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SHORT COMMUNICATION

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# Depolymerization of hyaluronate by the phototoxic drugs phenothiazines and sulfacetamide

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**Abstract** Phenothiazines (promazine, promethazine, chlorpromazine) and sulfacetamide, known as phototoxic drugs, depolymerize aqueous sodium hyaluronate (HA) on exposure to light. The reduction in the HA molecular weight was followed by size-exclusion chromatography with low-angle laser light scattering. In the low-concentration region of the drugs below 0.05 mM, the rate constants of depolymerization increased. The molecular weight of HA was practically unchanged without UV irradiation in the presence of drugs or with UV irradiation in the absence of drugs, indicating the phenothiazines and sulfacetamide require photoenergy to yield any kind of damaging chemical species for HA depolymerization. An involvement of active oxygen radicals in the effects of promazine and promethazine was evidenced by inhibition under anaerobic conditions. Further, addition of mannitol controlled the reaction in the presence of oxygen, pointing to hydroxyl radicals as the damaging agent. Chlorpromazine and sulfacetamide preferably depolymerized HA under anaerobic conditions, suggesting the participation of hydrated electrons.

**Key words** Hyaluronate · Depolymerization · Phenothiazines · Sulfacetamide · UV irradiation

## Introduction

Hyaluronate (HA), a glycosaminoglycan linear polymer with a molecular weight of $10^4$–$10^7$, has unique rheological properties, serving as a lubricant, protector and humectant in diverse body sites, such as joints, vitreous humor and skin. High-molecular-weight HA forms entangled molecular networks, which exert their tissue protective effects by reducing friction and wear in joints [1, 2], constructing an elastic three-dimensional structure in the skin and preventing damaging substances from approaching close to cells [3]; however, low-molecular-weight HA is less efficient at forming a network at similar concentrations. Moreover, HA can be depolymerized quite easily by various physical and chemical factors. In our previous studies, scission of HA polymer chains was observed during measurements with a cone-plate rotational viscometer by shearing stress [4] and by contact with solid-state metals without elution of metal ions [5]. In the latter case, hydroxyl radicals, generated at the surface of the cup and the cone of the viscometer, were concerned in the depolymerization. There are also reports of HA depolymerization induced by UV irradiation [6–10]. Hydroxyl radicals and hydrated electrons generated by light irradiation of aqueous solutions and singlet oxygen produced in the presence of photosensitizers have been suggested as the reactive species.

The clinically used phenothiazines are known to cause both phototoxic and photoallergic reactions in the skin and eyes of patients receiving these drugs for a long period. Upon UV irradiation phenothiazines yield a variety of free radicals, including cation radicals, hydrated electrons and some active oxygen species which could play roles in their phototoxicity [11–13]. As HA is

present in the skin and eye tissues, the possibility arises of depolymerization by radicals produced in photoexcitation processes.

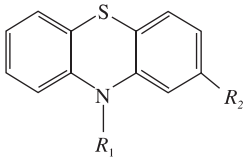
In the present study, we examined depolymerization of HA by phototoxic drugs, with especial attention being para to promazine and chlorpromazine, under both aerobic and anaerobic conditions. The radical species involved in the depolymerization were considered for each drug.

## Experimental

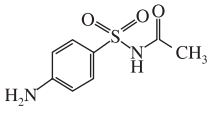
The sodium HA employed, extracted from a culture medium of *Streptococcus equi*, had a weight-average molecular weight ($M_w$) of about $3.0 \times 10^6$. Unless otherwise noted, a solution prepared at a final concentration of 0.1% HA in 0.2 M NaCl was used. For the experiments to investigate the effects of enzymatic radical scavengers, superoxide dismutase (SOD) and catalase, solutions were prepared in 50 mM phosphate buffer (pH 7.4). Phenothiazines and sulfacetamide were commercial products of Sigma Chemical Company and they were applied without further purification. The chemical structures of the three phenothiazines (promazine, promethazine and chlorpromazine) and of sulfacetamide are listed in Table 1. SOD from bovine erythrocytes (2990 units/mg solid) and catalase from bovine liver (2800 units/mg solid) were purchased from Sigma Chemical Company. Other reagents were of analytical grade. All the water used was distilled once and then filtered through Millipore filters.

Depolymerized HA sample solutions were obtained as follows. For the light source, two UV lamps (100 V, 5 W) for spot detection of thin-layer chromatography with different wavelengths (254 and 365 nm) were used. For the experiments with irradiation at 254 nm, square quartz cells were employed under aerobic conditions. For anaerobic conditions, a cylindrical quartz cuvette with optically flat windows and a narrow neck was used, and air was excluded from the samples by continuous flushing with nitrogen. For the experiments with 365-nm irradiation, glass ampules were used, since the glass could conveniently eliminate shorter wavelength UV. To generate anaerobic conditions, the samples were deaerated by 5-min nitrogen bubbling before irradiation, and the necks of the ampules were sealed immediately with a burner. The distance of the system vessels from the light source was set at 5 cm and their reverse sides were immersed in a thermostated water bath (37.0 °C). After the desired exposure, the sample solution was removed, appropriately diluted and the drugs were eliminated by solid-phase extraction with a Sep-Pak C18 cartridge before being subjected to molecular-weight measurement. This was achieved using size-exclusion chromatography (SEC) with low-angle laser light scattering (LALLS). SEC-LALLS (Tosoh Co.) was performed on three columns, a TSK-guard column $PW_{XL}$ letters ($6.0 \times 40$ mm), a TSK-G6000$PW_{XL}$ column ($7.8 \times 300$ mm) and a TSK-G3000$PW_{XL}$ column ($7.8 \times 300$ mm), with 0.2 M NaCl solution. The flow rate was 0.5 ml/min. A 500-$\mu$l portion of the original and depolymerized samples containing about 0.02% HA was injected, and the peak elution was monitored with a LALLS photometer (Tosoh LS-8000) and a differential refractometer (Tosoh RI-8012). Estimation of molecular weights was performed with the Tosoh GPC-LALLS data processing program in the LALLS mode. Each determination was carried out at least three times and the mean value was given as the result.

**Table 1** Structures of phenothiazines and sulfacetamide




| Name | $R_1$ | $R_2$ |
|---|---|---|
| Promazine | $(CH_2)_3N(CH_3)_2$ | H |
| Chlorpromazine | $(CH_2)_3N(CH_3)_2$ | Cl |
| Promethazine | $CH_2CH(CH_3)N(CH_3)_2$ | H |




sulfacetamide

## Results and discussion

### Requirement of photoenergy for HA depolymerization by phenothiazines

Changes in the $M_w$ of HA in the presence of each of the three phenothiazines in the dark or light, expressed as percentage residues of $M_w$ are shown in Fig. 1. All three phenothiazines required UV irradiation to depolymerize HA, and no decrease in $M_w$ was observed in the dark in line with reports that phenothiazines need photoenergy to generate radicals [11–13]. Shorter wavelength UV light was more effective, presumably related to differences in the strength of the photoenergy, the absorption spectrum of phenothiazines and the wavelength dependence of related radical species. The energy to induce photochemical reactions, such as excitation of chemicals, bears a reciprocal linear relationship to wavelength. Further, aqueous solutions of all the phenothiazines studied showed absorption spectra with peaks at 254 nm

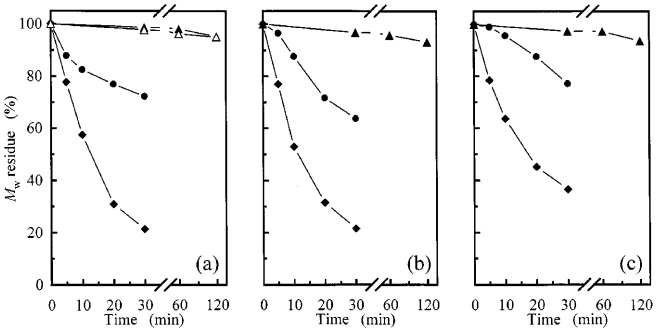



**Fig. 1** Aerobic depolymerization of hyaluronate (HA) by **a** promazine, **b** promethazine and **c** chlorpromazine under light and dark conditions with 0.1 mM phenothiazines and 365-nm irradiation (●), with 0.1 mM phenothiazines and 254-nm irradiation (◆), with 1 mM phenothiazines in the dark (▲) and with 1 mM preirradiated promazine in the dark (△)

and at around 300 nm, with that at 254 nm being several times higher. It was thus concluded that photoexcitation of phenothiazines would be optimal wih 254-nm, irradiation.

Although there are reports of depolymerization of HA by UV irradiation [6, 8, 14], in the present study the $M_w$ of HA remained practically unchanged in light without phenothiazines. After 240 min, the $M_w$ was 94 ± 3% of the initial value with irradiation at 254 or 365 nm and was almost equal to that obtained in the dark. Hvidberg et al. [6] reported that the relative viscosity of potassium HA prepared from umbilical cords was reduced to about 32% of the initial value after 120-min irradiation. Lapcik et al. [8] reported that the $M_w$ of HA (extracted from rooster combs) changed from $1.12 \times 10^6$ to $0.18 \times 10^6$ on 300-min irradiation. The power of the light sources used in our study was, however, weaker than that of the mercury-quartz high-pressure burner of 125 W employed by Hvidberg et al. and the 500 W high-pressure mercury lamp used by Lapcik et al. Furthermore, the wavelengths of our UV lamps were relatively long compared to the 184.9, 194.2 and 253.7 nm used by Khan et al. [14]. The 253.7-nm irradiation resulted in little polymer chain scission. Irradiation of water with 184.9-nm UV light is known to produce radicals, such as hydrated electrons, hydrogen atoms and hydroxyl radicals, which could depolymerize HA.

On UV irradiation, phenothiazines take on a pale red or blue-purple coloration; however, these stable photoproducts did not seem to act as activated species, because addition of preirradiated phenothiaziones did not cause depolymerization of HA in the dark. A typical result when promazine solution previously irradiated at 365 nm for 2 h was added to the HA solution is illustrated in Fig. 1a. The degree of change in $M_w$ was the same as that with nonirradiated promazine in the dark. Thus, photoinduced radicals or intermediates with short decay times participated in the HA depolymerization.

## Quantitative dependence of the aerobic HA depolymerization rate on promazine concentration

Next, let us discuss the kinetics study of the degradation. The type of chain scission can be suggested by changes in the molecular-weight distribution [4, 15], that is generally obtained from division of $M_w$ by the number-average molecular weight. During the course of depolymerization, the molecular-weight distribution of HA samples showed only slight variation. For example, when the $M_w$ decreased to about one-fifth of the initial value on 30-min irradiation at 254 nm with 0.1 mM phenothiazines as illustrated in Fig. 1, the distribution varied between 1.03 and 1.40. If depolymerization occurs nonrandomly, the molecular-weight distribution would be expected to show a broad variation. The small value observed suggested random depolymerization. Moreover, as the plot of the percentage residue of $M_w$ on a log scale versus time indicated a linear relationship at the very beginning of the reaction, a pseudo-first-order reaction was considered, and rate constants were estimated from the slopes of the straight lines. The dependence of the initial depolymerization rate on the concentration of promazine with 365-nm irradiation under aerobic conditions is shown in Fig. 2. Up to 0.05 mM the depolymerization rate increased in proportion to the concentration. In the range 0.05–1 mM, a plateau appeared to be reached; therefore, the maximum drug concentration was set at 1 mM.

## HA depolymerization under anaerobic conditions

HA can be depolymerized by many kinds of radicals, such as hydroxyl radicals, singlet oxygen, superoxide and hydrated electrons [3, 5–10, 14, 16], and phenothiazines are know to yield a variety of radical species including the corresponding cation radicals, hydrated electrons and active oxygen [11–13]. Many phototoxic effects in vitro have been reported, such as hemolysis [17], inhibition of enzyme activity [18, 19], mutagenesis [20] and strand breakage in DNA [21, 22]. It has not been verified in all cases which specific radical species are responsible. Reports of phototoxicity by chlorpromazine have been more frequently published than reports of phototoxicity by other phenothiazine derivatives. We concentrated on whether or not there are differences in HA depolymerization effects depending on the phenothiazine species.

First, the participation of oxygen was examined using samples deaerated with nitrogen. As shown in Fig. 3, promazine and chlorpromazine demonstrated opposite

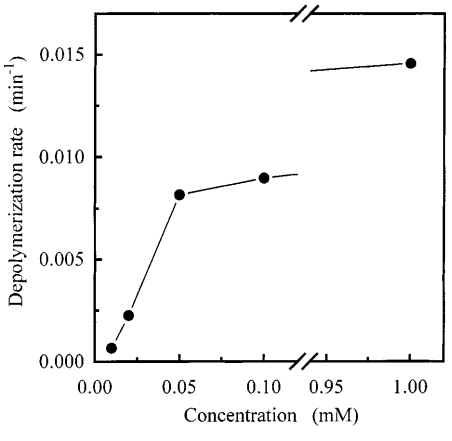



**Fig. 2** Dependence of the HA depolymerization rate on the promazine concentration with 365-nm irradiation under aerobic conditions

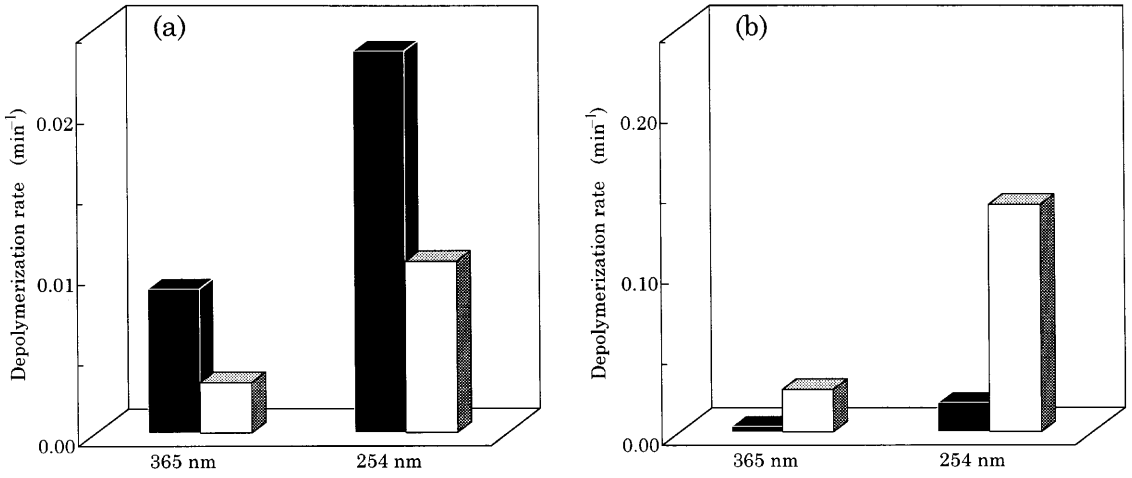



**Fig. 3** Effect of nitrogen bubbling on the HA depolymerization rate with **a** promazine and **b** chlorpromazine under aerobic conditions (*filled columns*) and under anaerobic conditions (*open columns*)

oxygen requirements for HA depolymerization. The depolymerization was inhibited by nitrogen saturation in the case of promazine, regardless of the UV wavelength. The depolymerization rate reduced to less than half of that before bubbling nitrogen through the sample for 5 min. With promethazine, the oxygen requirement was similar (data not shown). In contrast, chlorpromazine accelerated the depolymerization rate by about 8 times under anaerobic conditions; the degree was almost the same with 254-nm and 365-nm irradiation. Differences in oxygen dependence could be attributed to the means of photochemical radical formation [11, 13]. According to Motten et al. [13], excitation of promazine into the singlet state produces no significant radical adducts in nitrogen-bubbled solutions, but in the presence of oxygen, an oxygen-centered radical is produced which gives peroxy and hydroxyl adducts as decay products at neutral pH. The signal intensity of the hydroxyl adduct decreased in the presence of SOD, suggesting the formation of superoxide. On the other hand, with chlorpromazine, various pathways to radical formation have been proposed. Under aerobic conditions, a peroxy adduct was trapped on photolysis at 330 nm. In nitrogen-bubbled solutions, carbon-centered adducts were detected. The chlorpromazine triplet undergoes direct homolytic fission to yield a chlorine atom and a neutral promazinyl radical; these abstract hydrogen from surrounding donors. In addition, hydrated electrons were presumably formed with the generation of cation radicals as suggested by Navaratnam et al. [11] from flash photolysis studies. From our result, participation of hydrated electrons could be concluded, because nitrogen bubbling accelerated the HA depolymerization in the presence of chlorpromazine (Fig. 3b). The rate of the reaction of hydrated electrons with oxygen is 2 orders of magnitude faster than that with HA [11, 23]. Therefore, under aerobic conditions, hydrated electrons react preferentially with oxygen to form superoxides, lowering their ability to depolymerize HA [23].

The higher phototoxicity of chlorpromazine is consistent with its ability to form various kinds of reactive species regardless of the existence of oxygen. Further, in the context of HA depolymerization, it is of interest that in vitro experiments by Matsumoto and coworkers [25, 26] have shown dimer and higher-molecular-weight photoproducts of chlorpromazine, generated by UV irradiation, to activate hyaluronidase. This naturally leads to a reduction in the HA molecular weight. The relation of the HA depolymerization to the phototoxicity in the skin and eyes is still unclear, since associated pathological events are complicated and various factors exert an influence on photosensitivity. However, a reduction in the HA molecular weight would be expected to result in a predisposition to disorders, because low-molecular-weight HA provides less effective physiological protection in living tissues.

## Active oxygen species involved in HA depolymerization by promazine

Promazine apparently cooperates with oxygen in depolymerizing HA, because the decrease in $M_w$ was suppressed by nitrogen bubbling as shown in Fig. 3a. The results of adding various kinds of radical scavengers are summarized in Table 2, with the values being expressed as percentage residues of $M_w$ after 30-min irradiation. Bovine serum albumin was examined as a control for the competitive inhibition of protein against radical attacks. The HA depolymerization was obviously inhibited by mannitol, a scavenger of hydroxyl

**Table 2** Effects of radical scavengers on the depolymerization of hyaluronate by 1 mM promazine with 365-nm irradiation

| Scavengers | Concentration ($ml^{-1}$) | $M_w$ after 30 min (%) |
|---|---|---|
| None | | 74.3 |
| Bovine serum albumin | 0.7 mg | 69.3 |
| Superoxide dismutase | 10 units | 63.2 |
| | 100 units | 63.7 |
| | 500 units | 71.0 |
| Catalase | 500 units | 65.7 |
| | 1000 units | 69.4 |
| Superoxide dismutase + catalase | 1000 units each | 85.8 |
| Mannitol | 0.91 mg | 76.1 |
| | 1.82 mg | 75.1 |
| | 3.64 mg | 94.2 |
| | 18.2 mg | 97.1 |

radicals, at 3.64 mg/ml, corresponding to 20 mM. SOD and catalase had no effect when they were used separately. In combination at 1000 units/ml of each, depolymerization was retarded. After 30 min the $M_w$ remained at 85.8% of the initial value, however, it decreased to 19.2% after 240 min of prolonged irradiation, even with SOD and catalase. The value of 19.2% was same as that obtained without scavengers, whereas $M_w$ remained at 56.9% with 3.64 mg/ml mannitol. From these results, it could be concluded that the principal species depolymerizing HA with promazine was the hydroxyl radical. Since SOD showed no inhibition effect, the superoxide itself must be unimportant, even if it was formed, as observed with aerobic photolysis of promazine at 330 nm by Motten et al. [13]. The reason why the depolymerization was accelerated by adding a small amount of SOD might be that the hydrogen peroxide generated by SOD and the surplus superoxide promote the formation of hydroxyl radicals, which have a high ability to depolymerize HA.

### HA depolymerization by hydrated electrons generated photochemically with sulfacetamide

The participation of hydrated electrons in HA depolymerization was suggested by the anaerobic photodegradation of chlorpromazine. We, therefore, expected that other drugs, which yield hydrated electrons photochemically, would also depolymerize HA. Sulfacetamide, which is known to form hydrated electrons on photolysis [24], has been used as an antibacterial drug for dermatological and ophthalmic treatment but often causes phototoxic and photoallergic reactions.

As expected, in the presence of sulfacetamide, HA was depolymerized with but not without 254-nm irradiation (Fig. 4). In addition, 365-nm irradiation induced no change in $M_w$ even at concentrations of sulfacetamide up to 1 mM. The first step in the photochemical reaction must be absorption of light, but the absorbance by sulfacetamide at 365 nm is almost zero; the maximum is seen at 265 nm. With photolysis at 254 nm, the rate of depolymerization increased in proportion to the sulfacetamide concentration up to 0.05 mM (Fig. 5), but above 0.05 mM the rate constant decreased. The depletion of light in a saturated solution might be responsible.

The results prove that HA depolymerization by hydrated electrons could be obtained under anaerobic conditions; the depolymerization was accelerated by nitrogen bubbling (Fig. 4).

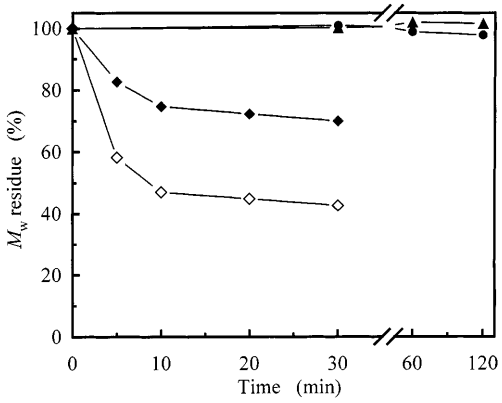



**Fig. 4** HA depolymerization profiles with sulfacetamide. 1 mM sulfacetamide and 365 nm irradiation (●), 0.015 mM sulfacetamide and 254 nm irradiation (◆), 0.015 mM sulfacetamide and 254 nm irradiation under anaerobic conditions (◇), 1 mM sulfacetamide in the dark (▲)

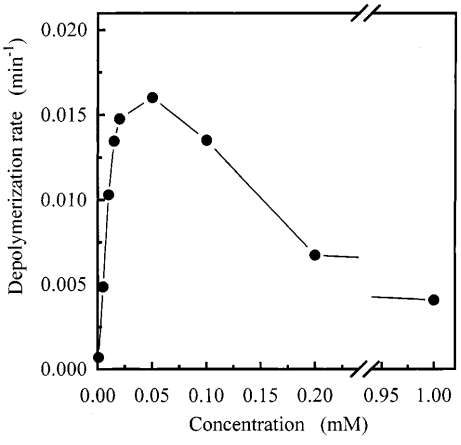



**Fig. 5** Dependence of the HA depolymerization rate on the sulfacetamide concentration with 254-nm irradiation under aerobic conditions

## Conclusions

HA was depolymerized by UV irradiation with phenothiazines and sulfacetamide due to their production of

different kinds of radicals on photodegradation. The actual radical species taking part in the depolymerization differ depending on the drug. Promazine and promethazine cooperate with oxygen to depolymerize HA. Hydroxyl radicals are responsible for the damage, the reaction being inhibited under anaerobic conditions and being controlled by the addition of mannitol in the presence of oxygen.

Chlorpromazine depolymerized HA not only under aerobic but also under anaerobic conditions, with diversity of radical species involved: in the presence of oxygen, active oxygen species, probably hydroxyl radicals, depolymerized HA, and in its absence, hydrated electrons appeared responsible. HA depolymerization by photochemically generated hydrated electrons was also suggested with sulfacetamide.

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