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CORRELATION BETWEEN THE AUTOXIDATION OF FERROUS IONS OR OF L-ASCORBIC ACID AND THE DEPOLYMERIZATION OF HYALURONATE*

A. HERP, M. HARRIS, AND T. RICKARDS
New York Medical College, New York, New York 10029 (U. S. A.)
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ABSTRACT

The depolymerization of hyaluronic acid by reductants in the presence of a variety of buffer systems has been investigated. Many organic buffers inhibit the depolymerization. A parallelism was observed between absorption of oxygen, oxidation of L-ascorbic acid, and depolymerization of hyaluronic acid.

INTRODUCTION

Autoxidation of the mucopolysaccharide hyaluronic acid has been the subject of considerable interest ever since the observation[1] that the polymer is prone to nonenzymic depolymerization. The compounds leading to degradation of hyaluronic acid *in vitro* were found[2,3] to be, in general, reducing agents, and their effects are completely arrested[3,4] in the absence of oxygen. Consequently, the reactions were termed oxidative–reductive depolymerizations (ORD reactions) in an earlier paper[4] from this laboratory. The reactions are known to be inhibited by some metal-chelating agents, by both organic and inorganic free-radical scavengers, and by many organic compounds[3–6]. One facet of such reactions seems to be their inefficiency in chemical terms. However, when viscous, polymeric material is dealt with, the effect of autoxidants is readily followed by recording the change in viscosity with time.

EXPERIMENTAL

General. — Water doubly distilled from glass was used for final rinsing of all glassware employed in the preparation of solutions, and reagent-grade chemicals were used. The L-ascorbic acid was a gift (Hoffmann-La Roche Inc., Nutley, N. J.).

Solutions having appreciable buffering capacity were adjusted to pH 7.2–7.4. A phosphate buffer was adjusted by mixing equimolar proportions of mono- and di-potassium salts, whereas the Veronal (sodium 5,5-diethylbarbiturate) and tris(hydroxymethyl)aminomethane (Tris) buffers were adjusted to neutrality with equi-ionic,

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reagent-grade hydrochloric acid. Salts having no buffering capacity were used without adjustment of the pH. Veronal buffer was used at a final ionic strength of 0.07, because of its low solubility at pH 7 and its high ionic strength.

Hyaluronic acid was prepared by the method described earlier[7] involving precipitation from bovine synovial fluid with cetylpyridinium chloride. Samples from several preparations were used, having intrinsic viscosities ranging from 23 to 48 dl/g. Each purified product contained <3% of protein.

Viscometry was conducted in Cannon–Manning glass viscometers (size 100) thermostated at 30 ±0.01° in a Sargent water-bath. The flow times for the buffers used (0.5 ml) were approximately 60 sec. For each experiment, hyaluronic acid appropriately diluted in buffer was studied simultaneously. For 24-h incubations, the controls retained 98 to 100% of their initial, specific viscosity.

In the standard procedure, hyaluronic acid solution (0.5 ml) was transferred from a blow-out pipet to a test tube, and thoroughly mixed with buffer (0.5 ml). The tube was covered with Parafilm, and incubated for 60 min at room temperature. On addition of an aqueous solution of autoxidant (0.5 ml), a stop-watch was started, the contents of the tube were mixed, and an aliquot (0.5 ml) was introduced into the viscometer at 30.00°. Viscosity measurements were started after about 4 min, and at frequent time-intervals thereafter, usually over a period of 2 h. The results were expressed either as percent diminution in specific viscosity or as change in specific fluidity.

For estimation of L-ascorbic acid in the incubation mixtures, 2,6-dichlorophenolindophenol was used[8]. Dehydro-L-ascorbic acid was determined by the method of Roe and Kuether[9]. Hydrogen peroxide was analyzed by use of horse-radish peroxidase and 3,3′-dimethoxybenzidine dihydrochloride[10].

Oxygen absorption was measured with an RGI Compensated Syringe Manometer (Roger Gilmont Instruments, Inc., Great Neck, N.Y.), which determines evolution or absorption of up to 200 µl of a gas in 0.2-µl divisions. The manometer was immersed in a water bath at 30 ±0.01°. The reaction vessel connected to the instrument was a conventional, 30-ml, Warburg flask having a side arm and a central well. Experiments were conducted at atmospheric pressure. Calibration of the instrument by means of hydrazine sulfate, ferricyanide, and sodium hydroxide revealed that the calibration marks on the micrometer were sufficiently accurate (within 3%) to be used uncorrected. The experimental reproducibility of the results was 3%. As initial, duplicate experiments conducted with and without sodium hydroxide in the central well gave the same results, an absorbent for carbon dioxide was unnecessary. The reaction vessel was shaken at ~100 oscillations per min by means of an electric motor fitted above the waterbath and connected to the instrument.

RESULTS

Degradation of hyaluronate by ferrous ions; influence of the buffering medium. — Ferrous ion is the most potent inorganic reductant in the oxidative–reductive degra-

dation of hyaluronic acid. On mixing ferrous ions with buffered solutions of hyaluronic acid, an immediate drop in viscosity occurs, followed by a slower decrease.

The percent changes in viscosity produced by final concentrations of 40μM, 200μM, and 3.3mM ferrous sulfate were found to vary considerably from one buffer to another, and with the order in which the ferrous ions, buffer, and hyaluronic acid were mixed (see Table I). When the iron ions (Fe) were incubated with hyaluronic acid

TABLE I

EFFECT OF BUFFER AND ORDER OF MIXING ON THE DEGRADATION OF HYALURONIC ACID[a] BY FERROUS SULFATE

Buffer[b]	$FeSO_4$ (μM)	*Diminution*[d] *in* η_{sp} (%)		
		Order of mixing[c]		
		$(HA+Fe^{2+})+B$	$(HA+B)+Fe^{2+}$	$(B+Fe^{2+})+HA$
0.2M Phosphate	3,300	92 (3)	85 (2)	45 (20)
	200	87 (8)	79 (10)	3 (6)
	40	67	79	0
0.2M KF	200	85	81	52
	40	41	41	30
0.2M KCl	3,300	95	93	90
	200	74	35	16
0.2M K_2SO_4	3,300	83	60	66
	200	68	32	31
0.2M Citrate	3,300	98	99	94
	200	92	95	67
0.2M McIlvaine	3,300	97	97	90
	200	44	81	16
0.07M Veronal	3,300	77	71	71
	200	9 (44)	13 (29)	7 (25)
	40	2 (20)	9 (20)	0 (26)
0.2M Tris	3,300	56	57	−3 (8)
	200	10 (16)	−2 (4)	
0.2M NaOAc	3,300	87	67	74
	200	90	91	1

[a]Final concentration of hyaluronic acid: 0.4 mg/ml. [b]Buffers were adjusted to pH 7.3; [c]HA = hyaluronic acid; B = buffer. [d]Measurements were made after 2 h at 30 ±0.01°. Figures in parentheses indicate additional decrease in viscosity after 24 h.

(HA) for 1 h and then added to the buffer (B) (Fe+HA+B), the greatest initial degradation occurred in phosphate and fluoride buffers, and the least degradation in Tris and Veronal buffers. The order was the same when the samples of hyaluronic acid were incubated with buffers for 1 h before being mixed with the ferrous ions (HA+B+Fe). Incubation of the ferrous ions with phosphate buffer for 1 h, followed by addition of hyaluronic acid, namely, (Fe+B+HA), greatly lessened the depolymerization; but, with the other buffer systems studied, the order of mixing had little effect.

TABLE II

EFFICIENCY OF REACTION OF FERROUS IONS[a] IN PHOSPHATE BUFFER

Conc. of Fe^{2+} *(μM)*	η_{sp} *(extrapolated) after 2 h*	η_{sp} *after 24 h*	η_{sp} *after addition of further* Fe^{2+} *(24μM)*	*Chainbreaks per HA molecule*	*Chainbreaks per* Fe^{2+}	*Additional chainbreaks per* Fe^{2+}
0	1.56	1.52	—	—	—	—
6.7	1.49	1.44	0.60	0.05	0.015	0.13
13.7	1.40	1.30	0.57	0.15	0.022	0.13
33.0	0.94	0.85	0.49	0.90	0.054	0.10
67.0	0.61	0.50	0.29	1.70	0.051	0.15
330.0	0.23	0.22	—	5.60	0.034	—

[a]Ferrous iron added to solution (having $[\eta]$ = 23 dl/g) of hyaluronic acid in phosphate buffer, to give a final concentration of 0.4 mg/ml of hyaluronic acid (HA) in 0.2M buffer of pH 7.4 at 30.000°. The second addition of iron was made to the incubated mixture after 24 h, leading to a slight change in viscosity through increase in the volume of the 2-ml samples by 0.1 ml; this increase was not corrected for in the calculation of the number of chainbreaks/molecule of hyaluronic acid.

Unbuffered solutions of hyaluronic acid were also susceptible to depolymerization by ferrous sulfate, but the rate of degradation of the polymer followed an exponential course and did not undergo a rapid, initial phase. Addition of potassium sulfate to such a solution (to overcome the electroviscous effect) gave a specific viscosity (η_{sp}) of 0.66 after 18 h, whereas the control solution had η_{sp} 1.82. This corresponds to a decrease of 64% in the specific viscosity in the absence of any buffering system.

Efficiency of chain cleavage by metal ions in phosphate buffer at pH 7.2. — For polyelectrolytes that obey the Huggins relationship between reduced viscosity and specific viscosity (as does hyaluronic acid in buffered solutions), it is possible to calculate, from a knowledge of the initial and final specific viscosities, the approximate number of chainbreaks that take place during depolymerization*. To calculate maximum efficiency, hyaluronic acid was incubated in phosphate buffer with various concentrations of ferrous salt, and the viscosities were measured after 2 and 24 h. Under the experimental conditions, the process was rather inefficient, with a maximum efficiency for concentrations of ferrous ions of 30–60μM (see Table II). To those solutions that retained at least 60% of their initial specific viscosity after 24 h (see Table II), more ferrous sulfate (24μM) was added, and the additional number of chainbreaks per atom of iron was determined. A pronounced increase in efficiency during the second period was observed in each instance.

Degradation of hyaluronic acid by L-*ascorbic acid; the effect of different buffers.* — The extent of degradation of hyaluronic acid by L-ascorbic acid was affected by the type of buffer employed. Buffer was added to a sample of hyaluronic acid, followed by L-asorbic acid.

In an ionic milieu of phosphate, sulfate, or nitrate ions (pH 7.2), and in distilled water (for solutions in which the initial and final viscosities were corrected for the electroviscous effect), a decrease of 80 to 90% in the initial viscosity occurred during 2 h of incubation (see Table III); bicarbonate (pH 8.3), Veronal, and Tris buffers gave a much lower rate of degradation. In the next 24 h, a further diminution in viscosity of about 10% occurred in Tris buffer, and 40% in Veronal buffer.

As with ferrous salt, the rate of depolymerization of hyaluronic acid by L-ascorbic acid is dependent on the order of mixing. Notably, preincubation of L-ascorbic acid (0.33mM) with phosphate buffer (0.2M, pH 7.2) for various periods of time affected the degree and the rate of the degradation induced in hyaluronic acid (0.4 mg/ml), as illustrated in Fig. 1.

Effect of metal ions on the degradation of hyaluronic acid that is induced by L-*ascorbic acid.* — These results are recorded in Table IV. Addition of catalytic

*For accurate calculation of the number of chainbreaks occurring, it is necessary to calculate the changes in $MW_{visc.}$ from the changes in intrinsic viscosity. In the treatment of polysaccharides that obey the Huggins relationship for polyelectrolytes, plots of reduced viscosity are made, and, from them, intrinsic viscosities are taken to be the value of the intercept on the ordinate. For polymers that give parallel straight lines for degraded and undegraded solutions (as does hyaluronic acid, approximately), changes in intrinsic viscosity may be considered to be reflected in their percent changes in specific viscosity.

quantities of cupric or ferrous ions to the phosphate buffer, to L-ascorbic acid, or to hyaluronic acid caused no enhancement of the final fluidity value, regardless of the

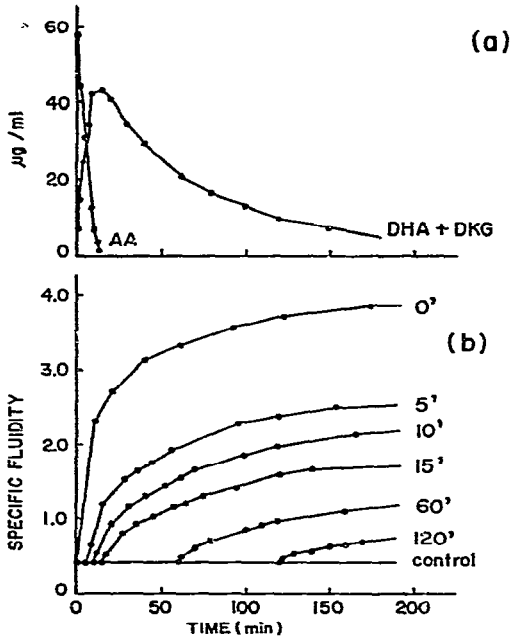

Fig. 1. (a) Consumption of L-ascorbic acid and formation of dehydro-L-ascorbic acid and L-*threo* 2,3-hexodiulosonic acid in the system described in (b) for zero time. (b) Depolymerization of hyaluronic acid (0.04%) after pre-incubation of L-ascorbic acid (0.33mM) in 0.2M phosphate buffer of pH 7.2 for the periods indicated. AA, L-ascorbic acid; DHA, dehydro-L-asorbic acid; DKG, L-*threo*-2,3-hexodiulosonic acid.

TABLE III

DEGRADATION OF HYALURONIC ACID BY L-ASCORBIC ACID (0.33mM); EFFECT OF BUFFER OR ADDED POTASSIUM IONS

Solvent	*Hyaluronic acid (mg/ml)*	*Initial* η_{sp}	*Degradation after 2 h (%)*
Distilled water	0.4	2.9[a]	90
	0.8	4.8[a]	93
0.2M Phosphate buffer	0.4	2.9	88
pH 7.4	0.8	4.6	87
0.2M Sulfate[b]	0.4	2.9	85
0.2M Nitrate	0.4	2.9	87
0.2M Bicarbonate[c]	0.8	3.9	43
0.2M Tris buffer			
pH 7.1	0.8	4.4	24
pH 8.3	0.8	5.3	21
0.1M Veronal buffer	0.8	5.4	6

[a]Corrected for electroviscosity. [b]No buffering properties. [c]Prepared by addition of 0.2M HCl to 0.2M potassium hydrogen carbonate (pH 8.3).

sequence of mixing. However, at the concentration of L-ascorbic acid used here (0.33mM), no increase could be expected from an addition of metal ions, in view of the extensive diminution in viscosity produced by L-ascorbic acid alone.

TABLE IV

DIMINUTION IN SPECIFIC VISCOSITY OF HYALURONIC ACID[a] BY L-ASCORBIC ACID, AND THE EFFECT OF FERROUS OR CUPRIC IONS, OR BOTH

Buffer system	*L-Ascorbic acid (mM)*	*Fe^{2+} (μM)*	*Cu^{2+} (μM)*	*Decrease in viscosity after 2 h (%)*
Phosphate buffer,	0.33			88 ±1.7[b]
0.2M, pH 7.4	0.33	3.3		88
	0.33	4.3		89
	0.33	1.7	1.7	92
	0.33		3.3	88
	0.33		6.7	71
	0.33		43.0	91
	3.3			95
	3.3	0.3		94
	3.3	0.7		93 ±2.8[b]
	3.3	1.3		95
	3.3	6.7		92
Tris buffer,	0.33			24
0.2M, pH 7.3	0.33	8.0		35
	0.33		8.0	62
Veronal buffer,	0.33			8
0.07M, pH 7.1	0.33	4.0		50
	0.33		4.0	65
Bicarbonate,	0.33			43
0.2M, pH 8.2	0.33	8.0		46
	0.33		8.0	53
Sulfate, 0.2M	0.33			85
Nitrate, 0.2M	0.33			87

[a] Concentration, 0.8 mg/ml; intrinsic viscosity, 34 dl/g at 30°. [b] Standard deviation.

With Tris and Veronal buffers, addition of copper or iron salts to the hyaluronate–ascorbate system resulted in an increase in the percent diminution of viscosity, as compared to that of the samples that contained no added metal ions. In no case, however, was the viscosity lowered to the levels observed when phosphate buffer was employed.

Activity of L-ascorbic acid and dehydro-L-ascorbic acid in phosphate buffer at pH 4.2 and 7.2. — With dehydro-L-ascorbic acid (0.33mM) in 0.2M phosphate buffer of pH 7.2, hyaluronate was degraded extensively (~90%), as measured over a 2-h incubation period. At pH 4.2, dehydro-L-ascorbic acid was inactive, but L-ascorbic acid retained much of the activity that it showed at pH 7.2 (see Fig. 2).

Oxygen absorption of solutions of L-ascorbic acid in 0.2M phosphate buffer; effect of metal ions and of hyaluronic acid. — Unbuffered solutions of L-ascorbic acid (about pH 2.5) had negligible rates of oxygen absorption. At pH 7.2, the rate of

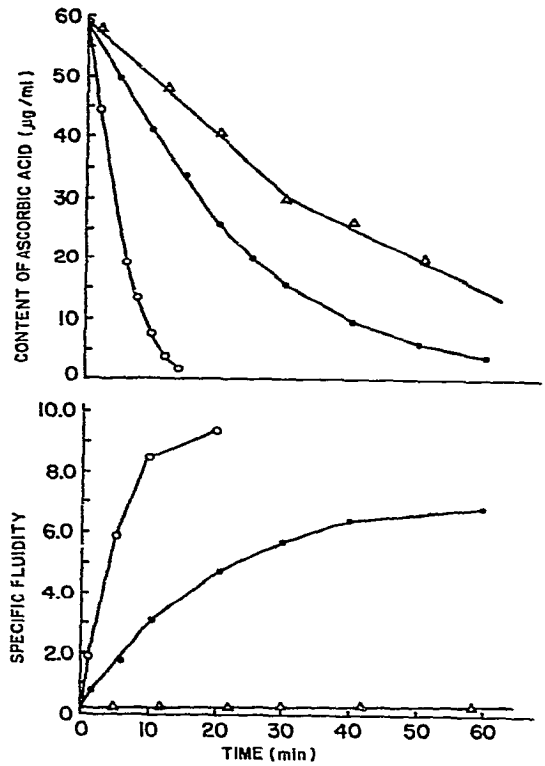

Fig. 2. Consumption of L-ascorbic acid (0.33mM) and fluidity–time curve for the depolymerization of hyaluronate by L-ascorbic acid (0.33mM). (0.2M phosphate buffer, pH 7.2, O—O; 0.2M phosphate buffer, pH 4.2, ●—●; 0.2M Tris buffer, pH 7.2, Δ—Δ.)

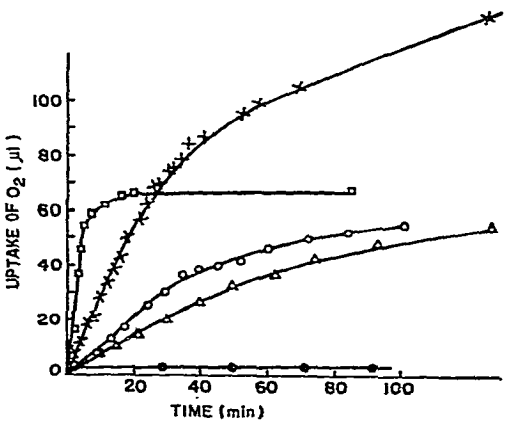

Fig. 3. Oxygen absorption at 30°. Values were corrected for temperature difference. [$FeSO_4$ 9.8μM in 4.0 ml of 0.2M phosphate buffer of pH 7.2, □—□; ditto in distilled water, ●—●; L-ascorbic acid 4μM in 4.0 ml of 0.2M phosphate buffer of pH 7.2,X—X; ditto in phosphate buffer of pH 4.2, O—O; dehydro-L-ascorbic acid 4μM in 4.0 ml of 0.2M phosphate buffer of pH 7.2, Δ—Δ; ditto in phosphate buffer of pH 4.2, ●—●.]

oxidation was linear (2.5 μl/min) over a period of about 20 min, at a 1mM concentration of L-ascorbic acid (see Fig. 3). According to these data, the complete oxidation of L-ascorbic acid and of its oxidation products consumes 3 gram-atoms of oxygen per molecule of reducing agent at pH 7.2. At pH 4.2, the amount absorbed is 1 gram-atom of oxygen per molecule of L-ascorbic acid, indicating that the oxidation does not proceed beyond the state of dehydro-L-ascorbic acid (see Table V). No difference was observed in the volume of oxygen absorbed in the presence of hyaluronic acid (0.04%), at a 1mM concentration of L-ascorbic acid.

TABLE V

ABSORPTION OF OXYGEN BY L-ASCORBIC ACID, DEHYDRO-L-ASCORBIC ACID, AND FERROUS SULFATE AT 30°

Reactant[a]	*pH*	*Total volume (ml)*	*Oxygen uptake (μl)*	*Gram-atom per molecule of reactant*[b]	*Time (min)*
L-Ascorbic acid	7.2	4	121	2.46	164
	7.2	4	151	3.0	∞
	4.2	4	50	1.0	∞
Dehydro-L-ascorbic acid	7.2	4	99	2.0	∞
	4.2	4	0	0	∞
Ferrous sulfate (2.5mM)	7.2	4	62	0.50	100
	water	10	0	0	∞

[a]Concentration, 1mM in 0.2M phosphate buffer, unless otherwise stated. [b]Values are corrected for temperature difference by a factor of 1.11.

Solutions of dehydro-L-ascorbic acid in phosphate buffer of pH 7.2 gave two-thirds the oxygen uptake of the same concentration of the reduced form; at pH 4.2, no oxygen consumption was observed. Unbuffered solutions of ferrous sulfate did not absorb any oxygen, but when such a solution was added to a phosphate buffer at pH 7.2, an oxygen uptake of 0.5 gram-atom per molecule of the iron salt was measured (see Table V); this would correspond to the scheme: 4 Fe(II) $\xrightarrow{O_2}$ 4 Fe(III).

The oxidative–reductive depolymerization of hyaluronic acid was investigated for possible formation of hydrogen peroxide. Consistent with the results of our studies of oxygen absorption, neither in the presence of L-ascorbic acid, nor of iron salt, nor of a combination of the two, was any hydrogen peroxide detected at pH 7.2, even when (ethylenedinitrilo)tetraacetic acid was added to the reaction mixture. However, with L-ascorbic acid as the autoxidant, any hydrogen peroxide that might have been produced during the course of the depolymerization of hyaluronic acid would have been destroyed as fast as it was formed, as the addition of hydrogen peroxide to the mixture gave a negative test.

Disappearance of L-ascorbic acid in 0.2M phosphate buffer, and depolymerization of hyaluronic acid. — Because of the insufficiency of oxygen present, highly concentrated solutions of L-ascorbic acid in phosphate buffer, kept in stoppered containers,

remain stable even after several months. Inadequate attention to aeration of L-ascorbic acid solutions may be responsible for divergent conclusions regarding the stability of L-ascorbic acid that are reported in the literature[11].

Mixtures of hyaluronic acid (0.04%) in 0.2M phosphate buffer (pH 4.2 or 7.2) to which L-ascorbic acid (0.33mM) had been added were periodically analyzed for formation and consumption of dehydro-L-ascorbic acid and L-*threo*-2,3-hexodiulosonic acid. These data and the corresponding fluidity curves for the complete reaction mixtures are presented in Figs. 2 and 4.

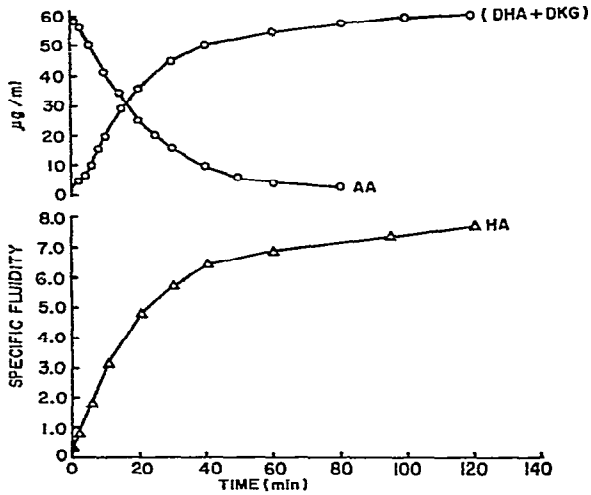

Fig. 4. Depolymerization of hyaluronic acid (HA) (0.04%) in 0.2M phosphate buffer of pH 4.2, and the corresponding formation of dehydro-L-ascorbic acid (DHA) and L-*threo*-2,3-hexodiulosonic acid (DKG) from L-ascorbic acid (AA) (0.33mM).

In phosphate buffer at pH 7.2, wherein depolymerization of hyaluronic acid is rapid, L-ascorbic acid is rapidly oxidized to dehydro-L-ascorbic acid and L-*threo*-2,3-hexodiulosonic acid. At this pH, these products are subject to further decomposition. At pH 4.2, where the changes in fluidity of the polymer in the presence of L-ascorbic acid are slowed, the rate of oxidation of L-ascorbic acid is substantially diminished; furthermore, at the acid pH, the autoxidation stops at the stage of dehydro-L-ascorbic acid or hexodiulosonic acid; even after 24 h at 30°, their combined level is still unaffected.

The results of these experiments demonstrate that the rate of depolymerization of the polymer depends on the rate of conversion of L-ascorbic acid into its oxidation products (which, at the appropriate pH, are also efficient depolymerization agents).

In Tris buffer, the rate of disappearance of L-ascorbic acid proceeds at a much lower rate than in phosphate buffer (see Fig. 2); correspondingly, L-ascorbic acid is significantly less efficient as a depolymerizing agent in the organic buffer than in phosphate buffer. As the rate of degradation of the polysaccharide is still lower than would be expected from the rate of disappearance of the L-ascorbate from the reaction

medium, it is possible that the organic buffer system acts not only by slowing the oxidation of L-ascorbic acid but also as a free-radical scavenger in the oxidative–reductive depolymerization reaction.

*Experiments in an oxygen-free atmosphere**. — By using a modification of the equipment described elsewhere[12] and the standard conditions (0.33mM autoxidant in 0.2M phosphate buffer of pH 7.3), we investigated the effect of the following systems on the stability of hyaluronic acid: dehydro-L-ascorbic acid; copper(II); iron(II)+ L-ascorbic acid; and hydrogen peroxide. Of these, only hydrogen peroxide lowered the viscosity of the polymer. The decrease in specific viscosity was ~30% after 2 h; this diminution in viscosity is comparable to that observed when the same agent at the same concentration (0.33mM) was assayed in the air with buffered hyaluronic acid.

DISCUSSION

In the present work, the rate of depolymerization of hyaluronic acid, as determined by changes in fluidity or viscosity, was examined in relation to the rate of oxidation of reductants in the presence of each of a variety of buffer systems.

Under standard experimental conditions (phosphate buffer, pH 7.2), the rapid depolymerization of hyaluronic acid by added ferrous ions is associated with a rapid uptake of oxygen by the reductant (see Fig. 3), and both processes are essentially complete within 10 min. This conversion of ferrous into ferric ions occurs because of the smaller redox potential for valence changes of the phosphate complex of iron[13]. Pre-equilibration of ferrous ions in phosphate buffer causes their oxidation to ferric ions, thereby abolishing the depolymerizing activity.

Although, by manometry, we observed no measurable oxidation for aqueous solutions of ferrous ions (see Table V), a perceptible change in the viscosity of the polymer was still found.

As regards the depolymerization by L-ascorbic acid, a parallelism exists between the rate of change in fluidity of hyaluronic acid and the rate of disappearance of L-ascorbic acid (see Fig. 2) under the standard experimental conditions (that is, in phosphate buffer).

The oxidation of L-ascorbic acid is facilitated in neutral and slightly alkaline solutions of phosphate buffer, in which the mono-dissociated ion is present. However, at progressively lower pH values <5, the mono-dissociated ion becomes replaced by the undissociated acid which, being neutral, is less susceptible to the electron removal required for oxidation. This behavior is reflected in the redox potentials for the oxidation of L-ascorbic acid at pH 7.25 and 4.0, namely, E_o' +0.051 and +0.154, respectively[14], and is in agreement with the finding that degradation of hyaluronic acid by L-ascorbic acid at pH 4.2 proceeds at a much lower rate than at neutral pH (see Fig. 4).

*Helium of high purity (The Matheson Company, Inc.) was further purified by bubbling it through a 10% solution of L-ascorbic acid in phosphate buffer at pH 7.2.

Under conditions of mild acidity, the products of the autoxidation of L-ascorbic acid are at their maximum stability[14,15] and, consequently, depolymerization is due solely to the conversion of L-ascorbic acid into dehydro-L-ascorbic acid; as this compound is not subject to hydrolysis or to further oxidation at the low pH, no change in viscosity occurs when hyaluronic acid is treated with dehydro-L-ascorbic acid at pH 4.2.

At pH 7.2, depolymerization continues to occur after the oxidation of L-ascorbic acid is complete (see Fig. 2). It must, therefore, be concluded that the products formed after the oxidation of L-ascorbic acid cause further depolymerization; this conclusion is consistent with the claims that L-*threo*-2,3-hexodiulosonic acid is a strong reductant[16] and an active depolymerizing agent[17]. The results of our experiments with pre-equilibrated L-ascorbic acid in phosphate buffer substantiate this interpretation (see Fig. 1).

It is, therefore, not surprising that Swann[18] reported slow changes in viscosity for the depolymerization of hyaluronic acid induced by L-ascorbic acid, as his experiments were, on one occasion, conducted at pH 3. In another experiment, he performed the reaction at pH 7.5, but used Tris buffer (which inhibits the oxidative–reductive depolymerization of hyaluronic acid; see Table III). This inhibitory effect seems to be due not only to the protective effect that Tris exerts against the oxidation of L-ascorbic acid but also to its ability to act as a free-radical scavenger.

As pointed out in earlier reports[3,4], the depolymerization of hyaluronic acid by reductants is oxygen-dependent; among the various agents examined in this system under an inert atmosphere (nitrogen or helium), only hydrogen peroxide seems to exhibit depolymerizing activity. This phenomenon is explained by the ability of hydrogen peroxide to afford hydroxyl free-radicals in the absence of atmospheric oxygen.

In conclusion, the oxidative-reductive depolymerization of hyaluronic acid is influenced by the nature of the autoxidant, the buffer system, and the order in which the various ingredients of the system are mixed. Metal impurities in the distilled water or in the reagents used greatly affect the course and the rate of this reaction. The latter aspect will be discussed elsewhere.

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