CLXXIX. FACTORS PREVENTING OXIDATION OF ASCORBIC ACID IN BLOOD SERUM

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The extreme readiness with which ascorbic acid undergoes oxidation *in vitro*, in presence of traces of Cu, leads one to suppose that under the conditions encountered in animal organisms, i.e. in presence of O_2 and of salts of Cu and Fe, this process would also proceed rapidly were it not for the existence of certain protective mechanisms. These mechanisms, which act *in vitro*, but which may also apply *in vivo*, may be classified into two types:

(*a*) Inhibition of conversion of ascorbic to dehydroascorbic acid by proteins, amino-acids and salts [Kellie & Zilva, 1935; Stotz *et al.* 1937; Barron *et al.* 1937].

(*b*) Prevention of conversion of dehydroascorbic acid into further irreversibly oxidized products by glutathione and possibly other thiol derivatives [De Caro & Giani, 1934; Borsook *et al.* 1937].

The action of factors of the first class depends on binding by proteins and amino-acids of Cu, which catalyses this process, whilst the action of factors of the second class depends on oxidation-reduction processes causing reconversion of dehydroascorbic acid. No satisfactory explanation of the action of salts has been put forward.

The present paper describes experiments on the action of proteins, amino-acids and salts on oxidation of ascorbic acid *in vitro*.

Experimental

Methods. The systems were prepared in Erlenmeyer flasks, and contained 16 ml. of water or phosphate buffers, 2 ml. of a 0·05–0·1 % solution of ascorbic acid, and 2 ml. of the added substances whose influence was under examination. The concentration given in the tables are those attained in the solution after addition of the substances, e.g. salt. The systems were left in open flasks at a temperature of 25°. At the beginning of the experiment and after a given time (stated in the tables), 1 ml. samples were taken and, after acidification with acetic acid, the ascorbic acid content was determined by titration with dichlorophenolindophenol solution. The figures given in the tables are means of 3–5 determinations. The solutions of ascorbic acid were prepared immediately before the experiment.

In the experiments for the examination of the influence of protein we added horse serum globulin obtained by 1/2 saturation with ammonium sulphate, followed by dissolving of the precipitate and dialysis for 4 days against distilled water. Globulins prepared by other methods and other proteins were also used, but the effect obtained did not differ greatly. Deproteinization was effected with the help of a solution containing 8 % trichloroacetic acid and 2 % metaphosphoric acid.

In the experiments on the influence of blood components, efforts were made to maintain the same relation of the various constituents as in the blood, i.e. the amount of haemolysed corpuscles in 5 ml. of solution corresponds to 10 ml. of whole blood.

The ascorbic acid was generously given to us by Roche, Ltd., Warsaw.

Results

(1) *Effect of proteins.* Our first experiments had as their object the establishment of the least concentration of protein still affecting oxidation of ascorbic acid, as well as to demonstrate whether the inhibitory action of protein was proportional to its concentration. The velocity of oxidation of ascorbic acid in these experiments depended on the concentration of protein as well as on the Cu content of the distilled water used. This was constant in our experiments, and amounted to about 0·01 p.p.m.

Table I. *Influence of serum globulin on oxidation of ascorbic acid*

Conc. of globulin %	Conc. of $CuSO_4$ %	mg. ascorbic acid remaining after				
		0	45 min.	120 min.	165 min.	24 hr.
0·002	—	0·625	—	0·33	—	—
0·011	—	0·625	—	0·53	—	—
0·023	—	0·625	—	0·55	—	—
0·063	—	0·625	0·55	—	0·51	0·24
0·125	—	0·625	0·51	—	0·51	0·22
0·625	—	0·625	0·52	—	0·52	0·14
1·250	—	0·625	0·55	—	0·47	0
0·625	0·000125	0·625	0·32	—	0·14	0
0·625	0·000250	0·625	0·33	—	0·13	0
0·625	0·001250	0·625	0·22	—	0·09	0
Control	—	0·625	0·34	0·25	—	0

It appears from Table I that the inhibitory action of serum globulin is evident at a concentration of 0·011 %. With increasing concentration of protein this effect augments only to a certain limit, being smaller with 0·1 % protein than at lower concentrations. In systems to which $CuSO_4$ was added the concentrations of protein did not suffice to bind the entire amount of Cu, and in these experiments the inhibitory action of protein was not evident.

The effects obtained with whole serum were similar to those with globulin; highly diluted sera had a greater action than undiluted or slightly diluted sera.

In view of the possibility that the preparations of serum globulin taken contained Cu, which might catalyse the reaction of oxidation of ascorbic acid, the ash remaining after combustion of 2 g. protein was dissolved in a dilute solution of H_2SO_4 in triply distilled water, and the solution was added to aqueous solutions of ascorbic acid. Considerable activation of oxidation was found in such systems, for no ascorbic acid could be found after 50 min., as compared with over 12 hr. in the control systems. It may be concluded that the fall in protective action of protein with its increasing concentration is ascribable to its inorganic content, and hence probably to Cu, the presence of which in serum proteins has repeatedly been demonstrated [cf. Guillemot, 1932; Warburg & Krebs, 1927; Mann & Keilin, 1938]. This finding is of considerable interest with reference to the relation of proteins to Cu in the catalysed oxidation of ascorbic acid. Thus we know that proteins bind Cu, which catalyses this reaction. On the other hand, this binding does not totally abolish the catalytic activity of Cu. As was pointed out by Stotz *et al.* [1937], the Cu-protein complex is of such a

type that transformation of Cu^{I} to Cu^{II}, or the reverse, is possible. Cu combined with protein retains its catalytic properties, albeit to a smaller extent than in the ionic state. Stotz *et al.* even consider Cu-protein complexes as a kind of enzyme model.

A comparison of different proteins showed that globulins prepared from the serum of animals immunized to diphtheria did not differ in their activity from those from normal animals. Gelatin, as has already been shown by Stotz *et al.*, has a feebler protective action than has casein, which also acts slightly more strongly than does serum globulin. The experiments with casein had to be performed under somewhat different conditions in view of its insolubility at ordinary *p*H. Its inhibitory action at the higher *p*H was, however, greater than that of other proteins studied.

(2) *Effect of amino-acids.* Glycine inhibits oxidation in concentrations of 0·25 %. The effect does not diminish with rising glycine concentration, as was the case with proteins. Leucine and aspartic acid were also investigated. These amino-acids are with difficulty soluble in water, but the concentrations taken sufficed to establish a definite inhibitory action on the reaction. Acetamide had no action.

Table II. *Effects of glycine, leucine, aspartic acid and acetamide on the oxidation of ascorbic acid*

	*p*H	mg. ascorbic acid found after			
		0	1 hr.	2 hr.	24 hr.
Control	7·0	0·44	0·14	0	0
Glycine 0·66 %	7·0	0·44	0·42	0·40	0·18
,, 3·30 %	6·9	0·44	0·43	0·42	0·18
,, 6·60 %	6·5	0·44	0·44	0·43	0·35
Control	7·1	0·44	0·12	—	0
Leucine 0·1 %	7·2	0·34	0·28	—	0
,, 0·5 %	7·1	0·34	0·28	—	0·13
,, 0·9 %	7·0	0·34	0·32	—	0·16
Aspartic acid 0·1 %	7·0	0·34	0·23	—	0·05
,, 0·5 %	7·5	0·34	0·31	—	0·20
,, 0·9 %	7·1	0·34	0·32	—	0·27
Acetamide 0·2 %	7·1	0·34	0·15	—	0
,, 1·0 %	7·1	0·34	0·13	—	0
,, 1·8 %	7·1	0·34	0·19	—	0

(3) *Effect of blood constituents.* As has been mentioned, oxidation of ascorbic acid proceeds much more slowly in serum than in aqueous solutions of the same concentration. The inhibitory effect of undiluted serum is less than that of diluted serum, e.g. 1 : 15.

Oxidation in solutions of haemolysed erythrocytes is also slower than in aqueous solutions. Addition of even small amounts of haemolysed blood to serum causes, however, fairly considerable acceleration of oxidation.

(4) *Effect of salts.* It has been shown by Kellie & Zilva [1935] that oxidation of ascorbic acid is retarded in presence of NaCl. The effect of adding a number of salts, at various concentrations and *p*H, is given in Table IV.

It appears that of the salts studied only chlorides exert a retarding influence on the reaction. It is of interest that NaF, which inhibits a number of enzymic processes taking place in the organism, has no action in this case. The action of NaCl appears to be related to the catalytic action of copper, as is shown by the results of experiments in which the relative concentrations of Cu and NaCl

Table III. *Effect of blood constituents on oxidation of ascorbic acid*

	mg. ascorbic acid found after					
	0	1 hr.	3 hr.	6 hr.	24 hr.	48 hr.
A. Whole blood	1·0	—	—	—	0·88	0·81
Serum	1·0	—	—	—	0·91	0·74
Laked erythrocytes	1·0	—	—	—	0·35	0·11
Laked erythrocytes + serum	1·0	—	—	—	0·53	0·37
Phosphate buffer *p*H 7	1·0	—	—	—	0	0
B. Serum diluted 1 : 15	0·5	0·47	0·47	—	0·33	—
Serum diluted 1 : 7	0·5	0·48	0·44	—	0·39	—
Serum diluted 1 : 3	0·5	0·47	0·47	—	0·39	—
Serum undiluted	0·5	0·43	0·42	—	0·16	—
Phosphate buffer *p*H 7	0·5	0·13	0	—	0	—
C. Serum + water	0·4	0·33	—	0·29	—	—
Serum + laked erythrocytes	0·4	0·25	—	0·05	—	—
Phosphate buffer *p*H 7	0·4	0·15	—	0·05	—	—

The experiments of group A were performed in test tubes of equal size completely filled with the system, covered with paper and sealed with paraffin wax. Oxidation thus took place under conditions of limited O_2 supply, i.e. that dissolved in the solution. Each system contained 10 ml. of whole blood, serum or laked erythrocytes. In the case of a mixture of serum with erythrocytes 5 ml. of laked erythrocytes + 5 ml. of serum were taken. Three parallel determinations were made in each series. To each test tube 1 mg. of crystalline ascorbic acid was added. The experiments of groups B and C were performed as usual in Erlenmeyer flasks, and contained 16 ml. of serum, 2 ml. of erythrocytes and 2 ml. of ascorbic acid solution.

Table IV. *Influence of various salts on oxidation of ascorbic acid at* p*H 6·9*

	mg. ascorbic acid found after			
	0	1 hr.	5 hr.	24 hr.
Phosphate buffer control	0·4	0·30	0	0
0·5 *N* KCl	0·4	—	0·4	0·37
0·5 *N* NaCl	0·4	—	0·4	0·35
0·5 *N* $CaCl_2$	0·4	—	0·4	0·31
0·5 *N* $MgCl_2$	0·4	—	0·4	0·35
0·5 *N* $(CH_3COO)_2Mg$	0·4	—	0	0
0·5 *N* $MgSO_4$	0·4	—	0	0
0·5 *N* Li_2SO_4	0·4	—	0	0
0·5 *N* Na_2SO_4	0·4	—	0	0
0·5 *N* KNO_3	0·4	—	0	0
0·5 *N* NaF	0·4	—	0	0
0·003 % $CuSO_4$ in 1·3 % NaCl	0·4	0·21	—	0
0·003 % $CuSO_4$ in 12·0 % NaCl	0·4	0·40	—	0·40

were varied. The effect of chlorides cannot be ascribed to impurities present in the salts as the action of ordinary preparations of KCl did not differ from that found for samples of KCl specially purified by repeated recrystallizations from water and alcohol.

Discussion and summary

The protective action of proteins in the reaction of oxidation of ascorbic acid by atmospheric O_2 is less than might be expected were it not that protein-Cu complexes retain a certain catalytic activity. Natural protein-Cu complexes, such as are obtained from serum, thus act on the one hand as feeble catalysts; on the other hand, they are still able to bind a certain amount of copper or iron, and can in this way protect ascorbic acid from oxidation.

Proteins are not the only constituents of serum having an inhibitory action on oxidation of ascorbic acid; amino-acids and chlorides have a similar action.

The chloride ion is perhaps the more important, for its action is exerted irrespective of the cation, and so may act both in the serum and in the tissues. The action of amino-acids is probably far smaller, in view of their low concentration in serum; it may, however, become considerable during resorption from the digestive tract, and during transport to the tissues.

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