Hyaluronate Depolymerization Following Thermal Decomposition of Oxytetracycline

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Depolymerization of sodium hyaluronate (HA) by tetracyclines was investigated. Reduction in HA molecular weight was followed by size exclusion chromatography with a low angle laser light scattering detector. On mixing with oxytetracycline hydrochloride (OTC) solution and incubating at 37 °C, HA was gradually depolymerized. OTC, a representative antibiotic, is known as a photosensitizer, and phototoxic side effects relevant to radicals have been reported. However, HA depolymerization required no irradiation. As time passed, OTC solution incubated at 37 °C got colored reddish brown, even in the dark. With reversed-phase HPLC separation, several peaks derived from decomposed OTC appeared. One of the peaks had an absorbance in the visible range. A quantitative correlation between the discoloration and the HA depolymerization rate was obtained. On the other hand, when samples were incubated below 25 °C, change of color was slight, and practically no HA depolymerization was observed after up to 4 h. Oxygen depletion by nitrogen saturation or addition of mannitol also prevented the depolymerization. Under anaerobic conditions, the color of the solution did not change, whereas it turned red under aerobic conditions in the presence of mannitol. The mannitol did not inhibit the OTC decomposition, but it preserved HA from damage. On the basis of the known decomposition of OTC and the results of HPLC separation, anhydrooxytetracycline can be proposed as the derivative causing HA depolymerization.

Key words hyaluronate; depolymerization; oxytetracycline; thermal decomposition; molecular weight

Hyaluronate (HA) is a glycosaminoglycan ubiquitously distributed in organisms. It has a linear structure without side chains, and a molecular weight of 10^4 to 10^7. The huge molecular mass characterizes the physiologic and physicochemical properties of HA. High-molecular-weight HA forms entangled networks, having unique viscoelasticity and water-holding ability.[1] The characteristics are molecular-weight-sensitive, and yet, high molecular weight HA is easily depolymerized by various physical and chemical factors. There are many reports about HA depolymerization by photo,[2,3] thermal,[2,4] mechanical[2,5] and chemical actions.[6,7] Previously, we described HA depolymerization by phenothiazines and sulfacetamide with UV irradiation.[7] These drugs are known to cause dermatological or ophthalmic phototoxicity in patients receiving them in the long term. We have revealed the hydroxyl radicals and hydrated electrons generated during photodegradation of the drugs to be the active chemicals for HA depolymerization.

Tetracyclines have also been reported to exhibit phototoxic and photoallergic reactions, with certain radicals proposed to account for *in vivo* and *in vitro* toxicity.[8,9] In this paper, the depolymerization of HA by tetracyclines, expressly oxytetracycline (OTC), was investigated. From depolymerization profiles under various conditions, the probable causative derivative was identified.

Experimental

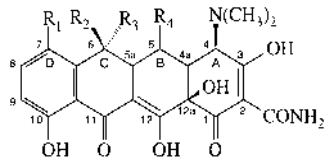
Materials Two sodium HA samples were used: one was extracted from culture medium of *Streptococcus equi*, with a weight average molecular weight (M_w) of 2.6×10^6, and the other was isolated from rooster comb, with a M_w of 1.1×10^6. Sodium alginate of medium viscosity from kelp was a commercial product of Sigma Chemical Co. Tetracycline hydrochlorides were purchased from Sigma Chemical Co. or Wako Chemical Co. They were used without further purification. Their chemical structures are listed in Table 1. Other reagents were of analytical grade. All the water used was distilled once and then Millipore filtered.

Sample Preparation Polymer solutions were mixed with tetracycline solutions to make at final concentrations of 0.1% in 0.2 M NaCl. They were incubated in test tubes in a thermostated water bath. At the desired time, a portion was removed, appropriately diluted and after drugs were eliminated by solid-phase extraction on Sep-Pak C_{18} cartridges, the molecular weight was measured. For studies under anaerobic conditions, glass ampules were used in place of test tubes. Solutions in ampules were de-aerated by 10-min nitrogen bubbling, immediately before the necks of the ampules were sealed with a burner. For UV irradiation, lamps (100 V, 5 W) with 254 nm or 365 nm for TLC spot detection were employed. The distance of the vessels from the light source was set at 5 cm. In the experiments with 254 nm irradiation, the vessels were squared quartz cells.

Molecular Weight Measurement M_w of HA was measured using a size exclusion chromatography (SEC) equipped with a low angle laser light scattering (LALLS). SEC-LALLS (Tosoh Co. Ltd.) was performed on three columns, a TSK-guard column PW_{XL} (6.0×40 mm), a TSK-G6000PW_{XL} (7.8×300 mm) and a TSK-G3000PW_{XL} (7.8×300 mm), with 0.2 M NaCl solution. The flow rate was 0.5 ml/min. A 500 μl aliquot of 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). The estimation of M_w was performed with the Tosoh GPC-LALLS data processing program in the LALLS mode.

HPLC Conditions Chromatographic separation of OTC and its decomposition products was performed. The HPLC system consisted of an LC-6A Liquid Chromatograph (Shimadzu), an SCL-6A System controller (Shimadzu) and an SPD-6A UV-Spectrophotometric detector (Shimadzu). The detector was set at 265 nm. The data were collected on a C-R4A System controller (Shimadzu). For 3-dimensional chromatogram and contour map

Table 1. Structures of Tetracyclines



Name	R_1	R_2	R_3	R_4
Oxytetracycline	H	CH_3	OH	OH
Doxycycline	H	CH_3	H	OH
Tetracycline	H	CH_3	OH	H
Chlortetracycline	Cl	CH_3	OH	H
Minocycline	$N(CH_3)_2$	H	H	H

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Table 2. Percentages of HA Molecular Weight Incubated with1 mM Tetracyclines at 37 °C under Various Conditions

Tetracyclines	Irradiation	Aerobic/ Anaerobic	Other additives	Time (h)				
				0.5	1	2	4	10
Doxycycline	—	Aerobic	—	99.5	100.4	100.1	100.0	99.1
Tetracycline	—	Aerobic	—	99.3	100.5	98.2	100.2	96.9
Chlortetracycline	—	Aerobic	—	100.1	100.2	100.0	100.1	—
Minocycline	—	Aerobic	—	96.5	91.1	90.7	92.7	92.8
Oxytetracycline	365 nm	Aerobic	—	100.8	97.7	92.1	73.8	—
	254 nm	Aerobic	—	100.3	97.8	92.8	67.5	—
	—	Aerobic	—	100.0	96.6	89.9	70.0	24.3
	—	Aerobic	5 mM mannitol	98.7	98.8	98.5	91.5	—
	—	Aerobic	20 mM mannitol	99.2	99.4	98.9	95.2	—
	—	Anaerobic	(Nitrogen saturated)	98.7	98.1	96.5	90.7	—

measurements, a Hewlett-Packard (Palo Alto) Model 1100 liquid chromatograph equipped with a photodiode array high-speed spectrophotometric detector was used in combination with HP ChemStation software. In both systems, a GL-Pack Lichrosorb RP18-5 (GL Sciences Inc.) of 4.6 mm i.d.× 250 mm was used. The mobile phase was methanol–acetonitrile–aqueous 0.01 M oxalic acid/0.001 M disodium–EDTA (12 : 12 : 76, v/v%). The flow rate was 0.8 ml/min. All the measurements were made at 40 °C. Sample solutions were prepared by 5-fold dilution of heated 1 mM OTC with mobile phase.

Spectra Measurement UV and visual absorption spectra of OTC solutions were measured with a spectrophotometer (Hitachi U-3210) at ambient temperature.

Results and discussion

HA Depolymerization by Tetracyclines Table 2 summarizes data for changes in M_w of HA from *Streptococcus equi*, during incubation with each tetracycline analog (1 mM) at 37 °C. The values are percentages of the initial M_w. The measurements did not vary widely, therefore, only the mean values from at least three repeated determinations are given as the results. Of the five tetracyclines studied, only OTC obviously depolymerized HA. Tetracyclines are known as photosensitizers.[8,9] From recent clinical reports, doxycycline appears to be the most phototoxic. However, with 365 nm irradiation, 1 mM doxycycline did not exert a major influence on the HA molecular weight. Similar results were obtained with 254 nm irradiation. In contrast, OTC depolymerized HA, even in the dark, and the reduction in M_w was almost to the same degree as in the light. For cases of other tetracyclines than OTC, as the light and its wavelength did not affect the HA molecular weight, only the results obtained in the dark are tabulated. Although phototoxicity and oxygenation of tetracyclines have been reported, such reactions were observed under neutral or alkaline conditions. In our study using non-buffered 0.2 M NaCl solution, the pH of the system was slightly acidic (pH 3—4). In consequence, the irradiation would not be expected to induce photochemical reactions.

To check for the existence of impurities in the OTC reagent, a sample from another supplier was also examined. The percentage of M_w was reduced to 89.2% after 4-h incubation, and 20.1% after 10-h incubation. Thus, contaminating impurities were not likely to be responsible for the observed affects but rather OTC itself or its decomposition products.

Depolymerization of Other Polysaccharides Table 3 shows data for reduction in M_w of low molecular weight HA from rooster comb and sodium alginate, with a different saccharide composition from HA. In both cases, the M_w decreased during incubation with OTC. As the depolymerization was observed irrespective of such factors as polysaccharide structure, initial M_w and origin, it was confirmed to chemically proceed through the action of non-specific active species in the OTC solution. The higher the initial M_w of HA, the more clear the depolymerization. Accordingly, henceforth we used high molecular weight HA in our experiments.

Table 3. Reduction in M_w of Low Molecular Weight HA and Sodium Alginate of Medium Viscosity

	M_w ($\times 10^4$)			
Time (h)	0	1	2	4
Low molecular weight HA	109.8	99.9 (91.0%)	93.6 (85.2%)	79.9 (72.8%)
Alginate	23.5	21.1 (89.8%)	18.4 (78.3%)	17.2 (73.2%)

0.1% polysaccharide with 1 mM OTC solutions were incubated at 37 °C. The numbers in the parenthesis indicate the percentage residue from the initial M_w.

Thermal Effects on OTC-induced Depolymerization of HA Tetracycline solutions are unstable when heated. Particularly, the OTC solution incubated at 37 °C visually changed from yellow to reddish brown over time. In contrast, with incubation at 10 or 25 °C the sample solutions scarcely colored, and practically no HA depolymerization was observed after up to 4 h (Fig. 1). Even at 37 °C, about a half-hour lag was observed before depolymerization became apparent. This time lag disappeared and the M_w immediately decreased, when an OTC solution, previously heated for 2 h at 37 °C and slightly reddened, was used for sample preparation (---●---). The reddish tinge continued to deepen during the experiment. When a sample, prepared with the same preheated OTC solution, was incubated at 10 °C thereafter, the reduction of M_w was decelerated (---○---), and the color of the solution hardly changed.

Thermal Decomposition of OTC The results described above imply that the depolymerization profiles were linked to discoloration of OTC, and therefore decomposition products. The definite decomposition mechanisms of OTC have not yet to be elucidated, but several compounds are known to be generated: 4-*epi*-derivatives; anhydro-derivatives; and *apo*-oxytetracyclines (*apo*-OTC).[10—12] Epimerisation at C-4 yields 4-*epi*-tetracyclines. This rearrangement is slow in the case of OTC due to the stereochemical effect of the hydroxy

group of C-5. In the acidic region, tetracyclines having a hydroxyl group at C-6 readily eliminate water with concomitant aromatization of ring C to form anhydro-derivatives. With OTC, α- and β-*apo*-OTCs are formed in a series of successive reactions through anhydrooxytetracycline (anhydro-OTC). The anhydro-OTC, as a precursor, has not been isolated for studies in detail, probably because of its instability. However, anhydrotetracycline, whose chemical structure is very similar to anhydro-OTC, can be isolated, and is reported to have an absorption around 400—500 nm.[8,13] Incidentally, *apo*-OTCs have no absorption in the visible range. The known pathways of decomposition and rearrangements are illustrated in Fig. 2.

Separation, identification and measurement of OTC and some decomposition products have been described,[11,12] but simple, reliable methods have not yet to be established. Furthermore, reference substances for decomposition products are not readily available. In this study, isocratic reversed-phase HPLC was applied to separate the derivatives. A HPLC chromatogram of thermally decomposed OTC is shown in Fig. 3. Increase and decrease in peak areas during the incubation are plotted in Fig. 4. The main peak at 6.6 min was attributed to original OTC. The peak at 8.3 min was also found in samples without heating, and its area decreased rather than increased with prolonged incubation. This is probably 4-*epi*-OTC, because the peak area slightly increased when the sample solution was acidified to pH 1 with hydrochloric acid. Epimerisation is accelerated at low pH. The peak areas with the retention times at 4.8 and 12.9 min increased with the incubation period, but only the derivative eluting at 4.8 min had an absorption in the visible range. Judging from the spectrum of each peak, that at 4.8 min was suited to anhydro-OTC, and that at 12.9 min was suited to *apo*-OTC.[12] For subsequent investigations, the 480 nm absorbance of the solution was considered to reflect the extent of decomposition for convenience.

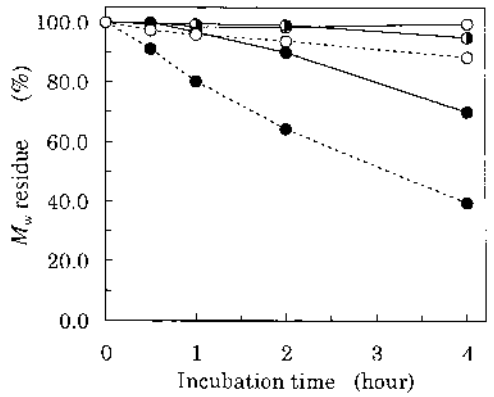

Fig. 1. Effect of Temperature on HA Depolymerization by OTC

—○—, ---○---, 10 °C; —◑—, 25 °C; —●—, ---●---, 37 °C. The results connected with dotted lines were obtained from the samples prepared with pre-heated OTC solution. HA concentration was 0.1%, and OTC concentration was 1 mM.

Quantitative Dependence of Depolymerization Rate on Concentration of OTC or its Decomposition Products The relation between the discoloration and the depolymerization rate was quantitatively investigated. First the OTC concentration of the sample preparation was changed. To eliminate the initial delay time, the OTC solutions were heated at 37 °C for 2 h before mixing with HA. It was confirmed in another test that the increase in 480 nm absorbance was independent of the OTC concentration. As semilogarithmic plots of the percentage of original M_w *versus* incubation time showed linear relations at the beginning of the reaction, pseudo-first order reactions were concluded. The rate constants were taken from the slope of the lines (Fig. 5). The depolymerization rate increased in proportion to the OTC concentration in the region of 1—4 mM.

Another possible measure to increase the decomposed derivatives was to prolong the pre-heating period. With incubation of the OTC solution at 37 °C under aerobic conditions, the absorbance around 480 nm increased with time. Changes in the absorption spectrum of 1 mM OTC are shown in Fig. 6(a), and the increase in 480 nm absorbance in Fig. 6(b). Exhaustively decomposed OTC became insoluble, and the solution was turbid. Therefore, over 16-h incubations, the supernatant was used after insoluble substances were removed by centrifugation. The absorbance almost reached a maximum after thirty hours. Sample solutions were prepared with OTC solutions, whose initial concentration was the same and pre-heated periods were different. Figure 7 indicates the relationship between the HA depolymerization rate and the 480 nm absorbance at the start of the reaction. Pre-heated periods of 2, 4, 8 and 16 h were in order of increasing absorbance and an adequate correlation was the result. In the sample pre-

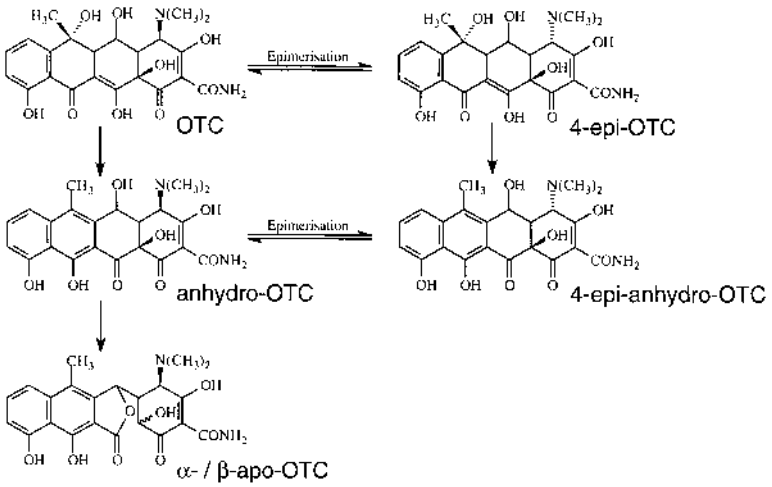

Fig. 2. Decomposition and Rearrangements of OTC

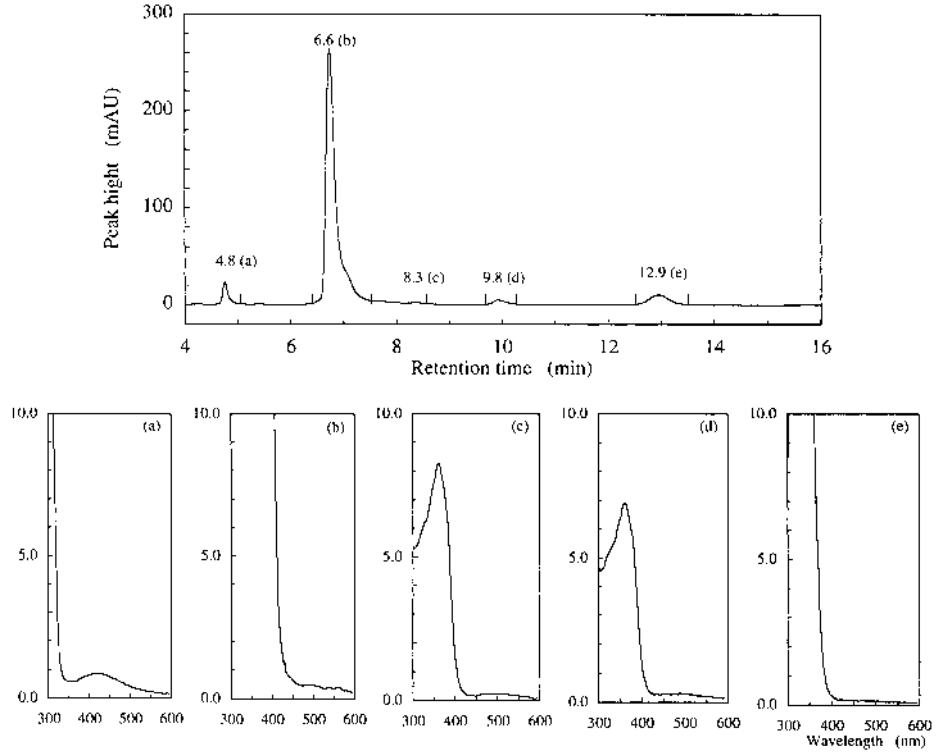

Fig. 3. Chromatogram of Thermally Decomposed OTC Solution, and UV-Visible Spectrum at the Top of Each Elution Peak

1 mM OTC was incubated at 37 °C for 24 h. HPLC separation was done with 5-fold diluted sample solutions.

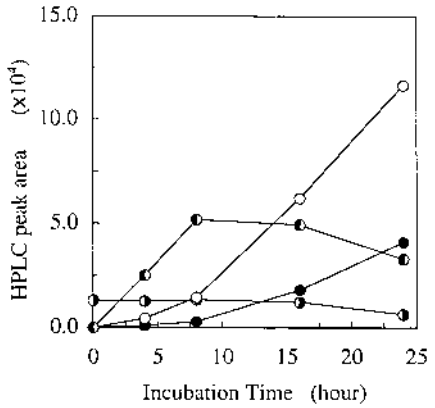

Fig. 4. Changes in HPLC Peak Area Eluted at Each Retention Time

○, T_R=4.8 min; ◑, T_R=8.3 min; ◐, T_R=9.8 min; ●, T_R=12.9 min. 1 mM OTC was incubated at 37 °C. HPLC separation was done with 5-fold diluted sample solutions.

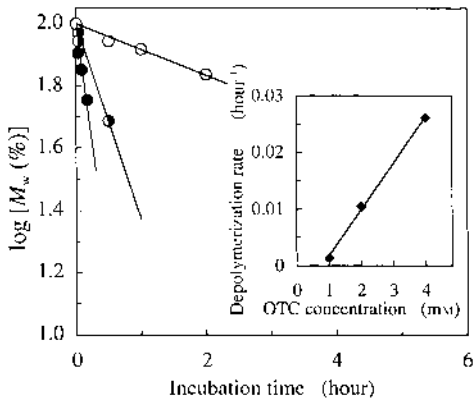

Fig. 5. Dependence of HA Depolymerization Rate on OTC Concentration

○, 1 mM; ◑, 2 mM; ●, 4 mM. 0.1% HA was incubated with OTC at 37 °C.

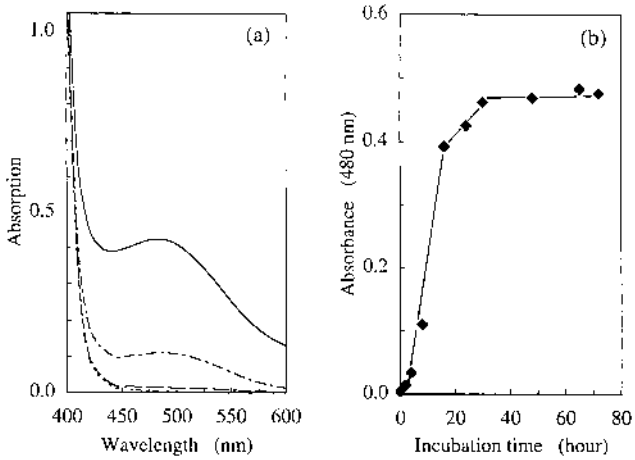

Fig. 6. Change in Absorption Spectra and 480 nm Absorbance of 1 mM OTC by Incubation at 37 °C

(a) Change in visible spectra: ------, initial; ———, after 2 h; —·—·—, after 8 h; ——, after 24 h. (b) Increase in 480 nm absorbance.

pared with 72-h pre-heated OTC solution, the HA depolymerization proceeded so rapidly that measurement was not possible. These findings proved that the HA was not depolymerized through the decomposition of OTC itself, but through processes occurring when reddish derivatives decomposed. The time lag, observed at the early stage of the reaction at 37 °C (Fig. 1, —●—), is in line with the delay until the active derivatives were thermally generated.

Active Chemical Species Involved in the Depolymerization To assess the mechanism of the HA depolymerization with OTC, changes in the M_w and the color of the solution were examined under various conditions. In Fig. 8, values for 480 nm absorbance of 1 mM OTC after 24-h incubation are

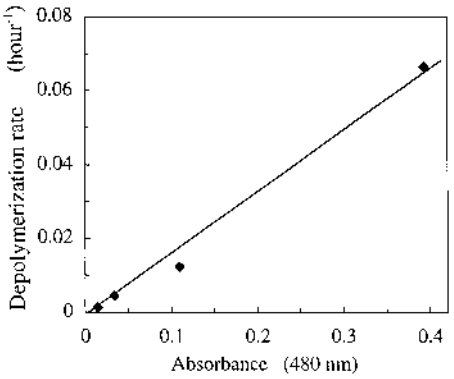

Fig. 7. Correlation between HA Depolymerization Rate and 480 nm Absorbance

0.1% HA was incubated at 37 °C after mixing with pre-heated OTC solution.

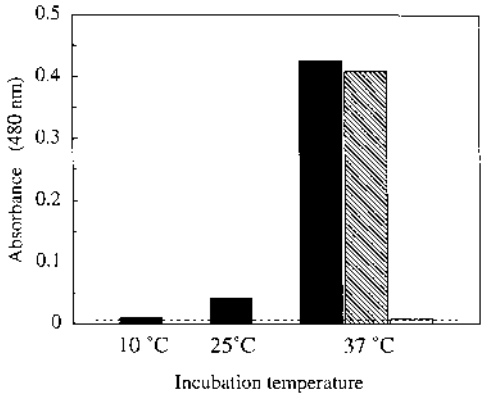

Fig. 8. Increase in 480 nm Absorbance after 24-h Incubation

Closed columns, under aerobic conditions; hatched column, with 20 mM mannitol under aerobic conditions; open column, oxygen depleted with nitrogen saturation. The dotted line in the figure indicates the initial value of 480 nm absorbance.

compared. The dotted line in the figure indicates the initial absorbance. The absorbance increased remarkably as the temperature was raised (closed columns). The presence of 20 mM mannitol, a scavenger of hydroxyl radicals, did not affect the discoloration (hatched column). On the other hand, under nitrogen saturated conditions, there seemed to be no change in the absorbance (open column). As shown in Fig. 1, practically no depolymerization occurred after 4 h at 10 °C and 25 °C, and no change in HPLC chromatograms was observed. Decrease in M_w was inhibited by both addition of mannitol and nitrogen saturation (Table 2). The addition of mannitol did not influence the peak elution and areas originating from OTC. With nitrogen saturation, the eluting peak area at 9.8 min was increased while those at 4.8 and 12.9 min had only slight if any increment. Since the M_w did not decrease under anaerobic conditions, it was considered that the derivative eluting at the retention time of 9.8 min was not involved in the HA depolymerization.

Considering the above results, the following can be proposed. OTC was thermally decomposed in the presence of oxygen. Because of the positive correlation observed between the discoloration and the depolymerization rate, and the findings for separated HPLC peaks, anhydro-OTC is the most likely causal derivative. Anhydro-OTC, which readily forms *apo*-OTC, would be expected to have high reactivity. It is not clear how the derivative might break polymer linkages, but an idea commonly reported for depolymerization might be applicable: polymer scission is initiated by abstraction of a hydrogen atom by free radical action.[14] This can also be imagined in the case of HA depolymerization by OTC. From the fact that the reaction was inhibited by the addition of mannitol, active oxygen species, particularly hydroxyl radicals, are clearly candidates for playing a role in the depolymerization process. Mannitol did not inhibit the oxidative decomposition of OTC, but it preserved HA from damage.

It has been reported that the toxicity of tetracyclines is mostly due to their decomposition products.[8] From our experiments, anhydro-OTC can be suspected to depolymerize HA, which is present in many sites of the body, like the mucosa, skin and eye where OTC preparations are applied. Further, HA is not necessarily the only target substance since proteins, lipids and other constituents may be attacked by active species. Possible participation in clinical side effects is an interest for a future study.

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References

1) Welsh E. J., Rees D. A., Morris E. R., Madden J. K., *J. Mol. Biol*., **138**, 375—382 (1980); Morris E. R., Rees D. A., Welsh E. J., *ibid*., **138**, 383—400 (1980); Yanaki T., Yamaguchi T., *Biopolymers*, **30**, 415—425 (1990); Kobayashi Y., Okamoto A., Nishinari K., *Biorheology*, **31**, 235—244 (1994); Presti D., Scott J. E., *Cell Biochem. Funct*., **12**, 281—288 (1994).
2) Rehakova M., Bakos D., Soldan M., Vizarova K., *Int. J. Biol. Macromol*., **16**, 121—124 (1994).
3) Lapcik L., Jr., Chabrecek P., Stasko A., *Biopolymers*, **31**, 1429—1435 (1991).
4) Lowry K. M., Beavers E. M., *J. Biomed. Mater. Res*., **28**, 1239—1244 (1994); Tokita Y., Okamoto A., *Eur. Polym. J*., **32**, 1011—1014 (1996).
5) Miyazaki T., Yomota C., Okada S., *J. Appl. Polym. Sci*., **67**, 2199—2206 (1998).
6) Uchiyama H., Dobashi Y., Ohkouchi K., Nagasawa K., *J. Biol. Chem*., **265**, 7753—7759 (1990); Miyazaki T., Yomota C., Okada S., *Colloid Polym. Sci*., **276**, 388—394 (1998).
7) Miyazaki T., Yomota C., Okada S., *Colloid Polym. Sci*., **278**, 84—89 (2000).
8) Hasan T., Kochevar I. E., McAuliffe D. J., Cooperman B. S., Abdulah D., *J. Invest. Dermatol*., **83**, 179—183 (1984); Hasan T., Allen M., Cooperman B. S., *J. Org. Chem*., **50**, 1755—1757 (1985); Dos Santos H. F., De Almeida W. B., Zerner M. C., *J. Pharm. Sci*., **87**, 190—195 (1998).
9) Wiebe J. A., Moore D. E., *J. Pharm. Sci*., **66**, 186—189 (1977); Nilges M. J., Enochs W. S., Swartz H. M., *J. Org. Chem*., **53**, 5623—5630 (1991); Li A. S., Chignell C. F., Hall R. D., *Photochem. Photobiol*., **46**, 379—382 (1987); Lichszteld K. K., Michalska T., *Toxicol. Environ. Chem*., **35**, 167—173 (1992).
10) Durckheimer W., *Angew. Chem. Int. Ed. Engl*., **14**, 721—734 (1975).
11) Sina A., Youssef M. K., Kassem A. A., Attia I. A., *J. Pharm. Sci*., **60**, 1544—1547 (1971); Khan N. H., Roets E., Hoogmartens J., Vanderhaeghe H., *J. Chromatogr*., **405**, 229—245 (1987); Izer K., Torok I., Pinter-Magyar G., *Acta Pharm. Hung*., **64**, 63—66 (1994).
12) Morovjan G. Y., Horvath E., Vagi I., Fekete J., *Acta Pharm. Hung*., **64**, 67—73 (1994).
13) Ascione P. P., Chrekian G. P., *J. Pharm. Sci*., **59**, 1480—1482 (1970).
14) Herp A., Rickards T., Matsumura G., Jakosalem L. B., Pigman W., *Carbohydr. Res*., **4**, 63—71 (1967).