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Glutaraldehyde Nonfixation of Isolated Viral and Yeast RNAs[1]

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The RNAs of brome mosaic (BMV), barley stripe mosaic (BSMV), and tobacco mosaic (TMV) viruses were inactivated by reaction with buffered glutaraldehyde. Glutaraldehyde did not fix 4% BMV-RNA, 20% t-RNA, 5% polyadenylic acid, or 5% adenosine monophosphate into water-insoluble precipitates, or gels, in distilled water or in low or high ionic strength buffers nor did it change their ultraviolet (UV) spectra. Two SDS- and phenol-purified commercial yeast RNA preparations from different sources gave UV spectra typical of pure RNA, but could not be freed of a contaminant that reacted with glutaraldehyde by forming a precipitate. The yeast RNAs did not become water-insoluble after glutaraldehyde reaction. BMV-RNA precipitated by Mg^{2+} could not be cross-linked into an insoluble form by glutaraldehyde. Nonfixation of RNA by glutaraldehyde must be considered in interpretation of attempts to localize RNA by electron microscopy. KEY WORDS: Ribonucleic acid; Glutaraldehyde fixation; Electron microscopy; Viral RNA.

Introduction

Except for Hopwood's (1975) investigations of the reactions between glutaraldehyde and nucleic acids, not much is known of the effect of glutaraldehyde on nucleic acids. This lack of knowledge is surprising in view of the large body of information from electron micrographs of glutaraldehyde-fixed tissue. Glutaraldehyde has been widely used as the primary fixative in electron microscopy since its introduction in 1963 (Sabatini et al., 1963). Hopwood (1975) concluded that glutaraldehyde reacted with nucleic acids only when the temperature was raised to their melting points, but that even at high temperature the glutaraldehyde did not fix nucleic acids.

Reactions of formaldehyde and nucleic acids are somewhat better known. Staehelin (1958) showed that most amino groups in tobacco mosaic virus (TMV)-RNA were accessible to reaction with formaldehyde. In model RNA fixations he found that polyadenylic and polycytidylic acid reacted readily. Others (Stevens and Rosenfeld, 1966) reported that nearly all (90%) of the adenosine monophosphate groups in polyadenylic acid reacted with formaldehyde. Lindigkeit and Eckel (1962) showed that reaction of formaldehyde with RNA in the presence of Mg^{2+} produced no hyperchromic changes. Glutaraldehyde should also react with exposed bases in single-stranded portions of nucleic acids and possibly even cross-link nucleic acids when these occur in high concentration. Glutaraldehyde reaction with nucleic acid has been shown indirectly in the bismuth staining reaction of nucleoproteins (Brown and Locke, 1978). Bismuth apparently binds to nucleoproteins through amino and phosphate groups. Glutaraldehyde fixation blocked nucleolar staining.

I have investigated glutaraldehyde-nucleic acid interactions because of their potential importance in fixing tissues for electron microscopic ultrastructure analysis. A preliminary report has been presented elsewhere (Langenberg, 1979b).

Materials and Methods

Plant viruses. The following plant viruses were used: brome mosaic virus [BMV, type strain, American Type Culture Collection (ATCC) #PV47], barley stripe mosaic virus (BSMV, type strain, ATCC #PV43), and tobacco mosaic virus (TMV, common strain, ATCC #PV135). The BMV and BSMV were maintained in barley (*Hordeum vulgare* L. cv. Larker) and TMV was maintained in tobacco (*Nicotiana tabaccum* L. cv. Xanthi).

Virus purification. All plants were grown in a greenhouse. Virus-infected plants were ground in a Waring Blendor in 0.1 M dibasic ammonium citrate (pH 6.0 with solid KOH) containing 1% polyvinylpyrrolidone and 1% sodium dithionite. One to 3 ml of

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buffer was used per gram of diseased tissue. Plant sap was expressed through muslin and clarified by a low-speed centrifugation (10,000 rpm for 10 min in an SS 34 rotor of a Sorvall RC-2B centrifuge). Triton X-100 was added to the supernatant to 0.25%. The clarified sap was centrifuged at high speed (28,000 rpm for 90–180 min in a #30 rotor of a Spinco L-1 centrifuge). Pellets were dissolved in a few ml of 0.1 M ammonium citrate, pH 6.0, and insolubles were removed by low-speed centrifugation. The clarified and concentrated viral solution was then layered on 20% sucrose (8–10 ml) in distilled water or 0.01 M ammonium citrate, pH 6.0, in bottles of the Spinco #30 rotor and centrifuged again at high speed. Pellets were dissolved in distilled water or directly in a few ml of disruption buffer: 0.2 M ammonium carbonate, 0.3 M ammonium chloride, and 1% SDS (sodium dodecyl sulfate), pH 9.5, with NaOH.

Viral RNA extraction. Pellets (± 15 mg) of BMV, BSMV, and TMV were dissolved in 2–4 ml of disruption buffer. The solution was vortex stirred and extracted with an equal volume of water-saturated phenol. The emulsion was broken by low-speed centrifugation. The supernatant was removed, divided into two equal parts, and brought to 70% ethanol by addition of cold 100% ethanol. RNA was precipitated by placing the solution for 1 hr at −10°C. RNA was collected by low-speed centrifugation and the pellet was washed one or more times with cold 70% ethanol to remove phenol.

RNA inactivation by glutaraldehyde. Infectious viral RNA was dissolved in 1 ml of 0.1 M sodium borate, pH 9.2. A 1-ml solution of 1% glutaraldehyde (Sigma, St. Louis, Mo.) in the same buffer (at 25°C) was added and the preparation was vortex mixed. Five minutes later cold 100% ethanol was added to 70% and RNA was precipitated by incubating the mixture for 1 hr at −10°C. Precipitated RNA was collected with a low-speed centrifugation. The pellet was washed once with cold 70% ethanol, drained well, and dissolved in 1 ml of 0.1 M glycine, pH 9.1, containing 200 μg of bentonite and 1% celite (Brakke, 1972). The BSMV- and BMV-RNAs were inoculated onto young barley plants with cotton swabs and TMV-RNA was inoculated onto expanded leaves of "Glurk" tobacco, a local lesion host for TMV. Plants were rinsed with tap water immediately after inoculation.

Yeast RNA-glutaraldehyde reactions. *Torula* yeast RNA (Type VI, Sigma) and BDH yeast RNA (14% N, 8% P, Gallard-Schlesinger, N.Y.) were dissolved in disruption buffer and, after dissolution, extracted with an equal amount of water-saturated phenol. The emulsion was broken with a low-speed centrifugation and the water phase was extracted with an equal volume of phenol. A third or fourth phenol extraction was usually necessary to remove most of the colored impurities and leave the supernatant clear light-tan or amber. The RNA was precipitated by addition of 2 volumes of cold 100% ethanol to the water phase and incubation at −10°C for 1 hr. Precipitated RNA was pelleted with a low-speed centrifugation; the pellet was washed once with cold 70% ethanol, thoroughly drained, and dissolved in a small volume of sterile distilled water; 0.1 M cacodylate, pH 7.2; or 0.1 M sodium borate, pH 9.2. The concentration was determined by ultraviolet (UV) absorption of aliquots at 260 nm. Aliquots of RNA were allowed to react with buffered or unbuffered neutralized glutaraldehyde at 25°C as described under Results. In some experiments $MgCl_2$ or NaCl was added in various amounts to the reactants in cacodylate buffer as described under Results. With *Torula* RNA only, bases were blocked with fluorodinitrobenzene (Sanger, 1945) and the treated RNA was washed several times by dissolution in distilled water and precipitation with cold ethanol. The final blocked RNA preparation was dissolved in distilled water, neutralized, and mixed in equal volume with 2% neutral glutaraldehyde in distilled water. The pH was read after glutaraldehyde addition and intermittently for 1 hr at room temperature.

Baker's yeast t-RNA (Type x, Sigma) was used as received. Known concentrations of t-RNA or BMV-RNA in 0.1 M sodium borate, pH 9.2, 0.1 M cacodylate, pH 7.2, or 0.1 M phosphate-citrate, pH 7.2, were mixed with 2.5% glutaraldehyde in the same buffer as the RNA. Various concentrations of RNA were also layered on glutaraldehyde for fixation by diffusion. The UV absorption spectra of fixed and unfixed RNA were compared.

Model RNA fixation. Solutions of poly-l-lysine (Type VI, mol wt 4,000–15,000, Sigma), polyadenylic acid (type 1, Sigma), and adenosine monophosphoric acid (AMP, Type II, Sigma) in distilled water were layered on 2–5% glutaraldehyde in 0.1 M sodium borate, pH 9.1, 0.2 M phosphate-citrate, pH 7.2, or neutral distilled water at room temperature. The solutions were observed for fixation into a water-insoluble gel both without disturbing the interphase and after mixing.

Polyacrylamide gel electrophoresis. 2.5% glutaraldehyde-reacted and native BMV-RNA, t-RNA, and yeast RNAs were run on 2.8% polyacrylamide gels by the method of Peacock and Dingman (1967).

Briefly, samples were diluted to 1 mg/ml with TEB dissociation buffer [2.5% SDS, 1.25% mercaptoethanol, 20% sucrose in 2.5× TEB (1× TEB—0.09 M Tris, 1 mM Na_2 EDTA, 0.09 M boric acid; pH 8.3)]. Portions (25 μl) of the samples were applied to 2.8% polyacrylamide made up in 1TEB with 0.1% SDS and 3.6% sucrose. The gels also contained a trace of diethylpyrocarbonate (DEPC) as RNase inhibitor. Gel dimensions were 75 × 5 mm. Electrophoresis was for 2 hr at a constant voltage of 100 V at room temperature with 1 × TEB + 0.1% SDS + a trace of DEPC for the electrode buffer. The gels were stained for 2 hr in 0.5 μg/ml ethidium bromide, destained for 1 hr in distilled water, and then photographed.

Results

Plant Virus RNA and Glutaraldehyde

All three plant virus RNAs were inactivated by glutaraldehyde. None of the plants inoculated with glutaraldehyde-treated RNA developed symptoms typical of the disease. When BMV- and BSMV-RNAs were treated identically, but without glutaraldehyde, they were infectious and caused mosaic symptoms in all inoculated plants. When TMV-RNA was inoculated on Glurk tobacco, the local lesion host, typical water-soaked lesions appeared 2 days after inoculation (Figure 1) and became necrotic by the third day. Leaves inoculated with glutaraldehyde-treated TMV-RNA did not show a single lesion.

Solutions of fixed and unfixed BMV- and t-RNA were scanned from 320 to 220 nm. No differences were detectable between native RNA and glutaraldehyde-treated RNA (Figure 2). With unfixed BMV-RNA in the reference position of the spectrophotometer, glutaraldehyde-fixed BMV-RNA showed a flat baseline, indicating no increased UV absorption after glutaraldehyde reaction with RNA at 25°C (data not shown).

A solution of 4% BMV-RNA (0.2 ml) in 0.2 M phosphate-citrate buffer, pH 7.2, was layered on 0.2 ml of 2.5% glutaraldehyde in the same buffer. One hour later the solution was yellow and the pH was still 7.2, but no precipitate or gel had formed. The possibility that the high negative charge of RNA prevented cross-linking was investigated by

Figure 1. Leaves of a local lesion tobacco host plant inoculated with glutaraldehyde-treated TMV-RNA (left) and native TMV-RNA (right) 2 days after inoculation. Only the leaf inoculated with native RNA shows local lesions (arrows).

precipitating BMV- and TMV-RNAs with Mg^{2+}, collecting the pellets by low-speed centrifugation, and overlaying them with buffered 2.5% glutaraldehyde. The pellets rapidly dissolved. The solution initially was colorless but turned a clear yellow in 1 hr at room temperature. When glutaraldehyde fixative buffered with 0.1 M borate, pH 9.1, or 0.2 M cacodylate, pH 7.2, and containing sufficient Mg^{2+} to precipitate a 4% solution of BMV-RNA was layered over a Mg^{2+}-precipitated BMV-RNA pellet, the pellet again dissolved. The mixture was vortex stirred and left at room temperature for 1 hr without formation of a precipitate or gel and without change in pH. Magnesium-precipitated RNA did not dissolve when overlayered with buffered glutaraldehyde containing a fivefold or larger excess of Mg^{2+}. After 1 hr at room temperature the precipitated glutaraldehyde-reacted RNA pellet had changed from a white and opaque to nearly transparent and gelatinous appearance. The pellet readily dissolved in buffer, however, and RNA was not cross-linked.

Model fixations with high concentrations of RNA were further performed with *Torula* and BDH yeast RNAs and baker's yeast t-RNA.

Yeast RNA and Glutaraldehyde

Commercial yeast RNA could not be used as received. The RNA had to be purified from the purchased stock by dissolution in disruption buffer and by phenol extraction. The purified RNA was free of proteins, since a solution made to 1 N HCl hydrolyzed completely after standing at room temperature overnight. No visible precipitate remained. However, commercial purified yeast RNAs were severely degraded. No defined bands formed when the yeast RNAs were run on polyacrylamide gels. The RNA was heterogeneous and spread over the length of the gel.

To solutions of 5–20% *Torula* RNA in 0.1 M borate, pH 9.1, or 0.1 M phosphate-citrate, pH 7.2, were added equal volumes of 2.5–10% glutaraldehyde in the same buffer. In borate-buffered reactions the pH dropped instantly to about 7.5 with the lower concentrations of RNA and glutaraldehyde and dropped further to 5.5 with the higher concentrations. In phosphate-buffered reactions the pH dropped instantly to 6 with a more gradual drop to 5.5 within 5 min after mixing the reactants. At about pH 5.6 a white precipitate began to form; it became heavy at pH 5.5. The pH did not drop further. The precipitate was formed at the same pH in both buffers and could be collected by low-speed centrifugation. The amorphous low-speed pellet could not be dissolved in distilled water, 0.2 M phosphate, or 0.2 M borate buffer. The precipitate remained insoluble in these buffers for a week, the longest time tested. The RNA did not precipitate from solutions in which the pH was gradually lowered to 4.5 by acetic acid addition, nor did the color change.

Buffered glutaraldehyde-treated BDH yeast RNA preparations of 2.5% or higher were also turbid. An insoluble yellow-brown amorphous precipitate could be separated by low-speed centrifugation. The RNA concentration in the supernatant was the same as that at the start of fixation.

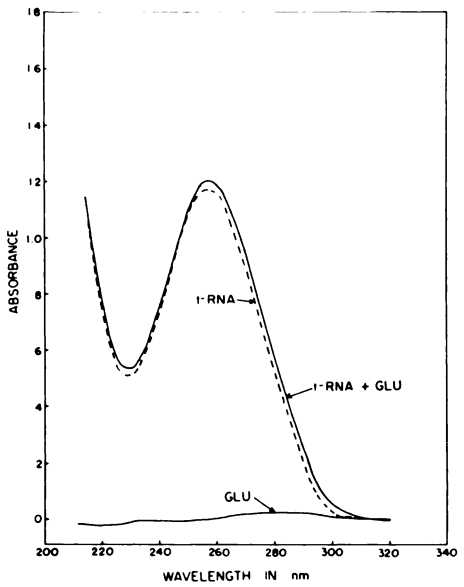

Figure 2. UV scan of glutaraldehyde-fixed yeast t-RNA (solid line) and native t-RNA (broken line). No contribution by bound glutaraldehyde to absorption spectrum. Bottom line is a scan of glutaraldehyde (GLU) in 0.1 M sodium borate, pH 9.1, at the same dilution from the stock as in the RNA solution.

Removal of the amorphous glutaraldehyde-fixed contaminant precipitate did not lower the effective RNA concentration or alter the normal RNA spectrum obtained for fixed or unfixed RNA. The concentration of the contaminant in BDH yeast RNA was lower than that in *Torula* yeast RNA preparations. Addition of glutaraldehyde dropped the pH in unbuffered, but not in buffered, solutions. The RNA and glutaraldehyde in distilled water were neutralized with NaOH to pH 7.5 just before mixing. Similarly, no pH drop was observed in buffered t-RNA preparations, but there was a pH drop the instant neutralized unbuffered solutions of t-RNA and glutaraldehyde were mixed. No pH drop was noted when unbuffered neutral solutions of BMV-RNA were mixed with unbuffered glutaraldehyde of the same pH. No decrease in pH was measured when neutral unbuffered glutaraldehyde was added to neutral fluorodinitrobenzene-treated *Torula* RNA in distilled water or in neutral 0.1 M NaCl. Treatment was continued for 7 hr. No drop in pH occurred. Release of acid (H^+) by glutaraldehyde reaction with *Torula* RNA is attributed to the unknown contaminant, which was not eliminated by the SDS and phenol purification of commercial yeast RNA.

Concentrations as high as 20% yeast RNA or t-RNA were not fixed into insoluble gels when the pH was maintained at pH 7.2 by adding KOH, although the amorphous precipitate of the fixed contaminant formed and settled out of solution.

Model RNA Fixation

On a solution of 10% poly-l-lysine in distilled water was layered an equal volume of 5% glutaraldehyde in 0.2 M phosphate-citrate buffer, pH 7.2. A yellow front rapidly spread downwards. The polylysine was almost immediately fixed into a hard yellow-orange gel that soon turned brown. When unbuffered 1, 2.5, or 5% polyadenylic acid or 5% adenosine monophosphate (AMP) in neutral distilled water was mixed with an equal volume of 2.5% neutral glutaraldehyde also in distilled water, no color reaction took place. No pH drop was evident as with unbuffered yeast RNA-glutaraldehyde mixtures. Even when the polyadenylic acid-glutaraldehyde mixture was heated to 65°C for 1 hr, the pH did not drop and color did not change.

Gel Electrophoresis of Glutaraldehyde-treated RNA

Solutions of glutaraldehyde-treated BMV- and t-RNAs were compared to native RNAs by electrophoresis on 2.8% polyacrylamide gels. Bands of RNA formed at the same distance from the source in both cases. No difference in electrophoretic behavior could be detected between RNA reacted with glutaraldehyde and native RNA. Purified commercial yeast RNA was badly degraded. No bands formed in stained gels of *Torula* RNA, while one band in addition to smearing was found in BDH-RNA (data not shown).

Discussion

The effect of standard OsO_4 postfixation on the immobilization of RNA was not determined in the described model systems for the following reasons. As discussed in an earlier article on the relative speed of fixation of glutaraldehyde and OsO_4 (Langenberg, 1978), these fixatives are mutually exclusive in their reactions with amine containing compounds. Since RNAs were glutaraldehyde-treated in the above experiments, glutaraldehyde-reacted amino groups in RNA would presumably be unavailable for reaction with OsO_4. Prefixation with OsO_4, or OsO_4 alone, has given poor preservation of ultrastructural detail of plant tissues and created known artifacts in a variety of animal tissues (Langenberg, 1978).

Glutaraldehyde-treated BSMV- and TMV-RNAs did not precipitate in buffered solutions. Concentrations of RNA were low, however, and did not exceed 750 μg/ml. Concentrations of BMV-RNA as high as 4% were still not precipitated by glutaraldehyde. In *Torula* RNA fixation experiments the pH of a 5% or higher buffered solution dropped when glutaraldehyde was added without addition of NaOH to maintain the pH. This indicates that a large amount of contaminant was carried along during the purification process. The contaminant did not absorb UV light, since removal of the precipitate did not alter the UV spectrum nor did it lower the concentration of RNA. The contaminant could not be detected by gel electrophoresis either.

It is not understood why glutaraldehyde in 0.1 M phosphate-citrate-buffered solutions failed to cross-link and precipitate RNA even at concentrations of *Torula* RNA as high as 20%. A plausible reason for the failure of glutaral-

dehyde to precipitate RNA is intramolecular cross-linking. However, poly-l-lysine, which also has the potential to form intramolecular cross-links with glutaraldehyde, was readily precipitated by glutaraldehyde when buffered. The Mg^{2+} precipitation of BMV-RNA to remove phosphate negative charges did not allow glutaraldehyde to cross-link the RNA. That a reaction had occurred was evident from the change of the pellet from opaque to translucent.

No RNA could be cross-linked by glutaraldehyde in high or low salt at high or neutral pH. Glutaraldehyde did react with BMV-RNA, BSMV-RNA, and TMV-RNA as evidenced by the inactivation of infectious RNA.

Higher concentrations of RNA than used by Hopwood (1975) were used here because glutaraldehyde does not precipitate low concentrations of BSA or gelatin (Millonig and Marinozzi, 1968; Langenberg, 1979a). Hopwood's finding that RNA is not cross-linked and precipitated by glutaraldehyde is confirmed for concentrations up to 20% RNA.

Glutaraldehyde does not precipitate nucleic acids. Glutaraldehyde does, however, react rapidly with available bases in single-stranded RNA (revealed by loss of infectivity); the rapid reaction is followed by a slower reaction that may take several hours. The slow reaction was visible in the slowly yellowing solutions of BMV-RNA and yeast RNA. The possibility that the yellowing of glutaraldehyde-RNA solutions resulted from a contaminant in RNA preparations cannot be excluded, since polyadenylic acid solutions did not yellow after glutaraldehyde fixation.

Since glutaraldehyde does not fix nucleic acids, their precise intracellular location in ultrathin sections could be in doubt unless they are trapped in a surrounding matrix of fixed protein or cross-linked to proteins as mentioned by Hopwood (1975).

In this respect it should be borne in mind that glutaraldehyde is unable to fix low concentrations of protein (Millonig and Marinozzi, 1968; Langenberg, 1979a) or low or high concentrations of RNA (Hopwood, 1975; this report).

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Literature Cited

Brakke MK (1972): Slowly sedimenting infectious entities of southern bean mosaic virus. Virology 50:669

Brown GL, Locke M (1978): Nucleoprotein localization by bismuth staining. Tissue Cell 10:365

Hopwood D (1975): The reactions of glutaraldehyde with nucleic acids. Histochem J 7:267

Langenberg WG (1978): Relative speed of fixation of glutaraldehyde and osmic acid in plant cells measured by grana appearance in chloroplasts. Protoplasma 94:167

Langenberg WG (1979a): Chilling of tissue before glutaraldehyde fixation preserves fragile inclusions of several plant viruses. J Ultrastruct Res 66:120

Langenberg WG (1979b): Ribonucleic acid fixation by glutaraldehyde. Implications for the interpretation of electron micrographs. Presented at the 71st Annual Meeting of the American Phytopathological Society, Washington, DC, August 5–11

Lindigkeit R, Eckel R (1962): Formaldehyde reaction with RNA (ribonucleic acid) in phosphate buffer and Mg binding solutions. Acta Biol Med Ger 8:581

Millonig G, Marinozzi V (1968): Advances in Electron Microscopy. Academic Press, New York, p 251–341

Peacock AC, Dingman CW (1967): Resolution of multiple ribonucleic acid species by polyacrylamide gel electrophoresis. Biochemistry 6:1818

Sabatini DD, Bensch K, Barrnett RJ (1963): The preservation of cellular ultrastructure and enzymatic activity by cytochemistry and electron microscopy. J Cell Biol 17:19

Sanger F (1945): The free amino groups of insulin. Biochem J 39:507

Staehelin M (1959): Reaction of tobacco mosaic virus nucleic acid with formaldehyde. Biophys Biochem Acta 29:410

Stevens ChL, Rosenfeld A (1966): The secondary structure of polyadenylic acid. Inferences from its reaction with formaldehyde. Biochemistry 5:2714–2721