CXXVII. THE CATALYTIC OXIDATION OF ASCORBIC ACID.

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As long ago as 1922, Hess [1922] demonstrated the possibility of destruction of vitamin C through the catalytic action of metals. He showed in this connection that when milk was pasteurised in glass containers it was antiscorbutically more active than when it was submitted to this treatment in copper vessels. Recently, Euler *et al.* [1933] found that pure ascorbic acid took up oxygen more readily in the presence of certain metals. The experiments described in this communication, which were the outcome of an observation on the instability of ascorbic acid in distilled water, yield further information on this subject.

The rate of oxidation of ascorbic acid dissolved in water of different degrees of purity.

In these experiments the spontaneous oxidation of ascorbic acid in tap water, ordinary laboratory distilled water obtained from a tin-lined copper still, water redistilled from glass apparatus and water distilled from and received in quartz apparatus was investigated. 15 mg. of *l*-ascorbic acid were dissolved in 40 ml. of each sample of water contained in conical flasks of 100 ml. capacity. In the case of the quartz-distilled water round-bottomed flasks of 50 ml. capacity were used. The reaction in each case was adjusted to p_H 7·4 by addition of the requisite quantity of sodium hydroxide and the flasks were kept at 37°. At the end of 20 hours the reaction in each case fell to about p_H 6·7. 5 ml. of the solutions were withdrawn at various intervals and the ascorbic acid was determined by titration with indophenol. The results are given in Table I, from which it will

Table I.

mg. of ascorbic acid per 100 ml. of water.

Hours	Tap	Ordinary distilled	Glass-distilled	Quartz-distilled
0	35·0	35·4	37·7	37·9
1	25·6	25·8	37·6	37·5
2	15·1	15·2	37·5	37·2
3	6·9	6·3	36·8	37·0
4	1·4	1·9	36·3	36·7
20	0·0	0·0	25·6	31·6

be seen that the rate of disappearance of ascorbic acid in tap water and in laboratory distilled water was fairly quick but that the oxidation in glass-distilled and quartz-distilled water was very much slower. It should be mentioned, however, that unless the distillation of the water in glass was carried out three times and the product used immediately the low rate of oxidation was not observed. On the other hand, when oxygen was bubbled for one hour through

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this water the rate of oxidation of the vitamin was not significantly accelerated. These results suggested that the increase in the rate of oxidation of ascorbic acid in tap water or in ordinary distilled water was due to the presence of traces of metal. Subsequent experiments proved this view to be true.

The catalytic action of traces of metals present in ordinary distilled water on the oxidation of ascorbic acid.

The first step taken in order to ascertain whether the higher rate of oxidation of ascorbic acid in tap water and ordinary distilled water was due to the catalytic effect of substances dissolved in the water, was to establish whether the addition of such substances to glass-distilled water would accelerate the oxidation of ascorbic acid. For this purpose the incinerated and untreated residues left on evaporation of ordinary distilled water were employed. These were obtained in the following way. Ten litres of ordinary distilled water were evaporated to about 100 ml. The liquid was then divided into two equal quantities, which were evaporated to dryness on a water-bath; one of the residues was then incinerated. In each case the residue was taken up in 100 ml. of glass-distilled water and the oxidation of ascorbic acid dissolved in these two samples was determined by the procedure mentioned above. These results which are given in Table II show that both residues accelerated the oxidation.

Table II.

mg. of ascorbic acid per 100 ml. of water.

Hours	Glass-distilled	Glass-distilled + untreated residue	Glass-distilled + incinerated residue
0	37·9	36·7	35·1
¼	36·8	28·6	24·2
1½	34·9	19·8	13·7
3	33·3	8·8	6·3
20	26·5	0·0	0·0

In view of the fact that the inorganic residue increased the rate of oxidation in glass-distilled water an experiment was performed in which cyanide was added to the ordinary distilled water. The results (Table III) showed that the rate of

Table III.

mg. of ascorbic acid per 100 ml. of water.

Hours	Ordinary distilled	*M*/25,000 sodium cyanide in ordinary distilled
0	33·0	37·0
1	23·5	36·7
2	18·1	36·1
3	13·7	35·3
4	11·1	34·6
20	0·0	27·4

disappearance of the ascorbic acid fell to about the same level as in glass-distilled water thus suggesting that at least some of the inorganic constituents contained in the normal sample were catalysing the oxidation. This water was therefore submitted to an examination for iron and copper, since it has been shown by Euler *et al.* [1933] that traces of these metals, especially of the latter, were capable of catalysing the absorption of oxygen by ascorbic acid in a Barcroft apparatus. The presence of such traces could well be expected in

distilled water obtained from a laboratory still. The detection of iron was carried out with analytically pure potassium thiocyanate according to Sakuma [1923]. The reaction was found to be positive in water concentrated 200 times, the concentration of the iron being, however, too low for quantitative determination. After precipitating the iron with ammonium hydroxide the copper was determined colorimetrically with sodium diethyldithiocarbamate and was found to be present in a concentration of 0·001 mg. per 100 ml. of water.

It was then possible to demonstrate that when these metals were added to quartz-distilled water the oxidation of ascorbic acid proceeded at an increased rate (Table IV). This was particularly marked in the case of the added copper

Table IV.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Fe/40 ml.	Quartz-distilled + 0·01 mg. Cu/40 ml.
0	37·0	34·5	34·1
1	35·2	31·8	24·2
2	33·0	29·6	21·2
3	30·9	28·2	15·2
4	29·4	25·8	8·0
20	25·7	10·3	0·0

which is in consonance with the results of Euler *et al.* [1933]. It may be mentioned that when quantities of copper were added to bring up the concentration to that found in the ordinary distilled water, the rate of oxidation was also raised but was less than that observed in the above experiment.

The next experiment was instituted with the object of establishing whether by treating ordinary distilled water by the usual procedures employed in the removal of the bulk of dissolved oxygen the rate of oxidation of the vitamin could be significantly modified. The results obtained (Table V) showed that with the quantities of ascorbic acid used, this was not the case. Furthermore, as already mentioned, when the catalysts are absent, the water (fresh glass-distilled)

Table V.

mg. of ascorbic acid per 100 ml. of water.

Hours	Ordinary distilled	Ordinary distilled. Air removed by boiling	Ordinary distilled. Air removed by evacuation	Ordinary distilled. Air replaced by passing nitrogen
0	34·9	34·7	33·0	34·4
¼	28·4	30·2	28·8	30·2
1½	15·8	16·5	18·1	17·2
3	5·4	8·1	10·4	4·7
5	0·4	2·5	4·7	1·6
20	0·0	0·0	0·0	0·0

Table VI.

mg. of ascorbic acid per 100 ml. of water.

Hours	Glass-distilled	Glass-distilled saturated with air	Glass-distilled saturated with oxygen
0	37·7	36·4	36·6
¼	37·6	36·2	36·5
1½	37·0	35·1	35·2
3	36·3	34·3	34·1
5	35·8	33·3	33·2
20	27·4	24·4	23·9

may be saturated by previously passing through it a current of air or oxygen without significantly accelerating the disappearance of the dissolved ascorbic acid (Table VI).

The inhibiting effect of animal tissues and other substances on the oxidation of ascorbic acid in ordinary distilled water.

It has recently been observed [Quastel and Wheatley, 1934; De Caro and Giani, 1934; Mawson, 1934] that when animal tissues or their extracts were added to a solution of ascorbic acid the oxidation of the vitamin was greatly inhibited. In other words the action of the tissue is similar to that of cyanide in this connection. Similar observations had already been made in this laboratory independently, but as a detailed investigation on this subject has already been published by De Caro representative experiments only will be reported here for the purpose of continuity.

The technique employed in these experiments was as follows. One g. of tissue (guinea-pig) was thoroughly ground with 10 parts by weight of ordinary distilled water. After centrifuging, the supernatant liquid was added to 30 ml. of an aqueous solution containing 15 mg. of ascorbic acid thus bringing up the total volume to 40 ml. The incubation and determination of the ascorbic acid were carried out as above. The results of such an experiment are given in Table VII

Table VII.

mg. ascorbic acid per 100 ml. of water.

Hours	Liver	Kidney	Muscle	Spleen	Plasma	Ordinary distilled water
0	38·2	37·5	37·0	37·5	36·8	32·0
1	37·7	36·3	36·1	37·4	36·1	24·9
2	37·4	35·6	35·1	37·0	35·4	15·2
3	37·0	34·7	34·6	36·7	35·2	9·8
4	36·8	34·2	33·7	36·3	34·6	5·3
20	31·1	25·6	28·8	23·5	24·0	0·0

from which the stabilising action of tissue extracts becomes evident. Extracts from the large and the small intestine were found by us also to be effective. Like De Caro, we found that extracts from the tissues of scorbutic animals exercised the same effects as those from tissues of normal guinea-pigs.

That the inhibiting action of animal tissues and their extracts was due to their influence on the catalytic action of metals present in solution was proved by the following experiments in which graded amounts of iron and copper were added to solutions containing ascorbic acid and liver tissue extracts prepared as above. The oxidised ascorbic acid was determined in the usual way. The results (Table VIII) showed that although copper is more effective as a catalyst

Table VIII.

mg. of ascorbic acid per 100 ml. of water.

Hours	Tissue extract	Tissue extract +0·01 mg. Fe/ 40 ml.	Tissue extract +0·02 mg. Fe/ 40 ml.	Tissue extract +0·05 mg. Fe/ 40 ml.	Tissue extract +0·10 mg. Fe/ 40 ml.	Tissue extract +0·05 mg. Cu/ 40 ml.	Tissue extract +0·10 mg. Cu/ 40 ml.	Tissue extract +0·20 mg. Cu/ 40 ml.	Tissue extract +0·30 mg. Cu/ 40 ml.	Tissue extract +0·50 mg. Cu/ 40 ml.
0	39·3	39·3	39·5	39·8	39·1	39·1	39·5	39·7	39·5	36·7
1	39·1	39·1	38·8	37·0	35·3	39·0	39·3	39·5	39·1	29·8
2	39·0	39·0	37·7	34·4	32·4	38·6	39·1	39·0	37·0	25·1
3	38·8	38·7	37·2	32·4	29·5	37·9	38·4	38·4	35·4	20·4
4	37·7	37·7	35·8	30·7	26·4	37·7	37·7	36·5	33·2	15·4
20	33·2	31·2	24·7	12·6	4·6	32·4	32·1	24·9	6·0	1·4

than iron in this process, the inhibiting action of the tissue extract is very much more marked in the former case. It should be mentioned here that ordinary distilled water which contains copper and iron was used in these experiments and consequently the actual amount of catalyst put out of action was rather higher than that suggested by the figures in Table VIII.

De Caro observed further the interesting fact that Ringer's solution and the salts of which it is made up had also a stabilising effect on ascorbic acid. We have repeated this part of his work since the appearance of his paper and have been able to confirm his results. The protective action of the salts will be seen from the results given in Table IX. It was of interest to ascertain whether this

Table IX.

	mg. of ascorbic acid per 100 ml. of water.				
Hours	$N/10$ KCl	$N/10$ NaCl	$N/10$ $CaCl_2$	Ringer solution	Ordinary distilled water
0	35·4	33·3	32·8	31·8	35·6
1	32·8	30·0	30·6	27·0	27·0
2	30·5	27·0	26·8	23·0	16·4
3	28·6	25·6	24·2	17·7	6·8
4	26·7	22·8	21·1	14·6	1·4

protective action was, as in the case of animal tissues, due to the suppression of the catalytic action of metals. The rate of oxidation was therefore studied in the presence and in the absence of NaCl in quartz-distilled water to which copper was added. The results obtained were clear cut and as will be seen from Table X

Table X.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + $N/10$ NaCl	Quartz-distilled + $N/10$ NaCl + 0·01 mg. Cu/40 ml.	Quartz-distilled + 0·01 mg. Cu/40 ml.
0	38·6	37·4	37·7	35·4
1	38·1	35·8	31·8	28·6
2	37·4	33·9	28·6	21·2
3	35·4	32·8	26·1	15·6
4	33·5	32·3	22·8	7·7

the oxidation catalysed by Cu was very much slower when NaCl was present. It may be noted that the addition of $N/10$ NaCl did not reduce the velocity of the reaction to that of the control solutions of ascorbic acid which did not contain any copper.

The influence of blood on the catalytic oxidation of ascorbic acid.

Particular attention has been given to this point owing to the fact that it is claimed that in the blood vitamin C is present as dehydroascorbic acid [Emmerie *et al.*, 1933]. The effects of plasma, intact and haemolysed red corpuscles and leucocytes were therefore investigated. It was mentioned above (Table VII) that plasma had an inhibiting action on the catalytic oxidation of ascorbic acid. In the case of the red corpuscles an equivalent of 1 ml. and in that of the leucocytes an approximate equivalent of 10 ml. of blood was added to the same quantity of ascorbic acid and under the same conditions as mentioned above. In both cases the cells were washed three times with an isotonic solution of sodium citrate. It will be seen from Table XI that whilst the erythrocytes offered very marked protection, the leucocytes had no perceptible influence on the rate of oxidation of the ascorbic acid.

Table XI.

mg. of ascorbic acid per 100 ml. of water.

Hours	Intact red corpuscles	Leucocytes	Citrate control	Laked red corpuscles	Ordinary distilled
0	34·2	33·9	34·7	30·7	32·3
1	33·0	28·2	28·9	30·4	23·0
2	31·9	21·9	22·1	30·2	16·7
3	31·2	16·3	17·2	27·4	7·9
4	30·5	11·1	11·6	20·7	3·3

Laked red corpuscles were also found to be capable of protecting ascorbic acid from oxidation but in this case an initial disappearance of the vitamin was noted. It was possible to show that the quantity of ascorbic acid which disappeared was proportional to the amount of haemolysed blood added and further that when the red corpuscles were previously treated with CO the destructive action of the haemolysed erythrocytes was hardly appreciable (Table XII).

Table XII.

mg. of ascorbic acid per 20 ml. of solution determined immediately after addition.

	Control	1 ml.	2 ml.	4 ml.
Intact red corpuscles	7·0	7·1	7·5	7·8
Laked red corpuscles	6·7	5·8	4·9	3·0
Laked red corpuscles treated with carbon monoxide	6·8	6·8	7·0	7·5

It would therefore appear that on haemolysing a substance, most probably oxyhaemoglobin, is set free which is capable of interacting with ascorbic acid.

The product formed by the catalytic oxidation of ascorbic acid.

In the above experiments the disappearance of the ascorbic acid was followed up by titration with indophenol. This procedure did not therefore offer any evidence concerning the character of the oxidation product formed. In the experiments to be described an attempt was made to determine the amount of dehydroascorbic acid present at various intervals, namely after 4, 20, 24 and 28 hours' incubation. The dehydroascorbic acid was determined by titrating an aliquot portion of the solution with indophenol before and after treatment with hydrogen sulphide. The results revealed that after 4 hours' incubation in the cases of both the ordinary distilled water and the quartz-distilled water with added copper a significant part of the vitamin was still present as dehydroascorbic acid although the major part of the ascorbic acid was irreversibly oxidised. There was no dehydroascorbic acid present either in the solution to which cyanide was added or in the quartz-distilled water (Table XIII). After

Table XIII.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Cu/40 ml.	Cyanide	Ordinary distilled
0	38·6	35·4	34·2	36·1
1	38·1	28·6	32·8	28·1
2	37·4	21·2	31·9	14·7
3	35·4	15·6	30·9	7·2
4	33·5	7·7	30·2	2·6
4 hours after treatment with H_2S	34·9	15·4	30·5	7·5

Table XIV.

mg. of ascorbic acid per 100 ml. of water.

Hours	Quartz-distilled	Quartz-distilled + 0·01 mg. Cu/40 ml.	Cyanide	Ordinary distilled
0	38·8	36·0	36·7	37·0
4	35·3	8·6	33·3	2·8
20	30·2	0·0	26·7	0·0
20 hours, after treatment with H_2S	30·7	3·7	27·2	3·5
24	29·5	0·0	25·8	0·0
24 hours, after treatment with H_2S	30·0	1·8	26·1	2·1

20 and 24 hours (Table XIV) the quantity of dehydroascorbic acid present in the two above experiments was decidedly less and it was further found that after 28 hours it was only just appreciable in both cases. It would appear therefore that the dehydrogenation of ascorbic acid takes place first and is then followed by irreversible oxidation. This view is further strengthened by the fact that after a short time (about 10 minutes) the loss in the indophenol-reducing capacity of a solution of ascorbic acid can be almost entirely re-established by treatment with hydrogen sulphide.

Conclusions.

A notable feature in this investigation is the great stability of ascorbic acid in the presence of oxygen when metallic catalysts are absent from solution. The negligible deterioration of the vitamin in quartz-distilled water, previously saturated with oxygen, suggests that in absolutely pure water, ascorbic acid is as stable as in the solid condition; in other words oxygen without the aid of catalysts is inert towards it. In practice, however, it is extremely difficult to produce conditions which would favour such stability of the vitamin in solution. Even the purest distilled water, when allowed to remain in glass vessels for some time, becomes contaminated enough to catalyse the oxidation of ascorbic acid.

It is noteworthy that amongst all the tissues examined only the leucocytes were unable to inhibit the oxidation of ascorbic acid in the presence of catalysts. These cells had nevertheless no accelerating action on the oxidation of the vitamin. There seems, therefore, no indication that ascorbic acid is reversibly oxidised in any part of the animal organism. The observation of Johnson and Zilva [1934] that dehydroascorbic acid is excreted in the urine as ascorbic acid suggests that the tissues on the contrary reduce the reversibly oxidised form of the vitamin. The evidence seems to favour the view that dehydroascorbic acid has to be reduced in the organism before it can function as an antiscorbutic.

Summary.

Ordinary laboratory distilled water contains sufficient quantities of metals (copper and iron) to catalyse the irreversible oxidation of dissolved ascorbic acid. In the very early stages of the process dehydroascorbic acid is found in quantities almost equivalent to the amounts of ascorbic acid which are oxidised, but as the reaction proceeds the quantities of the dehydrogenated acid present diminish. This catalytic action is barely perceptible when the vitamin is dissolved in water which has been redistilled several times from glass apparatus and which is used immediately or in water distilled from and received in quartz. When this water is previously saturated with oxygen the disappearance of ascorbic acid is not significantly increased. The addition of aqueous extracts of

liver, kidney, muscle, spleen, large and small intestine and of plasma, intact or haemolysed erythrocytes and $N/10$ NaCl inhibits the oxidation of ascorbic acid in ordinary distilled water or in water to which iron or copper has been added. Leucocytes have no influence on the oxidation. There is a disappearance of ascorbic acid immediately after the addition of haemolysed erythrocytes which is proportional to the quantity of the added corpuscles. This disappearance does not take place when the haemolysed corpuscles are previously treated with carbon monoxide. It is suggested that dehydroascorbic acid has to be reduced in the organism before it can exercise antiscorbutic activity.

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