Scanning Electron Microscopy

Volume 4
Number 1 *The Science of Biological Specimen Preparation for Microscopy and Microanalysis*

Article 5

1985

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Science of Biological Specimen Preparation (pp. 51-62)
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GLUTARALDEHYDE FIXATION CHEMISTRY

A SCHEME FOR RAPID CROSSLINKING AND EVIDENCE FOR RAPID OXYGEN CONSUMPTION

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Abstract

The chemical literature suggests that pyridine derivatives are the major reaction products of amine-aldehyde reactions. A scheme is proposed for pyridine synthesis which is derived from a classic synthesis of pyridines from one amine molecule and 3 aldehyde molecules. The pyridine polymers are considered as a major class of cross-links in glutaraldehyde fixed cells. This scheme for pyridine synthesis accounts for the following observations of amine-glutaraldehyde reactions: 1) large quantities of acid are rapidly produced, 2) oxygen uptake occurs at a high rate, 3) a variety of products distinguished by molecular size are rapidly and irreversibly synthesized, and 4) the major reaction products share physical and chemical characteristics with highly substituted pyridine compounds. When formaldehyde is added to glutaraldehyde-amine reactions, oxygen uptake is sharply reduced and the product spectrum is changed in a complex manner. Formaldehyde may thus have an entirely different role in the mixed glutaraldehyde-formaldehyde fixatives than has previously been suggested.

KEY WORDS: Glutaraldehyde, glutaraldehyde fixation, glutaraldehyde-amine reactions, acid production, oxygen uptake, pyridines, crosslinking

Introduction

The use of the dialdehyde, glutaraldehyde, as a fixative for electron microscopy was popularized by Sabatini et al. (37). In that paper many aldehydes were examined for the purpose of introducing a fixation step prior to osmication which would preserve tissue morphology, yet permit cytochemical studies not possible after osmication. Sabatini and coworkers noted that dialdehydes were known to be rapidly acting crosslinking agents, but they did not propose a mechanism for chemical crosslinking. For some time it was widely assumed that tissue fixation by glutaraldehyde, an α,w-dialdehyde, was accomplished by crosslinking amino groups through α,w-bis-Schiff base formation (Fig. 1).

Many workers have explored the nature of the stable glutaraldehyde-induced crosslink in biological materials (21,28,33,35). Richards and Knowles (35) pointed out that the chemical properties of the glutaraldehyde-induced crosslinks were not compatible with the known chemistry of Schiff base formation. In aqueous solutions, for example, the glutaraldehyde crosslinks were irreversible (2,17,31), while Schiff base formation is reversible (note Fig. 1). Richards and Knowles suggested that aldol condensation products of glutaraldehyde (Fig. 2a and 2b) provided new reactive sites for irreversible cross-linking via a subsequent Michael reaction (Fig. 2c). The work of Richards and Knowles stimulated others to analyze aqueous glutaraldehyde solutions for evidence of the α,ß-unsaturated aldol condensates (13,21,44). Whipple and Ruta (44) offered data from ^{13}C NMR which showed that glutaraldehyde exists primarily as the cyclic monohydrate forms, with only small fractions present as linear forms (see 1, 2, 3, Fig. 6a for reactions of glutaraldehyde with

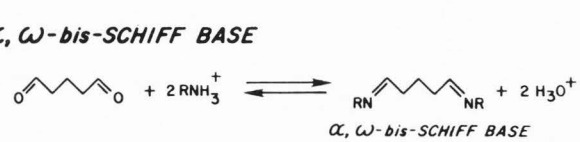


Figure 1. Bis-Schiff base formation. Schiff base formation is rapid and reversible in aqueous solution. Acid is a byproduct near pH 7 (19).

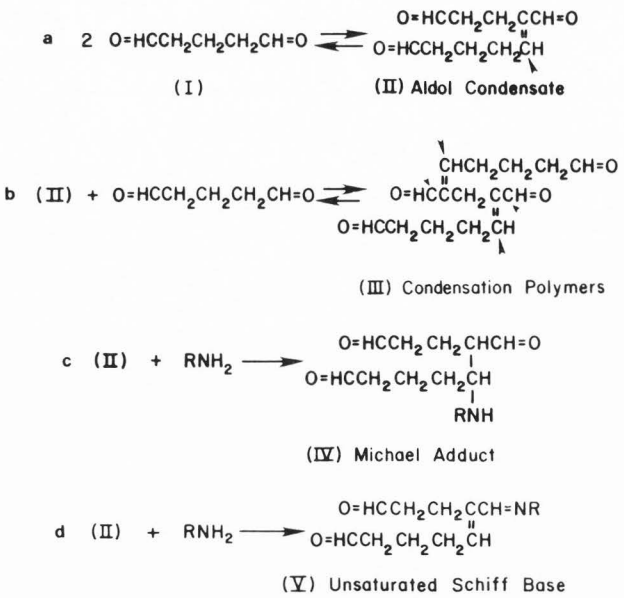


Figure 2. Previous proposals for irreversible crosslinking. The aldol condensation products of glutaraldehyde with itself (reactions a and b) yield structures (II) and (III). Higher order polymers (not shown) are possible. The α,ß unsaturated carbonyl groups of structures (II) and (III) react irreversibly with nucleophilic groups such as primary amines. The Michael reaction c yielding the Michael adduct (IV) is one class of crosslink (35). The large arrowhead marks the site of reaction with nucleophiles. Michael reactions with (III) and higher order polymers may crosslink nucleophilic groups. Alternatively, if an amine reacts at the carbonyl carbon of condensates (II) and (III) (small arrowhead) (reaction d), unsaturated Schiff bases (V) are formed. The aldol condensates permit many sites for crosslinking (28,33).

water). More importantly, however, aldol condensation products were not detected in aqueous solutions (13,21,44).

The nature of the stable glutaraldehyde-induced crosslink has been periodically reexamined (12,14,15,21,26,28,33). Monsan (28), for instance, has suggested that the stable crosslink is based upon formation of an α,ß-unsaturated Schiff base (Fig. 2d). Alternatively, Hardy and coworkers (12,14,15) suggested that derivatives of pyridine constitute the stable crosslink.

It has been recognized in the chemical literature since the turn of the century (40) or earlier (4) that pyridine derivatives can be isolated from aldehyde-amine reactions. Moreover, under appropriate conditions pyridine derivatives have been described as the major reaction products (4,7,9,30). However, these glutaraldehyde-amine reaction pathways received little attention in biological applications until the work of Hardy et al., (12,14,15). Hardy and coworkers isolated highly substituted pyridines (desmosine derivatives) from the products of model amino acid-glutaraldehyde and protein-glutaraldehyde reactions.

In this paper a scheme is proposed by which pyridines and pyridine polymers are produced from glutaraldehyde-amine precursors. The scheme accounts for the rapid production of acid (19) and the subsequent consumption of oxygen. In addition, it is proposed that these pyridine polymers are important crosslinking elements which bridge erratically spaced primary amino groups in cells. This study of model amine-glutaraldehyde reactions provides data which support many steps in this proposed scheme. Since these model reactions were studied under conditions commonly used for tissue fixation with glutaraldehyde, these observations likely apply to the fixation of cells and tissues.

Materials and Methods

In the experiments described below, the following chemicals were used: glycine (crystalline), aminoethyl cellulose (0.29 meq/g), sodium borohydride (98%+, crystalline), and Sephadex G-15 (40-120 μm, Pharmacia Fine Chem.) all from Sigma Chem. Co., St. Louis, MO; ammonium acetate (reagent grade, Baker, Philipsburg, NJ); ethanolamine (95%, Aldrich Chem. Co., Milwaukee, WI); paraformaldehyde (photo grade, Mallinkrodt, McGraw Park, IL); and 25% or 50% glutaraldehyde (ultrapure TEM grade, Tousimis, Rockville, MD).

The hydrochloride form of ethanolamine was prepared by the addition of HCl to ethanolamine to a pH 5-6. The solution was decolorized with activated charcoal. Aminoethyl cellulose was suspended in 1 M NaCl overnight, washed with 0.01 M NaOH to obtain the free base form, rinsed with water, washed with 0.2 M imidazole HCl (pH 7.2) until that pH was obtained, rinsed with water to remove the soluble salts and dryed over Drierite. Formaldehyde (10% solution) was prepared from a paraformaldehyde suspension by adjusting the pH to 8-9 with 5 M NaOH, heating it to 60°C, and readjusting the pH to 7 with 2 M HCl.

Oxygen uptake. A "Clark type" polarographic electrode was used to measure the initial O_2 uptake rates in model glutaraldehyde-amine reactions and in glutaraldehyde-homogenized tissue reactions. Dissolved O_2 was measured at 37°C with a YSI Model 50 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH). The glutaraldehyde-amine reactions were initiated by the addition of glutaraldehyde (final concentration of 1% or 0.1 M) to aerated and rapidly stirred amine solutions or to 10% aminoethyl cellulose suspensions in buffer A (0.075 M Na_2HPO_4, 0.025 M NaH_2PO_4, 0.20 M NaCl, pH 7.1) Dissolved O_2 was monitored before and after glutaraldehyde addition.

Quadriceps muscles from rat were homogenized in a Waring blender (2 minutes) using 9 parts of rat Ringers buffer to 1 part of muscle (w/v). The homogenate was further diluted 1:1 with additional rat Ringers buffer. After equilibrating the homogenate to temperature and thoroughly aerating it by vigorous stirring in the electrode cell, the rate of O_2 uptake of the homogenate was

measured in the absence and presence of glutaraldehyde as above. The measurements were repeated in the presence of cyanide. Tissue amine-glutaraldehyde reactions were initiated by the addition of 94 µl of 50% glutaraldehyde to 4.5 mls of homogenate.

Size exclusion chromatography. The glutaraldehyde-amine products were separated by gel filtration on a 1.33 x 48 cm column of Sephadex G-15 gel. The column was equilibrated with gel buffer (0.19 M acetic acid, 0.01 M sodium acetate, and 0.20 M NaCl, pH 3.4) prior to sample application, and developed with the gel buffer after the sample was applied. Column equilibration, sample application, and column development were accomplished with a Gilson minipuls peristaltic pump at 30 ml/h (21 cm/h). The absorbance of the column effluent was monitored at 254 nm with an ISCO UA-5 absorbance monitor. After 45-60 minutes of reaction, glutaraldehyde-amine product mixtures (37°C, 100% O_2 atmosphere) were diluted 10-fold with gel buffer and then 1.0 ml was applied to the gel.

The time course of the development of the UV and visible spectra of glutaraldehyde-ethanolamine reaction products (22°C, normal atmosphere) were recorded with a Hewlett Packard 8450 UV/VIS spectrophotometer with a scanning time of 1 second. The reactions were initiated by the addition of glutaraldehyde to the amine solution in buffer A (see above).

The UV spectra of the gel separated products were obtained in pH 3.4 gel buffer using a Gilford Model 250 spectrophotometer.

Borohydride reduction of glutaraldehyde-glycine products. After 45 minutes of reaction in buffer A, glutaraldehyde-glycine (0.20 M-0.05 M) products were adjusted to pH 9-10 with 5 M NaOH and reduced with $NaBH_4$ (final concentration of 0.17 M). After 10 minutes of reduction at 22°C, the solution was adjusted to pH 7 with 2 M HCl. The reduced products were separated by chromatography on Sephadex G-15 before examining them by UV spectroscopy.

Results

O_2 uptake in model glutaraldehyde-amine reactions. The following experiments were done to determine if the addition of glutaraldehyde to solutions of primary amines stimulates O_2 consuming reactions.

The addition of glutaraldehyde to a solution of ethanolamine, a primary amine, stimulated rapid depletion of dissolved O_2 (Fig. 3c and 3g). For example, dissolved O_2 has a half life of about 15 seconds (37°C) when glutaraldehyde (final concentration 1% or 0.1 M) is added to 50 mM ethanolamine (Fig. 3g).

Tissues contain soluble amines (e.g., free amino acids) as well as "solid state amines" such as amino lipids and proteins in membrane bilayers. Reactions of solid state amines and glutaraldehyde were studied to determine whether soluble amines and solid state amines would react similarly with glutaraldehyde. Aminoethyl cellulose was chosen as the model solid state

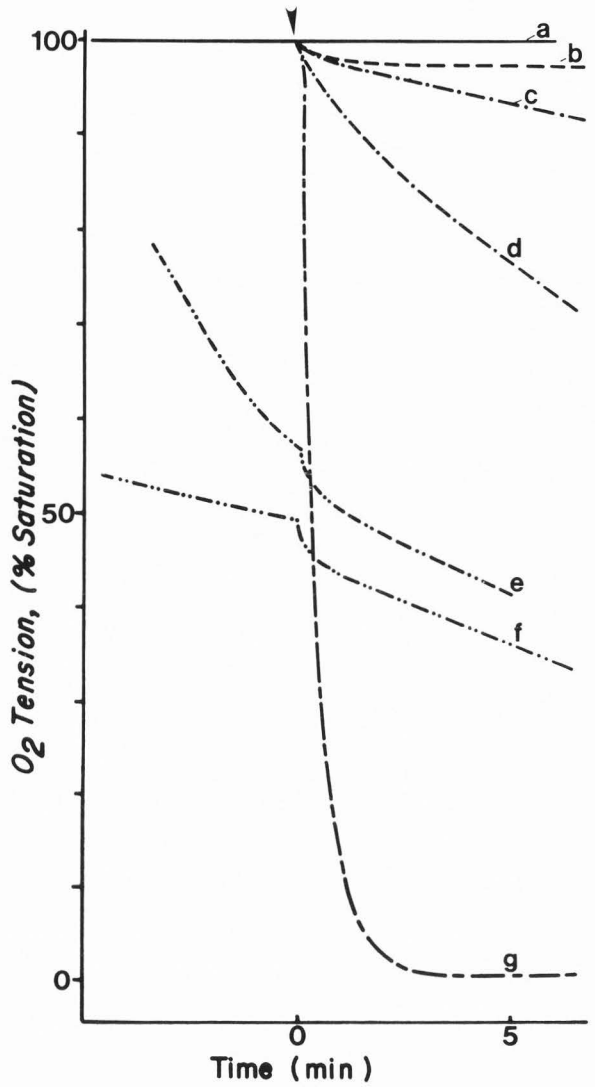


Figure 3. O_2 uptake kinetics. Glutaraldehyde-amine reactions (37°C) were initiated by the addition (arrowhead) of 120 µl of 25% glutaraldehyde (0.096 M or 0.96% final concentration) to an amine solution or a suspension of aminoethyl cellulose in 3.0 mls of buffer A. The initial concentration of dissolved O_2 in the buffered amine solutions was 0.175 mM, (curve a, no glutaraldehyde added). The effect of glutaraldehyde on the electrode is measured (curve b, no amine is added). O_2 uptake for 5 mM and 50 mM ethanolamine is illustrated (curves c and g). O_2 uptake for a 10% aminoethyl cellulose suspension is illustrated (curve d). The amine concentration in the suspension is about 27 mM. Similarly, the rate of O_2 uptake stimulated by the addition of glutaraldehyde to a homogenized muscle suspension (26°C) was monitored (curve e) and repeated in the presence of 4 mM cyanide (curve f).

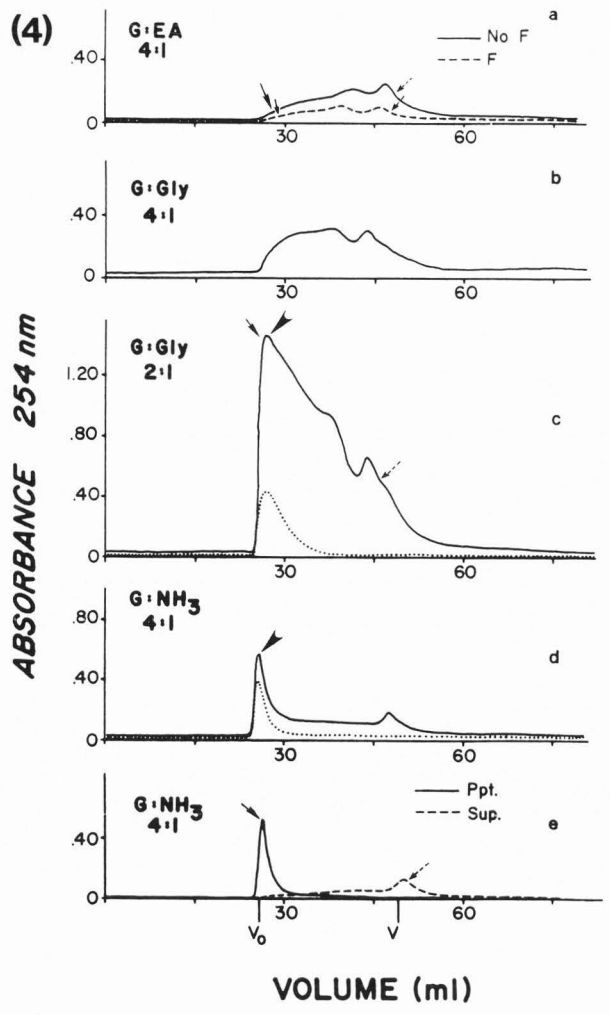


Figure 4. Size exclusion chromatography of glutaraldehyde-amine reaction products. The chromatograms in panels a, b, d and e were obtained by the application of glutaraldehyde (0.20 M, 2%)-amine (0.05 M) reaction products (37°C, 100 % O_2 atmosphere) to a Sephadex G-15 gel column. The reaction time is in parentheses in each panel description. In the chromatogram in 4c, the glycine concentration was 0.10 M.

4a. Ethanolamine products (60 min) in the absence (solid line) and in the presence of formaldehyde (dashed line). The concentration of formaldehyde in the reaction mixture was 0.33 M (1.0%).

4b. Glycine products (45 min).

4c. Glycine products (45 min). Excluded products (large arrowhead) were rechromatographed (dotted line).

4d. Ammonia products (60 min) (solid line). Excluded products (large arrowhead) were rechromatographed (dotted line).

4e. Separation of glutaraldehyde-ammonia products by precipitation. Some ammonia-glutaraldehyde products precipitate at pH 7. The initial precipitate was harvested by pelleting in a centrifuge, dissolved at pH 3, reprecipitated by adjusting the pH to 7, and again harvested by pelleting. This precipitate was dissolved in gel buffer with additional HCl to adjust the pH to 3. That material was chromatographed (solid line). The initial supernatant was also chromato-graphed (dashed line).

Key: G = glutaraldehyde; F = Formaldehyde; EA = ethanolamine; Gly = glycine; NH_3 = ammonia; V = column liquid volume; V_o = void volume.

amine because the ethanolamine moiety is present as a significant fraction of membrane lipids. Aminoethyl cellulose has one randomly distributed ethanolamine group for every ten glucose subunits on the cellulose backbone.

Addition of glutaraldehyde to suspensions of aminoethyl cellulose stimulated O_2 consumption (Fig. 3d). However, at equivalent amine concentrations, the rate of O_2 uptake by aminoethyl cellulose is an order of magnitude less than for its soluble analog, ethanolamine (Table 1). The extent of reaction of glutaraldehyde with the amino groups on aminoethyl cellulose was determined as follows: aminoethyl cellulose was exposed briefly (30 sec) to glutaraldehyde, followed by either fluorescamine (41) or o-phthalaldehyde (36). Glutaraldehyde blocked the reaction of all the amino groups of aminoethyl cellulose with these flourescent reagents (unpublished observations). Therefore, the rate difference between aminoethyl cellulose and ethanolamine was not attributed to the failure of the solid state amine groups to react with glutaraldehyde.

O_2 uptake in glutaraldehyde treated tissue suspensions. Respiration is likely the primary O_2 consuming process during the fixation of many tissues. The foregoing experiments suggested that the tissue amine-glutaraldehyde reactions would contribute significantly to the overall O_2 uptake during tissue fixation. Therefore, the rate O_2 uptake due to respiration was compared with that resulting from the reaction of tissue amines with glutaraldehyde.

As expected, respiration in homogenized muscle suspensions depleted dissolved O_2 at very high rates (Fig. 3). In less than one minute at 26°C, homogenized muscle consumed O_2 at a rate sufficient to deplete all of the dissolved O_2 in a volume of buffer equivalent to a volume of tissue (Table 1). Glutaraldehyde reduced the rate of O_2 uptake by a factor of 2-3 (Table 1). The depressed rate of O_2 uptake in the presence of glutaraldehyde was presumed to reflect the sum of the stimulated tissue amine-glutaraldehyde oxidative chemistry plus the residual respiration. The contribution of respiration to O_2 uptake was measured in the presence of sodium cyanide a respiratory inhibitor. In the presence of 4 mM cyanide, the respiratory O_2 consumption rate of homogenized muscle was decreased by 90%. The addition of glutaraldehyde stimulated the rate of O_2 uptake in the cyanide inhibited muscle suspension by more than 50% (Table 1).

The addition of 5 mM sodium cyanide to buffered ethanolamine solutions had no effect on the rate of O_2 uptake (unpublished observations). Thus, if the increment in O_2 uptake rate in the cyanide inhibited muscle suspension is assigned to tissue amine-glutaraldehyde oxidative

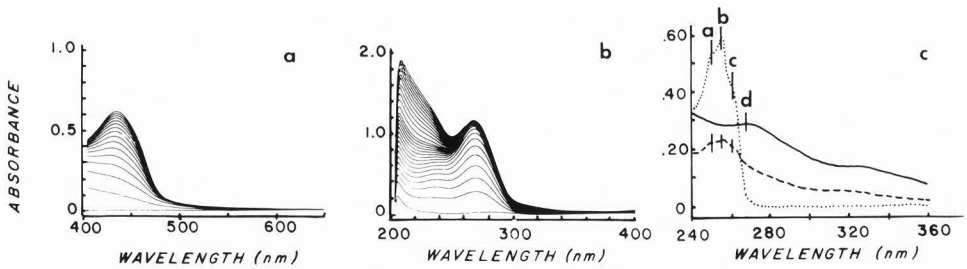


Figure 5. Ultraviolet and visible spectra of glutaraldehyde-amine products. In Figures 5a and 5b, the UV and visible spectra of glutaraldehyde-ethanolamine reaction products (22°C, normal atmosphere) were recorded. In Figure 5c the UV spectra of the glutaraldehyde-ammonia products were recorded.

5a. Visible spectra were recorded every 2 minutes for a total of 30 minutes, beginning 10 seconds after initiating the reaction (glutaraldehyde, 0.025 M [0.25%]; ethanolamine, 0.010 M).

5b. UV spectra were recorded every 2 minutes for the first 30 minutes of reaction, and every 5 minutes thereafter. The first spectrum was taken 10 seconds after initiating the reaction (glutaraldehyde, 0.005 M [0.05%]; ethanolamine, 0.002 M).

5c. The UV spectrum of the high molecular weight glutaraldehyde-amine products (dotted line) was obtained after 2 cycles of precipitation and gel filtration (solid arrow, Fig. 4e). The UV spectrum of the low molecular weight products (dashed line) was obtained after removal of the high molecular weight products by precipitation and gel filtration (broken arrow, Fig. 4e). The UV spectrum of reagent grade pyridine (10 µM) (dotted line) was compared to that of the low molecular weight products (solid line). The wavelengths at a, b, c, and d are 251, 256, 261, and 268 nm, respectively.

Table 1

O_2 Consumption Rates by Amines and Homogenized Muscle Suspensions after Glutaraldehyde Addition

Amine	Aldehyde[a]	Rate[b]
EA (25 mM)	G	9.7 ± 0.6
EA (12.5 mM)	G	2.0 ± 0.3
EA (5 mM)	G	0.28 ± 0.07
AEC[a]	G	0.15 ± 0.05
AEC[a]	G, F	0.017 ± 0.009
none	G	0.004
none	F	0.002
EA (25 mM)	none	0.003
HMS	none	130 ± 25
HMS	G	40 ± 12
HMS (CN^-)	none	15 ± 2.5
HMS (CN^-)	G	25 ± 3

Key: G = glutaraldehyde; F = formaldehyde; EA = ethanolamine; AEC = aminoethyl cellulose; HMS = homogenized muscle suspension; CN^- = cyanide.

[a]The aldehyde concentrations were: glutaraldehyde = 1% (0.10 M); formaldehyde = 0.6% (0.20 M). The amine concentration ≃ 27 mM (AEC suspension).

[b]For EA and AEC, The relative rates are % O_2 saturation/min/µeq/ml at 37°C. The relative rates for HMS are % O_2 saturation/min/g tissue/ml at 26°C. O_2 saturation levels for these experiments was ≃175-180 µM/ml.

pathways, then the O_2 uptake rate of these pathways is approximately 10% of the rate of respiration before glutaraldehyde addition.

O_2 uptake in mixed aldehydes. The addition of glutaraldehyde to a buffered aminoethyl cellulose suspension containing formaldehyde stimulated O_2 uptake at only 10% of the rate observed in the absence of formaldehyde (Table 1).

Size exclusion chromatography of glutaraldehyde-amine product mixtures. Glutaraldehyde-amine reaction products were heterogeneous as determined by size exclusion chromatography on Sephadex G-15 (Fig. 4). The ratio of high molecular weight to low molecular products varied with the amine (Fig. 4a, 4b, and 4d) and with the glutaraldehyde:amine ratio (Fig. 4b and 4c). In contrast to the random size distribution of the primary amine-glutaraldehyde reaction products, the ammonia-glutaraldehyde products exhibited a marked bimodal size distribution (compare Fig. 4d with Figs. 4a and 4b).

To determine whether the high molecular weight products were in equilibrium with the low molecular weight products, the excluded, high molecular weight fractions of the glycine-glutaraldehyde and the ammonia-glutaraldehyde reaction products (arrows, Figs. 4c and 4d) were rechromatographed on Sephadex G-15. Single peaks of excluded material were obtained in each case (dotted lines, Figs. 4c and 4d). Therefore, the formation of high molecular weight products was irreversible.

Some of the ammonia-glutaraldehyde reaction products precipitated 1-2 minutes after the reaction was initiated. Precipitation preceded

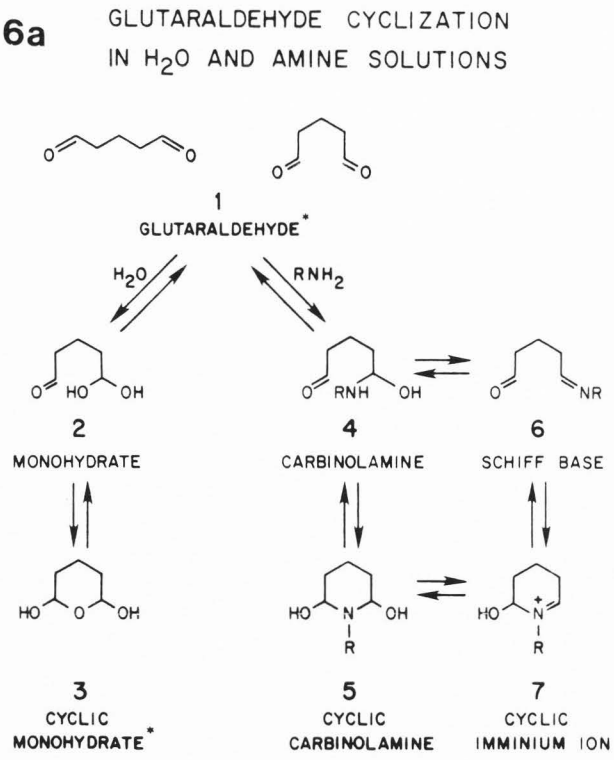
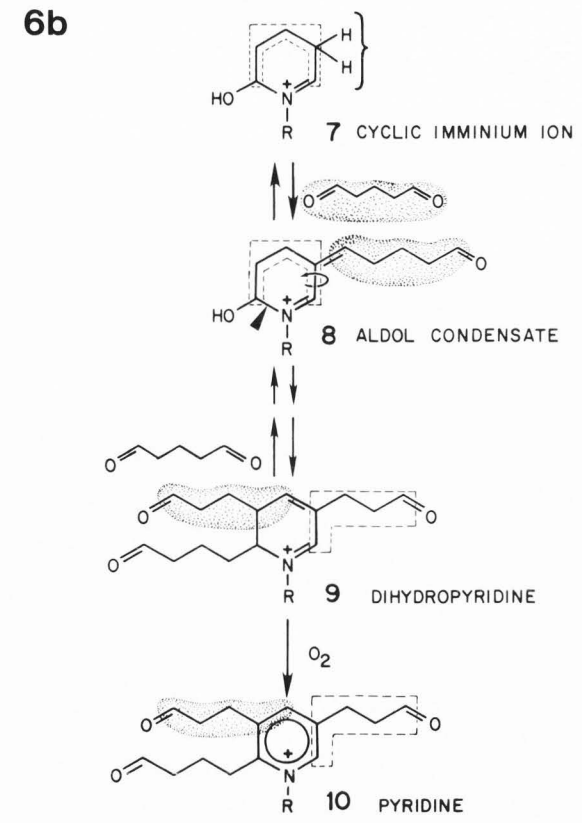


Figure 6. A scheme for the synthesis of pyridines from glutaraldehyde-amine precursors.

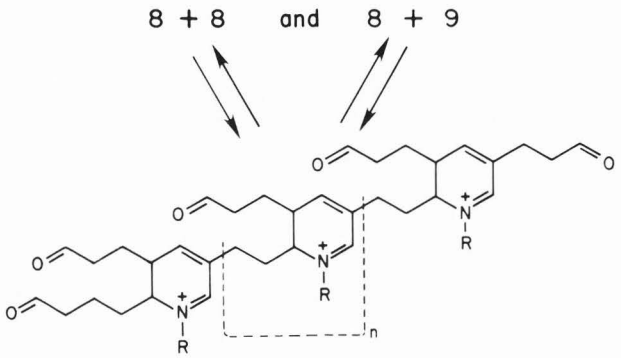
6a. Glutaraldehyde cyclization reactions with water and primary amines. In water glutaraldehyde readily cyclizes (44). The cyclic structures represented by 3 (boat and chair forms) account for more than 80% of aqueous glutaraldehyde (44). The open chain forms of glutaraldehyde account for virtually all of the remaining glutaraldehyde in water. These forms include glutaraldehyde, 1, the monohydrate 2, and the dihydrate, not shown (44). This scheme assumes that in the presence of primary amines, glutaraldehyde molecules cyclize rapidly, as well, generating a key intermediate, the cyclic imminium ion, 7.



6b. Low molecular weight pyridine products, 10. The formal positive charge on 7 permits easy abstraction of a proton (brackets) from the ß-carbon atom of 7. Thus, carbanion formation, which usually is a rate limiting step in condensation reactions, is enhanced (11). (The original glutaraldehyde molecule in the following condensation sequence is outlined by a dashed line.) The resulting carbanion condenses with the carbonyl group of a second glutaraldehyde molecule (stipled) yielding the aldol condensate, 8. A third glutaraldehyde molecule condenses with 8 after a series of steps including heterocyclic ring opening (arrowhead) and rotation around the indicated C-C bond yielding the dihydropyridine, 9. The carbon atoms of the central ring of 9 are derived from portions of 3 glutaraldehyde molecules which contribute 2, 2, and 1 C atoms, respectively, as they combine to form the heterocyclic ring. Oxidation rapidly and irreversibly converts 9 to 10.

6c. Polypyridine products, 12. Dihydropyridine polymers, 11, can be produced if the third glutaraldehyde molecule in the condensation sequence is replaced by any molecule which has a single aldehyde function such as species 8, 9, and 10. Polypyridines, 12, are afforded by the oxidation of 11 as in the 9 → 10 conversion. To the extent that the conversion of 8 to 9 is the rate limiting step of the overall process, the concentration of 8 should increase relative to other intermediates. Polymer formation should, therefore, be favored as the ratio of 8 to free glutaraldehyde increases, since the reactions leading to products 9 and 11 are competitive. Thus, high initial glutaraldehyde concentrations should favor low molecular weight products such as 10, while lower glutaraldehyde concentrations would favor higher molecular weight products (compare Figs. 4b and 4c). The pyridine polymers are polyaldehydes whose ionic character is defined by the R sidechain. If R is uncharged (i.e., ethanolamine), the polymer is a polycation. If the R is negative (i.e., glycine), the polymer is a polyzwitterion. R is a dissociable proton in the ammonia-glutaraldehyde products. Thus, at a pH below the pK_a of the pyridine moieties, the ammonia polymer is a polycation; while at a pH significantly above the pK_a, the ammonia polymer is uncharged. This property was exploited in Fig. 4e where high and low molecular weight products were separated by a pH dependent precipitation.

6c CROSSLINKING BY FORMATION OF PYRIDINE POLYMERS



11
"DIHYDROPYRIDINE POLYMERS"

O_2

12
"PYRIDINE POLYMERS"

significant uptake of O_2. The product mixture was soluble at pH < 3.0.[2] A fraction of the acid soluble products was reprecipitated by readjusting the pH to > 7.0. The ammonia-glutaraldehyde products were separated into high and low molecular weight fractions by the pH dependent, reversible precipitation (Fig. 4e). The precipitate consisted of high molecular weight products (Fig. 4e).

Mixed aldehyde-amine product mixtures. The addition of formaldehyde to the ethanolamine-glutaraldehyde reaction depressed the yield of 254 nm absorbing products (dashed line, Fig. 4a). Thus, formaldehyde inhibited the formation of the normal products of ethanolamine-glutaraldehyde reactions.

UV and visible spectra. The UV and visible spectra of an ethanolamine-glutaraldehyde reaction product mixture are illustrated as a function of the reaction time. In the visible regions of the spectrum, an absorbance maximum (λmax) of 434 nm developed (Fig. 5a). In the UV a λmax of 267-268 nm developed rapidly which replaced the initial, weak 280 nm λmax of glutaraldehyde (Fig. 5b).

Since the amine-glutaraldehyde products were heterogeneous with respect to molecular size (Fig. 4), the relative contributions of the high and low molecular weight products to the composite spectra of Fig. 5b were determined. The ammonia-glutaraldehyde products were separated on the basis of molecular size by a reversible, pH dependent precipitation (Fig. 4e). The UV absorbance spectra of the higher molecular weight ammonia-glutaraldehyde products (solid arrow, Fig. 4e) and the lower molecular weight products (dotted arrow, Fig. 4e) were compared in Fig. 5c. The fine structure around 256 nm was absent in

Table 2

UV Maxima of Glutaraldehyde-Amine Products

	λmax nm[a]	
Amine	V	V_o
EA	267 (300)	270
EA (F)	267 (300)	273
Gly	262 (300)	272 (330-340)
Gly (BH_4^-)[b]	- (302)	275, 300, 335
NH_3[c]	256 (320)	268 (330)

Key: G = glutaraldehyde; F = formaldehyde; EA = ethanolamine; Gly = glycine; BH_4 = borohydride; V = column liquid volume; V_o = void volume.

[a]The UV data were obtained after separating the glutaraldehyde-amine reaction products on Sephadex G-15 (Fig. 4). The UV maxima for the low molecular weight products (dashed arrow, Fig. 4a, 4c, and 4e) are listed under V. The UV maxima for the higher molecular weight products or excluded fractions (solid arrow, Fig. 4a, 4c, and 4e) are listed under V_o.

[b]Glutaraldehyde-glycine products were reduced with borohydride (BH_4^-). The reduced products were chromatographed as in Fig. 4.

[c]Data from Fig. 5.

the higher molecular weight products and the λmax was shifted 12 nm to the red (to 268 nm). The UV spectra of the low molecular weight ammonia-glutaraldehyde products was also compared to that for reagent grade pyridine (Fig. 5c). For the low molecular weight material the λmax = 256 nm and the secondary structure on either side of the λmax at 251 and 261 nm simulate that seen for pyridine. In addition, the ammonia-glutaraldehyde products have UV chromophores absorbing beyond 270 nm.

For the other amine-glutaraldehyde reaction products, the composite product spectra were simplified by separating the reaction products by gel filtration (Fig. 4). The high molecular weight products eluting at the excluded volume, V_o, had a λmax that was red shifted 3-10 nm when compared to the low molecular weight products, which eluted at the column liquid volume (Table 2). The fine structure found near the λmax for the low molecular weight products was absent in the spectra of the larger products.

Sodium borohydride reduction eliminated the 262 nm chromophore of the low molecular weight glycine-glutaraldehyde products (Table 2). A less strongly absorbing chromophore with a λmax of 302 nm remained. The UV spectra and the destruction of the 262 nm chromophore by sodium borohydride are properties shared with the highly substituted pyridine compounds prepared by Hardy et al. (9,14).

Discussion

Pyridine derivatives from glutaraldehyde and amine precursors: a scheme for low molecular weight pyridines. A scheme by which pyridine derivatives are produced from glutaraldehyde-amine precursors is proposed. This scheme is based upon the cyclization of the α,w-dialdehyde with primary amines (Fig. 6a) and the subsequent condensation reactions with two additional glutaraldehyde molecules yielding dihydropyridines, 9 (Fig. 6b). Pyridine derivatives, 10, are afforded by the oxidation of the dihydropyridines, 9. Analogs of the dihydropyridines, 9, and pyridines, 10, are well documented products of amine-aldehyde reactions (5,7,9,14,30,40). The conversion of the dihydropyridines, 9, to the pyridines, 10, is accomplished by a facile oxidation in the presence of O_2, I_2, Ag^+ (9), or Cu^{++} (7). There is, in addition, ample precedent for each of the intermediates proposed in the mechanism except for 8.

The initial cyclization of glutaraldehyde with amines is analogous to reactions of glutaraldehyde with water (1, 2, 3, Fig. 6a). A key intermediate in this scheme, the cyclic imminium ion 7, has been trapped with sodium cyanoborohydride and recovered in good yield as N-methylpiperidine from the reaction of methylamine with glutaraldehyde (1). Many N-substituted piperidines have been prepared by reductive amination of glutaraldehyde (43). The formal positive charge on the cyclic imminium ion, 7, facilitates the abstraction of a proton from the ß-carbon atom of 7 (Fig. 6a). The newly formed carbanion condenses with a second glutaraldehyde molecule to yield the aldol condensate, 8 (11). A third glutaraldehyde molecule is required in the condensation scheme to obtain dihydropyridines, 9.

In the 2-2-1 condensation scheme depicted in Fig. 6, three glutaraldehyde molecules contribute 2,2, and 1 carbon atoms, respectively, to the 1,2,3,5 substituted pyridine ring (5). A 2-1-2 scheme yielding 1,3,4,5 substituted pyridines is also possible (not shown, but see 5,14). Substituted pyridines have also been obtained from the cyclic imminium ion by pathways other than those in Fig. 6, including n-alkylpyridines which are afforded by dehydration and oxidation of the cyclic imminium ion, 7 (Fig. 7a) (9). In the absence of O_2, pyridines and tetrahydropyridines have been produced from dihydropyridines by disproportionation (Fig. 7b) (7).

A scheme for pyridine polymers. The scheme depicted for obtaining the pyridine derivatives, 10, does not provide for crosslinking. A scheme for the synthesis of pyridine polymers is illustrated in Fig. 6c. Since only one aldehydic function is required for the third glutaraldehyde molecule to participate in the condensation reaction 8 → 9, any molecule having an aldehydic function may participate. Thus, pyridine precursors condense with each other yielding polydihydropyridines, 11, which when oxidized yield polypyridines, 12. Polymer growth is favored under conditions where there is a high probability that intermediates such as 8 will react with each other, that is, when glutaraldehyde is limiting. If excess glutaraldehyde is present, conditions for forming pyridine monomers are favored.

Linear polymers, in which the repeating unit contains 2 glutaraldehyde molecules per amine, are depicted for convenience. However, the aldehyde sidechains allow branched polymer growth. The branched pyridine polymer network is an attractive candidate to be the major crosslinker in glutaraldehyde fixed tissue.

A proposal for tissue crosslinking. For the following discussion, tissue primary amines are segregated into two categories: "soluble" amines, such as amino acids, and "solid state" or insolubilized amines, such as the aminolipids and proteins in membranes or other organelles. In tissue fixation, glutaraldehyde and soluble primary amines provide the necessary precursors for the pyridine polymer bridges between "solid state" amines (Fig. 8). These reactions may account for the nonspecific binding of radiolabelled primary amines such as amino acids in autoradiography experiments using glutaraldehyde fixatives (18).

The pyridine polymer network is capable of crosslinking erratically spaced "solid state" primary amines (Fig. 8b). The extent and rate of tissue crosslinking by reactions based on this model depends upon the availability and ratio of polymer precursors (Fig. 8b). In the absence of sufficient amine precursors, slower crosslinking reactions may occur via aldol condensation between the aldehyde sidechains. These slower reactions may also explain secondary hardening of tissues which are stored in glutaraldehyde.

This scheme for pyridine and pyridine polymer formation contains four relevant features for the process of glutaraldehyde fixation of biological tissues: 1) Acid (19) is an unavoidable side product. 2) Pyridines, 10 and 12, are afforded from their immediate precursors by an irreversible oxidation step. This step competes with respiration for available O_2. 3) Rapid tissue hardening or crosslinking may be accomplished through multiple, interconnected linkages provided by the branched pyridine polymers, 12 (Figs. 6 and 8). These polymers are capable of spanning variable distances between the erratically spaced primary amino groups of tissues (Figs. 6c and 8b). 4) The extent of polymerization or crosslinking in cells should vary according to the soluble amine concentration or the glutaraldehyde/amine ratio.

Evidence for pyridine products of glutaraldehyde-amine reactions. Glutaraldehyde-collagen reactions were characterized by increased adsorption at 265 nm accompanied by losses of lysine (2). Hardy and coworkers (12,14,15) provided the first evidence that glutaraldehyde-primary amine reaction products were mixtures of substituted pyridines (desmosine analogs) and pyridine polymers. The products which Hardy et al. described in these reports were isolated from glutaraldehyde-amine reactions at pH 6.0 and 9.3. Related compounds were later

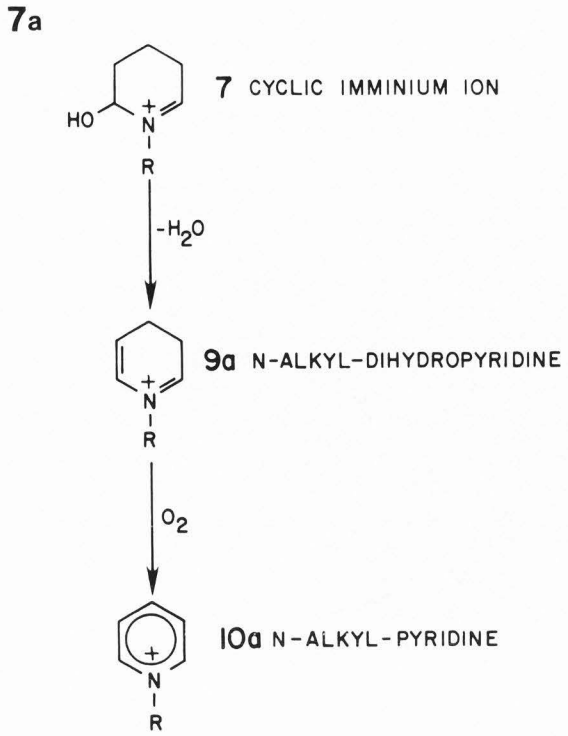


Figure 7. Alternate routes of pyridine synthesis. 7a. Dehydration and oxidation. Pyridines, 10a, may be obtained by dehydration of the cyclic imminium ion, 7, and oxidation of the resulting dihydropyridine, 9a (9).

7b. Disproportionation. In the absence of oxidizing agents, dihydropyridines, 9, can oxidize and reduce each other yielding pyridines, 10, and tetrahydropyridines 13a, and 13b (7).

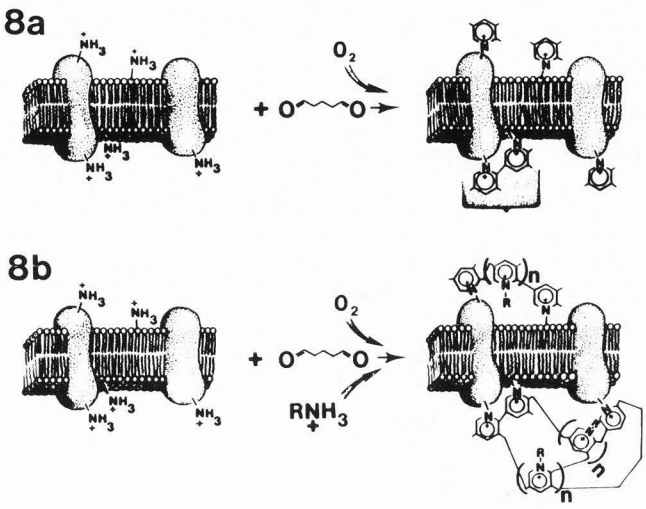
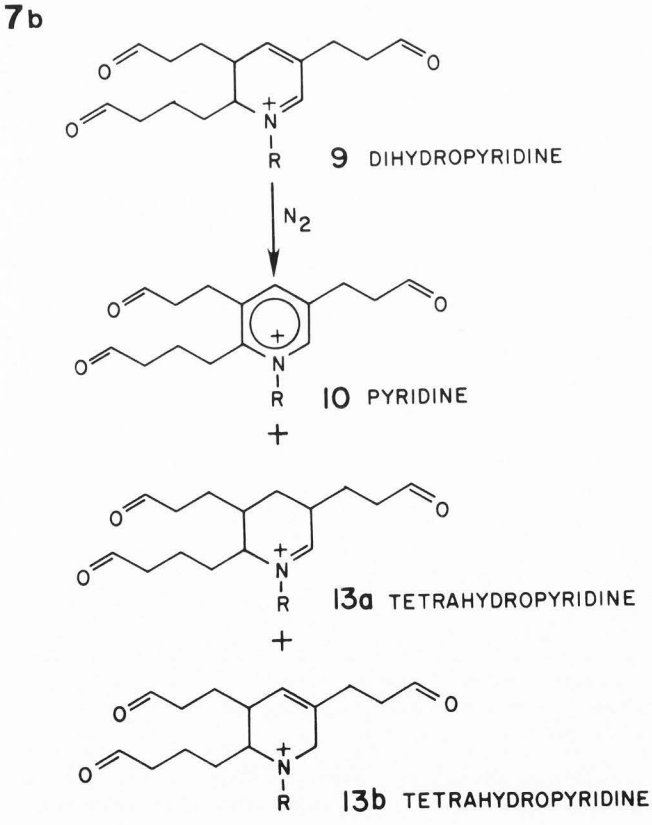


Figure 8. A model for tissue fixation based upon polypyridine crosslinking. Glutaraldehyde-tissue amine reactions are considered in two parts: a) glutaraldehyde-"solid state amine" reactions and b) glutaraldehyde-soluble amine reactions. For this model solid state amines are considered to have restricted mobility. These include membrane amino lipids and membrane proteins. If solid state amines react with glutaraldehyde in the absence of soluble amines, occasional crosslinks can occur between the solid state amines when they are sufficiently close to permit the linkage of two pyridine moieities (stipled bracket), (panel a). Soluble amines permit crosslinking of more distant solid state tissue amines via complex polypyridine networks (panel b). Therefore, for rapid tissue fixation or crosslinking, solid state amines, soluble amines, and glutaraldehyde are all necessary precursors.



isolated from acid hydrolysates of ovalbumin which had reacted with 12.5% glutaraldehyde in the presence of ammonium acetate buffer (pH 4.7) (12,15). The latter compounds were thought to be the actual crosslinks between protein molecules reacted with glutaraldehyde. The glutaraldehyde-amine products had a borohydride sensitive chromophore with an absorbance maximum at 266 nm (2) and appeared to be polyzwitterions from their chromatographic behavior on SE-Sephadex and DEAE-Sephadex. However, Hardy et al. (14) did not claim that pure products were isolated. Therefore, final characterization remains incomplete. The products described in this paper have a borohydride sensitive chromophore in the 265 nm range and appear to be related to pyridine (Fig. 5 and Table 2).

Polymer studies. It is noteworthy that Hardy et al.(14) found that for reactions of N-acetyllysine with glutaraldehyde, the absorbance at 266 nm, which is attributed to the pyridinium chromophore, increased with the glutaraldehyde:amine ratio to a maximum when the ratio was 2:1. The repeating unit of the pyridine polymer has 2 glutaraldehyde molecules per amine (12, Fig. 6c).

A complex product spectrum was produced by primary amine-glutaraldehyde reactions (Fig. 4). The molecular size of the products was dependent upon the specific amine reacting with glutaraldehyde and upon the glutaraldehyde:amine ratio (Fig. 4). The large increase in the higher molecular weight products observed for the change in the glutaraldehyde:amine ratio from 4:1 to 2:1 (Figs 4b and 4c) lends support to the mechanism illustrated in Fig. 6c for the irreversible production of polymers. These experiments suggest that the extent of crosslinking in tissue during fixation may be manipulated by changing the glutaraldehyde concentration in the fixative. Also, since various tissues have different amine concentrations, the concentration of glutaraldehyde used in fixatives should be varied to suit the tissue.

The dependence of the amine-glutaraldehyde product size upon the specific amine was not unexpected since the ammonia polymer is uncharged (at pH $\geq$ 7.0), the glycine polymer is polyzwitterionic, and the ethanolamine polymer is polycationic (see the R group of structure 12, Fig. 6c). The addition of uncharged subunits to neutral polymers (e.g., ammonia products) requires far less chemical work than the addition of positively charged subunits to a polycation (e.g., ethanolamine products). A similar comparison can be made for glycine and ethanolamine products. The ethanolamine products were found to be smaller than the glycine or the ammonia products (Fig. 4).

Amine concentrations in tissue: tissue crosslinking. The concentration of free amines in brain tissue (8) even after exposure to glutaraldehyde and formaldehyde is sufficient to support the proposed polymerization scheme. In aldehyde treated brain tissue, the concentration of glutamate drops initially, but then remains constant at 10 mM (8). Presumably, transaminase activity continuously regenerates the amino acid from its cognate keto acid as the amino acid reacts with glutaraldehyde (22). The total free amine concentration in brain tissue during the aldehyde exposure may be as high as 35 mM (8). As the cellular primary amine content varies from cell to cell, one might expect that the rate and extent of crosslinking would vary.

Oxygen consuming glutaraldehyde-amine reactions. The stoichiometry of the oxidation step is: 9 + 1/2 O_2 $\rightarrow$ 10 + H_2O (Figs. 6a and 6b). Model primary amine-glutaraldehyde reactions consume O_2 very rapidly (Fig. 3). There is sufficient O_2 uptake to account for the stoichiometric conversion of amines to pyridines (unpublished observations). These results support the mechanism presented in Fig. 6 and strongly suggest that O_2 consuming pathways are the major reaction routes in glutaraldehyde-primary amine reactions. The O_2 consuming pathways (Figs. 6 and 7a) predominate kinetically over other pathways such as disproportionation (Fig. 7b) (7), at least when O_2 is available.

"Solid state" tissue models. O_2 uptake was stimulated when glutaraldehyde was added to a suspension of aminoethyl cellulose. When compared to its soluble analog, ethanolamine, the rate of O_2 uptake by aminoethyl cellulose was far less (Table 1). This experiment suggests that tissue primary amines with restricted mobility can be expected to participate in O_2 consuming reactions with glutaraldehyde, but that their limited mobility significantly reduces the rate of O_2 uptake.

Tissue suspensions. The experiments with homogenized muscle suspensions suggested that two oxygen consuming processes occur during glutaraldehyde fixation (Fig. 3 and Table 1). Respiration and the chemically induced O_2 uptake compete for a limited supply of O_2. While the effect of this on the polymerization process is not very dramatic (unpublished observations), the effects of hypoxia on ultrastructure should be explored more thoroughly.

Role of tissue enzymes which oxidize aldehydes. The increase in the rate of O_2 uptake induced by the addition of glutaraldehyde to cyanide-inhibited muscle suspensions was attributed to the oxidation of tissue amine-glutaraldehyde reaction product intermediates. This assignment was based upon the assumption that the nicotinamide-containing aldehyde dehydrogenases and metallo-flavin aldehyde oxidases did not contribute to the O_2 uptake rate in the presence of cyanide (3,10,25,29,38,39).

Function of fixative additives. In the past microscopists have added additional reagents to glutaraldehyde solutions, thereby attempting to improve the quality of tissue fixation. The biochemical effects of these additives are poorly understood. The results presented in this paper suggest that some of these additives may function by relieving hypoxia. Oxygen is then available for either maintaining ultrastructure and/or the crosslinking chemistry. Some effects of hypoxia on morphology include mitochondrial swelling (27,34), caveolae changes (24), and, perhaps the disruption of supramolecular structure (23). Van Harreveld (42) described a rapid loss of extracellular material in mouse cerebellum after O_2 deprivation. Unfortunately, a systematic analysis of morphological changes induced by hypoxia is not available.

Glutaraldehyde fixation protocols which increase available O_2.

Glutaraldehyde plus oxygenation. Available O_2 may be increased by continuously aerating fixation buffers with 100% O_2 (or with 95% O_2, 5% CO_2 when bicarbonate-based buffers such as Ringers solution are used). An oxygenation period of 15 minutes is recommended based upon the time it takes to reduce the rate of O_2 uptake in model amine-glutaraldehyde reactions to less than 10% of the initial rapid rate (unpublished observations).

Glutaraldehyde plus hydrogen peroxide. Perrachia and Mittler used a mixture of 25-130 mM hydrogen peroxide (H_2O_2) and 3-6% glutaraldehyde to improve tissue preservation by immersion fixation (32).

Glutaraldehyde plus electron transport inhibitors. Higher oxygen tension within tissue may be obtained by inhibiting respiration. Minassian and Huang (27) have added sodium azide to glutaraldehyde based fixative solutions. Azide inhibits the transfer of electrons to O_2 at cytochrome aa_3 in the mitochondrial electron transport chain. They reported significant improvement in tissue preservation deep within immersion fixed tissue (up to several mm). The chemical uptake of O_2 in amine-glutaraldehyde reactions is not affected by azide or cyanide (unpublished observations).

Mixed aldehyde fixatives. It has been suggested that the role of formaldehyde in mixed formaldehyde/glutaraldehyde fixatives (20) is to rapidly penetrate and fix tissue (18). While the reactions of formaldehyde with tissues are recognized to be at least partially reversible, it has been proposed that formaldehyde may temporarily fix tissues until the slower penetrating and reacting glutaraldehyde irreversibly crosslinks the tissue (18). However, the present evidence suggests that formaldehyde may function in mixed formaldehyde-glutaraldehyde fixatives via modes not previously described. Formaldehyde alters the primary amine-glutaraldehyde chemistry by significantly decreasing O_2 uptake (Table 1) and by altering the yield of UV absorbing glutaraldehyde-amine products (Fig. 4). Although formaldehyde lacks the active α-hydrogen atoms required for carbanion formation in the aldol condensation process, (11), formaldehyde has the requisite carbonyl group which can react with the transient carbanion intermediates of the condensation reaction scheme illustrated in Fig. 6. The preliminary data presented here suggests that formaldehyde participates in these condensation reactions and changes the ultimate reaction products. In addition, the data show that part of the effect of formaldehyde in the mixed aldehyde fixative may be to reduce tissue hypoxia. Obviously, the chemical role of formaldehyde in glutaraldehyde based fixatives is not established. It will be of much interest to determine whether the "improved" tissue fixation sometimes claimed for formaldehyde-glutaraldehyde fixative is a result of decreased O_2 uptake and/or of the different products formed.

Buffers. Buffers used in aldehyde fixation must have sufficient buffering capacity to minimize pH decreases resulting from the rapid adduct formation between aldehydes and amines. Acid production results from the fact that the pK_a's of the parent amines are generally 2-3 pH units higher than that of the newly formed adducts (19). In order to minimize the pH decrease, the buffering species chosen for fixation should ideally have a pK_a of 0.2-0.3 pK_a units less than the pH at which fixation is to occur. The concentration of the buffering species should be 2-3X greater than the concentration of the primary amines exposed to glutaraldeyde. Finally, the buffering species should not have amino functions which will interact with the aldehyde functions. The buffering species should also not bind divalent cations. Some of the Good's buffers, such as MOPS and HEPES, which have pK_a's in the range of 7.0-7.3 (37°C) offer these desirable charcteristics.

Laboratory safety. Excess glutaraldehyde from experimental protocols or glutaraldehyde spills may be quickly and inexpensively controlled by adding solid glycine. Alternatively, a wash bottle containing 1 M glycine may be kept on hand to spray on the glutaraldehyde. One volume of the 1 M glycine will effectively and rapidly render an equivalent volume of 50% glutaraldehyde (or lesser concentrations) non-volatile. Ammonium salts are effective also.

Conclusions

Knowledge of the chemistry of the fixation processes intervening between the living tissue and the micrograph record is scant. This paper provides a framework for the biochemical understanding of the mechanism of glutaraldehyde fixation of tissues based upon the formation of pyridine monomers and polymers. It also supplies new data concerning O_2 uptake occurring in fixation processes as glutaraldehyde and amines react under conditions commonly used for fixation. These results suggest that the time course and extent of fixation may be altered by changing the concentrations of glutaraldehyde, primary amines, and oxidizing agents. These results may help interpret: 1) hypoxia-dependent changes in mitochondria (27,34); 2) intramembrane particle aggregation and dispersal (23); 3) artifacts of lipid mobility including membrane blistering, fusion, and stretching (4,6,16); and 4) caveolae changes in glutaraldehyde fixed tissue (24). These data have ramifications for preservation of biological samples for light microscopy, transmission and scanning electron microscopy, and freeze-fracture.

Acknowledgements

I thank Dennis Giddings for his illustrations, Theresa Borders for help in preparing the manuscript, and Barbara Johnson and John Rash for their comments. Financial support was provided by NIH grant NS15991 (to J. Rash and T. Johnson).

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