The Kinetics of Hydroxyl-Radical-Induced Strand Breakage of Hyaluronic Acid. A Pulse Radiolysis Study Using Conductometry and Laser-Light-Scattering

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Hyaluronic Acid, Strand Breakage, Pulse Radiolysis, Hydroxyl Radical, Superoxide Radical

Hydroxyl radicals were generated radiolytically in N_2O- and N_2O/O_2(4:1)-saturated aqueous solutions of hyaluronic acid. The hydroxyl radicals react rapidly with hyaluronic acid mainly by abstracting carbon-bound H atoms. As a consequence of subsequent free-radical reactions, chain breakage occurs the kinetics of which has been followed using the pulse radiolysis technique.

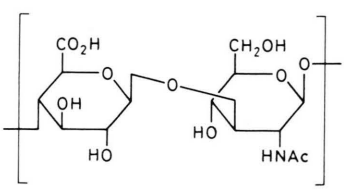
In the absence of oxygen, strand breakage was followed by the change in conductivity induced by the release of cationic counterions condensed at the surface of hyaluronic acid which is a polyanion consisting of subunits of glucuronic acid alternating with N-acetyl-glucosamine. It appears that strand breakage is not due to one single first-order process, however, the contributions of the different components cannot be adequately resolved. At pH 7 the overall half-life is 1.4 ms, in both acid and basic solutions the rate of free-radical induced strand breakage is accelerated (at pH 4.8, $t_{1/2} = 0.6$ ms; at pH 10, $t_{1/2} = 0.18$ ms). In the absence of oxygen there is no effect of dose rate on the kinetics of strand breakage.

In the presence of oxygen in addition to conductometric detection, strand breakage was also followed by changes in low-angle laser light-scattering. These two techniques are complementary in that in this system the conductometry requires high doses per pulse while the light-scattering technique is best operated in the low-dose range. In the presence of oxygen a pronounced dose-rate effect is observed, *e.g.* at pH 9.7 after a dose of 9.4 Gy the overall half-time is approx. 0.5 s, while after a dose of 6.6 Gy the half-time is approx. 0.23 s. Both the yield and the rate of strand breakage increase with increasing pH, *e.g.* at pH 7 G(strand breaks) $= 0.7 \times 10^{-7}$ mol J^{-1} and at pH 10.4, 4.8×10^{-7} mol J^{-7}.

The radiolytic yields of CO_2, H_2O_2, organic hydroperoxides, $O_2^{\cdot -}$ and oxygen consumption have been determined in γ-irradiated N_2O/O_2(4:1)-saturated solutions of both hyaluronic acid and β-cyclodextrin.

Introduction

Hyaluronic acid belongs to the group of mucopolysaccharides which form an integral part of animal connective tissue. It may be isolated from umbilical cord, synovial fluid and skin but is present in all tissues so far analyzed, even though mostly in small amounts [1]. It is a straight-chain polymer consisting of alternating β-D-(1→4) linked 2-acetamido-2-deoxy-D-glucose and β-D-(1→3) linked D-glucuronic acid:



Its biochemistry has been reviewed recently [1].

In inflammatory diseases, free radicals including the highly reactive hydroxyl radical, are thought to be generated (*cf.* Ref. [2]). Hydroxyl radicals are well-known to cause the degradation of polymeric carbohydrates. A few strand breaks can reduce the molecular weight of a polymer to such an extent that the viscosity of a given system which is due to the presence of this polymer is drastically lowered [3]. Such free-radical-induced viscosity loss in syn-

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ovial fluid clearly has important biological consequences [4]. It is of interest to note that these processes are also of some concern to the oil industry where polysaccharides are used as adjuvants in drilling. Here, besides frictional disruption, polysaccharide degradation may also be partly due to the action of free radicals formed, in this instance by ultra-sound (around collapsing gas microbubbles, *cf.* [5, 6]).

The mechanisms involved in the hydroxyl-radical-induced scission of the glycosidic linkage have been studied in some detail with di- and oligosaccharide model systems (for reviews see [7, 8]). In the hyaluronic acid system the radiation-chemical yields of strand break formation have been measured by steady-state radiolysis both in the absence and presence of oxygen [9]. It was, however, impossible to follow the kinetics of these processes, *i.e.* to give the time-scale at which the radical-induced strand break occurs.

To measure the kinetics of strand breakage we use the technique developed by Bothe and Schulte-Frohlinde [10]. Their technique, first applied to polyuridylic acid (poly(U)), is based on the fact that the high electric field of polyelectrolyte anions causes the condensation of cationic counterions around the polyelectrolyte and only a fraction of the counterions remain free. When a strand break occurs the electric potential at the newly formed ends is lowered and counterions are released, leading to an increase in conductivity. At neutral pH, hyaluronic acid (pK_a = 2.9 [11]), like poly(U), is a single-stranded polyelectrolyte and hence the formation of strand breaks can be measured conductometrically. In order to measure the kinetics of strand breakage the initial damage must be produced relatively rapidly, and the increase in conductivity then measured as a function of time. This can be achieved using the pulse radiolysis technique [8].

A more direct method of detecting strand breakage is by means of low-angle laser light-scattering. This technique has been applied only in the case of oxygenated solutions due to the fact that our newly installed apparatus is not yet fully air-tight.

When aqueous N_2O-saturated solutions containing hyaluronic acid are subjected to ionizing radiation such as 3 MeV electrons from a Van-de-Graaff electron accelerator the water is decomposed (reaction (1)). The solvated electrons are converted by N_2O into additional OH radicals (reaction (2)).

$$H_2O \xrightarrow[\text{radiation}]{\text{ionizing}} {}^{\cdot}OH, e^-_{aq}, {}^{\cdot}H, H^+, H_2O_2, H_2 \quad (1)$$

$$e^-_{aq} + N_2O \longrightarrow {}^{\cdot}OH + N_2 + OH^- \quad (2)$$

Under these conditions the yield of OH radicals is $G({}^{\cdot}OH) = 5.6 \times 10^{-7}$ mol J^{-1} whereas H atoms are formed with a G value of only about 0.6×10^{-7} mol J^{-1}. Both radicals react with hyaluronic acid by abstracting carbon-bound hydrogen atoms. The OH radicals react with hyaluronic acid with a rate constant of $k = 9 \times 10^8$ dm^3 mol^{-1} s^{-1} while the reactivity of the H atom is an order of magnitude lower ($k = 7 \times 10^7$ dm^3 mol^{-1} s^{-1}) [9]. The OH radical, in particular, is not very selective and the available carbon-bound hydrogen atoms are most likely abstracted more or less at random (*cf.* Ref. [12]).

In the presence of oxygen (N_2O/O_2(4:1 v:v)-saturated solutions) the majority of the H atoms from reaction 1 will be scavenged by oxygen (reaction (4); $k_4 = 2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}) and the carbohydrate radicals from reaction 3 are converted into the corresponding peroxyl radicals (reaction 5; $k_5 \approx 2 \times 10^9$ dm^3 mol^{-1} s^{-1}; *cf.* Ref. [13]).

$$ {}^{\cdot}OH + RH \longrightarrow H_2O + R^{\cdot} \quad (3)$$

$$ H^{\cdot} + O_2 \longrightarrow HO^{\cdot}_2 \quad (4)$$

$$ R^{\cdot} + O_2 \longrightarrow RO^{\cdot}_2 \quad (5)$$

In the absence of oxygen some of the various radicals R$^{\cdot}$ undergo reactions leading to strand breakage while in the presence of oxygen the precursors of strand breakage are the peroxyl radicals $RO^{\cdot}_2$. In the pulse radiolysis experiments to be reported the ionizing radiation was applied within times ≤ 4 μs so that reactions 1 and 2 are essentially complete within the pulse duration and reactions 3–5 are over only a few microseconds later. In pulse radiolysis the most common detection of free radicals is by UV/VIS-spectrometry. This method is not applicable in our case, because carbohydrate-derived radicals have only rather featureless absorption spectra in the UV. Any changes that might be observed cannot be ascribed to strand breakage with certainty, because other unimolecular processes such as water elimination occur as well [7, 8]. As mentioned above, the conductivity change is quite decisive in this respect and has the additional advantage that it operates

effectively over a large time range: from microseconds to seconds.

Experimental

Hyaluronic acid (high purity) was a gift from Pharmacia, Uppsala, Sweden. Dissolution of the hyaluronic acid (weight-average molecular weight ca. 2 MDalton) was achieved by gentle overnight stirring in either dilute phosphate buffer pH 7 or water adjusted to pH 9.5–10 with potassium hydroxide. When radiolytic yields of carbon dioxide were to be measured, solutions were made up in dilute solutions of sodium sulphate to avoid the possibility of raising background levels of carbonate. Hyaluronic acid concentrations are given in terms of its dimeric repeating unit, molecular weight 401 Dalton (Na-salt). β-Cyclodextrin was purchased from Sigma and Fluka; results from the two samples showed no significant differences. Concentrations of β-cyclodextrin are expressed in terms of complete molecules, using a molecular weight of 1134 Dalton. All other chemicals used were of analytical grade, water was Millipore Milli-Q-filtered.

Steady-state γ-radiolysis was performed using a ^{60}Co-source, the dose-rate, 0.41 Gy s^{-1}, was determined by Fricke dosimetry.

Pulse-conductivity experiments were performed using a 100 kHz AC bridge, details of which have already been described [10]. Light-scattering measurements with aqueous solutions of hyaluronic acid were performed using a commercially available low-angle laser light-scattering instrument (Chromatix KMX-6). For the molecular weight determination of the unirradiated hyaluronic acid, the instrument was operated with the built-in He/Ne-laser ($\lambda = 633$ nm) at an angle of scattered light collection of 6–7°, in the manner described earlier for poly(U) [14, 15]. For the pulse radiolytic experiments the He/Ne-laser was replaced by an Ar-ion laser ($\lambda = 488$ nm) of higher output power (40 mW) in order to improve the signal-to-noise ratio. Here the scattering angle was reduced to 4.5–5.5°.

The quantity obtained from light-scattering measurements is the Rayleigh factor, R_θ ($R_\theta = r^2 i_\theta / I_0$, where i_θ is the scattered light intensity, I_0 the incident light intensity, and r is the distance from the scattering volume. At low scattering-angles and in the limit of zero polymer concentration (usually measured in g cm^{-3}), the excess Rayleigh factor ΔR_θ ($\Delta R_\theta = R_\theta$(solution) $- R_\theta$(solvent)), is related to the weight-average molecular weight, $\overline{M}_w$ (equation (6)),

$$\left(\frac{Kc}{\Delta R_\theta}\right)_{c \to o} = \frac{1}{\overline{M}_w} \tag{6}$$

where, K is a constant whose value depends on the wavelength of the light, the angle of scatter, the refractive index of the solution (n), and the rate of change of refractive index with changing polymer concentration (dn/dc). For our apparatus (λ = 633 nm) $K = 4.08 \times 10^{-6}$ mol cm^{-4} n^2 $(dn/dc)^2$. Reported values of dn/dc for aqueous hyaluronate solutions are 0.15 [16] and 0.17 cm^3 g^{-1} [17]. Taking the average value of 0.16 cm^3 g^{-1} along with the value of 1.34 for n enables K to be calculated. From a plot of $Kc/\Delta R_\theta$ against c, $\overline{M}_w$ can be determined and was found for our hyaluronic acid sample to be 2×10^6 Dalton (in agreement with viscosity measurements).

In the kinetic experiments, solutions were pulse-irradiated directly in the cell of the light-scattering photometer. The output voltage of the instrument's photomultiplier tube (which is proportional to the intensity of the scattered light) was recorded as a function of time before and after the electron pulse, using a transient recorder (Biomation model 8100) on line with the computer system. From the stored data, values of $\overline{M}_w$ and molar strand-break concentrations ([sb]) were calculated using equations (6) and (7).

$$[\text{sb}] = 2(1/M_D - 1/M_0) \times 1000 \times c \tag{7}$$

In equation (7) M_0 and M_D denote the weight average molecular weights of unirradiated and irradiated hyaluronic acid (dose = D). Details of equation (7) have been given elsewhere [18]. The dose absorbed by the solution in the cell of the light-scattering photometer was estimated by measuring the height of the signal obtained with single-stranded DNA (ssDNA; concentration 1.4×10^{-4} g cm^{-3} in aqueous N_2O/O_2(4:1)-saturated solution, 10^{-2} mol dm^{-3} $NaClO_4$, pH 7.5). The G value of strand break formation of ssDNA has been determined earlier for ^{60}Co-γ-irradiation conditions using the same instrument [19]. A value of 0.62×10^{-7} mol J^{-1} was obtained on making the measurement at least 30 min after ending irradia-

tion. In the pulse radiolysis experiments for the determinations of the dose, the reading of the light-scattering intensity was taken 8 sec after the irradiation pulse. At this time the light-scattering signal had not declined completely to the final value. Measurements at much longer times are difficult to perform due to the instability of the base line. It has been concluded, however, that roughly 25% of the strand breaks occur at times much longer than 10 sec after the pulse [20]. Consequently the signal 8 sec after the pulse is due to strand break formation in ssDNA with a G value of 0.5×10^{-7} mol J^{-1}. From the molar concentration of strand breaks (equation (7)) and this G value for strand breakage, the dose absorbed by the solution in the light-scattering cell was calculated.

In the determination of the yields of carbon dioxide, solutions were irradiated in gas-tight vessels fitted with a short side-arm sealed with a serum cap. After irradiation, acid was injected through the serum cap to give a final pH of *ca.* 3.5. Any carbon dioxide was then flushed out and quantitatively analyzed by gas chromatography [21]. Oxygen uptake measurements were carried out using an oxygen-sensitive electrode (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) and γ-irradiating *in situ* (dose rate 0.4 Gy s^{-1}). Results obtained in the irradiation of N_2O/O_2(4:1)-saturated dilute aqueous solutions of 2-propanol demonstrated that the method was reliable over the entire pH range used.

Total peroxide was determined with Allen's reagent (iodide/molybdate) [22], organic hydroperoxide was measured in the same way after the removal of hydrogen peroxide by the addition of catalase. In those cases in which the pH was >9, it was adjusted to 7 by the addition of tris buffer prior to the catalase treatment. The radiolytic yields of $O_2^{\cdot}$ in N_2O/O_2(4:1)-saturated solutions of hyaluronic acid and β-cyclodextrin were measured by γ-irradiating (maximum dose = 17 Gy) these substrates (1×10^{-3} mol dm^{-3}) in the presence of tetranitromethane (TNM). TNM reacts rapidly ($k = 2 \times 10^9$ dm^3 mol^{-1} s^{-1}) with $O_2^{\cdot}$ to form the nitroform anion (NF^-), which is readily quantified spectrophotometrically (ε(350 nm) = 15,000 dm^3 mol^{-1} cm^{-1} [23]). Any competition between O_2 and TNM for reaction with the carbohydrate radicals was avoided by using a low TNM concentration (1×10^{-5} mol dm^{-3}); *cf.* $[O_2] = 2.5 \times 10^{-4}$ mol dm^{-3}.

Results and Discussion

Oxygen-free solutions

When N_2O-saturated solutions containing 0.2–0.4 g dm^{-3} ($0.5-1.0 \times 10^{-3}$ mol dm^{-3} in subunits) hyaluronic acid are subjected to electron pulses of 0.4–1 μs duration a subsequent *increase* in conductivity is observed, both at pH 4.8 and 9.8 (Fig. 1 and 2) as well as at pH 7.0 in the presence of 1×10^{-4} mol dm^{-3} phosphate buffer (data not shown).

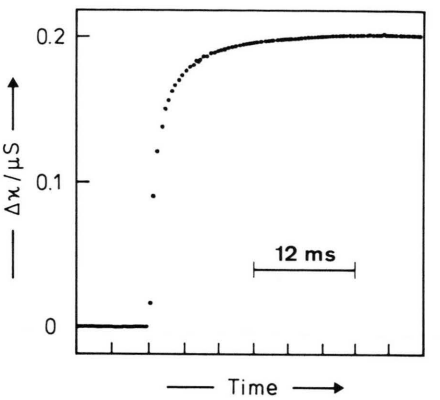


Fig. 1. Conductivity change (μSiemens) as a function of time on pulse-irradiating an N_2O-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic acid solution pH 4.8 (dose = 30 Gy).

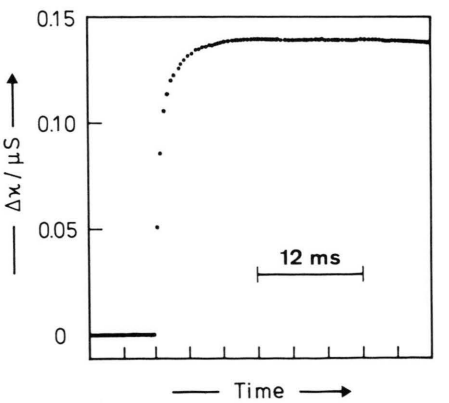


Fig. 2. Conductivity change (μSiemens) as a function of time on pulse-irradiating an N_2O-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic acid solution pH 9.8 (dose = 30 Gy).

This is an indication that the observed change in conductivity is indeed due to the release of counterions as a consequence of strand breakage. If the conductivity change were due to the formation of an acid (formation of protons and the corresponding anions) a conductivity *increase* would have been observed at pH 4.8, however there would have been a *decrease* at pH 9.8 due to the neutralization of the protons ($\lambda^{\circ}(H^+) = 315$ Siemens cm^2 mol^{-1}) by the hydroxide ions ($\lambda^{\circ}(OH^-) = 170$ Siemens cm^2 mol^{-1}) leaving behind the anion of the acid which might have been formed ($\lambda^{\circ}(A^-) \approx 40-60$ Siemens cm^2 mol^{-1}). In the buffered solution practically no conductivity change would occur as a result of acid formation.

This conductivity increase is clearly not due to one single process (Fig. 3).The overall half-life is independent of the dose rate and hence the processes involved are kinetically of first order.

At pH 6.95 (10^{-4} mol dm^{-3} phosphate) the overall half-life is 1.4 ms. This value decreases to 0.1 ms at pH 10.2 (Fig. 4), showing that the processes leading to strand-breakage contain a base-catalyzed component. An overall half-life of 0.6 ms at pH 4.8 suggests that acid catalysis of the strand breaking process also occurs. Around neutral pH, the rate of strand breakage is not significantly altered by the presence of 10^{-4} mol dm^{-3} phosphate buffer, suggesting that at this concentration phosphate does not significantly catalyse chain scission. Attempts have been made to simulate the kinetics for six pH values by assuming two first-order reactions and allowing both the rate constants and the relative contributions of each to vary. The results are incompatible with such a simple model and at the present time it is felt that the system cannot be described in detail and is best portrayed by only using overall half-lives.

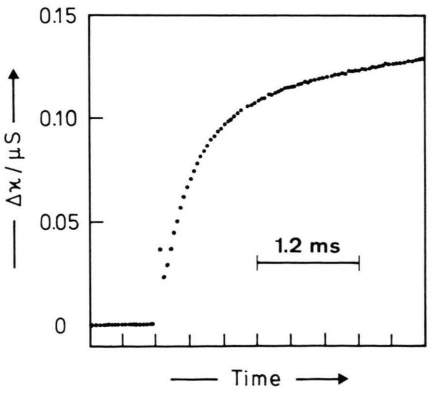


Fig. 3. Conductivity change (μSiemens) as a function of time on pulse-irradiating an N_2O-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic acid solution pH 9.8 (dose = 30 Gy). (As in Fig. 2, but shorter time scale.)

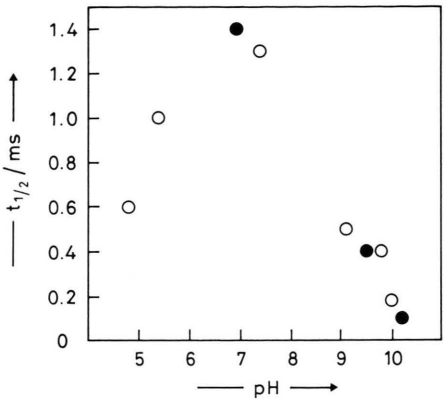


Fig. 4. Dependence of counter ion-release half-times on pH upon pulse radiolysis of aqueous N_2O-saturated hyaluronic acid (0.25 mmol dm^{-3}) solutions. (○) unbuffered. (●) 10^{-4} mol dm^{-3} phosphate buffer. (dose, 5–35 Gy.)

There is the possibility that carbon-centered radicals at the site of the glycosidic linkages facilitate the hydrolysis of the glycosidic bond and hence lead to strand breakage. This hydrolysis could be acid/base-catalyzed and could be responsible for the observed pH dependence. However, in simple disaccharides other processes also lead to scission of the glycosidic linkage [24–26], and at this stage it is not possible to correlate the observed strand breakage with specific free-radical reactions.

At longer times at pH > 9 there is a conductivity decrease (Fig. 5) which is independent of both dose-rate and pH up to pH 10.5 ($k = 8$ s^{-1}). It is certainly not the reaction of OH^- with radiolytically produced CO_2, for which a G value of 0.8×10^{-7} mol J^{-1} was measured after steady-state γ-radiolysis. The observed rate constant for OH^- reacting with CO_2 would be pH-dependent, the bimolecular rate constant for this reaction being

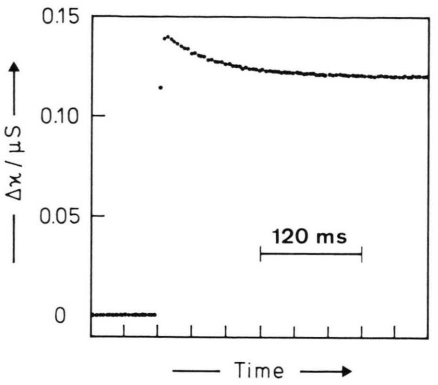


Fig. 5. Conductivity change (μSiemens) as a function of time on pulse-irradiating an N_2O-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic acid solution pH 9.8 (dose = 30 Gy). (As in Fig. 2, but longer time scale.)

6.9×10^3 dm^3 mol^{-1} s^{-1} [27]. Thus the OH^-/CO_2 reaction is only mixed-up with the observed pH-independent reaction at higher pH values, where in fact both processes can be observed to occur on the same time-scale. At the moment the identity of the pH-independent reaction is not known.

Oxygen-containing solutions

In the presence of oxygen the carbohydrate radicals are converted into the corresponding peroxyl radicals and the subsequent reactions of these peroxyl radicals can lead to strand breakage. For this study, in addition to the pulse conductivity technique, pulse low-angle laser light-scattering has been used. These two techniques are in many ways complementary. However, due to experimental restrictions, the low-angle laser light-scattering is best performed in the low-dose range, whereas for hyaluronic acid which is only a weak polyelectrolyte (at 25 °C, the charge density parameter (ε) = 0.7 [11]), high doses are required for a reasonable signal-to-noise ratio in the conductivity measurements.

As can be seen from Fig. 6a, after the pulse the intensity of scattered light decreases as a function of time. These data have been converted into the concentration of strand breaks as a function of time (Fig. 6b).

The final yields of strand breaks as measured 1.5–2.5 sec after the pulse increased linearly with dose in the low-dose range. The slopes of plots of

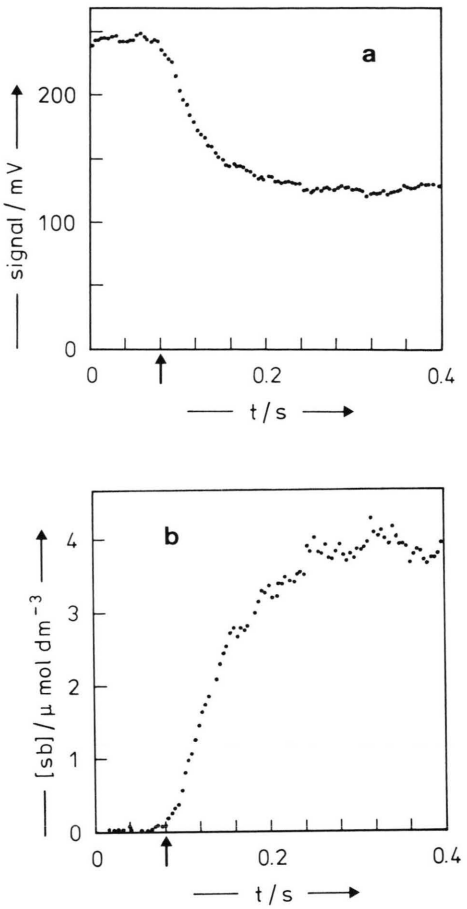


Fig. 6a and 6b. Time dependence of the low-angle laser light-scattering signal (PMT output voltage in mV) (a), and of molar concentration of strand breaks formed (in μmol dm^{-3}) (b), as observed in the pulse radiolysis of aqueous N_2O/O_2(4/1)-saturated solutions of hyaluronic acid (2.5×10^{-4} mol dm^{-3}), containing 10^{-2} mol dm^{-3} Na_2SO_4, at room temperature and pH 10.4 after a dose of 1.0 Gy.

the number of breaks against the dose (G values) depend on the pH (Fig. 7). Calibrating our apparatus using DNA solutions (see Experimental Section), gives G(strand breakage) of 0.7×10^{-7}, 2.6×10^{-7} and 4.8×10^{-7} mol J^{-1} at pH 7, 9.7 and 10.4, respectively. At pH 7 a value for G(strand breakage) of 1.9×10^{-7} mol J^{-1} has been measured by viscometry after steady-state γ-radiolysis (N_2O/O_2 (4/1)) (D. J. Deeble, S. Blake, and G. O. Phillips unpublished results). Further experiments are being undertaken to endeavour to explain this difference.

At pH 7 it has been possible to extend the pulse radiolysis measurements to a higher dose range and it is apparent that the slope is reduced at these larger doses. As the number of strand breaks increases the relative change in scattered light intensity decreases, consequently it is not possible to determine strand breakage at high doses in alkaline solution. Nevertheless at pH 9.7 there is an indication that again the yield of strand breakage is reduced at higher doses (dose-rates). It should be remembered that in pulse radiolysis experiments dose and dose-rate are inseparable.

The kinetics of the formation of strand breaks are not straightforward. As can be seen from Fig. 6b the buildup of strand breaks is slightly *S*-shaped. At this stage a detailed analysis of the kinetics has not been attempted and only overall half-lives have been calculated. Their inverse values are plotted as a function of dose in Fig. 8. The dependence of the reciprocal of the overall half-life on the dose points to the involvement of bimolecular reactions of radiation-induced species in the processes giving rise to strand breakage. Just as in the anoxic system, the rate of strand breakage seems to be base-catalyzed (Fig. 8). At present it is too soon to speculate on the precise nature of these reactions, although bearing in mind the relatively low mobility of radicals fixed to the hyaluronic acid chain, it may be that the more mobile superoxide anion radicals *(vide infra)* take part in the bimolecular process which eventually results in chain scission.

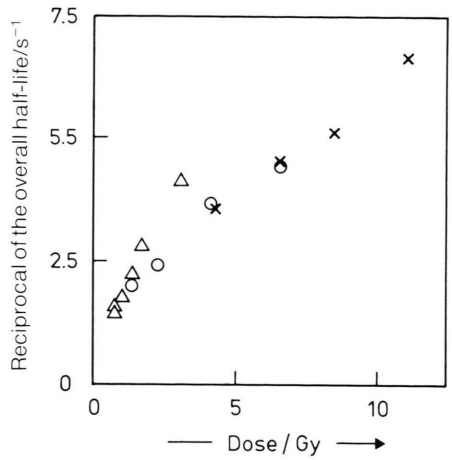


Fig. 8. The reciprocal of the overall half-life for strand break formation, measured by low-angle laser light-scattering for pulse-irradiated $N_2O/O_2(4/1)$-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic acid solutions as a function of dose: x, pH 7; o, pH 9.7; Δ, pH 10.4.

A large proportion of the peroxyl radicals formed are α-hydroxyalkylperoxyl radicals which are known to undergo $HO_2^{\cdot}$-elimination [28, 29], a reaction which is base-catalyzed and therefore speeded up in alkaline solutions [29]. Since $HO_2^{\cdot}$ has a pK_a value of 4.8, its formation gives rise to a change in conductivity. In basic solutions the conductivity decreases due to the replacement of OH^- by $O_2^{\cdot-}$ ($\lambda^o(O_2^{\cdot-}) = 60$ Siemens cm^2 mol^{-1}) (*cf.* Fig. 9).

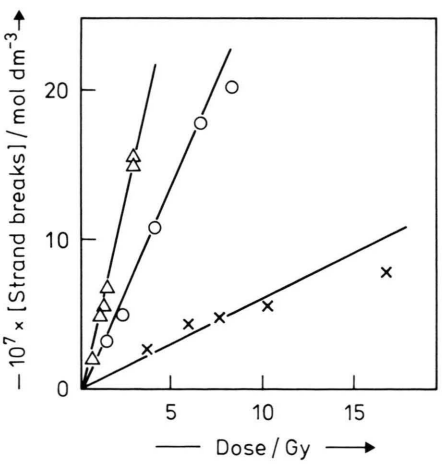


Fig. 7. The concentration of strand breaks produced on pulse-irradiating an $N_2O/O_2(4/1)$-saturated 2.5×10^{-4} mol dm^{-3} hyaluronic solution as a function of dose: x, pH 7; o, pH 9.7; Δ, pH 10.4.

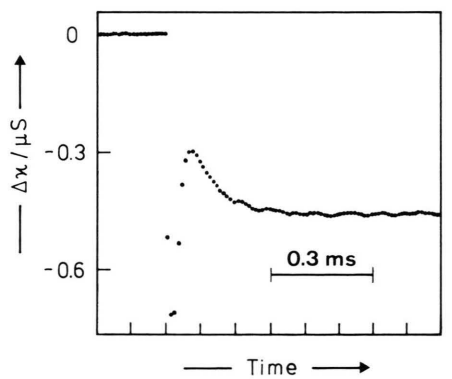


Fig. 9. Conductivity change (μSiemens) normalized to a dose of 10 Gy as a function of time on pulse-irradiating an $N_2O/O_2(4/1)$-saturated 1.0×10^{-3} mol dm^{-3} hyaluronic acid solution pH 9.9 (dose = 8 Gy).

At high pH, $O_2^{\cdot-}$ is long-lived, and at short times any conductivity increase due to counterion release would be masked (Fig. 10).

To overcome this problem, superoxide dismutase (SOD) has been added at a concentration of 27 mg dm^{-3}. In this way $O_2^{\cdot-}$ is replaced by OH^- within 4 ms (Fig. 11), so any conductivity increase resulting from counterion release can be monitored (*cf.* Fig. 12–14).

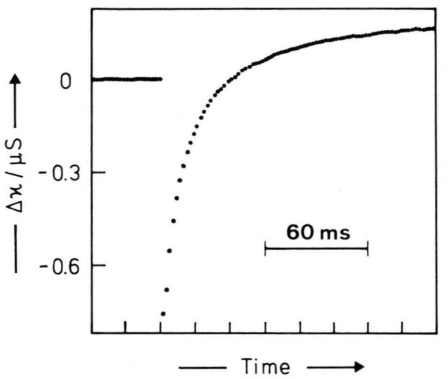


Fig. 10. Conductivity change (μSiemens) as a function of time on pulse-irradiating an $N_2O/O_2(4/1)$-saturated 1.0×10^{-3} mol dm^{-3} hyaluronic acid solution pH 9.9 (dose = 40 Gy).

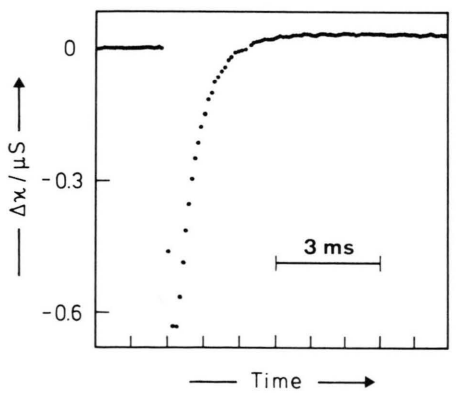


Fig. 11. Conductivity change (μSiemens) as a function of time on pulse-irradiating an $N_2O/O_2(4/1)$-saturated 1.0×10^{-3} mol dm^{-3} hyaluronic acid solution pH 9.9 containing 27 mg dm^{-3} superoxide dismutase. Dose: 70 Gy.

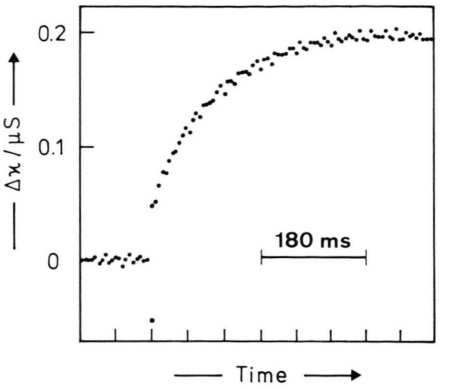


Fig. 12. Experiment as in Fig. 11 but with a dose of 37 Gy.

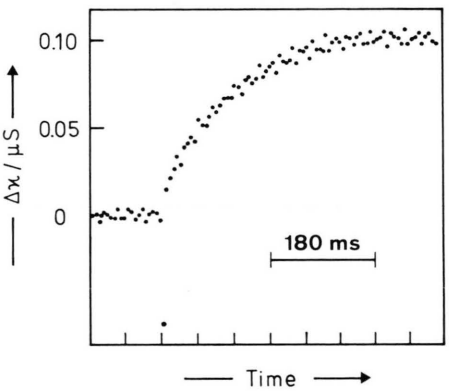


Fig. 13. Experiment as in Fig. 11 but with a dose of 22 Gy.

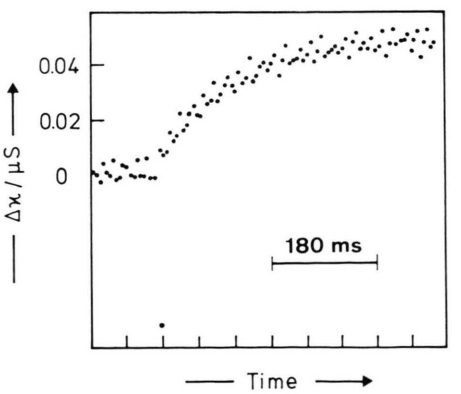


Fig. 14. Experiment as in Fig. 11 but with a dose of 12.5 Gy.

It is evident (Fig. 12–14) that following $O_2^{\cdot-}$ dismutation there is an increase in conductivity at pH 9.9, the overall half-life for which is approximately 60 ms. In contrast to the light-scattering data (Fig. 8), there is no marked dependence of the overall half-life for conductivity increase on dose, nevertheless there is a dose-dependent component which can be seen in Fig. 12, where the dose given was 37 Gy, as an initial step-like increase in conductivity following the pulse. With a dose of 22 Gy this step is smaller in relation to the total conductivity change (Fig. 13) and after a lower dose of 12.5 Gy it is no longer discernible (Fig. 14). The data from eight pulse-conductivity traces in which the dose was varied between 12 and 70 Gy were simulated using a simple mono-exponential function, $\Delta\varkappa = Ae^{-kt} + C$, where A, k and C are constants and $\Delta\varkappa$ is the change in conductivity at time t after the pulse. On examining the values for these constants giving the best fit for each trace, A, as expected, was directly proportional to the dose, whilst k, a first-order rate constant, lay in the range 8.2 to 9 s^{-1}. The values of C, the step-like conductivity increase seen in Fig. 12, exhibited a somewhat complex dependence on the dose, being approximately proportional to the dose raised to the power of 1.4. No attempt will be made at this stage to interpret these results, however it should be mentioned that although at first sight the overall half-life observed in the conductivity experiments appears too short to be compatible with the light-scattering data, account must be taken of the large difference in doses applied in the two sets of measurements. As already mentioned, the overall half-life for strand breakage as measured by light scattering is strongly dose-dependent (Fig. 8), and a linear extrapolation of this data to the lowest dose used in the conductivity studies would give a value for the half-life in the same range as that found from conductivity. The fact that the overall conductivity half-life is essentially independent of dose could be reconciled with the light scattering results by postulating that at low doses the rate constant for strand breakage is dose dependent but that this dependence no longer exists at high doses (*i.e.* if it were possible to extend the dose range, a plot of the type shown in Fig. 8 would plateau out).

When conductivity changes at pH 9.9 were monitored over longer periods following the pulse, a large decrease was observed (Fig. 15). The slow conductivity decrease occurring in the pulse experiments is explicable as being due to the reaction of radiolytically produced carbon dioxide with hydroxide ions *(vide supra)*.

Steady-state radiolysis

Steady-state γ-radiolysis of N_2O/O_2-(4/1)-saturated hyaluronic acid solutions (pH *ca.* 5) gave linear yield-dose plots for carbon dioxide formation, from which the radiolytic yield of carbon dioxide was calculated to be 1.4×10^{-7} mol J^{-1}. The mechanism for the formation of carbon dioxide has not yet been established; it would perhaps seem quite reasonable to suppose that the carboxyl group of the glucuronic acid subunit is involved. β-Cyclodextrin is a cyclic oligosaccharide consisting of seven 1–4′-linked glucose subunits and as such provides a good model for the study of free-radical processes in high-molecular-weight polysaccharide chains (a cyclic oligomer to some extent approximates an infinitely long polymer). Under anoxic conditions (2 mmol dm^{-3} β-cyclodextrin, pH 5.5, N_2O-saturated), the radiolytic yield of carbon dioxide was extremely low (0.08×10^{-7} mol J^{-1}). The absence of carboxyl groups in β-cyclodextrin taken in conjunction with this low radiolytic yield of carbon dioxide in comparison to that found for hyaluronic acid (0.8×10^{-7} mol J^{-1}) is consistent with the participation of the hyaluronic acid carboxyl groups in car-

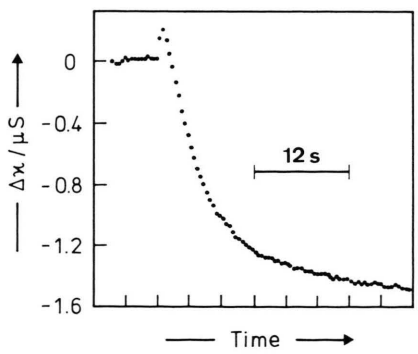


Fig. 15. Conductivity change (μSiemens) normalized to a dose of 10 Gy as a function of time on pulse-irradiating a N_2O/O_2(4/1)-saturated 1.0×10^{-3} mol dm^{-3} hyaluronic acid solution pH 9.9, containing 27 mg dm^{-3} superoxide dismutase (dose = 37 Gy).

bon dioxide formation in anoxic radiolysis. In the presence of oxygen (N_2O/O_2 (4/1)) the radiolytic yield of carbon dioxide in hyaluronic acid solutions is also considerably higher than in β-cyclodextrin solutions (Table I), again perhaps indicating the involvement of the hyaluronic acid carboxyl groups. However, the carbon dioxide yield (0.34×10^{-7} mol J^{-1}) in N_2O/O_2-saturated β-cyclodextrin solutions is appreciable and points to an additional process for carbon dioxide production in which carboxyl groups take no part. Since the carboxyl groups confer on hyaluronic acid its polyelectrolyte character, even low levels of isolated decarboxylation might lead to some release of condensed counter-ions. The relative size of this effect in comparison to that produced by chain scission is not known, although it might be expected to be smaller. However it is important to remember that its contribution is included in the pulse-conductivity measurements.

A particularly striking result is the strong pH dependence of the radiolytic yield of hyaluronic acid strand breaks in N_2O/O_2-saturated solutions measured by pulse low-angle laser light-scattering *(vide supra)*. Whereas at pH 10.4 strand breakage is around 80% of the primary radical yield, at pH 7 it is only 12%. A similarly dramatic pH dependence can be seen in the radiolytic consumption of oxygen in N_2O/O_2 saturated β-cyclodextrin solutions (Table I). In both systems the processes taking place at high pH seem to be very much different from those occurring in the neutral pH region. It is not known whether there is any connection between these two pH dependent phenomena.

As already mentioned many of the radicals formed following the reaction of ·OH with polysaccharides in the presence of oxygen are α-hydroxyperoxyl radicals which are likely to eliminate $HO_2^{\cdot}$, in addition there is evidence from studies on model compounds to suggest that peroxyl radicals at the carbon atoms participating in the glycosidic linkage might also eliminate $O_2^{\cdot-}$ [30, 31]. Employing the TNM method (see Experimental section), yields of $O_2^{\cdot-}$ at pH 7 were found to be 3.8 and 4.9×10^{-7} mol J^{-1} for hyaluronic acid and β-cyclodextrin respectively. Thus around 75% of β-cyclodextrin peroxyl radicals and some 60% of hyaluronic acid peroxyl radicals give rise to $O_2^{\cdot-}$. If random OH radical attack takes place, then in the case of β-cyclodextrin only about 50% of the peroxyl radicals are α-hydroxyperoxyl radicals (the radicals at C-2, C-3, and C-6), hence, with the major assumption that there is no contribution from the oxidation of diamagnetic products, it would seem that here some $O_2^{\cdot-}$ is indeed produced from the other peroxyl radicals (*i.e.* at C-1, C-4, and C-5). The total primary radical yield (H· and OH·) is 6.2×10^{-7} mol J^{-1}, and this equals the amount of oxygen initially consumed. In these systems, $O_2^{\cdot-}$ might be expected to dismutate or to react with peroxyl radicals, producing in both instances oxygen along with either H_2O_2 or an organic hydroperoxide. On this basis alone, from the measured yields of $O_2^{\cdot-}$, the net radiolytic uptake of O_2 should be 4.3×10^{-7} and 3.75×10^{-7} mol J^{-1} for hyaluronic acid and β-cyclodextrin respectively at pH 7. It is clear from the results given in Table I that even such an over-simplified model, in which no account is taken of the possibility of O_2 formation from reactions between peroxyl radicals, is incompatible with the experimental results. Again, assuming $O_2^{\cdot-}$ dismutation (or reaction with a per-

Table I. Radiolytic yields expressed in terms of 10^{-7} mol J^{-1} for oxygen consumption and the formation of superoxide radical anions, peroxides and carbon dioxide in the steady-state γ-radiolysis of N_2O/O_2(4/1)-saturated solutions of hyaluronic acid (1×10^{-3} mol dm^{-3}) and β-cyclodextrin (2×10^{-3} mol dm^{-3}). Hyaluronic acid concentration in terms of dimeric subunits.

	Hyaluronic acid			β-Cyclodextrin		
	pH 7	pH 9.5	pH 10.4	pH 7	pH 10.3	pH 10.6
Oxygen consumption	5.2[a]	6.9	6.5[b]	4.3[a]	10.6[b]	11.5
Hydrogen peroxide	2.1[a]		2.1	3.5[a]	2.1	
Organic hydroperoxide	<0.1[a]		0.15	0.7[a]	0.2	
Carbon dioxide	1.3			0.34		
Superoxide radical anion	3.8			4.9		

[a] = 10^{-3} mol dm^{-3} phosphate, [b] = 5×10^{-3} mol dm^{-3} borate.

oxyl radical), the total peroxide yield in the hyaluronic acid system is somewhat lower than that expected (*i.e.* after allowing for the molecular yield of H_2O_2 0.7×10^{-7} mol J^{-1}), however in the β-cyclodextrin system the total peroxide yield is much larger than would be predicted on this simple basis (Table I). At high pH values, oxygen consumption increases by about 25% in the hyaluronic acid system and by 160% in the case of β-cyclodextrin, while the corresponding yields of peroxides hardly change (Table I). It will be the aim of future research to attempt to elucidate mechanisms to explain these results and the marked influence of pH on the free radical processes occurring in polysaccharide solutions.

Possible mechanisms

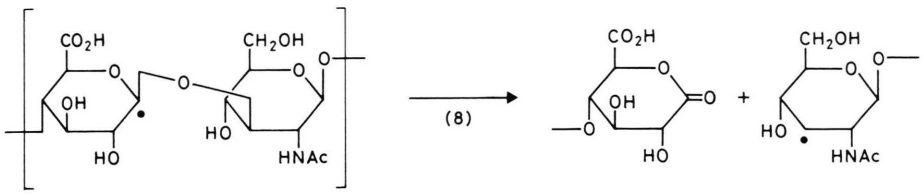
From product studies on the free-radical chemistry of disaccharides [24, 26, 30] it is clear that the scission of the glycosidic bond proceeds by more than one route. It is not yet possible to assign which of these are responsible for chain scission in the hyaluronic acid. Nevertheless, in order to give the reader some idea of what might be happening strand breakage resulting from the radical at C(1) will be discussed.

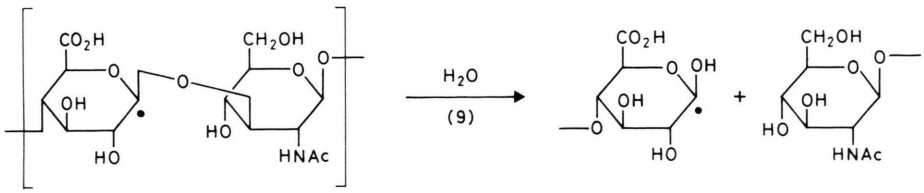
In the absence of oxygen, there are two ways in which the C(1) radical can induce scission, β-fragmentation (reaction (8) and hydrolysis (reaction (9)). The latter reaction is likely to be acid/base catalyzed which would fit in with our observations.

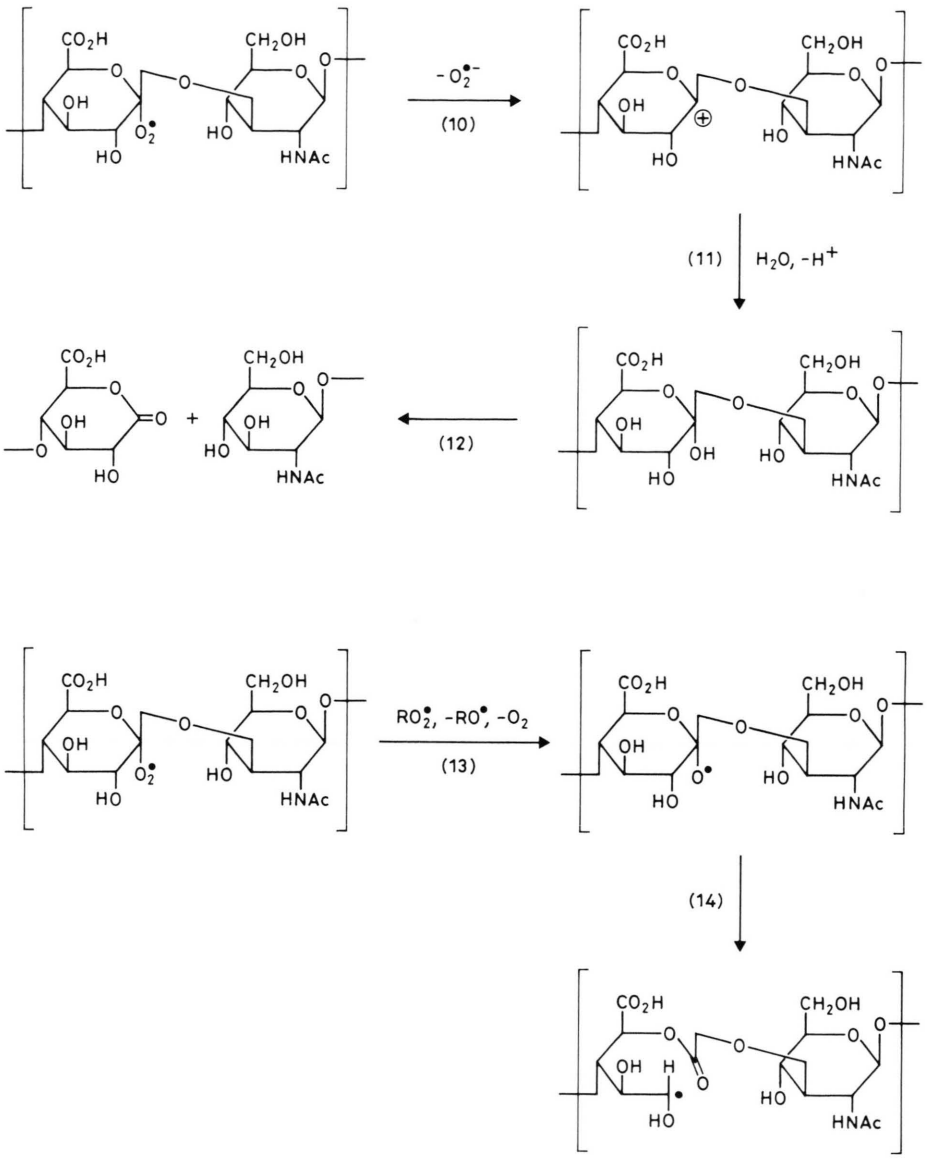
In the presence of oxygen, the C(1) radical is rapidly converted into the corresponding peroxyl radical which could eliminate $O_2^{\cdot -}$ (reaction (10); *cf.* Ref. [31]) with the resulting carbocation undergoing rapid reaction with water (reaction (11)). The ensuing product would not be stable and could lead to strand scission (reaction (12)), this process would also be base catalyzed. The results showed the rate of strand breakage increased in basic solution.

In competition with this unimolecular $O_2^{\cdot -}$-elimination there is the bimolecular reaction with another peroxyl radical. Tertiary peroxyl radicals such as this produce oxyl radicals (reaction (13)), these subsequently fragment (*e.g.* reaction (14)). The product formed *might* then undergo base-induced hydrolysis to give chain scission.

Starting from the tertiary peroxyl radical at the other side of the glycosidic linkage (C(3) for N-acetylglucosamine unit; C(4) for the glucuronic acid unit) a similar reaction sequence would lead







to an activated ester which would undergo rapid hydrolysis (*cf.* Ref. [31]).

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