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The effect of heating by microwave irradiation and by conventional heating on the aldehyde concentration in aqueous glutaraldehyde solutions

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Summary

The effect of short time heating of aqueous solutions of glutaraldehyde (GA) on relative aldehyde concentration was determined using spectrophotometric analysis. Because free monomeric GA absorbs U.V. light at 280 nm, whereas the alpha, beta polymeric forms absorb at 235 nm, the purity of GA solutions can be expressed as the ratio: A 235 nm / A 280 nm (purification index, P.I.).

Heating of 4 ml aliquots of 0.85% distilled aqueous GA solution resulted in an increase of the absorption at 280 nm which is correlated positively with temperature. No increase of absorption at 235 nm was found when solutions were kept at 40° C for several hours. The increase of absorption at 280 nm is caused by a rapid decyclization of hemiacetals producing an increase in free aldehyde concentration.

No major differences in absorption were found between the solutions heated by microwave and by conventional heating. However, because microwave irradiation is known to produce an homogeneous rise in temperature, especially in bulky samples, it is expected that the results of fixation procedures will improve by the combined effect of higher temperature and enhanced diffusion rates of the fixating species.

Introduction

In the last two decades, glutaraldehyde (GA) has been widely used for various purposes such as the tanning of leather, fixation of cells and tissues for electron microscopy, preservation of connective tissue for transplantation purposes, and chemosterilisation of hospital instruments.

Commercially available aqueous solutions of GA are complex mixtures of free aldehyde, mono- and dihydrated glutaraldehyde, monomeric and polymeric cyclic hemiacetals and various alpha, beta unsaturated polymers (Table 1). The content of free aldehyde is usually not more than 4%. Recently, cyclic glutaraldehyde oligomers, having a trioxane type skeleton, have been described (Tashima *et al.*, 1987). In addition, impurities can be detected in small quantities, such as ethanol, methanol, acrolein, glutaraldoxime, and glutaric acid. Glutaraldehyde solutions can be purified in order to extract impurities and to obtain a maximum amount of the free monomeric form, by vacuum distillation, by filtering through activated charcoal, or by ion exchange techniques. When very high purity is needed, the method of choice is vacuum distillation, either at atmospheric pressure or at reduced pressure (Gillett & Gull, 1972).

With increasing purity of a GA solution, the amount of monomeric forms of GA will increase with respect to the cyclic, oligomeric and polymeric forms. This will increase the concentration of carbonyl groups in the solution because free glutaraldehyde contains two of such groups per molecule whereas the derivatives carry none or less per unit molecular weight. Carbonyl groups absorb U.V. light at 280 nm and consequently, an increase in free aldehyde concentration will cause an increase of the 280 nm absorption peak. The alpha, beta unsaturated forms absorb U.V. light at 235 nm, due to their C=C double bonds. The purity of a GA solution is often defined as the ratio of absorbance at 235 nm to the absorbance at 280 nm, the so called purification index (P.I.). Lower P.I. values represent higher purity as far as the unsaturated polymers are concerned.

In spite of the enormous amount of literature about

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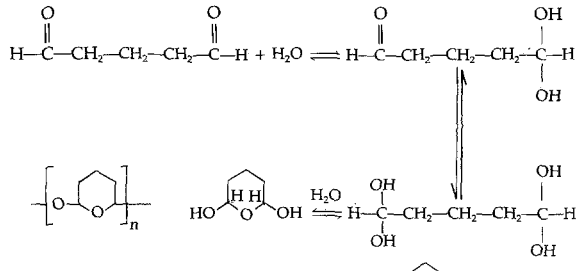
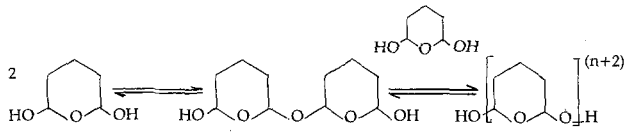
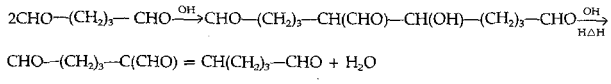
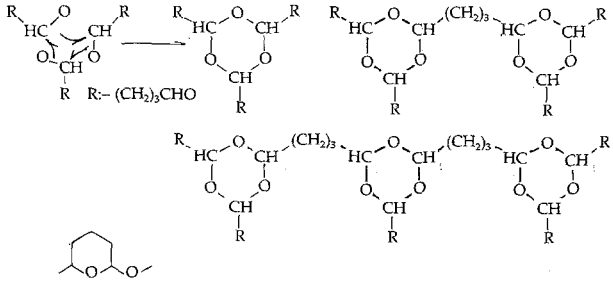
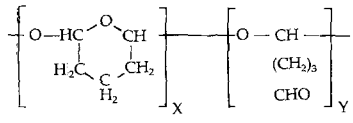
Table 1. Various polymerization products of glutaraldehyde.

	Type of reaction	*References*
	Mono and dihydrate cyclic hemiacetal formation	Aso and Aito, 1962
	Formation of polymers of hemiacetals	Korn *et al.*, 1972
	Aldol condensation	Rasmussen & Albrechtsen, 1974
	Paraglutaraldehyde pentamer, heptamer formation	Tashima *et al.*, 1987
	Cyclic polymerization	Yokota *et al.*, 1965
	Inter–intra-molecular propagation polymerization	Aso and Aito, 1962

the use of GA as a fixative for cells and tissues there is still no agreement about the main reactive species of GA solutions during the cross-linking process. Hayat (1981) states that free monomeric glutaraldehyde is capable of reacting at acidic or neutral pH and that purified solutions containing mostly free aldehyde are quite effective in the cross-linking of proteins. However, because in fixation conditions at slightly alkaline pH the presence of oligomers of glutaraldehyde is promoted, it must be assumed that these compounds also introduce cross-links into tissue.

Studies on the chemical reactions of GA with proteins have revealed that it is mainly the free monomeric form that is capable of effective penetration into the tissue (Nimni *et al.*, 1987). Polymerization of GA depletes free GA from the solution making it unavailable for further penetration into the tissue. This could result in an uneven distribution of cross-linking GA throughout the tissue, which may contribute to uneven distribution of physico-chemical properties in tissues fixed for electron microscopy or to be used as bioprostheses. Finally, especially in the case of bioprosthesis one may fear that oligomeric crosslinks will prove to be unstable and to decompose after some time. In the case of GA-processed bioprostheses introducing oligomeric or polymeric crosslinks has been shown to cause leaching of GA molecules out of the implant. This is a well known disadvantage of GA as a fixation agent for biomaterials since it may cause inflammatory reactions sometimes after years of implantation (Nimni *et al.*, 1987). Quality of fixation with GA will thus be increased by enhancing diffusion of the free monomeric form. Diffusion will increase exponentially as temperature rises. Temperature rise,

however, will also have an influence on various reactions between GA derivatives (Table 1). It would be of interest to know whether heating of glutaraldehyde solutions during fixation would be a good strategy. In this study we determined the effect of short time heating on the relative amount of free aldehydes in aqueous GA solutions.

A particular way of heating a system is by the use of microwave irradiation. It is well known that, as compared to conventional external heating in for instance a waterbath, microwaves cause a more homogeneous rise of the temperature throughout the irradiated system. In bulky systems therefore microwaves also enhance the rapid diffusion of polar compounds when concentration gradients are present as has been shown for histological techniques by Boon and Kok (1987). Because of these aspects it might be very attractive to carry out fixation procedures under microwave irradiation instead of using external ways of heating.

In preparation of our studies of microwave fixation techniques we included microwave irradiation as a means of heating in the present study.

Materials and methods

Commercially obtained 25% (w/w) glutaraldehyde solution (Janssen Chimica) was distilled through a 10 cm Vigreux column at 18 mm Hg. A fraction (9.5 g, b.p. 99–103° C) was collected in 44 g ice-cold distilled water. Thus 53.5 g of a 17–18% (w/w) GA solution was obtained. The aldehyde concentration was determined by iodometric titration according to the method of Frigerio and Shaw (1969). The distilled solution was diluted until an ultraviolet absorption spectrum could be made in the 200–300 nm region (Ultrospec 4050 LKB Biochrom). The concentration of the GA solution used was 0.85% (w/w).

Heating procedure

Aliquots of 4 ml GA solution were heated in a glass vessel either by means of a water bath (Tamson TC–3) or in a microwave oven (Bio-Rad H2500 Microwave Processor). The solutions were heated to 65° C and kept at this temperature for 5 min.

The Bio-Rad H2500 Microwave Processor is controlled by a programmable microprocessor reacting on a temperature probe. The glass vessel was placed on a rotating turntable. Thereby, the temperature probe stirred the fluid, resulting in a homogeneous rise in temperature. Excessive radiation was absorbed by a water load of 400 ml placed in a corner of the oven. The power level of the oven was set at 50%, the radiation cycle at 2 s.

The GA solution in the water bath was heated inside a stoppered 50 ml Erlenmeyer flask.

Measuring procedure

Before heating the GA solutions an ultraviolet absorption spectrum was made in the region of 200–300 nm and the purification index calculated (Fig. 1).

In the setting used, the GA solutions to be heated could not be thermostated inside the spectrophotometer. Therefore, the preheated solutions were allowed to cool inside the spectrophotometer while measuring the absorbances at 280 and 235 nm and monitoring the actual temperature in the cell with a fibre optic thermometer (ASEA 1010, Asea Innovation, Sweden). Other measurements comprised the determination of absorbance at 280 nm as a function of time when the solutions were held at a constant temperature of 40° C either in a waterbath or in the microwave oven for periods up to 2 h.

Results

The distilled glutaraldehyde solution showed only one absorption peak at 282 nm (A = 0.450 – 0.500) at 25° C. The absorption at 235 nm measured 0.04 – 0.06 without showing a real peak in the spectrum (Fig. 1). The purification index, A 235 nm / A 280 nm, therefore was in the order of 0.1.

Higher temperatures of the GA solutions caused a rather large increase in the 280 nm absorption peak, as is shown in Fig. 1. The relation between temperature and increase of absorbance is given in Fig. 2. No significant differences in absorption could be detected between the two methods of heating. This was confirmed in repeated experiments. Although a small increase of absorption was seen at 235 nm at higher temperatutes this did not prove to be a permanent effect and vanished when the solution was cooled down to room temperature.

When the GA solution was kept on a certain higher temperature, say 40° C, the corresponding higher absorbance at 280 nm was maintained for periods up to at least 2 h. Thus the purification index of the solution remained improved for prolonged times. Again, the method of heating – waterbath or microwave oven, had no influence on the results obtained.

Discussion

As early as 1967 Hopwood reported that the extinction of material absorbing at 280 nm increased exponentially with temperature when GA solutions were heated. On the basis of C–13 NMR studies Whipple and Rutta (1974) showed a similar correlation between free aldehyde groups and temperature in GA solutions. In our experiment we found also a rather substantial increase in absorbance at this wavelength with temperature and, by absence of absorption increase at 235 nm, an improvement of the purification index of the distilled GA solution (Fig. 2).

It can be assumed that the increase of extinction of material absorbing at 280 nm is caused by the formation of free monomeric glutaraldehyde. The breakdown of cyclic hemiacetals to glutaraldehyde is known to be enhanced by heating. Various workers found the cyclic hemiacetal to be the major component of a commercial 25% glutaraldehyde solution at 25° C

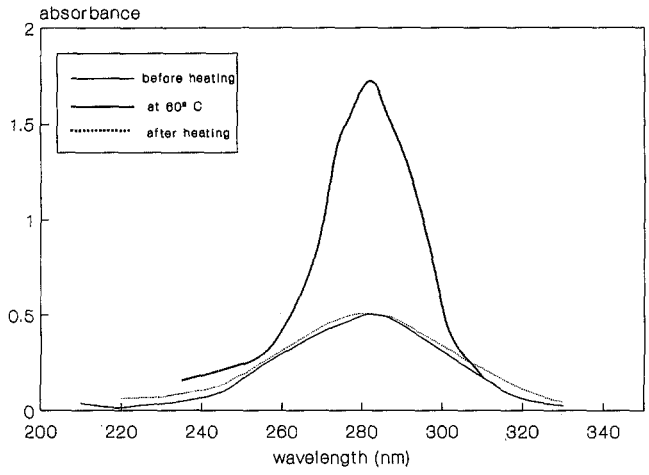


Fig. 1. U.V. spectrum of a freshly distilled 0.85% GA solution before heating, at 60° C and at 25° C after 2 h being kept at 40° C. There is no peak formed at 235 nm.

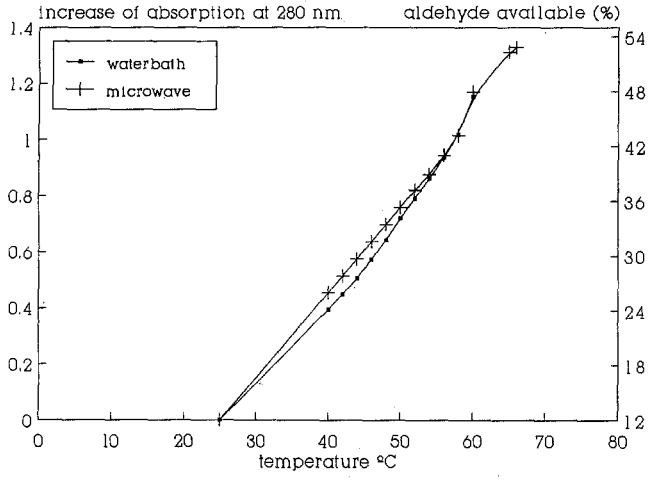


Fig. 2. Increase of absorbance at 280 nm of a GA solution as a function of temperature. The actual temperature in the cell was measured with a fibreoptic thermometer while the solution was cooling down from 65° C to 25° C in the spectrophotometer. Along the right Y-axis the percentage of the theoretical available aldehyde groups, as calculated by Whipple and Ruta (1974) for a 25% Ga solution, are depicted.

(Whipple *et al.*, 1974; Halloway *et al.*, 1975). The mono- and dihydrates formed will absorb U.V. light at 280 nm only very weakly, if they do so at all (Woodroof, 1977). Thus the peak at 280 nm is mainly due to the presence of free glutaraldehyde and an increase in absorption at 280 nm is anticipated when such a solution is heated.

An increase of material absorbing at 280 nm by the depolymerization of oligomers, having a trioxane type skeleton (Table 1) is unlikely at the temperatures of our experiments. Tashima *et al.* (1987) report these structures to decompose only at temperatures above 150° C.

The effect of temperature rise on glutaraldehyde solutions has been described in the past as a shift towards the formation of alpha, beta unsaturated polymers (Gillet & Gull, 1972; Rasmussen & Albrechtsen, 1974). When this occurs, the increase in polymer concentration is demonstrated by an increase in absorption at 235 nm, thereby lowering the purification index of the solution. This reaction, in which a water molecule has to be released through an aldol condensation reaction proceeds relatively slowly. A considerable amount of alpha, beta unsaturated polymers is therefore only seen after prolonged heating at high temperature. Also storage for several months at room temperature will cause this impurity to be formed, depending on other factors such as pH and initial concentration of the solution (Table 1).

No significant increase in absorption at 235 nm following heating was found by us. This can be explained by the heating time being too short to produce a significant amount of alpha, beta unsaturated polymers.

We conclude that heating for a short period of time produces only an increase in absorption at 280 nm. This increase was the same for the two methods of heating, that is by microwave irradiation and by conventional heating in a water bath.

During fixation of cells and tissues, the fixative molecules often have to travel considerable distances by means of diffusion. As is stated earlier, a major disadvantage of GA as a fixative is its slow penetration into tissue (Johannessen, 1978; Hopwood, 1972; McDowell & Trump, 1976).

Because both water and glutaraldehyde consist of polar molecules they absorb energy while being irradiated with microwaves. The homogeneous rise in temperature which results is expected to lead to a rapid increase of diffusion of glutaraldehyde similar to what Boon and Kok (1987) described for the case of dehydrating with alcohol and other histotechnical procedures. Therefore, although no major differences in purification index improvement by both methods of heating have been shown, we expect that fixation procedures, especialy of bulky samples, will benefit from microwave irradiation as a heating source. In further studies we intend to explore the advantage of microwave enhanced diffusion for the fixation of collageneous materials to be used as bioprostheses.

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