99. THE DEGRADATION OF MUCINS AND POLYSACCHARIDES BY ASCORBIC ACID AND HYDROGEN PEROXIDE[1,2]

BY WILLIAM v. B. ROBERTSON, MARIAN W. ROPES AND WALTER BAUER

From the Medical Clinic of the Massachusetts General Hospital, the Department of Medicine, Harvard Medical School, and the Massachusetts Department of Public Health, Boston, Massachusetts

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IT has been demonstrated [Robertson *et al.* 1940] that fresh vitreous humour of steers contains a labile substance capable of reducing the viscosity of synovial fluid mucin. The fact that the vitreous humour lost this activity upon standing, upon dialysis or upon addition of oxidizing agents, and the high vitamin C content of vitreous humour (approximately 0·5 mg./ml. as determined by 2:6-dichlorophenolindophenol titration), suggested that ascorbic acid might be an important factor in the breakdown of mucin.

This hypothesis was supported by the results of an experiment in which 100 mg. of ascorbic acid added to 25 mg. of mucin in *M*/20 phosphate buffer *p*H 7·2 caused a degradation of mucin in 2 hr., as shown by a reduction of the relative viscosity from 40 to *ca.* 1. Upon addition of acetic acid, a flocculent precipitate was obtained instead of the characteristic cohesive, fibrous precipitate of normal mucin. That the reduction in viscosity was irreversible, due to a change in the size of the mucoprotein molecule, and not caused by altered characteristics of the solvent, was proved by redissolving the precipitate in phosphate buffer at *p*H 7·2. The degraded mucin did not produce a viscous solution as does the fibrous precipitate of normal mucin. Reduction in the size of the mucin molecule was further shown by the fact that the degraded mucin passed through a Seitz filter (EK 6) which held back normal mucin. The characteristics of the altered mucin indicated that ascorbic acid had split the original macromolecules. This breakdown may or may not have been due to hydrolysis. However, the absence of free amino-sugars or of an increased concentration of reducing substances, even after a prolonged reaction period, demonstrated that there was no further hydrolysis.

The present investigation was undertaken to determine the role of ascorbic acid in the breakdown of mucin and to establish the degree of specificity of the reaction.

Methods

Ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol in HPO_3. H_2O_2 and traces of Cu salts, one or both of which were present in some of the ascorbic acid solutions, did not affect the determinations.

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H_2O_2 was determined by titration with $KMnO_4$ in acid solution. When H_2O_2 was to be determined in the presence of ascorbic acid, which also reduces $KMnO_4$, titrations were made before and after the addition of catalase and the H_2O_2 calculated by difference.

Phosphate was determined according to Tschopp & Tschopp [1932].

The method for determining the time required for degradation of mucin was based on the change in the type of precipitate obtained with acetic acid [Robertson *et al.* 1940]. Portions of the reacting ascorbic acid-mucin mixture were added, at frequent intervals, to dilute acetic acid until the resulting precipitate was flocculent.

The role of ascorbic acid in the degradation of mucin

In an attempt to determine whether reduced or oxidized ascorbic acid is active in the degradation of mucin, the two primary oxidation products were tested. Neither dehydroascorbic acid[1] nor the irreversible oxidation product formed by opening of the lactone ring[2] [Herbert *et al.* 1933] had any effect on mucin. However, the time of breakdown of mucin by ascorbic acid could be decreased some 250-fold by addition of dilute H_2O_2. The degradation was not due to a change in the *p*H of the buffered mucin solution, and H_2O_2, when present alone, caused no change in mucin.

By studying the activity, in the presence of H_2O_2, of ascorbic acid oxidized by $K_3Fe(CN)_6$, it was demonstrated that equimolar concentrations of ascorbic acid and dehydroascorbic acid together cause no degradation, and that dehydroascorbic acid even in the presence of H_2O_2 is not capable of splitting mucin. This inactivity is not due to inhibition by $K_4Fe(CN)_6$.

The above results suggested that reduced ascorbic acid and H_2O_2 are both essential for the breakdown of mucin. Corroboration was obtained by experiments in which ascorbic acid, partially autoxidized in the presence of Cu, was the active agent. The activation in this case is presumably due to the presence of H_2O_2, which according to Barron *et al.* [1935] is formed during the autoxidation. 5 mg. of ascorbic acid in 1 ml. *M*/100 $CuSO_4$ added to 3 ml. (15 mg.) of mucin solution caused a breakdown in 8 min. at *p*H 7·2 and 30°. A similar solution of ascorbic acid containing catalase (crystalline preparation [Sumner & Dounce, 1937]) produced no degradation. The addition of catalase, which destroys the H_2O_2 as it is formed, completely prevented the degradation of mucin, demonstrating the indispensability of H_2O_2 in this reaction.

In order to test the specificity of this action with respect to ascorbic acid, several organic reductants, some of which contained the ene-diol grouping, were substituted for ascorbic acid. *d-iso*Ascorbic acid,[3] acetone ascorbic acid[3] and *l*-saccharogulonoascorbic acid[3] were equally active. Dihydroxymaleic acid, reductic acid, reductone and pyrogallol were less efficacious, having activities which decreased in the order named. Monomethyl ascorbic acid, diacetone gulosonic acid,[4] catechol, adrenaline, glutathione, cysteine, glucose, oxalic acid and formaldehyde were inactive.

[1] Dehydroascorbic acid was formed by the oxidation of ascorbic acid at *p*H 5·0 with $K_3Fe(CN)_6$. At *p*H 5·0 and 28° dehydroascorbic acid has a half life of about 3 hr. [Ball, 1937].

[2] Irreversibly oxidized ascorbic acid was formed by aerating ascorbic acid overnight in the presence of traces of Cu^{++} at *p*H 7·4 and 38°.

[3] We wish to thank Merck and Company for supplying these products.

[4] We wish to thank Hoffmann-La Roche for supplying the diacetone gulosonic acid and the ascorbic acid used in this investigation.

In an attempt to clarify the synergistic action of ene-diols, such as ascorbic acid, and H_2O_2 in causing degradation of mucin, the oxidation of ascorbic acid by H_2O_2 was studied.

Ascorbic acid and H_2O_2 react mol. for mol. as is clearly shown by Table 1, which gives the results of an experiment in which the molar ratio of H_2O_2 to ascorbic acid was varied from 0 to 4.

Table 1. *Oxidation of ascorbic acid by H_2O_2*

	0.00 hr. m.equiv. present		50.00 hr. m.equiv. present		m.equiv. reacted	
Experiment	H_2O_2	Ascorbic acid	H_2O_2	Ascorbic acid	H_2O_2	Ascorbic acid
A	0·00	0·56	0·00	0·53	0·00	0·03
B	0·13	0·56	0·00	0·44	0·13	0·12
C	0·25	0·56	0·00	0·28	0·25	0·28
D	0·50	0·56	0·03	0·13	0·47	0·43
E	1·00	0·56	0·30	0·06	0·70	0·50
F	2·00	0·56	1·50	0·06	0·50	0·50

The H_2O_2 and ascorbic acid were dissolved in 40 ml. and kept under oil at 34°. No attempt was made to remove traces of heavy metals.

The time required for degradation of mucin was shortened by increasing the concentration of ascorbic acid in the presence of excess H_2O_2 (Table 2). The change in rate, however, was not directly proportional to the increase in ascorbic acid concentration. Similarly a change in H_2O_2 concentration in the presence of a constant amount of ascorbic acid did not affect the rate of breakdown proportionately (Table 2).

Table 2. *Effects of ascorbic acid and H_2O_2 concentrations on the time of degradation of mucin*

Exp.	H_2O_2 mg.	Ascorbic acid mg.	Time for breakdown of 15 mg. mucin at *p*H 7·2 and 30° min.
1	30	0·156	55
		0·312	25
		0·625	15
		1·25	12
		2·5	8
		5·0	5
2	60	0·156	84
		0·312	35
		0·625	17
		1·25	11
		2·5	6½
		5·0	4
3	0	2·5	>120
	0·13	—	9
	0·65	—	5½
	7·8	—	3
4	0	2·5	>120
	0·13	—	9
	0·26	—	7
	0·52	—	5
	0·78	—	4

The requisite amounts of H_2O_2 and ascorbic acid were dissolved in water and 1 ml. of the solution was added to 3 ml. of the mucin solution.

Cysteine and cyanide which inhibit the autoxidation of ascorbic acid are not inhibitors of the degradation caused by ascorbic acid and H_2O_2. Neither appreciably affects the rate of breakdown of mucin.

No constant temperature coefficient for the degradation of mucin could be obtained. It varied with temperature and with the concentration of H_2O_2 and ascorbic acid. The measured coefficient was a function of at least two reactions, the change in the macromolecule and the oxidation of ascorbic acid. At temperatures above 60° the oxidation of ascorbic acid predominated so that degradation was complete only when high concentrations of ascorbic acid were used.

Similarly the effect of hydrion concentration on the degradation was the resultant effect on the aforementioned reactions. However, in this case the data indicated a consistent trend: the activity diminished rapidly as the *p*H increased, as illustrated by the following experiment.

1 ml. 0·005 *M* ascorbic acid in 0·25 % H_2O_2 was added to 3 ml. mucin solution (15 mg.) in *M*/20 phosphate buffers of different hydrion concentrations from *p*H 5·35–8. At acidities greater than *p*H 5, mucin precipitates, thus precluding an extension of these experiments to a more acid range.

*p*H (glass electrode)	5·35	5·9	6·4	6·85	7·2	8·1
Rate of mucin degradation mg./min. (at 30°)	3·55	2·5	0·96	0·62	0·3	0·06

Specificity of the ascorbic acid-H_2O_2 system

The action of ascorbic acid was tested on mucins of mesothelial and epithelial origin, on several viscous polysaccharides, on pneumococci and on β-glycerophosphate.

(*a*) *Mucins of mesothelial origin.* Degradation of a mesothelial mucin was demonstrated in the above experiments in which synovial mucin was the substrate. Ascorbic acid plus H_2O_2 caused degradation of other mesothelial mucins (umbilical cord and connective tissue [Robertson *et al.* 1940]) and of vitreous humour, with resulting loss of viscosity and change in the characteristic acetic acid precipitability.

(*b*) *Mucins of epithelial origin.* Ascorbic acid and H_2O_2 caused a similar alteration of the viscosity and precipitability of epithelial mucins (salivary and gastric) as shown by the following experiments.

10 g. commercial gastric mucin (Wilson) were shaken overnight with 100 ml. *M*/15 Na_2HPO_4 and then centrifuged. To 10 ml. of mucin solution were added 1 ml. 1 % H_2O_2 and 20 mg. ascorbic acid. The relative viscosity of 85·6 at 5 min. was reduced to 13·4 after 30 min. and to 5·3 after 16 hr.

Salivary mucin was prepared by precipitating saliva with 2 volumes of alcohol and redissolving the mucin in 0·2 volume of phosphate buffer *p*H 7·2. To 10 ml. of this mucin solution were added 20 mg. ascorbic acid in 1 ml. 1 % H_2O_2. The relative viscosity after 1 hr. was 1·61 in contrast to 2·05 for the control containing 1 ml. H_2O_2. Upon precipitation with alcohol, the control yielded the typical ropey precipitate, whereas the precipitate of the digest was finely flocculent.

(*c*) *Polysaccharides.* Because of the apparent lack of specificity of the ascorbic acid-H_2O_2 system, the action was tested also on starch paste, pectin, flaxseed mucilage, the polysaccharide of synovial mucin and chondroitin sulphuric acid from cartilage. There was a marked reduction in viscosity and change in characteristic precipitability when present.

Potato starch was dissolved in hot phosphate buffer. To 10 ml. of the cooled starch paste 1 ml. 1 % H_2O_2 and 20 mg. ascorbic acid were added. The relative viscosity after 1 hr. was 4·05 in contrast to 497 for the control containing 1 ml. H_2O_2. The iodine reaction remained unchanged after 24 hr.

A pectin solution (Certo) was dialysed against $M/20$ Na_2HPO_4. The *p*H was 6·2. 3 ml. $M/5$ buffer *p*H 6·5 containing 100 mg. ascorbic acid and 1% H_2O_2 were added to 10 ml. of the pectin solution. The relative viscosity after 10 min. was 2·3 compared with 22·4 for the control containing 3 ml. H_2O_2.

Flaxseed mucilage prepared by alcoholic precipitation of an aqueous extract of flaxseed was dissolved in phosphate buffer *p*H 7·2. 1 ml. of ascorbic acid (5 mg.) in 1% H_2O_2 was added to 10 ml. of this solution. After 10 min. the relative viscosity at 38° was 1·22 in contrast to 5·45 for the control solution containing 1 ml. 1% H_2O_2.

The polysaccharide prepared by tryptic digestion of synovial mucin [Robertson *et al.* 1940] was dissolved in phosphate buffer. 1 ml. 1% H_2O_2 containing 20 mg. of ascorbic acid was added to 10 ml. of polysaccharide solution. After 15 min. the relative viscosity was 1·25 in contrast to 59·6 for the control containing H_2O_2.

Two samples of chondroitin sulphuric acid from xyphoid cartilage were studied. One was prepared by 10% $CaCl_2$ extraction according to Meyer & Smyth [1937], the other by 2% KOH extraction. 1 ml. 1% H_2O_2 containing 20 mg. ascorbic acid was added to 5 ml. of chondroitin sulphuric acid solution buffered at *p*H 6·8. The controls contained H_2O_2 instead of ascorbic acid plus H_2O_2. The results with both samples were essentially the same, the relative viscosity after 1 hr. averaging 2·9 in contrast to 6·9 for the controls.

(*d*) *Pneumococci.* In view of the polysaccharide content of the pneumococcus capsule, the action of ascorbic acid and H_2O_2 on pneumococci was studied.[1]

Suspensions of heat-killed encapsulated pneumococci were prepared according to the method of Dubos [1937]. 0·5 ml. of this suspension was pipetted into precipitin reaction tubes and one drop of chloroform was added. 0·25 ml. each of buffered ascorbic acid, H_2O_2 and/or water was added and the tubes were incubated overnight. Microscopic examination of film preparations of each culture showed destruction of the capsule only in those cases in which both ascorbic acid and H_2O_2 were present, as shown below. The *p*H remained approximately 7 in all cases.

	Microscopic examination of pneumococci					
Type	I*	II	III*	VIII	XII	XVIII
Ascorbic acid and H_2O_2	Lysis	Lysis	Lysis	Lysis	Lysis	Lysis
Ascorbic acid (5 mg.)			Gram stain positive			
3% H_2O_2			Gram stain positive			
Water			Gram stain positive			

* Two different cultures of types I and III were tested.

(*e*) *Dephosphorylation of β-glycerophosphate.* The number of substances affected by ascorbic acid and H_2O_2 suggests action on a common linkage. The resemblance of the degradation of mucin to the reduction of the viscosity of starch paste by an amylophosphatase [Waldschmidt-Leitz & Mayer, 1935], the breakdown of synovial fluid mucin by serum phosphatase [Robertson *et al.* 1940] and the dephosphorylating activity of ascorbic acid [Thannhauser *et al.* 1938] suggest that a dephosphorylation may be responsible for the breakdown of mucins and polysaccharides reported above. This hypothesis is supported by the

Table 3

Reactants	mg. P liberated
1. 50 mg. β-glycerophosphate + 10 mg. ascorbic acid	0·000
2. 50 mg. β-glycerophosphate + 30 mg. H_2O_2	0·025
3. 50 mg. β-glycerophosphate + 10 mg. ascorbic acid + 30 mg. H_2O_2	0·525
4. 10 mg. ascorbic acid + 30 mg. H_2O_2	0·000

The reactants were dissolved in boiled sterile acetate buffer *p*H 5·5 and kept under oil. The total volume was 15 ml., the temperature 37° and the reaction time 24 hr.

[1] We are indebted to Dr Helen Arnold for bacteriological assistance.

observation that the dephosphorylation of β-glycerophosphate by ascorbic acid under anaerobic conditions requires H_2O_2. These experiments are presented in Table 3.

Although various types of substance are acted upon by ascorbic acid and H_2O_2, several compounds whose solutions are viscous (ovomucin, agar-agar and gelatin) were not affected by this system.

Discussion

The data presented show that ascorbic acid causes degradation of mucins only in the reduced form and in the presence of H_2O_2. However, it is impossible to conclude from the results whether ascorbic acid and H_2O_2 act separately and successively on the substrate or whether H_2O_2 is a sufficiently mild oxidant to form an active intermediary oxidation product such as that postulated by Bezssonoff & Woloszyn [1938] or by Dekker & Dickinson [1940].

Ascorbic acid has been considered a possible functional entity in the oxidation-reduction system of cells because of its strong reducing properties, yet no experiments have indicated its role either as an enzyme or coenzyme in such a system. The degradation of mucins and polysaccharides, as described in this paper, should be considered as a possible function of vitamin C in physiological and pathological processes.

Summary

1. Ascorbic acid and H_2O_2 react to cause degradation of synovial fluid mucin. This degradation involves a breakdown of the macromolecules without the liberation of detectable amounts of reducing substances or amino-sugars.

2. This action is not limited to mucins of mesothelial origin as is that of mucinase. The ascorbic acid-H_2O_2 system acts on gastric and salivary mucins, and on polysaccharides, such as starch, pectin, flaxseed mucilage and the polysaccharides of synovial mucin and cartilage, and destroys the capsules of various types of pneumococci. However, it causes no change in ovomucin, agar-agar or gelatin.

3. Ascorbic acid and H_2O_2 cause a dephosphorylation of β-glycerophosphate.

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