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Polymerization Reaction in Aqueous Solution of Glutaraldehyde Containing Trioxane-Type Oligomers under Sterilizing Conditions

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The precipitate, deposited in a 2% alkaline aqueous solution (pH 7.5—8.5) of glutaraldehyde (GA) used for chemosterilization of medical instruments, was characterized by means of thermogravimetry, pyrolysis gas chromatography, chemical ionization mass spectrometry and high-performance liquid chromatography. It was found that the precipitation could be attributed to the polymerization reaction based on aldol condensation involving both GA monomer and trioxane-type GA oligomers. The polymers produced had an average molecular weight higher than those obtained from GA alone. The relation between the turbidity of GA solution and the molecular weight of the polymer indicated that the precipitates included polymer with a molecular weight exceeding approximately 12000.

Keywords glutaraldehyde; polymerization reaction; aldol condensation; trioxane-type oligomer; paraglutaraldehyde; precipitation; disinfectant; chemosterilization; alkaline condition

Introduction

In recent years, the risk of transmission of pathogenic bacteria and viruses in hospitals has become greater with the increasing use of endoscopes.[1,2] Hepatitis B virus (HBV) infection is a particularly serious problem.[3,4] A 2% alkaline aqueous solution (pH 7.5—8.5) of glutaraldehyde (GA) has been widely used to sterilize medical instruments, since it is strongly bactericidal and inactivates viruses including HBV.[5,6]

However, a certain grade of commercial GA solution deposits white precipitates under sterilizing conditions, and damages the instruments. In the previous paper, we found that the precipitation is caused by the trioxane-type GA oligomers existing in GA solution as an impurity.[7,8] To cope with this problem, we developed a method to purify GA solution by using alkylated silica adsorbents[9] and also a method to prevent the precipitation by addition of sulfur oxyacid salts such as sodium bisulfite, sodium dithionite and rongalite to the alkaline GA solution.[10]

However, these sulfur oxyacid salts tarnished the surface of certain metals such as copper and brass, probably because of their reductive properties. In order to find a better additive than the sulfur salts, it is important to know how trioxane-type GA oligomers are involved in the formation of precipitates. The present paper aims to clarify the role of trioxane-type GA oligomers in the formation of precipitates in the GA solution under sterilizing conditions.

Materials and Methods

Chemicals Twenty percent and 25% aqueous GA solutions of electron microscopy (EM) grade were purchased from Wako Pure Chemicals (Osaka, Japan) and Ishidzu Pharmaceutical (Osaka, Japan), respectively. Chromatographic analysis[7,11] of these GA solutions indicated that the 20% GA solution contained no impurity and the 25% GA solution comprised trioxane-type GA oligomers (trimer 1.70%, pentamer 0.36%, heptamer 0.11%). Various polystyrene standards (molecular weight (MW), 1050—34000) were supplied by Polysciences (Warrington, PA). Other chemicals used were of analytical reagent grade. Water was purified using a Nihon Millipore (Yonezawa, Japan) Milli-Q II apparatus.

Preparation of Sample Solutions Containing GA and/or GA Oligomers The Ishidzu EM grade GA solution was diluted with water to a 10% GA content, and a 1500 ml portion was applied to a column packed with C8 silica adsorbent[9] at a flow rate of 6.7 ml/min. The first 500 ml of eluate was discarded and the following eluate was collected (solution 1). Then, the adsorbent was washed with 500 ml of water to remove GA monomer, and a 100 ml portion of 90% acetonitrile aqueous solution was passed through the column. The first 50 ml portion of the eluent was collected and diluted with 100 ml of water (solution 2). This solution contained trioxane-type GA oligomers (trimer 0.98%, pentamer 0.26%, heptamer 0.08%) and no GA monomer.

A 700 ml portion of solution 1 was applied again at 6.7 ml/min to the C8 column which had been regenerated by washing with water and methanol, and a 300 ml portion of water was passed through the column. Then, acetonitrile was fed to the column. The first 50 ml of eluate was discarded and the following 100 ml fraction was collected (solution 3). This solution contained 3.0% para-GA alone.

The sample solutions used for the reverse-phase liquid chromatographic (RPLC) and the size-exclusion chromatographic (SEC) studies were prepared by blending the Wako (20%) and Ishidzu (25%) GA solutions, water, acetonitrile and the 3.0% para-GA solution (solution 3) at the volume ratio given in Table I.

Preparation of Precipitates The precipitates A to D were prepared by the following procedures. Precipitate A was obtained by storing the 10% GA solution prepared by dilution of the Wako EM grade GA solution with water at initial pH 11.0 at room temperature for 1 h. Precipitate B was obtained by storing the solution 2 containing GA oligomers (trimer 0.98%, pentamer 0.26% and heptamer 0.08%) at initial pH 8.0 at room temperature for 4 d. Precipitate C was obtained by storing the 2% GA solution prepared by dilution of the Ishidzu EM grade GA solution at initial pH 8.0 at 40 °C for 4 d. Precipitate D was obtained by storing the 0.30% para-GA solution prepared by dilution of the 3.0% para-GA solution (solution 3) with water at initial pH 11.0 at 40 °C for 1h. After filtration through a 0.45 μm membrane, each precipitate was well washed with water and dried *in vacuo*.

Thermogravimetry (TG) TG analyses were done by using a Rigaku (Tokyo, Japan) Thermoflex 8100 series thermal analyzer in a nitrogen

Table I. Preparation of Sample Solutions Used in RPLC and SEC Studies

Materials	RPLC			SEC	
	A	B	C	A	B
20% Wako EM grade	50	—	50	10	5
25% Ishidzu EM grade	—	—	—	—	4
Water	20	70	20	90	91
Acetonitrile	30	20	20	—	—
3% para-GA solution (solution 3)	—	10	10	—	—

—: not blended.

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atmosphere at a flow rate of 10 ml/min. Thermograms were recorded at a heating rate of 5 °C/min.

Pyrolysis Gas Chromatography (Py-GC) Py-GC experiments were performed by using a Nihon Bunseki Kogyo (Tokyo, Japan) JHP-3 Curie-point pyrolyzer coupled to a Hewlett-Packard (HP, Palo Alto, CA.) 5890A gas chromatograph equipped with a flame ionization detector. Pyrograms were obtained by using a Shimadzu (Kyoto, Japan) C-R4A data processor. The precipitate (*ca.* 1 mg) was applied to the Py-foils (Curie-point temp., 358 °C) and heated for 3 s. The GC conditions were as follows: column, ULTRA #2 (HP, 25 m × 0.31 mm i.d., d_f 0.52 μm); splitting ratio, *ca.* 1/60; injection port temperature, 260 °C; oven temperature, 80 °C (2 min holding) to 250 °C at 5 °C/min; carrier gas, N_2 (23 cm/s).

Chemical Ionization Mass Spectrometry (CI-MS) CI-MS studies were carried out by using a JEOL (Tokyo, Japan) DX-303 gas chromatograph-mass spectrometer (GC-MS) system. The precipitates were introduced into the ion source by means of a direct insertion probe and heated from room temperature (1 min holding) to 300 °C and 256 °C/min. The CI-MS conditions were as follows: electron energy, 200 eV; emission current, 300 μA; ion source temperature, 240 °C; scanning range, 60 to 800 amu; scanning time, 0.5 s; accelerating voltage, 3 kV; reagent gas, ammonia.

High Performance Liquid Chromatography (HPLC) Pretreatment for Analyses: The alkaline solutions were filtered through a 0.45 μm membrane and 20 μl portion of the filtrates were subjected to RPLC.

The ethyl acetate extract of GA solution was prepared according to the following procedure. A 3 ml portion of the 2% GA solution containing precipitates was shaken vigorously with 3 ml of ethyl acetate for 30 s, and centrifuged for 5 min at 3000 rpm. A 2 ml portion of the organic layer was withdrawn and the solvent was evaporated at room temperature. The residue was dissolved in 2 ml of tetrahydrofuran (THF) and the THF solution was subjected to SEC after filtration.

The THF-soluble parts of the precipitates were obtained as follows. Each precipitate (A 50 mg and others 10 mg) was added to a 10 ml portion of THF. After vigorous shaking for 1 h, each THF-soluble part was subjected to SEC after filtration.

Instruments and Analytical Conditions: RPLC separation was carried out according to the method reported previously.[7]

SEC separation was performed by using a JASCO (Tokyo, Japan) TRIROTAR pump system. The operating conditions were as follows: column, Shodex KF-803 (exclusion MW size 70000, 300 mm × 8 mm i.d.); mobile phase, THF; flow rate, 0.5 ml/min; column temperature, 50 °C; detection, 235 nm; injection volume, 20 μl. The calibration of molecular weight was done by using polystyrene standards.

Results and Discussion

Characterization of Various Precipitates TG, Py-GC and CI-MS studies were carried out to characterize the various precipitates obtained from GA monomer and/or trioxane-type GA oligomers.

Figure 1 shows the TG curves of the precipitates A to C. The precipitate A from GA alone lost its weight most gradually between 150 and 300 °C (curve A). The precipitate B from the GA oligomers exhibited rapid weight-loss in the same temperature range (curve B). These results suggest that the precipitates A and B have different bonding characteristics. The precipitate B may have a trioxane skeleton as a component unit, because it gave a weight-loss *vs.* temperature curve similar to that of trioxane-type GA oligomers.[7] The precipitate C from the mixture of GA and GA oligomers showed a TG curve intermediate between A and B (curve C). Therefore, it can be speculated that the precipitate C may be composed of polymers derived from both GA and trioxane-type GA oligomers.

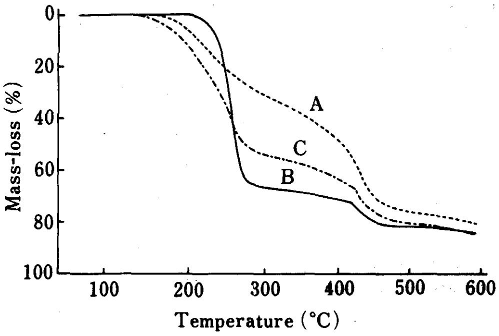


Fig. 1. TG Curves of Various Precipitates Obtained from Alkaline Solutions Containing GA and/or Trioxane-Type GA Oligomers

A (----), from GA monomer; B (——), from GA oligomers; C (—-—), from GA monomer and oligomers.

The pyrograms obtained from the precipitates A to C are shown in Figs. 2A to 2C, respectively. Para-GA was detected from the precipitate B at 36 min (asterisked peak in Fig. 2B). The assignment of this peak was performed in a CI-mode by Py-GC-MS using ammonia as the reagent gas (*m/z* 318).[7,8] The precipitate A did not give the peak due to para-GA, but many unknown peaks appeared between 15 and 25 min (Fig. 2A). On the other hand, the precipitate C exhibited a pyrogram common to the precipitates A and B, as shown in Fig. 2C. These results supported the speculation based on the TG study.

Figures 3A to 3C show the ammonia CI-MS of the precipitates A to C, respectively. As shown in Fig. 3B,

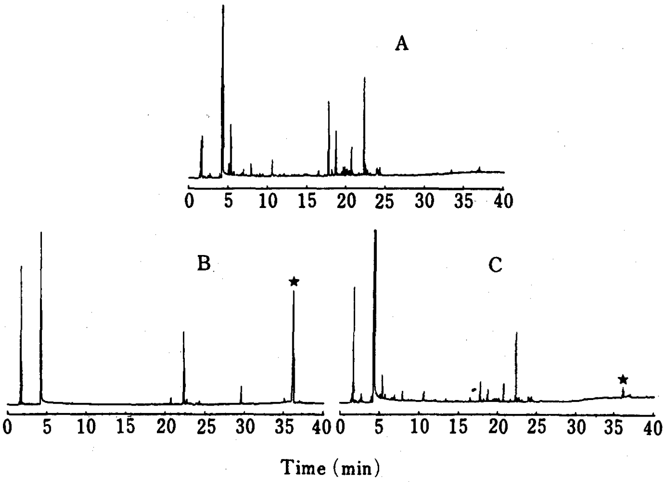


Fig. 2. Pyrograms of Various Precipitates Obtained from Alkaline Solutions Containing GA and/or Trioxane-Type GA Oligomers

A, from GA monomer; B, from GA oligomers; C, from GA monomer and oligomers.

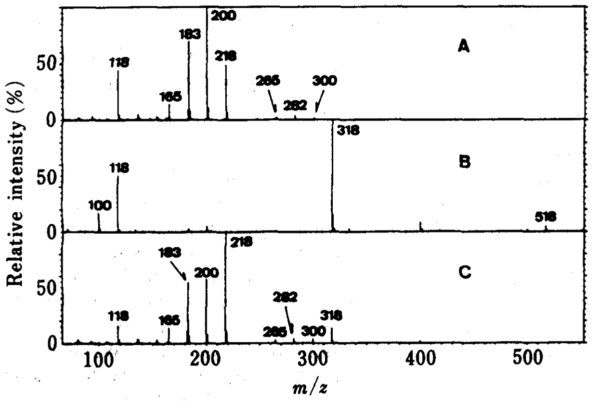
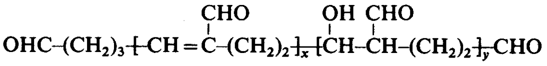


Fig. 3. Ammonia CI-MS of Various Precipitates Obtained from Alkaline Solutions Containing GA and/or Trioxane-Type GA Oligomers

A, from GA monomer; B, from GA oligomers; C, from GA monomer and oligomers.

the characteristic ammonium adduct molecular ions, $(M+NH_4)^+$, due to trioxane-type GA oligomers were found at m/z 318 and 518 from the precipitate B. The ion at m/z 318 may be related to para-GA (trioxane-type trimer) and the latter to the pentamer.[7] The precipitate A did not give these ion species, while it gave ion groups near m/z 200 and 300, as shown in Fig. 3A. Among them, the ions at m/z 165, 183, 200 and 218 and the three ions at m/z 265, 282 and 300 may be attributed to the dimers and trimers formed by the aldol condensation reaction of GA, respectively.[12,13] The two ions at m/z 183 and 265 may be due to the protonated ions, $(M+H)^+$, of the dimer (MW 182) and the trimer (MW 264), respectively. The ion peaks at m/z 200 and 282 are also attributed to the ammonium adduct ions of the dimer and trimer. Margel and Rembaum[12] proposed the chemical structure given in Fig. 4 as a possible structure of poly-GA. This indicates that dehydration may not always occur at every consecutive step of the aldol condensation reaction of GA. Hence, the ions at m/z 218 and 300 may be due to the ammonium adduct ion of the dimer (MW 200) and the trimer (MW 282) having hydroxyl group in their structures, respectively. The ion peak at m/z 165 is also generated by dehydration of the protonated ion of the dimer (MW 182). In the case of the precipitate C, there were the ion species related to para-GA and the aldol condensation products of GA alone, as shown in Fig. 3C.

From the above results of the TG, Py-GC and CI-MS analyses, it followed that the precipitates observed in a GA solution under sterilizing condition may be formed mainly by the aldol condensation reaction involving GA monomer and trioxane-type GA oligomers. The occurrence of the aldol condensation reaction is supported by the existence of two absorptions at 1720 and 1680 cm^{-1} assignable to $\nu_{C=O}$ of saturated and unsaturated aldehydes, respectively, in the infrared spectrum of the precipitates obtained from a slightly alkaline 2% GA solution.[9]



$$\mathrm{OHC{-}(CH_2)_3{-}[CH{=}\overset{CHO}{C}{-}(CH_2)_2]_x{-}[\overset{OH}{CH}{-}\overset{CHO}{CH}{-}(CH_2)_2]_y{-}CHO}$$

Fig. 4. Previously Proposed Chemical Structure of Polymers Formed by Aldol Condensation Reaction of GA Monomer

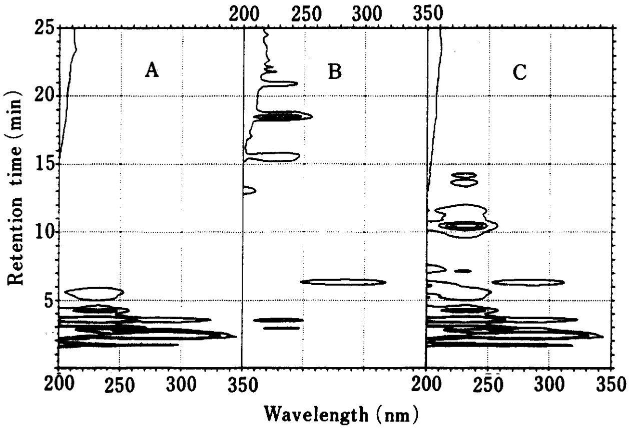


Fig. 5. Contour Plots in RPLC Separations of Water-Soluble Polymers in Various Alkaline Solutions Containing GA and/or Para-GA

A, GA monomer (10%); B, para-GA (0.3%); C, GA monomer (10%) and para-GA (0.3%). The sample solutions were stored at 40 °C after alkalization (pH 8.0) by adding 1.5 g of $NaHCO_3$ and a few drops of 0.1 M NaOH to a 100 ml portion of each sample. The storage times of three solutions A to C were 2, 6 and 2 h, respectively.

Polymerization Reaction Involving GA Monomer and Trioxane-Type GA Oligomers To examine in more detail the polymerization reaction involving GA and trioxane-type GA oligomers, we carried out the RPLC separation of the water-soluble polymers formed in alkaline solutions containing GA (10%) and/or para-GA (0.3%). When the solution containing GA alone was stored for 2 h at 40 °C, several polymers exhibiting λ_{max} at 235 nm were eluted with retention times between 3 and 7 min, and GA monomer having λ_{max} at 285 nm had the retention time of 2.2 min, as shown in Fig. 5A. These polymers seem to be α, β-unsaturated aldehydes, since their λ_{max} at 235 nm is assignable to π–π^* transition of the C=C bond[12,14] conjugated with an aldehyde group. However, λ_{max} near at 285 nm assignable to n–π^* transition of the C=O bond[12,14] was almost absent. This may be because the extinction coefficient of the C=C bond is very much larger than that of the C=O bond.[15-17] Figure 5B shows a contour plot of the solution containing para-GA alone when it was stored for 6 h at 40 °C. Para-GA was eluted at 6.5 min with λ_{max} at the same wavelength as that of GA monomer, and a few polymers having λ_{max} at 235 nm were found at retention times > 15 min. These polymers may be produced by the aldol condensation reaction of para-GA itself, because they have λ_{max} at 235 nm. When the sample solution containing GA and para-GA was stored at 40 °C for 2 h, there was no peak due to polymers formed by the polymerization of para-GA alone, whereas a new peak exhibiting λ_{max} at 235 nm appeared at 10.5 min, as shown in Fig. 5C. This peak may be due to one of the polymers involving GA and para-GA, because it did not appear in the case of the polymerization of GA alone. Additionally, the polymerization reaction of para-GA alone seemed not to occur, probably because of the much smaller content of para-GA than GA.

Degrees of Polymerization We tried to dissolve the aforementioned precipitates A to C in THF for comparison of their molecular weight by means of SEC. The precipitate A was readily dissolved into the solvent. A large part of the precipitate C was soluble in THF, but the precipitate B was hardly dissolved. The precipitate D obtained from para-GA alone was used instead of the precipitate B, because of its slightly higher solubility to THF than the precipitate B. Figure 6 shows the SEC elution profiles A to C of the THF-soluble parts of three precipitates A, D and C, respectively. From the degree of solubility of each precipitate to THF

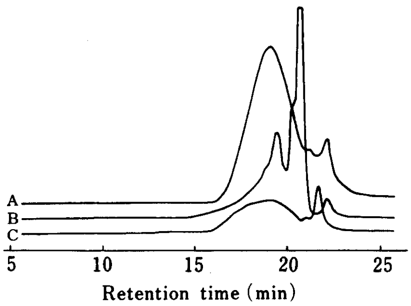


Fig. 6. SEC Elution Curves of THF-Soluble Parts of Various Precipitates Obtained from Alkaline Solutions Containing GA and/or Trioxane-Type GA Oligomers

A, from GA monomer; B, from para-GA; C, from GA monomer and oligomers.

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mentioned above, it was considered that the precipitate D may be comprised of higher polymers than the others. However, as shown by elution curve B in Fig. 6, most of the polymers existing in the THF-soluble part of this precipitate were distributed in a lower molecular weight region than those of the other precipitates (curves A and C). On the other hand, the elution curve B (*ca.* 14 min) showed a faster elution time than the curves A (*ca.* 16 min) and C (*ca.* 15.7 min), as shown in Fig. 6. This suggests that higher polymers (MW *ca.* 25000) may be produced by polymerization involving trioxane-type GA oligomers rather than by that (MW *ca.* 9000) of GA alone. Therefore, it can be assumed that the precipitation in the alkaline GA solution used as a disinfectant may be attributed to the formation of high polymers, since the GA oligomers are responsible for the precipitation.[7,8]

To confirm this assumption, we carried out SEC separations of the ethyl acetate extracts of 2% alkaline GA solutions stored for 48 h at 40 °C. Figure 7 shows the SEC elution curves A and B of the ethyl acetate extracts obtained from the GA solutions without and with GA oligomers, respectively. The comparison of two SEC profiles indicated that higher polymers had been formed in the alkaline GA solution containing GA oligomers. Therefore, the formation of high polymers is also responsible for the precipitation in the slightly alkaline GA solution.

Figure 8 shows the SEC elution curves of the ethyl acetate extracts from the 2% GA solution containing GA oligomers stored for different periods at 40 °C after alkalization. These elution curves were obtained by injecting the same volume and by detecting at the same sensitivity. The intensity of peaks in the SEC profiles obtained after storage for 2 and 4 h increased gradually with the storage time. The front of each SEC profile was shifted gradually to faster elution time as the storage time increased, but this shift was hardly found when the storage time was longer than 24 h. Even though the detection was done at higher sensitivity, there was a similar tendency to that mentioned above. Probably, the higher polymers than those eluting at near 14 min were not extracted with ethyl acetate. Therefore, we tried to estimate the size of polymer chains responsible for the precipitation by using the molecular weight calculated from the retention time when the front of the elution curve was detectable.

Figure 9 shows the time courses of the increment of the molecular weight and the formation of the turbidity in the 2% alkaline GA solutions with and without trioxane-type GA oligomers. The molecular weight of polymers formed by the polymerization of GA alone increased in the first 10 h and approached about 3500, as shown by the open

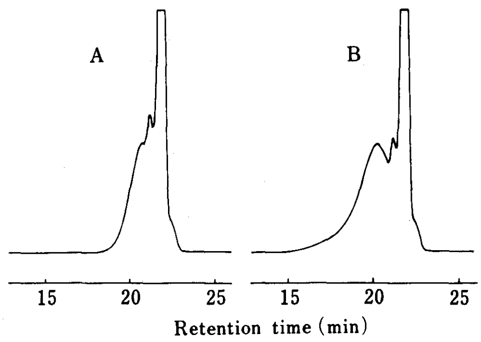

Fig. 7. SEC Elution Curves of Ethyl Acetate Extracts Obtained from Different 2% Alkaline GA Solutions

A, GA monomer; B, GA monomer and oligomers. The sample solutions were stored for 48 h at 40 °C after alkalization (pH 8.0) by adding 300 mg of $NaHCO_3$ and a few drops of 0.1 M NaOH to a 100 ml portion of each sample.

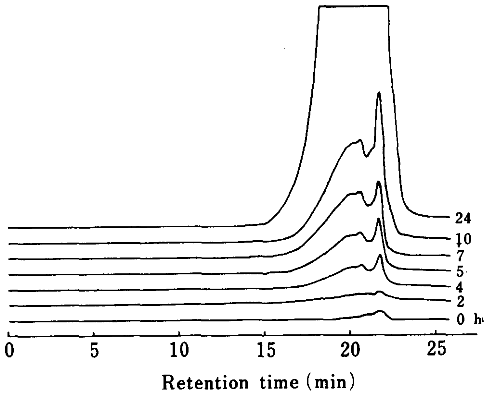

Fig. 8. SEC Elution Curves of Ethyl Acetate Extracts Obtained from 2% Alkaline GA Solutions Containing Trioxane-Type GA Oligomers after Standing for Different Periods at 40 °C

The 2% alkaline GA solution was obtained by the same method as that used for the sample solution B in Fig. 7.

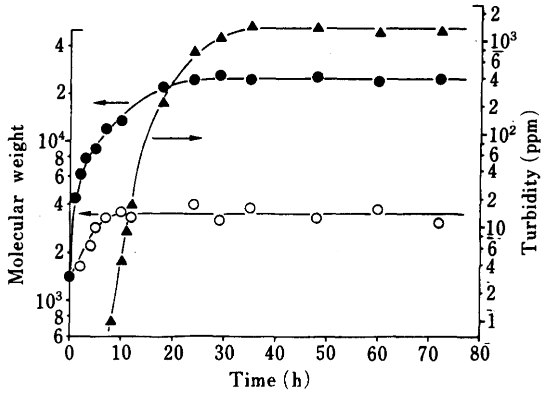

Fig. 9. Time Courses of Change in Molecular Weight and Turbidity in 2% Alkaline GA Solutions with and without Trioxane-Type GA Oligomers

Molecular weight: ○, GA monomer; ●, GA monomer and oligomers. Turbidity: ▲, GA monomer and oligomers. The 2% alkaline GA solutions were obtained by the same methods as used for the sample solutions A and B in Fig. 7.

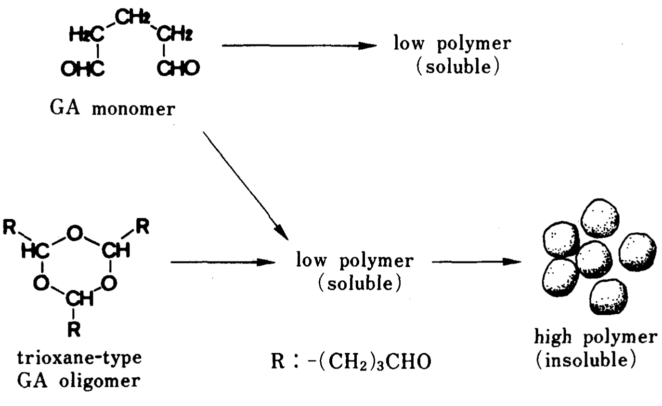

Fig. 10. Proposed Polymerization Mechanism for Formation of Precipitates in a Slightly Alkaline Low-Concentration GA Solution Containing Trioxane-Type GA Oligomers

TABLE II. Influences of GA Concentration, Temperature and Initial pH on Precipitation

Sample No.	GA conc. (%)	Temp. (°C)	Initial pH	Turbidity (ppm)
1	2	25	8.0	131
2	5	25	8.0	245
3	2	40	8.0	654
4	2	25	7.6	83

The concentration of para-GA in each sample was 0.05%. The turbidity value was measured after storing each sample for 4 d. Each sample was prepared by using water, the Wako GA solution (20%) and the 10% aqueous GA solution containing 0.7% para-GA obtained previously by purification of GA solution using C8 silica.[11] The initial pH values were adjusted by using 0.25 M NaOH or 0.25 M HCl after addition of 300 mg or 750 mg of $NaHCO_3$ to a 100 ml portion of 2% or 5% GA solution, respectively.

circles. This alkaline solution gave no precipitation. In contrast, the turbidity value of the alkaline GA solution containing GA oligomers increased rapidly during the first 10 to 20 h, followed by a gradual approach to about the 1400 ppm level (the closed triangles in Fig. 9). Similarly, an increment of molecular weight was observed during the storage time of 24 h, and the final value was about 25000, as shown by the closed circles. From the comparison of the time courses of the molecular weight and the turbidity, we speculated that the precipitation seems to appear at the time of formation of polymers in the molecular weight range of about 12000.

Mechanism of Formation of Precipitates From the above results, it is considered that the precipitation in an alkaline GA solution containing trioxane-type GA oligomers may occur by the mechanism given in Fig. 10. Alkaline aqueous solution containing 2% GA alone does not produce precipitates,[7,8] though GA readily polymerizes under alkaline conditions.[12,18] This means that GA homopolymers formed in a dilute alkaline GA solution do not have a molecular weight high enough to produce the precipitates. In contrast, when the trioxane-type GA oligomers exist as impurities in a GA solution, GA polymerizes with trioxane-type GA oligomers and produces copolymers with higher molecular weight than GA homopolymers. The formation of high polymers may be responsible for the precipitation in a 2% GA solution under sterilizing conditions. The polymerization reaction of GA oligomers themselves may hardly occur, because of their very small content compared with GA monomer.

GA polymerizes mainly by the aldol condensation reaction under alkaline conditions.[12-14] Therefore, pH, temperature and GA concentration are expected to affect the formation of the precipitates. Table II shows the influences of these conditions. The higher the values of initial pH, GA concentration and temperature, the greater the amount of precipitates.

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