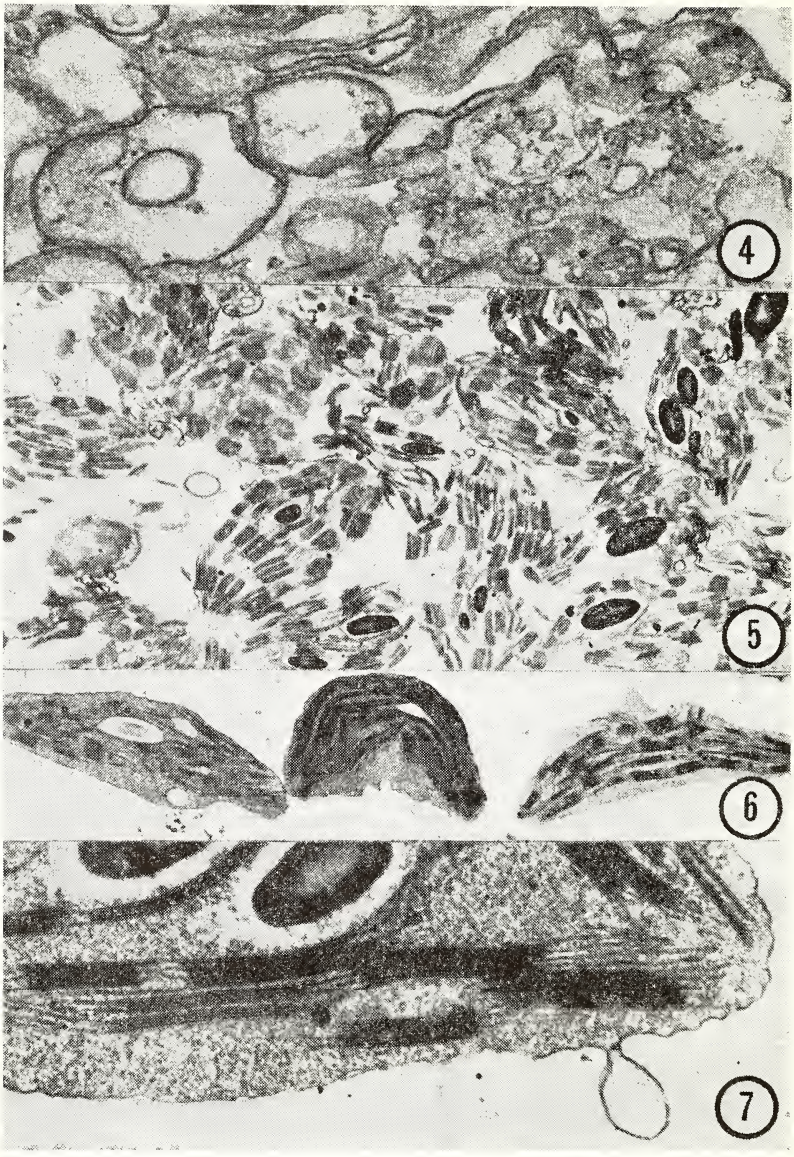


Figure 4. Smooth membrane fraction from onion stem stabilized by addition of glutaraldehyde to the homogenization medium. Many of the large vesicular profiles are derived from fragments of the plasma membrane. Osmium post-fixation. X 49,000.

Figure 5. Chloroplast fraction isolated from bean leaves in the absence of glutaraldehyde. Glutaraldehyde-osmium fixation. X 6,900.

Figure 6. Chloroplasts isolated from bean leaves in the presence of glutaraldehyde. Osmium post-fixation. X 6,900.

Figure 7. Glutaraldehyde-stabilized chloroplast at higher magnification.

nization medium (0.5 M sucrose; 0.1 M sodium phosphate, pH 7.1; 1% dextran). Various fractions including nuclei (Fig. 1), mitochondria (Fig. 2), Golgi apparatus (Fig. 3), endoplasmic reticulum (Figs. 3 and 8) and smooth membranes, including plasma membrane (Fig. 4), were isolated from onion stem and other plant and animal tissues in the presence of glutaraldehyde. These cell components were morphologically indistinguishable from organelles fixed *in vivo*. Nuclei had intact outer membranes and organized chromatin (Figs. 1 and 8). Mitochondria were dense and unswollen (Fig. 2). In the absence of glutaraldehyde, plant Golgi apparatus vesiculated and the stacked cisternae separated. With glutaraldehyde, breakdown of both Golgi apparatus (Fig. 3) and endoplasmic reticulum (Figs. 3 and 8) was retarded. With chloroplasts isolated without glutaraldehyde (Fig. 5), outer membranes, osmiophilic granules and stroma (chloroplast matrix) were missing and the lamellae were distorted and swollen. With the addition of 2.5% glutaraldehyde to the homogenization medium (Fig. 6), the outer membranes, osmiophilic granules and lamellae were well preserved and clearly defined. The stroma was retained in the uniformly dense aspect characteristic of chloroplasts fixed *in vivo*.

To illustrate a similar effect of glutaraldehyde on mammalian tissues, Figure 8 shows a pellet of an unfractionated homogenate of mouse liver prepared in the presence of glutaraldehyde. Organelles including nuclei, mitochondria, microbodies and endoplasmic reticulum were obtained with morphological characteristics resembling those of whole, fixed tissues.

Amount of glutaraldehyde bound. With onion stem treated with buffer alone, the dry weight from 12 determinations was 11.3±0.3% of the fresh weight. With 2.5% buffered glutaraldehyde followed by four 15 minute buffer rinses, the final dry weight was 12.4±0.5% of the fresh weight. The gain in dry weight due to glutaraldehyde treatment was 1.1% of the fresh weight or about 10% of the dry weight. The increase in dry weight, if due to bound glutaraldehyde, would amount to 110 μmoles of glutaraldehyde per g fresh weight.

Reaction of glutaraldehyde with lipids and proteins. Total lipid extracted by chloroform-methanol-ether consisted of about 40% phospholipid and 60% neutral lipid and was unchanged by glutaraldehyde treatment of the tissue (6.1% of the dry weight) as shown by data of Table 1. In contrast, total protein extracted by 0.1 N sodium hydroxide declined from 14% of the dry weight (in untreated controls) to 3.5% of the dry weight after glutaraldehyde treatment. These data demonstrate that 75% of the protein was sufficiently cross linked to prevent extraction into alkali. Total nitrogen was unchanged at 3.2% of the dry weight.

When glutaraldehyde was added to protein in solution (Fig. 9), the bulk of the protein was rendered insoluble with a reaction half-time of approximately 7 min. As with whole tissue, about 75% of the protein was sufficiently cross-linked to become insoluble in 0.1 N sodium hydroxide following glutaraldehyde treatment.

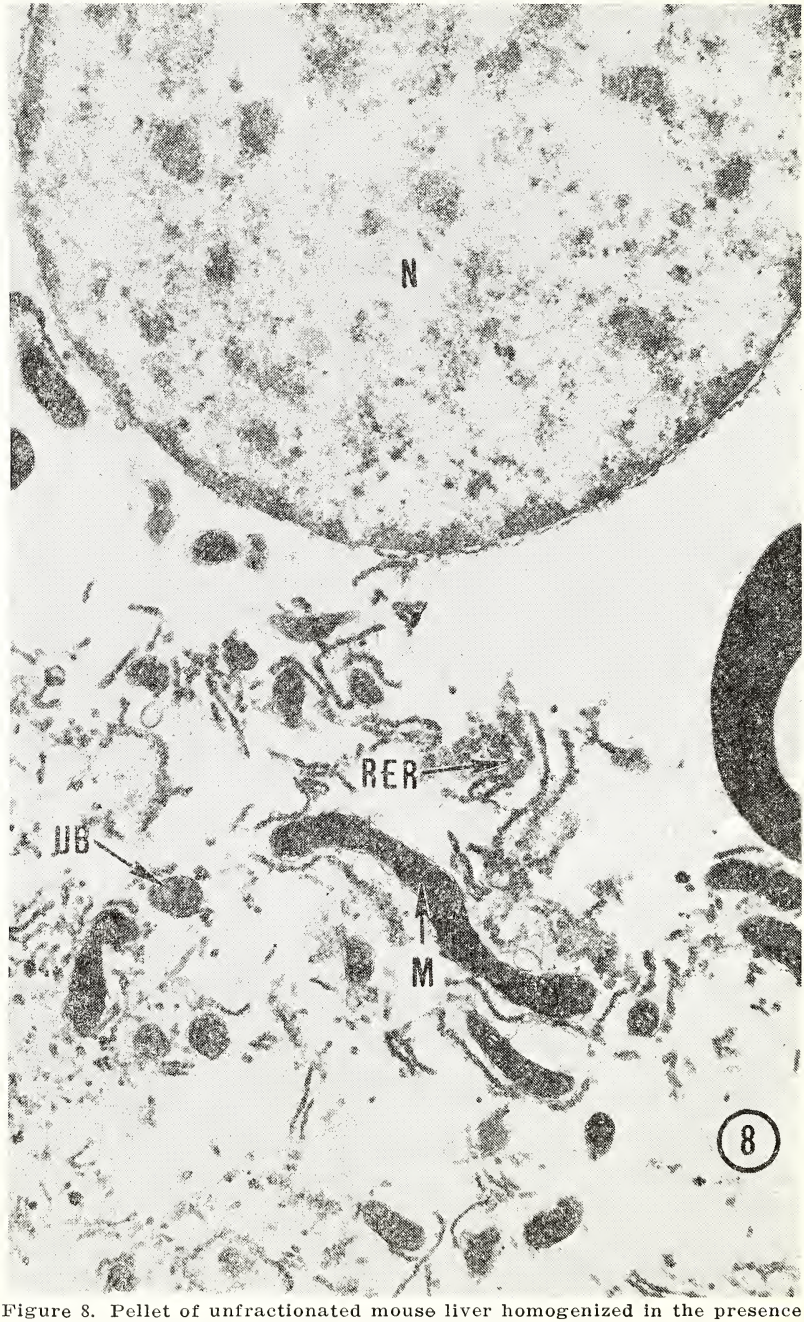


Figure 8. Pellet of unfractionated mouse liver homogenized in the presence of glutaraldehyde. A nucleus (N), segments of rough endoplasmic reticulum (RER), a microbody (μB) and numerous mitochondria (M) are shown. Osmium post-fixation. X 16,500.

Although lipid extractability was unchanged by glutaraldehyde treatment (Table 1), it was of interest to consider the possibility that certain lipids might react with glutaraldehyde and yet remain lipid soluble and be extracted into the chloroform-soluble fraction. To test this possibility, the phospholipid fraction was separated by thin layer chromatography into low R_f phospholipids (glyco- and sulfolipids), lecithin (phosphatidylcholines), ethanolamine phospholipids (phosphatidylethanolamines) and two phospholipid fractions containing serine (phosphatidylserines). As shown by data of Table 2, lecithin accounted for about 30% of the total lipid phosphorous and was unchanged by glutaraldehyde treatment. In contrast, the amino phospholipids (those containing serine and ethanolamine) were greatly reduced in amount at positions corresponding to their characteristic R_f's on the thin layer plates. Recovery of serine phospholipids was 35% and recovery of ethanolamine phospholipids was 66% in contrast to 95% recovery for lecithin. An apparent increase in low R_f phospholipids following glutaraldehyde treatment approximately compensated for the decrease in ethanolamine and serine phospholipids. Total recovery of lipid phosphorus was 94% of that of unfixed control tissue.

Reaction of glutaraldehyde with small molecules. Glutaraldehyde reacted rapidly with the primary amino groups of all twenty protein amino acids tested (Table 3) as evidenced by loss of ninhydrin reactivity. Paralleling the loss of chemical reactivity of the α-amino group, was the

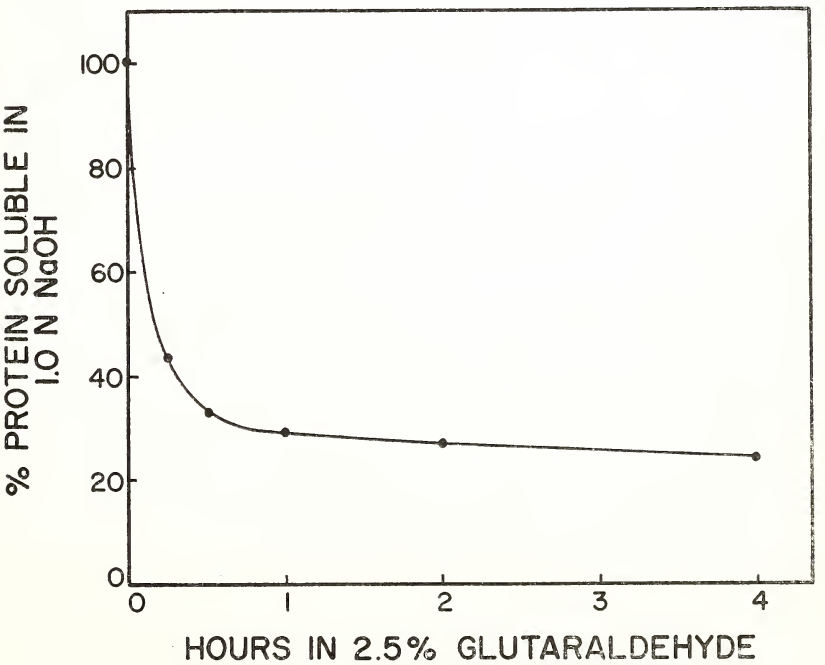


Figure 9. Solubility of bovine serum albumin in 1.0 N sodium hydroxide as a function of time in a 2.5% glutaraldehyde solution at 0 to 4°.

TABLE 1. *Composition of Onion Stem Determined in the Presence or Absence of Glutaraldehyde (GA).*

Constituent	% of Dry Weight	
	—GA	+GA
Total Lipids	6.1	6.1
40% Phospholipids		
60% Neutral Lipids		
Alkali Extractable Protein	14.0	3.5
Total Nitrogen	3.2	3.2

TABLE 2. *Phospholipid Composition of Onion Stem Determined in the Presence or Absence of Glutaraldehyde (GA).*

Constituent	Lipid Phosphorous (μg/g dry weight)					
	$CHCl_3$-Soluble		Residual		Total	
	—GA	+GA	—GA	+GA	—GA	+GA
Low R_f Phospholipids	292	346	57	65	349	411
Lecithins	258	244	16	14	274	258
Serine Phospholipids A	78	34	2	T	80	34
Ethanolamine Phospholipids	54	27	7	12	61	39
Serine Phospholipids B	78	20	T	2	78	22
Sterols and Neutral Lipids	27	41	7	14	34	55
Total	787	712	89	107	876	819

appearance of a yellow chromogen with an absorption maximum near 440 mμ and suggestive of Schiff base formation. Cysteine, in addition to forming the yellow coloration given by all amino acids, rapidly produced an insoluble precipitate not given by cysteine. The imino acids, proline and hydroxyproline, retained ninhydrin reactivity following glutaraldehyde treatment. Proline did not react.

Of the nucleotide bases tested, adenine and guanine were most reactive with the formation of an ultraviolet light-absorbing adduct and the yellow coloration. Neither cytidine nor uridine produced a visible reaction at room temperature but upon heating reacted in a manner similar to adenine.

Role of protein cross-linking in cytomembrane stabilization. To test the relative contributions of protein and lipid in maintaining the form of cytomembranes, a cell fraction containing dictyosomes and a few fragments of endoplasmic reticulum (Fig. 3) was prepared by glutaraldehyde stabilization and lyophilized. After drying to constant weight

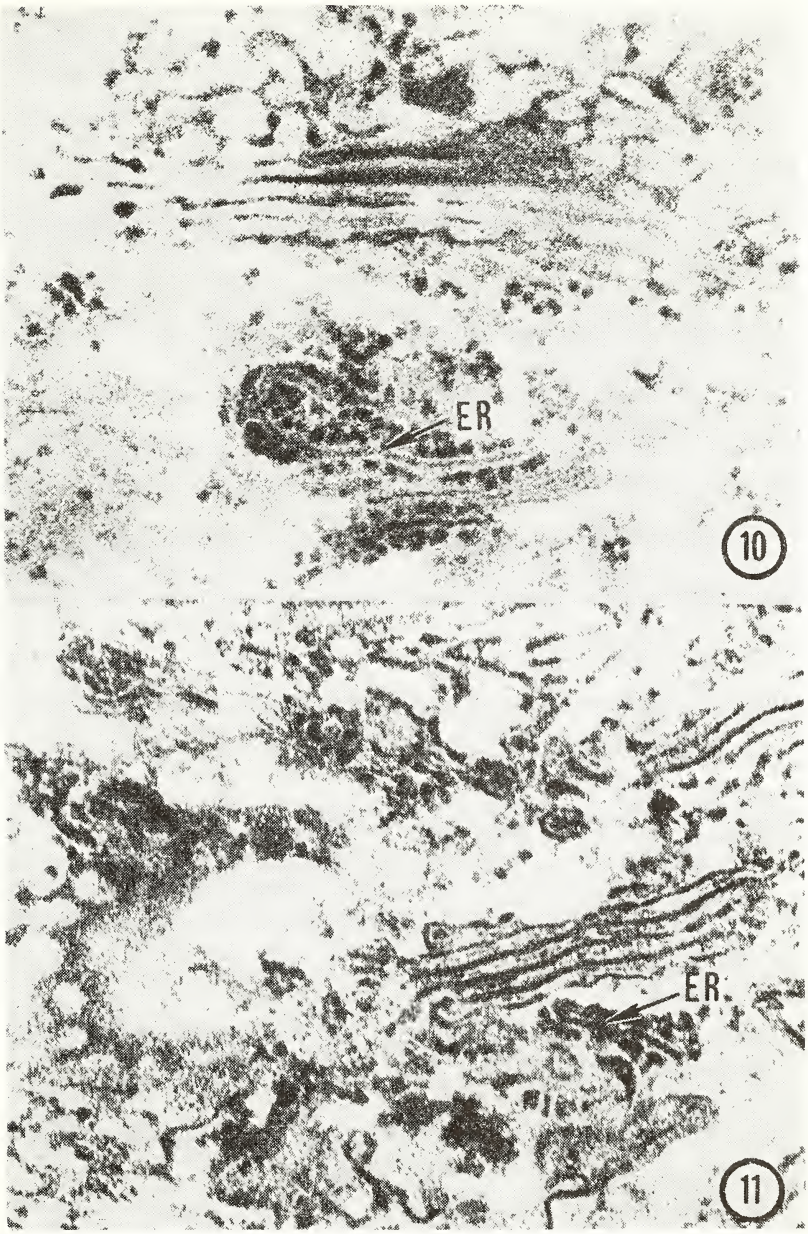


Figure 10. Golgi apparatus fraction from onion stem stabilized by addition of glutaraldehyde to the homogenization medium, freeze dried, stained with osmium vapor and embedded in epon-araldite. The field was chosen to contain a fragment of endoplasmic reticulum (ER) as well as a dictyosome showing cisternae sectioned both tangentially and in cross section. X 120,000.

Figure 11. As in Figure 10 except lipids removed by chloroform-methanol-ether extraction prior to exposure to osmium. X 120,000.

over calcium chloride, the lipids were extracted with chloroform-methanol-ether from one half of each pellet as described for whole tissue. The other pellet half served as an unextracted control. Prior to examination in the electron microscope, both pellets were stained by exposure to osmium tetroxide vapor and embedded directly in an epon-araldite mixture (10). Figure 10 shows a portion of a control pellet with a single dictyosome and a segment of endoplasmic reticulum. With both cell components, the osmium was localized largely in the lumina of dictyosome cisternae and with both the lumina and ribosomes of the endoplasmic reticulum.

In the extracted pellet (Fig. 11), the appearance of the membrane was similar to that of the unextracted controls except that the dictyosomes and endoplasmic reticulum were more clearly defined. It is clear that chloroform extraction of lipids did not alter the form of glutaraldehyde-stabilized dictyosomes and endoplasmic reticulum, and that the reactions of glutaraldehyde with protein of the membrane were apparently sufficient to stabilize the form of the organelle as viewed in the electron microscope.

Binding of small molecules to membranes in homogenates. Since glutaraldehyde is a dialdehyde, it was possible that reactive small molecules were bound to cytomembranes through reaction of the second aldehyde to reactive groups on large molecules. To test this possibility, radioactive metabolites were thoroughly mixed with homogenized onion stem

TABLE 3. *Reactivity of Amino Acids, Imino Acids and Nucleotide Bases With Glutaraldehyde.*

Amino Acids[a]			
Alanine	++	Phenylalanine	++
Arginine	++	Threonine	++
Aspartic Acid	++	Tryptophan	+
Cysteine	++[b]	Tyrosine	+
Cystine	++	Valine	+
Glutamic Acid	+	*Imino Acids*[a]	
Glycine	+		
Histidine	++	Proline	—
Isoleucine	++	Hydroxyproline	±
Leucine	++	*Nucleotide Bases*[c]	
Lysine	++	Adenine	+
Methionine	++	Guanine	+
		Cytosine	—
		Uracil	—

[a] Determined from loss of ninhydrin reactivity following paper chromatography and from increase in absorbance at 440 $m\mu$ during reaction in solution.

[b] In addition to the yellow chromogen given also for cystine, cysteine gave a copious precipitate not given by cystine suggesting a reaction of glutaraldehyde with the cysteine -SH.

[c] Determined from altered chromatographic properties and absorbance at 440 $m\mu$.

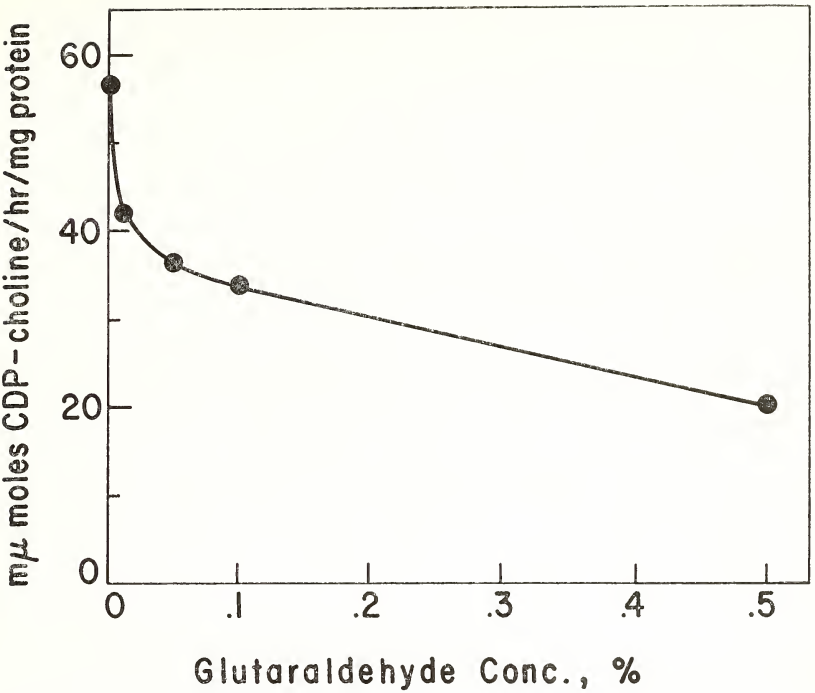


Figure 12. Inactivation of the enzyme CDP-choline-cytidyl transferase of homogenates of onion stem stabilized with varying concentrations of glutaraldehyde.

(same medium as for organelle isolation) after which glutaraldehyde was added to a final concentration of 2.5%. After 30 minutes at 0 to 4°, the homogenates were pelleted at 12,000 g for 30 min and washed with distilled water through several resuspension-centrifugation cycles until constant specific activity was achieved. With C^{14}-acetate, a molecule not expected to react with glutaraldehyde, the level of radioactivity in the homogenate was 1.8 mμmoles/μmole/mg N. Choline gave a similar value which suggested little or no binding. The value for C^{14}-uridine, although obtained from mung bean stem homogenates rather than onion, was sufficiently low to suggest little or no binding. Radioactivity from C^{14}-glucose bound to the 12,000 g pellet was about 3 times that of acetate showing a small but significant reactivity of glutaraldehyde with a sugar. Of the compounds tested, leucine was most reactive, binding at 5 times the amount of glucose. In competition experiments, it was found that the level of radioactive leucine bound to components of the homogenates was little influenced by a 100-fold excess of unlabeled leucine.

Loss of enzymatic activity. Typical denaturation kinetics are shown in Figure 12 for the enzyme CDP-choline-cytidyl transferase of onion stem. This enzyme catalyzes the formation of CDP-choline, an intermediate in the biosynthesis of lecithin, from phosphorylcholine (P-choline) and cytidine triphosphate (CTP).

Discussion

Although glutaraldehyde provides superior structural preservation for electron microscopy (1, 9, 14, 16), its use in organelle stabilization or in metabolic studies may modify enzymatic activity. Loss of enzymatic activity is generally associated with protein cross-linking by glutaraldehyde (3). Examples of losses of enzymatic activity are given by Hopwood (6), Sabatini *et al.* (14), Barnett (1) and in Figure 12. Not all enzymatic activities decline following treatment with glutaraldehyde. Some activities appear to be unchanged (15), and De Jong *et al.* (3) have reported activation by glutaraldehyde of an acid phosphatase of nuclei in cultured plant cells. Preliminary results indicate an activation of a CTPase of dictyosomes isolated from onion stem following treatment of homogenates with glutaraldehyde (unpublished observations).

For a fixative to be of general use in preparing tissues or organelles for chemical analysis, the chemical moiety under study must remain unmodified. With onion stem, lipids are recovered quantitatively after glutaraldehyde stabilization and the phospholipid lecithin appears to be unchanged. The lipid content after treatment appears to reflect the lipid content before fixation. Glutaraldehyde-stabilized dictyosome preparations from onion stem contain 50 to 60% protein, 20 to 30% phospholipids (rich in lecithin) with the remainder consisting mostly of sterols and neutral lipids (12).

Studies of Park *et al.* (13) suggest that quantum conversion and electron transport in photosynthesis takes place within the rigid framework of glutaraldehyde-stabilized plastids and that these processes do not require conformational changes in proteins. They showed that chloroplasts isolated from leaves previously fixed in 6% glutaraldehyde retain optical rotatory dispersion activity and absorption spectra of unfixed chloroplasts and the capability of limited photochemical activity. Ferricyanide-Hill activity measured by O_2 evolution or indophenol reduction was 25% of that of unfixed chloroplasts. The oxygen evolution was light dependent and sensitive to various photosynthetic inhibitors.

That the morphological integrity of glutaraldehyde-treated organelles is maintained after lipid extraction suggests that the cross-linked protein framework of the organelle is the critical feature of glutaraldehyde stabilization. Golgi apparatus fractions containing small fragments of endoplasmic reticulum when isolated by glutaraldehyde stabilization showed little or no morphological response to changes in osmotic environment. They maintained their *in vivo* shapes even after freeze drying (Fig. 10) or freeze drying followed by extraction of total lipids using chloroform-methanol-ether (Fig. 11). Except for slight increases in osmium deposition after lipid extraction such preparations were indistinguishable from each other.

Associated with the reaction of glutaraldehyde with tissue, tissue homogenates or isolated cell fractions is a yellow coloration which increases in intensity for about 1 hour after addition of glutaraldehyde at 0 to 4°C. Various protein, amino acid or amine solutions treated with

glutaraldehyde assume a similar color. The color is probably due to the formation of Schiff bases, which in proteins would be with basic amino acids. Cystine which consists of two cysteine residues linked by a disulfide bond did not precipitate with glutaraldehyde as did cysteine. This is evidence for a reaction of glutaraldehyde with the free sulfhydryl group of cysteine. The imino acids proline and hydroxyproline contain an amino group in the ring which is apparently unreactive with glutaraldehyde. Hydroxyproline appeared to react slowly with glutaraldehyde possibly through an involvement of the hydroxyl group.

Being a dialdehyde, glutaraldehyde is capable of forming cross-linking bridges in proteins involving both of the aldehyde groups, or under certain circumstances only one of the aldehyde groups may react with protein. Hopwood (6) demonstrated qualitatively that free carbonyl groups are introduced into proteins following reaction with glutaraldehyde. The free aldehyde could then react with other cell components including free amino acids. That this type of reaction occurs is shown by data of Table 4. Radioactive leucine added to total homogenates of onion stem cross-links to organelles sedimenting at 12,000 g. This binding is apparently nonspecific and in competition with a variety of reactive molecules since addition of excess unlabeled leucine had little effect on the amount of labeled leucine bound by the organelles. Other metabolites including choline, acetate and uridine are not bound significantly. Glucose is bound to the organelle fraction, presumably via cross-linking reactions involving the free hydroxyls, at a level 1/10 that of leucine.

These results show that glutaraldehyde fixation of tissues or organelle fractions incubated with radioactive metabolites may lead to nonspecific binding of the metabolites via the cross-linking reactions. This possibility must be taken into account in autoradiographic or biochemical studies involving radioactive metabolites and especially with amino acids when supplied in conjunction with glutaraldehyde stabilization.

TABLE 4. *Binding by Glutaraldehyde of C^{14}-Labeled Metabolites to the 12,000 o Particulate Fraction of Onion Stem*[a].

Metabolite	mμMoles/μMole/Mg Nitrogen
Acetate-2-C^{14}	1.8
Choline-2-C^{14}	1.8
Uridine-U-C^{14}[b]	0.7
Glucose-U-C^{14}	5.5
Leucine-U-C^{14}	50.4

[a] Homogenates were mixed with isotope (1.5 to 30 μμmoles/5 g fresh weight of tissue in 6 ml of total homogenate) followed by glutaraldehyde (final concentration of 2.5%) and equilibrated 30 minutes. After clearing the mixture of cellar debris, the 12,000 g pellet was washed with buffer to constant specific activity on a total nitrogen basis. Values are ± 30%.

[b] Mung bean stem.

To summarize our findings, calculations of the distribution of potential glutaraldehyde binding sites among various cell constituents is provided in Table 5. From the dry weight changes associated with reaction with glutaraldehyde, approximately 110 μmoles of glutaraldehyde are bound per g fresh weight of onion stem. Of this 110 μmoles, less than 15 might be expected to bind with protein on the basis of lysine and cysteine content. Other basic or hydroxy amino acids may react with glutaraldehyde but evidence presented here and elsewhere (2) suggests the α-amino group of lysine and the β-SH group of cysteine as potentially the most reactive groups in the interiors of protein chains (presumably the N-terminal residue would also be available for reaction with glutaraldehyde). The potential contribution of nucleic acid to the amount of glutaraldehyde bound in onion stem is probably less than 2 μmoles due to the small amounts of nucleic acid present. Adenine and guanine (A + G) are considerably more reactive in solution as the free base than either uridine or cytidine (U + C). Amino lipids react with glutaraldehyde but represent only 25% of the lipid phosphorus and therefore account for less than 1 μmole of the total glutaraldehyde bound. The largest proportion of glutaraldehyde bound to amino groups would be to free amino acids and other small molecules containing primary amines. Assuming that 80% of the nonprotein nitrogen consists of amino nitrogen (8), this class of molecules could account for up to 55 μmoles of glutaraldehyde per g fresh weight or approximately 50% of the total glutaraldehyde bound by the tissue.

We have no estimate of the amount of glutaraldehyde bound to cell wall, storage polysaccharides and small molecules such as sugars or alcohols. Assuming maximal binding to amines, this fraction would represent about 37 μmoles of the 110 μmoles total. However, it appears that under usual conditions of fixation no more than 75% of the available

TABLE 5. *Relative Proportions of Potential Glutaraldehyde Binding Sites of Onion Stem.*

Constituent	μ Moles Glutaraldehyde Bound per G Fresh Weight
Total (By Weight)	~110
Protein (Calculated on the basis of 6.5 g lysine N and 1 g cysteine N per 100 g protein N) (8)	<15
Nucleic Acid (Calculated on the basis of 0.45% of the dry weight; 50% A + G) (8)	<2
Free Amino Acids and Primary Amines (Calculated on the basis of 6.6% of the dry weight; average M. W. 120)	~55
Amino Lipids (Calculated on the basis of 25% of the total lipid P; average M. W. 750)	<1
Unaccounted For (Cell wall, storage polysaccharides, small molecules other than primary amines)	>37

sites react with glutaraldehyde (Fig. 9, Table 2 and estimates from reaction of amino acids and nucleic acids in solution). If 75% is used as the extent of reaction, less than half the bound glutaraldehyde is accounted for by reaction with amino and sulfhydryl groups (both as polymers or small molecules). Thus, binding of glutaraldehyde to cell wall and other polyhydroxycompounds might be more extensive than the 37 μmoles/g fresh weight estimated and, in plant tissues, might account for binding equal in magnitude to that by primary amines.

Summary

Chemical evidence suggests that glutaraldehyde stabilization of cytomembranes occurs primarily by cross-linking free amino groups of adjacent polypeptide chains. Hydroxyl and sulfhydryl groups may also contribute to the cross linking. However, 90% or more of the total lipid of glutaraldehyde treated tissue is recovered by solvent extraction. The bulk of the glutaraldehyde bound by tissue appears to be the result of its binding to small molecules and to large molecules other than protein. Cross-linking of protein, however, appears critical to glutaraldehyde fixation of cytomembranes.

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