II. THE PHOTOCHEMICAL DECOMPOSITION OF *L*-ASCORBIC ACID. II

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IN a recent investigation [Kellie & Zilva, 1938] it was shown that in the presence of air, *L*-ascorbic acid in aqueous solution, when exposed to ultraviolet light, was oxidized without the aid of sensitizers to dehydroascorbic acid. Furthermore, it was found that even in the absence of atmospheric oxygen the exposure to this light brought about a destruction of the vitamin. In the anaerobic experiments in which small quantities of ascorbic acid were used the amount of dehydroascorbic acid present after the exposure at pH 7 was so small as to throw doubt on its formation. At pH 3 on the other hand although the quantity of ascorbic acid which was decomposed by the ultraviolet light was less than at pH 7, the reduction of the residual solution with H₂S produced an appreciable rise in the indophenol-reducing capacity of the solution. That this actinic decomposition of *L*-ascorbic acid under anaerobic conditions was not due to oxidation brought about by the last traces of O₂ which could not be removed by repeated exhaustion and refilling with N₂ was shown by the fact that the quantities of ascorbic acid which disappeared after prolonged exposure required considerably larger amounts of O₂ for their oxidation than those which could possibly be present in solution even without previously removing from it the dissolved air.

This observation on the anaerobic photolysis of *L*-ascorbic acid required further elucidation and experiments for this purpose are described below. In the original investigation ascorbic acid was determined by titration with 2:6-dichlorophenolindophenol and any rise in the indophenol-reducing capacity after reduction with H₂S was assumed to be dehydroascorbic acid. The determination of pure *L*-ascorbic acid in aqueous solution by titration with indophenol is beyond criticism and great reliance can also be placed on the interpretation that the rise in the indophenol-reducing capacity in the *L*-ascorbic acid solutions exposed to the light in the presence of air after treatment with H₂S is due to the reduction of the reversibly oxidized form of the compound produced by the oxidation of some of the ascorbic acid. In the case of the anaerobic experiments, however, it was considered desirable to obtain additional evidence for the assumption that the increase in the reducing capacity of the exposed solutions after treatment with H₂S was really due to the formation of *L*-ascorbic acid. A method for the determination of ascorbic acid based on the production of furfuraldehyde was therefore employed in the present investigation. The results obtained with this method were similar to those observed when indophenol was used, thus supporting the view that even under anaerobic conditions oxidation of ascorbic acid with the formation of dehydroascorbic acid takes place during the exposure of the reduced compound to ultraviolet light.

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Determination of l-ascorbic acid as furfuraldehyde

The procedure adopted by us was based on the observation of Herbert *et al.* [1933] that by heating *l*-ascorbic acid with boiling 12 % HCl it readily gave furfuraldehyde which could be determined as the phloroglucide yielding 87 % of the theoretical value. As the application of the customary formula for the correction of the solubility of the phloroglucide yielded fictitious results with the quantities of ascorbic acid with which we worked we had recourse to the following empirical method which was found to be sufficiently accurate for our purpose. To a solution calculated to contain about 30 mg. of ascorbic acid were added half the volume of conc. HCl and sufficient 12 % HCl to bring the volume up to 100 ml. This solution was then distilled over a free flame at first slowly and then at a rate yielding 30 ml. of distillate per 10 min. until 360 ml. of distillate were collected. The volume in the distilling flask was kept constant by periodical additions of 50 ml. 12 % HCl. A quantity of phloroglucinol (about $2\frac{1}{2}$ times the weight of the expected furfuraldehyde) dissolved in 12 % HCl was added to the filtered distillate with stirring. The solution was made up to 400 ml. and allowed to remain at room temperature overnight to allow the black precipitate to separate completely. The precipitate was then collected by filtration in a sintered-glass crucible, washed with 150 ml. of water without being allowed to become dry, dried at 100° for 4 hr. and weighed after cooling in a desiccator. The equivalent quantity of *l*-ascorbic acid was ascertained from a graph (Fig. 1)

Fig. 1.

which was constructed by plotting the weights of the phloroglucide against the weights of *l*-ascorbic acid from which they were obtained. We have ascertained that solutions of dehydroascorbic acid distilled under the above conditions yielded a small quantity of phloroglucide which corresponded approximately to 10 % of its equivalent of ascorbic acid. As will be seen later the quantities of dehydroascorbic acid involved in our experiments were of an order that could

not seriously vitiate the assessment of the *l*-ascorbic acid in the exposed solutions before they were reduced with H₂S. Roe [1936] claims that dehydroascorbic acid does not yield any furfuraldehyde at all when boiled with HCl.

*The formation of dehydroascorbic acid from l-ascorbic acid
by irradiation under anaerobic conditions*

As the rise in the indophenol-reducing capacity of irradiated *l*-ascorbic acid solutions after treatment with H₂S was more marked in acid than neutral solution these experiments were carried out at pH 3.6. 50 mg. of *l*-ascorbic acid were dissolved in 100 ml. of sodium acetate-acetic acid buffer solution prepared with quartz-distilled water which was boiled and cooled immediately before use, and the solution was irradiated in a quartz tube of 125 ml. capacity. Before irradiation the tube was evacuated 3 times and filled with pure N₂ 3 times and the solutions were therefore finally irradiated in N₂ at atmospheric pressure. The quartz tube was exposed for 4 hr. to a Hewittic Y 2 mercury lamp at a distance of 9 in. with constant turning. The temperature of the solution gradually rose to 46° during this time. After cooling 1 ml. 1 % NaCN was added to the irradiated solution and 40 ml. were removed for furfuraldehyde determination. At the same time 5 ml. were titrated with indophenol. A further 45 ml. were then placed in a wash bulb through which H₂S was passed for 5 min. and, after sealing the vessel, were allowed to remain overnight. After thoroughly displacing the H₂S by a current of pure N₂ the following day, the furfuraldehyde content was determined in 40 ml. and another 5 ml. were again titrated with indophenol.

Table 1. *Oxidation of l-ascorbic acid during anaerobic irradiation
(initial concentration of l-ascorbic acid, 50 mg. per 100 ml.)*

Exp.	Indophenol-reducing capacity (mg. <i>l</i> -ascorbic acid per 100 ml.)				Determination as furfuraldehyde (mg. <i>l</i> -ascorbic acid per 100 ml.)			
	After irradia- tion	After reduction	Differ- ence	Destroyed during irradiation	After irradia- tion	After reduction	Differ- ence	Destroyed during irradiation
1	30.4	36.3	5.9	19.6	29.8	32.6	2.8	20.2
2	32.4	37.6	5.2	17.6	31.2	36.9	5.7	18.8
3	32.25	36.8	4.55	17.75	32.0	33.9	1.9	18.0
4	30.8	34.4	3.6	19.2	30.8	33.8	3.0	19.2
5	30.3	35.5	5.2	19.7	31.0	33.5	2.5	19.0
6	31.2	36.7	5.5	18.8	30.6	35.5	4.9	19.4

Table 2. *mg. of l-ascorbic acid per 100 ml. determined by indophenol titration*

Initial concentration	Time of exposure hr.	After irradiation	After reduction	Difference	Destruction per hr.
50	4.0	33.2	39.8	6.6	4.2
50	6.5	24.0	30.5	6.5	4.0
50	6.5	22.8	29.2	6.4	4.2
50	8.0	18.0	27.4	9.4	4.0
100	4.0	84.1	88.2	4.1	4.0
100	6.5	74.0	84.0	10.0	4.0

It will be seen from Table 1 that although both methods yielded consonant results for the total quantity of *l*-ascorbic acid destroyed during irradiation, this was not the case when the *l*-ascorbic acid in the exposed solution was determined

by these methods after reduction with H_2S . The results obtained by the furfuraldehyde method show nevertheless that considerable quantities of dehydroascorbic acid were formed during irradiation. This point will receive comment later. It may be mentioned here that the disappearance of the ascorbic acid was due to the action of the light and not to the rise in temperature of the exposed solution since there was no significant loss in similar solutions kept at 47° for 4 hr. in the dark.

*The probable dependence of the disappearance of ascorbic acid
on the intensity of the irradiation*

In Table 2 are given figures for the disappearance of *l*-ascorbic acid in solution in two concentrations exposed during different periods of time. These experiments were carried out successively so that the actinic output was in all probability of the same intensity in each case. The fact which emerges from these results is that the quantity of *l*-ascorbic acid which disappeared per hour was the same in every experiment; the destruction was therefore proportional to the time of exposure. These results also indicate that the output of actinic energy by the lamp which we used was too low for the disappearance of *l*-ascorbic acid to be affected by variation in the concentrations employed. It would thus appear, as one would expect, that the chemical change is dependent on the intensity of the irradiation.

*The protection of l-ascorbic acid by glutathione during
anaerobic irradiation*

Experiments were then carried out with the purpose of ascertaining whether the formation of dehydroascorbic acid takes place in solutions in which glutathione is present. A protective action of glutathione could be explained

Table 3. *Protection of l-ascorbic acid by glutathione during anaerobic irradiation*

Solution	Time of irradiation hr.	mg. <i>l</i> -ascorbic acid per 100 ml.		
		Initial content	After irradiation	After reduction
With glutathione	0.5	47.5	47.8	46.5
Without glutathione	0.5	44.0	39.3	41.0
With glutathione	2.0	49.0	45.3	44.3
Without glutathione	2.0	45.0	35.0	34.8
With glutathione	4.5	48.8	40.1	38.8
Without glutathione	4.5	45.6	24.2	23.9
Control solution containing no ascorbic acid	4.5	0.5	2.5	1.0
With glutathione	Kept in dark in incubator at 47° for 4.5 hr.	52.5	53.0	52.3
Without glutathione		48.5	45.3	43.5

Table 4. *Screening of l-ascorbic acid during anaerobic irradiation*

Composition of screening solution	mg. <i>l</i> -ascorbic acid per 100 ml.		
	Initial content	After irradiation	After reduction
Water only	48.0	17.0	16.0
0.100 g. glutathione in 50 ml. recently boiled and cooled quartz distilled water	46.5	40.8	39.0
0.34 g. KH_2PO_4 , 2.0 ml. <i>N</i> NaOH in 50 ml. of solution, <i>pH</i> 7.4	47.3	17.0	17.5
0.34 g. KH_2PO_4 , 2.3 ml. <i>N</i> NaOH, 0.100 g. glutathione, in 50 ml. of solution, <i>pH</i> 7.4	47.3	42.5	41.5

either by its reduction of the formed dehydroascorbic acid or by its absorption of rays responsible for the photochemical oxidation of the *L*-ascorbic acid. Two experiments were performed with this end in view. In both experiments the solutions were exposed at the physiological pH of 7.4.

In the first experiment 50 mg. of *L*-ascorbic acid, 0.68 g. KH_2PO_4 and 4.23 ml. of *N*/1 NaOH were made up to 100 ml. with quartz-distilled water (recently boiled and cooled). To 50 ml. of this solution 0.1 g. of crystalline glutathione and 0.33 ml. of *N*/1 NaOH were added. Both solutions were then at pH 7.4 and were placed in quartz flasks which were evacuated, filled with nitrogen and irradiated as before. Aliquot portions of solutions were titrated with indophenol at pH 3 before and immediately after each exposure. The exposed solutions were also reduced with H_2S and titrated again. It will be seen from Table 3 that in the presence and in the absence of glutathione there was a progressive disappearance of *L*-ascorbic acid during the exposure but, whilst in presence of glutathione after $4\frac{1}{2}$ hr. irradiation the loss was of the order of 20%, in its absence the destruction amounted to about 45%. This observation leaves no doubt that the glutathione had a protective action on the actinic decomposition of the *L*-ascorbic acid. It may be noted that no dehydroascorbic acid could be demonstrated in these solutions since there was no increase in the indophenol titre after reduction with H_2S .

In order to show that the protective action of the glutathione was mostly, if not entirely, due to its absorptive capacity for the active radiation, the following experiment was devised. 10 ml. of a solution of *L*-ascorbic acid, prepared as in the preceding experiment and also buffered at pH 7.4, were placed in a small quartz tube (approximate capacity 15 ml.). This tube was evacuated and filled with N_2 as before and placed concentrically within a larger quartz tube as shown in Fig. 2. The *L*-ascorbic acid was then exposed for $4\frac{1}{2}$ hr.

when the screening solution consisted of water, buffer or solutions containing 0.2% of glutathione. It is seen from Table 4 that the glutathione had in this case a protective action of the same order as in the preceding experiment. Also in this experiment, owing to the high pH, no dehydroascorbic acid was found.

DISCUSSION OF RESULTS

The results show conclusively that about two-thirds of the increase in the indophenol titration values after reduction of the exposed solutions of *L*-ascorbic acid could be traced to the formation of dehydroascorbic acid. The remaining third may have been due either (a) to the formation of some unknown substance during irradiation which, like dehydroascorbic acid, on reduction with H_2S yielded an indophenol-reducing substance, or (b) to some chemical change incurred during the reduction of the solution which interfered with the furfuraldehyde determination. Whatever be the explanation of the lack of agreement

Fig. 2. A, layer of liquid paraffin employed to prevent oxidation when glutathione was present in the screening solution; B, atmosphere of nitrogen; C, screening solution; D, *L*-ascorbic acid solution.

in the ascorbic acid contents of the irradiated and reduced solutions as determined by the two methods, it is noteworthy that dehydroascorbic acid was formed beyond doubt during the exposure to ultraviolet light in quantities requiring more O_2 than the traces which could possibly have been present in the *l*-ascorbic acid solutions after repeated exhaustion and refilling of the tube with N_2 .

Since Tian [1911] claimed that traces of O_2 together with H_2O_2 are formed when water is exposed to ultraviolet light of 190 $m\mu$, attempts were made by us to detect the formation of O_2 and of H_2O_2 during irradiation of buffer solutions under our experimental conditions, in order to ascertain whether the oxidation was due to this agency. Utilizing the $Ti(SO_4)_2$ test for H_2O_2 and the $Mn(OH)_2$ test for O_2 we could not detect the presence of either. That O_2 and H_2O_2 formed from water during the irradiation could not be responsible for the production of dehydroascorbic acid was also shown by the fact that the reversible oxidation of *l*-ascorbic acid took place even when it was screened during exposure by water which according to Tian's claims should have filtered out the short wave ultraviolet light capable of decomposing water. One is, therefore, forced to the conclusion that the ultraviolet light decomposed some of the ascorbic acid in solution with the formation of an oxidizing substance or substances which in their turn dehydrogenated some of the undecomposed *l*-ascorbic acid. The additional observation that in the presence of glutathione *l*-ascorbic acid in solution is protected from this process of dehydrogenation suggests that such oxidation is not likely to take place *in vivo* even if ultraviolet light were to penetrate superficial tissues containing vitamin C. Furthermore, any dehydroascorbic acid formed would simultaneously be reduced by the glutathione present.

SUMMARY

The observation made by Kellie & Zilva that dehydroascorbic acid is formed by exposing *l*-ascorbic acid anaerobically in buffer solution to ultraviolet light has been confirmed by using the furfuraldehyde method for the determination of ascorbic acid. The evidence shows that the extent of the reaction is dependent on the intensity of the ultraviolet radiation.

It is assumed that some ascorbic acid is decomposed during the exposure with the formation of a substance or substances capable of dehydrogenating some of the undecomposed *l*-ascorbic acid.

Glutathione, whether present in solution with the *l*-ascorbic acid or acting as a screen in a buffer solution, exercises a protective action against dehydrogenation.

It is improbable that any ultraviolet light penetrating superficial tissues containing *l*-ascorbic acid would bring about the oxidation of the vitamin *in vivo*.

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