Histochemie 30, 162—167 (1972)

Glutaraldehyde—Its Purity and Stability

Roy Gillett and Keith Gull

Departments of Biology and Microbiology Queen Elizabeth College, London, England

Received March 3, 1972

Summary. Six commercially available glutaraldehydes were tested spectrophotometrically for their purity. These results were compared with glutaraldehyde purified by two experimental techniques. Ten sets of storage conditions were chosen and aliquots of purified glutaraldehyde were stored under these conditions for eight months. These samples enabled the reappearance of the main contaminant, absorbing strongly at 235 nm, to be examined with regard to temperature, light and the availability of oxygen. From the results the optimum criteria for storage of pure glutaraldehyde were elucidated. The results lend weight to the view that the main contaminent in stored glutaraldehyde is a polymer of glutaraldehyde and not glutaric acid.

Introduction

Since the introduction of glutaraldehyde as a fixative for electron microscopy and histochemistry (Sabatini *et al.*, 1963) concern has been shown about its purity, stability and precise composition (Anderson, 1967a, b; Fahimi and Drochmans, 1965, 1968; Robertson and Schultz, 1970; Richards and Knowles, 1968). Hardy, Nicholls, and Rydon (1969) have indicated that the cross linking properties of glutaraldehyde responsible for fixation are due to the glutaraldehyde monomer or its hydrates.

Glutaraldehyde can be purified by distillation (Fahimi and Drochmans, 1965), shaking with activated charcoal, or shaking with Barium carbonate (Pease, 1964). Histochemically, it has been widely shown that the purified product is a better fixative, being much less inhibitory to a wide range of enzymes (Fahimi and Drochmans, 1968; Anderson, 1967a; Smith and Farquhar, 1966). Many workers agree that the purified glutaraldehyde gives superior fixation for electron microscopy; this is certainly the experience of this laboratory.

The growing use of purified glutaraldehyde has been reflected by the appearance of commercially available "pure" glutaraldehydes. In the present study the relative purity of commercial glutaraldehydes was compared, the results of two methods of purification assessed and an ideal method of storage of the purified product was sought.

Materials and Methods

Glutaraldehyde in aqueous solution was obtained from several suppliers: Taab Laboratories, Reading Berks; B.D.H. Chemicals Ltd., Poole, Dorset; Koch Light, Colnbrook, Bucks; George Gurr, Cressex Industrial Estate, High Wycombe, Bucks; Edward Gurr, Michrome Laboratories, London, S.W. 14; Polysciences Inc., Paul Valley Industrial Park, Warrington, Pennsylvania, U.S.A. Ultraviolet (U.V.) absorbance spectra were obtained using a Pye Unicam SP 800 scanning spectrophotometer. Spectra were obtained from commercial glutaraldehydes as soon after purchase as possible.

The distillation was performed under vacuum at 15 mm. Hg pressure and the 80–85° C fraction was collected. This was a viscid clear liquid that was diluted to 25% with boiling water immediately and stored at this strength (Fahimi and Drochmans, 1968). Purification with activated charcoal was performed by shaking the commercial 25% glutaraldehyde with 10% w/v activated charcoal at 4° C for one hour. The charcoal was filtered off after each extraction and the filtrate volume measured. The process was repeated until a satisfactory U.V. absorbance curve was obtained for a dilution of the solution. Two to three extractions were usually necessary.

Ten different storage conditions were chosen to test the effect of temperature, the presence of oxygen and the effect of light on the deterioration of pure glutaraldehyde. Two aliquots were stored at 37° C, one under nitrogen the other under air. Four aliquots were stored at 20° C, one under nitrogen in the dark, one under nitrogen in the light, one under air in the dark and the fourth under air in the light. Two more aliquots were stored at 4° C, one under air and one under nitrogen. Finally two aliquots were stored deep frozen at —20° C under air and nitrogen respectively. The samples were not disturbed during a storage period of eight months.

Results

I. Commercially Available Glutaraldehydes

All the glutaraldehydes tested with the exception of the Polysciences and Taab E.M. grade products were yellow in colour. As an assay of purity the relative values of U.V. absorbance at 235 and 280 nm were used. Pure monomeric glutaraldehyde has an absorbance peak at 280 nm and the main impurity, possibly a polymer (Robertson and Schultz, 1970; Hardy *et al.*, 1969), has an absorbance peak at 235 nm. All the glutaraldehyde preparations tested, except the Polysciences product, had a higher absorbance peak at 235 nm than at 280 nm (Fig. 1a, b). The Polysciences glutaraldehyde, which was packed in ampoules under nitrogen, gave an absorbance peak at 280 nm about equal to that at 235 nm. None of the commercial glutaraldehyde preparations tested gave a U.V. absorbance spectrum that was as satisfactory as the pure glutaraldehyde prepared by either of the experimental methods.

II. Experimental Purification

Both methods used for the purification of glutaraldehyde, vacuum distillation and repeated extraction with activated charcoal, gave essentially the same result. A third method of purification described in the literature, shaking with Barium carbonate (Pease, 1964), was not attempted since it has been considered inadvisable on theoretical grounds (Robertson and Schultz, 1970). The U.V. absorbance spectra of the two purified samples were almost identical, showing only one absorbance peak at 280 nm. If the sample being extracted with activated charcoal is scanned after each extraction (Fig. 2) the 235 nm peak can be seen to fall progressively until it approximates to the level of that of the distilled glutaraldehyde. Prolonged use of glutaraldehyde purified by either of the above methods has shown it to be an excellent fixative for a wide variety of plant and animal tissues.

III. Storage of the Purified Glutaraldehyde

The experiments carried out to find an ideal way of storing the purified glutaraldehyde yielded much useful information. The most important storage criterion was definitely temperature. Those samples stored at —20° C showed

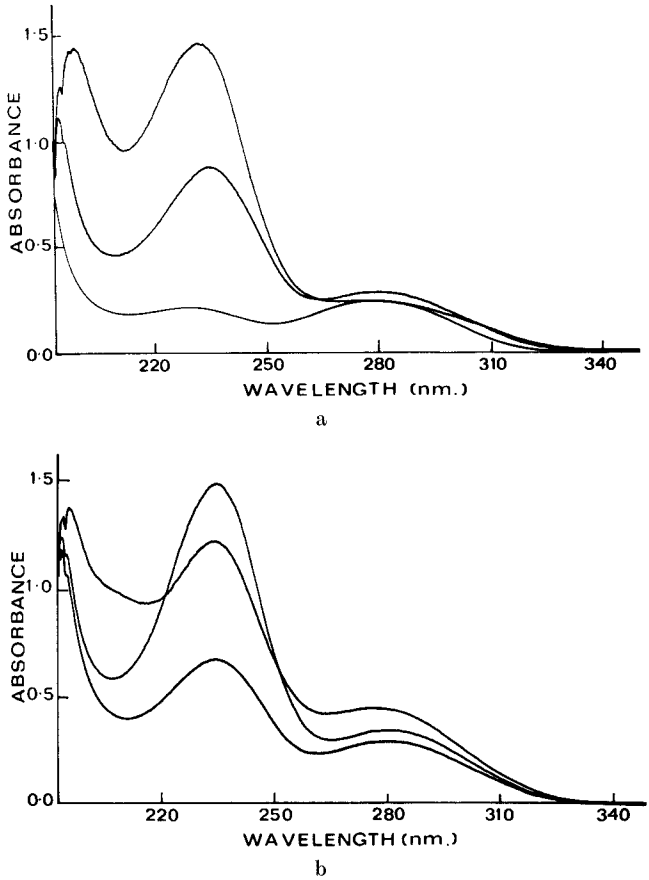

Fig. 1a and b. U.V. absorbance spectra from six commercially available glutaraldehydes, diluted from manufacturers stated concentrations to a nominal 0.5%. (Compare with Fig. 2, curve 3)

virtually no change in their U.V. absorbance characteristics, even after eight months storage. Those samples stored at 4°C showed a small peak of absorbance at 235 nm, though this was not as large as the peak at 280 nm. All samples stored at 20°C, however, showed considerable absorbance peaks at 235 nm, many times the size of the absorbance peaks at 280 nm. The effect of storage at 37°C on 235 nm absorbance peak height was even more pronounced; the 235 nm absorbance value being much greater even than in those samples stored at 20°C.

If the absorbance at 235 nm is plotted against storage temperature a direct relationship is obtained between storage temperature (above 0°C) and the appearance of 235 nm absorbing material (Fig. 3). If the slope of this line is taken to represent the rate of deterioration of the sample it was found that the slope for samples stored under nitrogen was only slightly less than for those stored under air. This would suggest that an inert atmosphere has only a very minor effect upon the stability of pure glutaraldehyde. There was no apparent difference

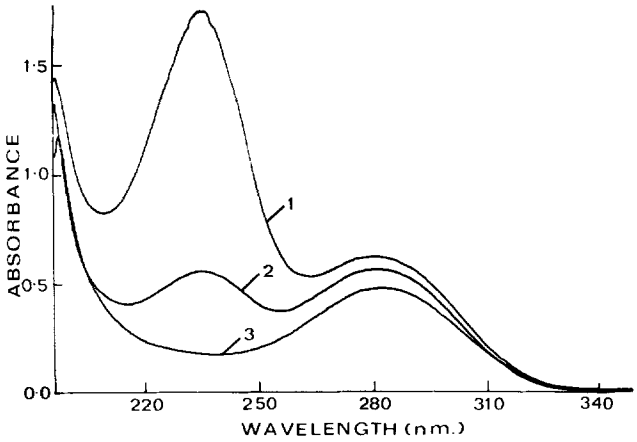

Fig. 2. U.V. absorbance spectra of 1:10 dilutions of glutaraldehyde after successive charcoal extractions. *1* Original commercial glutaraldehyde. (Taab Practical Grade); *2* After one extraction with activated charcoal; *3* After two extractions with activated charcoal

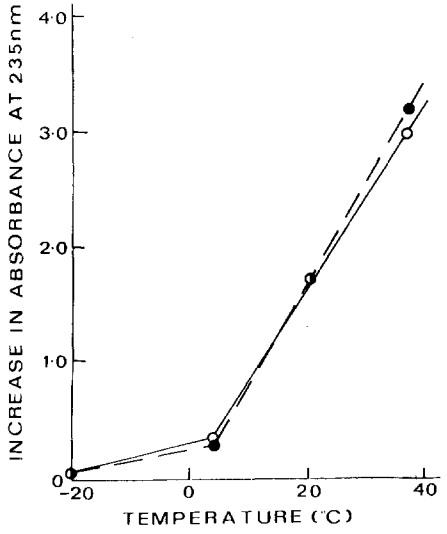

Fig. 3. The effect of varying storage temperature on the reappearance of the impurity absorbing at 235 nm in purified glutaraldehyde. o——o stored under nitrogen; •- -• stored under air

between the stability of those samples stored at room temperature in the light and those stored at the same temperature in the dark under either nitrogen or air. We consider, therefore, that light has no effect upon the process of deterioration.

Some authors have considered the main impurity in glutaraldehyde to be glutaric acid, formed by oxidation. Since the samples we stored under an inert atmosphere proved no more stable than those under air we tested a solution

of glutaric acid to find its U.V. absorbance maxima. In aqueous solution glutaric acid has an absorbance peak at 207 nm. Addition of purified glutaraldehyde had no effect on the position of this peak. Thus it would seem that the increase in 235 nm absorbing material on storage of glutaraldehyde is not due to the accumulation of glutaric acid.

Discussion

All commercially available preparations of glutaraldehyde tested contained considerable levels of impurities absorbing at 235 nm. These impurities could be removed by vacuum distillation or by treatment with activated charcoal. Glutaric acid was found to absorb maximally at 207 nm and therefore could not be the impurity which gives rise to the 235 nm peak. The increase in absorbance at 235 nm on storage of glutaraldehyde is temperature dependent, only slightly affected by the presence of oxygen and unaffected by the presence of light. These facts lend weight to the theory that the increase in absorbance at 235 nm is due to the formation of a polymer of glutaraldehyde rather than a product of oxidation (Robertson and Schultz, 1970; Richards and Knowles, 1969).

Storage of purified glutaraldehyde at —20° C greatly retards the appearance of the 235 nm absorbing material. Storage at 4° C is not as effective, but is still to be preferred to storage at room temperatures. Above 0° C there is a linear relationship between the storage temperature and the appearance of the 235 nm absorbing material.

Richards and Knowles (1968) attribute the protein crosslinking power of a glutaraldehyde solution to the presence in it of polymers rather than to monomeric glutaraldehyde itself; Robertson and Schultz (1970) claim that "aged" glutaraldehyde, containing polymers and absorbing strongly at 235 nm is a better cytological fixative than the pure distilled glutaraldehyde. Our experience runs contrary to the latter finding. Hardy, Nicholls, and Rydon (1969) have shown results at variance with those of Richards and Knowles in proving that it is the monomeric glutaraldehyde, or one of its hydrated forms, which is responsible for protein crosslinking. On empirical grounds we would support this view and many histochemical studies suggest that a purified monomeric glutaraldehyde is a better fixative, less inhibitory to a wide range of enzymes (Anderson, 1967a; Fahimi and Drochmans, 1968; Smith and Farquhar, 1966).

Our findings lead us to believe that for use as a histochemical or electron microscopical fixative commercial glutaraldehyde from any source should first be purified by vacuum distillation or repeated extraction with activated charcoal and then stored, unbuffered, in a 10–25% solution at —20° C until required for use.

Acknowledgements. One of us (K.G.) would like to acknowledge the support of an M.R.C. grant for part of this work.

References

Anderson, P. J.: Purification and quantitation of glutaraldehyde and its effects on several enzyme activities in skeletal muscle. J. Histochem. Cytochem. **15**, 652–661 (1967a).

Anderson, P. J.: Standardization of glutaraldehyde and evaluation of its enzyme inhibitory effects in skeletal muscle. J. Histochem. Cytochem. **15**, 789–790 (1967b).

Fahimi, H. D., Drochmans, P.: Essais de standardisation de la fixation au glutaraldehyde. I. Purification et determination de la concentration du glutaraldehyde. J. Micr. **4**, 725–736 (1965).

Fahimi, H. D., Drochmans, P.: Purification of glutaraldehyde. Its significance for preservation of acid phosphatase activity. J. Histochem. Cytochem. **16**, 199–204 (1968).

Hardy, P. M., Nicholls, A. C., Rydon, H. N.: The nature of glutaraldehyde in aqueous solution. Chem. Comm. 565–566 (1969).

Pease, D. C.: Histological techniques for electron microscopy, p. 58. New York: Academic Press 1964.

Richards, F. M., Knowles, J. R.: Glutaraldehyde as a protein cross-linking reagent. J. molec. Biol. **37**, 231–233 (1968).

Robertson, E. A., Schultz, R. L.: The impurities in commercial glutaraldehyde and their effect on the fixation of brain. J. Ultrastruct. Res. **30**, 275–287 (1970).

Sabatini, D. D., Bensch, K., Barnett, R. J.: Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. J. Cell Biol. **17**, 19–58 (1963).

Smith, R. E., Farquhar, M. G.: Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. J. Cell Biol. **31**, 319–347 (1966).

Keith Gull
Dept. of Microbiology
Queen Elizabeth College
Campden Hill Road
Kensington
London, W. 8
England