57. The Oxidation of Ascorbic Acid in the Presence of Copper

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The study of the catalytic activity of Cu under the influence of several common factors [Mystkowski & Lasocka, 1939] was extended in unpublished work carried out in Warsaw in 1939 to the inhibition of aldehyde dehydrogenase and amylase actions by Cu, and the detoxicating effect of NaCl.

In the present paper we give the results obtained in the oxidation of ascorbic acid under the influence of NaCl and proteins. During the course of these experiments we had to examine several other catalytic activities of Cu and these results are given here in brief.

Methods

The systems contained phosphate buffers in final concentrations 1/10–1/15 *M*, ascorbic acid 3–4 mg., substances whose influence was to be examined, and water up to the volume of 15 or 20 ml. The detailed composition of the systems is given in the appropriate tables.

The systems were left in open flasks at 24°. Samples were taken at intervals and after acidification with acetic acid, the amount of remaining ascorbic acid was determined by titration with dichlorophenolindophenol. In the experiments where the influence of temperature was examined the systems were kept in a water bath and then, after rapid cooling, the determinations were carried out.

In the experiments with proteins, potato extract and cucumber juice, the deproteinization was effected by a solution containing 8 % trichloroacetic acid and 2 % HPO_3.

Potatoes were either sliced with a knife or put through a meat mincer and then ground in a mortar with double their volume of water. After 30 min. the solid particles were centrifuged off and the supernatant fluid was used in experiments. 10 ml. of extract were used in each system, which in controls were replaced by 10 ml. of water. Cucumber juice was prepared according to Meiklejohn & Stewart [1941].

Experimental

(1) *Inhibition of ascorbic acid oxidation by NaCl*

The inhibition of ascorbic acid oxidation by NaCl was found first by de Caro & Giani [1934], and Kellie & Zilva [1935]. Mystkowski & Lasocka [1939] found that only the chloride ion is of importance and that the cation plays no part. This was confirmed by Mapson [1941].

In the present paper the oxidation of ascorbic acid in the presence of $CuSO_4$, $FeSO_4$, $Fe_2(SO_4)_3$ and $FeCl_3$ under the influence of NaCl was examined. The results of one typical experiment are given in Table 1.

This experiment shows great activation (30–75 %) of the oxidation of ascorbic acid by $CuSO_4$. The activation by $Fe_2(SO_4)_3$ is quite insignificant and never exceeds 10 %. In the systems containing only the Cu normally present in distilled water, inhibition caused by NaCl amounts to 65–90 %. NaCl also decreases distinctly the activation caused by added $CuSO_4$, and the decrease is proportional to the concentration of NaCl and inversely proportional to the concentration of $CuSO_4$.

In the presence of NaCl no catalytic activity of Fe salts was found. The inhibition by NaCl occurs here as in systems without any activation. Of all Fe salts examined, $FeCl_3$ shows the greatest activation.

Table 1. *Effect of NaCl on ascorbic acid oxidation in presence of Cu and Fe*

	O				NaCl				$CuSO_4$			
	30 min.		4 hr.		30 min.		4 hr.		30 min.		4 hr.	
*p*H	1	2	1	2	1	2	1	2	1	2	1	2
5·7	57·1	—	88·5	+55·0	5·7	−90·0	14·3	−75·0	74·3	+30·0	100·0	+75·0
6·0	62·8	+10·0	88·5	+55·0	8·5	−85·0	20·0	−65·0	74·3	+30·0	100·0	+75·0
6·4	—	+20·0	88·5	+55·0	8·5	−85·0	20·0	−65·0	74·3	+30·0	100·0	+75·0

	$CuSO_4$-NaCl				$Fe_2(SO_4)_3$				$Fe_2(SO_4)_3$-NaCl			
	30 min.		4 hr.		30 min.		4 hr.		30 min.		4 hr.	
*p*H	1	2	1	2	1	2	1	2	1	2	1	2
5·7	60·0	+ 5·0	91·4	+60·0	57·1	0	88·5	+55·0	11·4	−80·0	17·1	−70·0
6·0	60·0	+ 5·0	91·4	+60·0	62·8	+10·0	88·5	+55·0	17·1	−70·0	20·0	−65·0
6·4	62·8	+10·0	91·4	+60·0	62·8	+10·0	88·5	+55·0	17·1	−70·0	20·0	−65·0

The systems contained in 20 ml.: 5 ml. phosphate buffers $M/5$; 3·5 mg. of ascorbic acid; NaCl 0·14M; $CuSO_4$ and $Fe_2(SO_4)_3$ $2·5 \times 10^{-5}M$.
1. Decomposition, % 2. Activation (+) or inhibition (−) %.

Increase in temperature has comparatively little effect on the increase of the rate of ascorbic acid oxidation. Comparison of oxidations at 24 and 97° showed that although the increase of the velocity of reaction at 97° was not great, the inhibition by NaCl was smaller (13·6–25·0 %) in comparison with the inhibition at 24° (88·2 %). The activation by $CuSO_4$ is at the same level (13·0–25·0 % in comparison with 11·7 % at 24°). These experiments were performed in connexion with catalase activity of copper.

(2) *Correlation between appearance of Cu_2O and oxidation of ascorbic acid*

In experiments with the usual concentration of $CuSO_4$ ($2·5 \times 10^{-5}M$) a red precipitate of Cu_2O appears after a few minutes of ascorbic acid oxidation at 97°. In the system containing NaCl this precipitate does not appear.

The amounts of Cu_2O produced in the usual experiments at 24° were too small to be determined quantitatively. At $5 \times 10^{-4}M$ concentration a Cu_2O precipitate appears even at 24°.

To determine the amount of Cu_2O formed the last stage of the Bertrand method for sugar estimation was used. Cu_2O formed during reaction was centrifuged off, washed with distilled water, dissolved in a solution of $Fe_2(SO_4)_3$ in conc. H_2SO_4 and titrated with $M/50$ $KMnO_4$. The results in Table 2 are given in ml. $KMnO_4$ used in the titration.

Table 2. *Formation of Cu_2O during ascorbic acid oxidation*

		45 min.			3 hr. 30 min.			3 hr. 30 min.	
	NaCl conc.	1	2	3	1	2	3	$KMnO_4$ ml. $M/50$	Inhibition %
1	0	1·3	32·5	—	2·7	67·5	—	2·9	—
2	0·13M	1·2	30·0	7·7	2·4	60·0	11·1	1·7	41·3
3	0·4M	0·6	15·0	53·8	1·9	47·5	44·4	1·6	44·8
4	0·65M	0·4	10·0	69·2	1·0	25·0	62·9	1·5	51·7

*p*H = 6·5. Composition of system (in 30 ml.): 3 ml. phosphate buffer; 4 mg. ascorbic acid; $CuSO_4$ conc. $5 \times 10^{-4}M$.
1. Mg. of oxidized ascorbic acid. 2. Decomposition %. 3. Inhibition %.

In this experiment, performed at 24°, Cu_2O precipitate was visible after 1 hr. There is no strict parallel between the amount of ascorbic acid oxidized and Cu_2O formed. Nevertheless, in general throughout our experiments the greater the rate of ascorbic

acid oxidation, the greater the amount of Cu_2O formed. The inhibition of oxidation by NaCl was always accompanied by the diminution of Cu_2O formation. Thus the inhibition of the oxidation of ascorbic acid is accompanied by an inhibition of Cu^{II}–Cu^{I} transformation.

(3) *The influence of NaCl on the peroxidase and catalase actions of Cu*

Oxidation of ascorbic acid in the presence of H_2O_2 occurs with a much greater velocity than oxidation by atmospheric O_2. Experiments to demonstrate this are, however, complicated by the catalase activity of Cu, especially at higher temperatures.

In systems containing phosphate buffers, $CuSO_4$ and H_2O_2, no decomposition of H_2O_2 was visible at 20°. At 37° it was possible to observe a slow escape of bubbles of gas, which at 100° had become quite vehement.

Similar facts have already been observed by Karczag [1921] and others who examined the influence of salts of Cu and Fe on the decomposition of H_2O_2 and the decoloration of certain dyestuffs.

We found that NaCl has the same effect as $CuSO_4$ in activating the decomposition of H_2O_2. In the presence of $CuSO_4$, NaCl increases this decomposition greatly, so its action is quite opposite to its influence on the oxidation of ascorbic acid: here it activates the catalase activity of Cu.

When a solution of methyl red is added to the NaCl-$CuSO_4$-H_2O_2 system and the whole is brought to 50–80° no visible decomposition of H_2O_2 takes place. At the same time, however, the dye undergoes decoloration which is complete in 5–15 min. Only after this process is finished do gas bubbles appear.

This decoloration of methyl red by H_2O_2 in the presence of Cu is also activated by NaCl; the complete decoloration occurs two to three times as quickly as in the absence of NaCl (5 min. instead of 15 min.). All these experiments in which the presence of an acceptor for O_2 causes the complete inhibition of the evolution of O_2 from H_2O_2 were performed at pH 5·0–6·5.

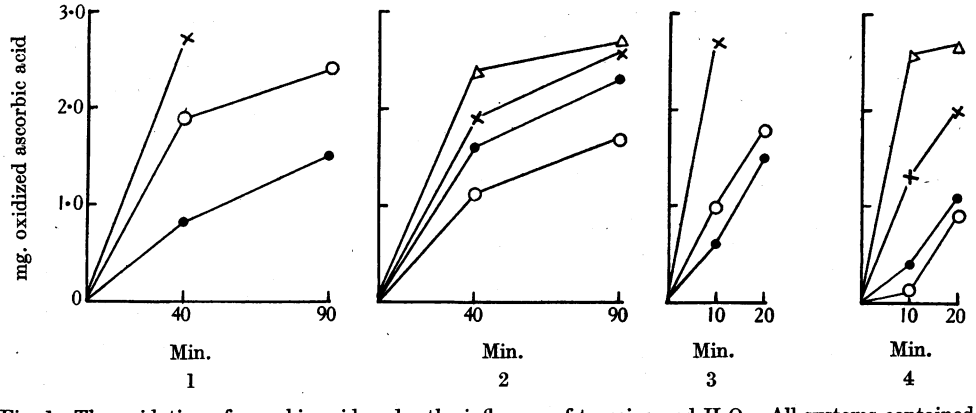

Fig. 1. The oxidation of ascorbic acid under the influence of tyrosine and H_2O_2. All systems contained in 15 ml.: 1·5 ml. phosphate buffer; 3 mg. ascorbic acid; pH = 6·3. 1, control curve; 2, system with 1 mg. tyrosine; 3, system with H_2O_2 $6 \times 10^{-4} M$; 4, system with tyrosine and H_2O_2. o, control; △, $CuSO_4$ $2 \times 10^{-4} M$; ×, $CuSO_4$ $4 \times 10^{-5} M$; •, NaCl 0·2 M.

Tyrosine has a similar influence. Its presence inhibits completely the O_2 evolution, and at the same time a reddish-brown colour appears, corresponding to quinone products of tyrosine oxidation. This oxidation of tyrosine by H_2O_2 is activated both by $CuSO_4$ and NaCl. In these systems the evolution of gas does not take place. Sometimes, how-

ever, slight visible decomposition of H_2O_2 was found in systems which passed their maximum of colour development and became colourless again.

Similar systems containing H_2O_2 and tyrosine were examined with regard to the oxidation of ascorbic acid. Their action was compared with systems containing $CuSO_4$ and NaCl only. The results are given in Fig. 1.

In systems without tyrosine NaCl inhibits the catalytic action of Cu in the presence of H_2O_2, so its action here is the same as in systems where the oxidation occurs with atmospheric O_2.

In our previous paper we found that other amino-acids inhibit the catalytic action of Cu. Tyrosine acts similarly (Fig. 1); in the presence of tyrosine the oxidation proceeds much more slowly and even the activation by added Cu is much diminished.

In the presence of tyrosine, NaCl shows none of its usual inhibition. As NaCl activates the oxidation of tyrosine, and by this intermediate action through quinones also the oxidation of ascorbic acid, the corresponding curve is a result of two actions, one of which is the oxidation by atmospheric O_2 (inhibited by NaCl), the second, oxidation by quinones (activated by NaCl). The same facts were observed in the presence of H_2O_2.

Another way in which tyrosine might possibly interfere with the oxidation of ascorbic acid is by competing with it for the available O_2.

(4) *The oxidation of ascorbic acid in the presence of enzymic systems*

Two enzymic systems were examined, viz. (*a*) extract from potatoes, (*b*) cucumber juice.

(*a*) *Potato extract.* The reactions occurring in potato extract are of a rather complicated nature and will be described separately in what follows. At least two major systems dealing with ascorbic acid oxidation were found in the potato. One of them causes a very quick disappearance of almost all the ascorbic acid present in potato after the structure of the latter has been destroyed by grinding or slicing. This rapid oxidation is not influenced by NaCl even in high concentration: 2–3 % trichloroacetic acid stops it.

The second system is connected with the oxidation of tyrosine by polyphenol oxidase. The products of tyrosine oxidation oxidize in turn ascorbic acid. NaCl activates slightly the oxidation of tyrosine and through this intermedially also the oxidation of ascorbic acid.

The interpretation of these results is difficult because of the different *p*H optima for ascorbic acid oxidation with Cu as catalyst, and for tyrosine oxidation by polyphenol oxidase. Also the different stages of oxidation of tyrosine interfere in many ways with the ascorbic acid oxidation. In consequence the facts observed in these experiments will require a more detailed examination.

(*b*) *Cucumber juice.* Stotz, Harrer & King [1937] have examined a number of compounds which inhibit the catalytic action of Cu. The results obtained by these authors show that both types of catalysis, by inorganic Cu in the presence of proteins and by 'enzymic copper', are inhibited by the added Cu-inhibitors. However, in cauliflower juice and cabbage juice the inhibition was smaller than in other systems. Experiments by Barron, De Meio & Klemperer [1935] show that cabbage juice and squash juice were not inhibited by *o*-hydroxyquinoline.

In our experiments the influence of $CuSO_4$ and NaCl in aqueous systems was compared with systems containing cucumber juice, fresh and inactivated by boiling. A difference in inhibiting actions of NaCl on these two systems was found.

This experiment of Fig. 2 shows that the oxidation of ascorbic acid is not inhibited by NaCl in systems containing active ascorbic acid oxidase. In systems containing boiled juice the inhibition caused by denatured proteins is so great that it masks completely any possible inactivation by NaCl.

$CuSO_4$ shows its greatest activating influence in the control system. This effect is abolished in cucumber juice, probably by the presence of proteins which bind Cu. In the

inactivated juice this effect of binding is even greater, so that here the activation by Cu is of quite a moderate order.

In connexion with these results we have examined a number of systems containing $CuSO_4$, NaCl and proteins (albumin or casein). The inhibition of the oxidation by proteins found in our previous paper was confirmed. Of native and denatured proteins the latter were found to inhibit much more strongly the effect of added copper sulphate. Summation of the two inhibitory effects, by proteins and NaCl, occurred.

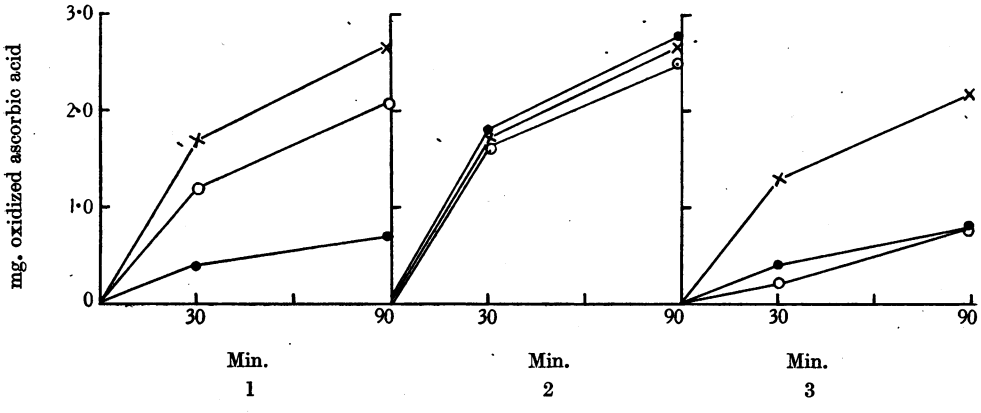

Fig. 2. The influence of NaCl and $CuSO_4$ in systems with ascorbic acid oxidase. All systems contained in 15 ml.: 3 ml. phosphate buffer; 3 mg. ascorbic acid; pH = 6·3. 1, control; 2, system with ascorbic acid oxidase (2 ml.); 3, system with inactivated ascorbic acid oxidase (2 ml.). o, control; ×, $CuSO_4$ $2{\cdot}5 \times 10^{-5} M$; •, NaCl 0·14 M.

The results obtained explain the slow rate of oxidation in systems containing inactivated enzyme. As a result of the denaturation of proteins, the Cu bound to a 'specific protein' loses its enzymic properties and the whole system behaves like inorganic Cu in the presence of a non-specific protein.

Discussion

The biological activity of Cu, like that of Fe and other metals, comprises two groups of reactions:

(i) inactivating influence on enzymic processes, both hydrolytic and redox;

(ii) catalytic action in many redox processes.

The catalytic action of iron in living organisms is connected with its presence in many enzymes. Except for a few compounds of an importance similar to those of iron, the role of Cu in enzymic processes is not so well established. Nevertheless the widespread presence of Cu in tissues and its catalytic activity observed *in vitro* suggest that it must be of considerable importance.

The catalytic action of Cu is very easily influenced by many factors including normal tissue components. The oxidation of ascorbic acid by atmospheric O_2 in the presence of Cu was found to be inhibited by NaCl, amino-acids and proteins.

In the presence of H_2O_2, however, the catalytic action of Cu is complicated by the fact that Cu can act as a model not only of peroxidase but also of catalase. It was found that the activity of Cu depends on the kind of substrate present. Thus at higher temperatures the catalase action of Cu was greatly diminished when the substrate suitable for the oxidation was available. The presence of methyl red or tyrosine, which undergo oxidation by H_2O_2 in the presence of Cu, stopped almost completely the latter's catalase function.

Both reactions, catalytic decomposition of H_2O_2 and oxidation of methyl red and tyrosine, are activated by NaCl. This is in contrast to the oxidation of ascorbic acid which is inhibited by NaCl in similar conditions.

These experiments show that the catalytic action of Cu is of two kinds and changes according to the presence of different substrates, and also that the influence of NaCl is of a quite opposite character in the two cases.

The influence of amino-acids on the catalytic action of Cu depends in the first place on their power to bind Cu. Tyrosine, however, shows another influence due to its participation in redox reactions. Under the influence of Cu, tyrosine undergoes oxidation by atmospheric O_2 and above all by H_2O_2. So in the presence of tyrosine the course of ascorbic acid oxidation is changed by the action of products of tyrosine oxidation. In such systems two different reactions take place; one, inhibited by NaCl, the oxidation by either O_2 or H_2O_2; the second, activated by NaCl, the oxidation by quinones.

The action of proteins on Cu in ascorbic acid oxidation is always of an inhibiting nature. Our previous experiments have shown how this influence depends on the concentration of Cu and protein. Although some Cu-protein preparations obtained *in vitro* may show certain properties in common with enzymes [Stotz *et al.* 1937], their action in the oxidation of ascorbic acid is less than that of corresponding concentrations of $CuSO_4$ alone.

The relation between the structure of Cu-protein complexes and their catalytic activity has yet to be examined. One fact is established, that proteins after denaturation have a much greater inactivating power on the catalytic action of Cu. It is probable that many 'enzymic' properties of the Cu-protein complexes depend on the colloidal state of the protein in solution.

On the other hand, in these complexes the catalytic activity of Cu in the oxidation of ascorbic acid is never completely abolished. All similar complexes (e.g. those isolated by Keilin & Mann [1939]) may therefore be of some significance, especially when their widespread occurrence is considered.

The influence of NaCl on the oxidation of ascorbic acid in systems containing enzymes is different from that on inorganic Cu. In potato extract, where at least two mechanisms of ascorbic acid oxidation were found, one of them is only slightly influenced by NaCl, whereas the second is quite insensitive to this factor. Similarly, in cucumber juice containing ascorbic acid oxidase, no influence of NaCl was found.

The lack of influence of NaCl on ascorbic acid oxidase, the inactivation of added Cu by an enzymically active juice, and the transformation of the latter into an 'inorganic system' after the denaturation of proteins, indicate that there must be a difference between 'enzymic' and inorganic Cu. This difference may consist either in the specificity of the enzymic protein, or in the special form of Cu-protein combination. The second possibility, which does not exclude the existence of a specific enzymic protein, is highly probable. The fact that the Cu present in the enzyme does not undergo dialysis [Meiklejohn & Stewart, 1941] as long as the protein is in its genuine state, confirms this conception. In acid solution the Cu becomes dialysable, but at the same time ascorbic acid oxidase loses its enzymic properties.

These facts indicate that even if a specific protein were not essential to an enzyme containing Cu, the linkage of these two components is of a nature which so far we have not been able to imitate.

These facts neither confirm nor contradict the existence of a specific ascorbic acid oxidase in cucumber juice. This particular enzyme seems to fall between the 'true' enzymes and the Cu-protein complexes described by Stotz *et al.* [1937].

The way in which chloride ions act on the catalytic activity of Cu is not known. In simple systems, where the oxidation of ascorbic acid runs parallel to Cu^{++}-Cu^{+} transformation, the inhibition of catalysis is accompanied by a diminution of Cu_2O formation. No influence of NaCl on Fe salts was found.

SUMMARY

1. The oxidation of ascorbic acid by Cu is inhibited by NaCl, amino-acids and proteins.
2. This inhibition was found in systems with either atmospheric O_2 or H_2O_2 as oxidant.
3. The influence of tyrosine is different from that of other amino-acids and is connected with its participation in redox processes.
4. NaCl shows either inhibiting (oxidation of ascorbic acid) or activating (catalase activity of Cu, oxidation of tyrosine) influence on the reactions catalysed by Cu.
5. NaCl has no influence on the action of ascorbic acid oxidase present in cucumber juice.

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