XCIII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. V.

By SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

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It was previously pointed out [Zilva, 1924] that even the purest antiscorbutic fractions possess the property of reducing ammoniacal silver nitrate in the cold and of decolorising potassium permanganate. In view of the fact that the antiscorbutic activity of solutions is destroyed by exposure to oxygen, it was not unreasonable to assume the existence of a possible connection between the two phenomena. The first obvious problem to solve was, therefore, whether the reduction of either or both of the above reagents was brought about by a substance or a grouping in a substance which was also responsible for the antiscorbutic potency. The experiments of Connell and Zilva [1924] pointed to the fact that this was not the case. In that investigation it was shown that although the conditions conducing to the preservation of the vitamin also conduced to the preservation of the reducing properties of an active solution, the destruction of the antiscorbutic activity and of the reducing properties proceeded at different rates.

Owing to the fact that antiscorbutic solutions lose their activity in the presence of atmospheric oxygen it is customary to imply that the destruction of the physiological activity is due to the direct oxidation of the vitamin. Although there is no experimental evidence against this conception one is not justified in excluding the possibility that the deleterious action of the oxidation process may be at least partly indirect, since the purest fractions so far obtained are impure in the chemical sense. In fact, the stability of the active principle may be conditioned by the presence of some of these accompanying "impurities." With the object of throwing some light on this subject the writer has been endeavouring, during the last few years, to modify active solutions in such a way as to destroy their capacity for reducing ammoniacal silver nitrate and at the same time leave the antiscorbutic activity mainly unimpaired. These efforts have not proved successful. Information, however, having a bearing on this problem has, in the meantime, been forthcoming from a different direction.

Phenolindophenol was found to be rapidly reduced in the air to its leuco-base by decitrated lemon juice and by active fractions derived from that

source. By using this indicator it is, therefore, possible to estimate the reducing capacity of such preparations titrimetrically. Substances with equal or higher oxidation intensities than phenolindophenol, if present, would, of course, not be accounted for by this method. In this empirical way it is nevertheless possible to obtain definite and consistent data of the reducing capacities of antiscorbutically active fractions under various conditions. An opportunity is thus afforded for correlating the two phenomena.

In this investigation it is proposed to show that the reducing agency (the word agency is used for convenience of expression without implying that a single substance is under discussion), although closely associated with the antiscorbutic factor, is not identical with it. There are, however, indications that its presence, most probably amongst other substances, in active solutions, may contribute to the stability of the antiscorbutic potency.

Experimental.

It was found convenient to employ, in the experiments to be described, a 0·02 % aqueous phenolindophenol solution. This indicator is red in acid and blue in alkaline solution. All titrations were carried out at p_H 7, since acid solutions decolorise the indicator independently of the reducing properties of the solution, whilst in alkaline solutions, as will be shown later, the reducing agency of the antiscorbutic solution deteriorates. The deleterious action of acidity can be demonstrated by adding a little of the indicator to distilled water adjusted to p_H 4·5 and p_H 3·4. After a few hours the colour of the solutions disappears. In the former case the addition of a few drops of ammonia regenerates the colour, in the latter case the colour is irretrievably lost.

Experiments were at first instituted with the object of studying the behaviour of the reducing agency under those conditions under which the behaviour of the antiscorbutic factor has already been established.

The influence of p_H *on the reducing agency.*

Three batches of decitrated lemon juice were adjusted to p_H 5·6, 7 and 9 respectively. Each of the solutions was titrated with the indicator immediately after adjusting the reaction, after 5 hours, and after 22 hours, no precautions having been taken to exclude air. It will be seen from Table I that in the case of the neutral and acid solutions there was comparatively little deterioration of the reducing agency in 22 hours. On the other hand, when the solution was made alkaline almost half of the reducing power was destroyed during the time taken for the adjustment of the reaction (the same batch of decitrated juice was used for the acid experiment). After 2 hours the reducing power was only about one-eighth of that of the original juice and after 22 hours the solution did not reduce the indicator at all. The reducing agency deteriorates, therefore, very quickly in alkaline solution in the presence of air. In this

respect its behaviour is similar to that of the antiscorbutic factor which is very unstable under such conditions [Zilva, 1923].

Table I.

	cc. of indicator per 5 cc. of solution		
p_H	At once	After 5 hours	After 22 hours
5·6	17·1	15	13·7
7	16·5	—	12·0
9	9·4	2	Nil

Influence of aeration on reducing agency.

Decitrated lemon juice which had not been previously precipitated with alcohol was adjusted to p_H 7 and air was aspirated through the solution at room temperature. Samples were removed at various intervals for titration purposes. The original preparation required 18 cc. of the indicator for 5 cc. of solution. After $2\frac{1}{4}$ hours the titre fell to 7 cc.; after 4 hours to 4·9 cc.; after 8 hours to 1·2 cc. No reduction of the indicator could be established after 17 hours. In this case, also, the behaviour of the reducing agency resembles that of the antiscorbutic factor [Zilva, 1922; Daubney and Zilva, 1926]. The decitrated juice at p_H 7·4 aerated for 15 hours was shown to become totally inactivated when tested on guinea-pigs.

Behaviour of the reducing agency in the process of fractionation.

In view of the information available concerning the behaviour of the antiscorbutic factor towards lead acetate as a precipitating reagent, it was of interest to follow the distribution of the reducing agency of decitrated lemon juice in the various fractions brought down by this reagent at different hydrogen ion concentrations. The best part of the vitamin is precipitated by lead acetate within the range of p_H 5·4–7·2. Traces, only, of the factor can be precipitated on the alkaline side of this range, whilst on the acid side, no demonstrable quantities are precipitated at all [Zilva, 1927]. The original decitrated lemon juice, the fraction precipitated by neutral lead acetate at p_H 5·4, and the fraction precipitated at p_H 5·4–7·2 after removal of the first (p_H 5·4) fraction from the solution, were titrated daily during a biological test for a period of about 6 weeks. Table II gives the cc. of indicator taken by equivalents of 5 cc. of each preparation in six representative cases chosen at random. It would appear, firstly, that only a part of the reducing agency is accounted for in the precipitates, secondly, that only about 30–50 % of it is present in the fraction which was shown to contain almost the entire antiscorbutic factor of the original juice (p_H 5·4–7·2), and thirdly, that the totally inactive fraction precipitated by lead acetate at p_H 5·4 shows even a higher reducing capacity than the active fraction. It is, therefore, obvious that the reducing power can have no *direct* connection with the antiscorbutic activity. This conclusion was strengthened by further evidence to be dealt with in a later section.

Table II.

Original decitrated juice	Fraction precipitated at p_H 5·4	Fraction precipitated at p_H 5·4–7·2
21	9·8	5·2
18·7	7·1	4·8
11·1	4·4	4·7
12·5	6·0	4·0
15	6·8	5·0
16·5	5	4·5

Fate of reducing agency in presence of quantities of indicator sufficient to oxidise it only partly.

When phenolindophenol is reduced and the leuco-base allowed to remain in the air, the latter becomes gradually oxidised. It was, therefore, to be assumed that if a quantity of indicator insufficient to oxidise the reducing agency were added, the reduced compound might possibly become oxidised in the air and eventually reduced again. This process would continue until the reducing agency became totally oxidised. In other words, the presence of a small quantity of the indicator could accelerate the oxidation of the reducing agency. This was actually found to be the case. To each of 5 batches of 5 cc. of decitrated lemon juice (not precipitated with alcohol) were added 6 cc. of indicator. 5 cc. of original juice required 13·6 cc. of the indicator and consequently a further 7·6 cc. was required to neutralise the reducing agency in each flask. The solutions, after standing in the air, were titrated at definite intervals. The first was titrated after 60 minutes and required 4·2 cc. of indicator; the second 3 cc. after 90 minutes; the third 2·3 cc. after 100 minutes; the fourth 1·5 cc. after 110 minutes; the fifth 0·6 cc. after 120 minutes. Soon afterwards, a solution treated as above became coloured, *i.e.* the re-oxidised leuco-compound was not again reduced owing to the disappearance of the reducing agency.

Behaviour of the antiscorbutic factor in the absence of the reducing agency.

It is seen from the foregoing experiments that it is possible to remove the reducing agency from antiscorbutic solutions by the addition of phenolindophenol. As this treatment is not drastic there was a likelihood that the antiscorbutic activity would not be destroyed at the same time, especially as it was seen that there is probably no direct connection between the two phenomena. This was actually demonstrated by the following two series of biological tests. In one case the indicator was added to lemon juice, decitrated without the alcohol treatment and adjusted to p_H 7, until it was no longer reduced. In the other case about 14 mg. of phenolindophenol were added per 30 cc. of the decitrated juice. This addition was calculated to oxidise about three-fourths of the reducing agency. Both preparations were then fed to the test guinea-pigs *with the least possible delay.* In both tests, doses of 1·5 cc., 3 cc. and 5 cc. with three animals (250–300 g.) on each dose were employed. Of the series receiving the totally oxidised juice, the three animals

receiving the 1·5 cc. dose died within 43, 49 and 57 days respectively, showing signs of scurvy *post mortem*. All the animals on 3 cc. and 5 cc. doses survived the 60 days of the test period and after being chloroformed were found to be normal at the autopsy. Of the animals receiving the three-fourths oxidised decitrated juice, all, with the exception of one which died of pneumonia after 57 days, survived the 60 days of the test period; only the animals on the 1·5 cc. and 3 cc. doses showed some signs of scurvy at the autopsy. The loss in the antiscorbutic activity in both cases was, therefore, not at all proportionate to the destruction of the reducing agency. These experiments, therefore, prove definitely that there is no direct connection between the two phenomena, and that the antiscorbutic activity persists at least for a short time after the total destruction of the reducing agency in the medium. Attention was consequently directed to the possible function of the reducing agency as a stabilising agency for the antiscorbutic factor and the relative behaviour of the two principles on storage and on heating was studied with this end in view.

The behaviour of the antiscorbutic factor and the reducing agency on storage.

Two sources were employed in this experiment, namely ordinary decitrated lemon juice obtained by removing the acids alone in the way already described in previous communications and alcohol-decitrated juice which was further purified by precipitation with alcohol after decitration and concentration. After the removal of the alcohol in the latter case, the decitrated lemon juice was made up to its original volume with a phosphate buffer solution p_H 6·9. The estimations of the reducing agency and the antiscorbutic potency were carried out in both cases in the original preparation, after 24 hours' storage, and after a week's storage. The storage took place in the cold room, the reaction being kept all the time as nearly as possible neutral. The phenolindophenol titrations took place daily shortly before the administration of the doses to the guinea-pigs during the entire period of testing. As one would expect, there was a certain amount of variation in the titres of corresponding preparations from day to day. Tables III and IV give the maximum, minimum and mean titres for 5 cc. of each of the preparations and a summary of the parallel biological tests. It is to be pointed out that although the differences between the maximum and the minimum titrations were great, most of the figures varied more or less in the neighbourhood of the mean.

The two tests can only be compared in a rough way since the antiscorbutic potency is assessed with much less precision by the biological method than the estimation of the reducing agency by titration with phenolindophenol. The results, however, show that the deterioration of the two principles on storage is more or less of the same order, possibly the loss is greater in the case of the reducing agency. This suggests a possible dependence of the antiscorbutic factor on the reducing agency. Further experiments pointed in the same direction.

Table III.

Ordinary decitrated juice.

	cc. indicator per 5 cc. of solution		
	Original cc.	After 24 hours' storage cc.	After 8 days' storage cc.
Mean	14·4	11·2	9
Maximum	19·9	18·6	15·8
Minimum	11·3	6·5	1·8
Alcohol-decitrated juice.			
Mean	11·5	8·8	5·5
Maximum	17·2	13·0	9·2
Minimum	6·5	5·5	2·8

Table IV.

Description of preparation	Dose cc.	Days alive	Remarks	Autopsy
Ordinary decitrated:				
Original	1·5	60	Chloroformed	Slight scurvy
,,	1·5	60	,,	Normal
,,	1·5	60	,,	,,
After 24 hours' storage	1·5	60	,,	Slight scurvy
,,	1·5	60	,,	,,
,,	1·5	60	,,	Normal
,,	3	60	,,	,,
,,	3	60	,,	,,
,,	3	60	,,	,,
After 8 days' storage	1·5	58	Died	No scurvy
,,	1·5	60	Chloroformed	Slight scurvy
,,	1·5	60	,,	Normal
,,	3	60	,,	,,
,,	3	60	,,	,,
,,	3	60	,,	,,
Alcohol decitrated:				
Original	1·5	58	Chloroformed	Slight scurvy
,,	1·5	58	,,	Normal
,,	1·5	58	,,	Slight scurvy
,,	3	58	,,	Normal
,,	3	58	,,	,,
,,	3	58	,,	,,
After 24 hours' storage	1·5	38	Died	Scurvy
,,	1·5	58	Chloroformed	,,
,,	1·5	53	Died	,,
,,	3	58	Chloroformed	Normal
,,	3	45	Died	Intercurrent disease no scurvy
,,	3	58	Chloroformed	Normal
,,	5	58	,,	,,
,,	5	58	,,	,,
,,	5	58	,,	,,
After 7 days' storage	1·5	40	Died	Scurvy
,,	1·5	33	,,	,,
,,	1·5	38	,,	,,
,,	3	58	Chloroformed	Slight scurvy
,,	3	58	,,	,,
,,	3	38	Died	,,
,,	5	58	Chloroformed	,,
,,	5	37	Died	,,
,,	5	58	Chloroformed	Normal

Influence of heat on the antiscorbutic factor in the absence of air.

In connection with another enquiry now in progress it was necessary to ascertain whether heating antiscorbutic solutions at high temperatures in the strict absence of air destroys their potency to any considerable extent. For this purpose ordinary decitrated lemon juice adjusted to p_H 7 was exhausted in an ampoule under a vacuum pump and washed out with nitrogen which was previously shown by absorption with alkaline pyrogallol to be free from appreciable traces of oxygen. This process was repeated three times and the evacuated ampoule, containing the antiscorbutic solution, was then heated for 1 hour in a steam autoclave under a pressure of one atmosphere and cooled before letting in the air. Such preparations made daily (except during the week-end) were tested for their potency in 1·5 cc., 3 cc. and 5 cc. doses, three guinea-pigs being used on each dose. The loss in the antiscorbutic potency was found to be very small since two of the animals on the lowest dose survived the test period of 58 days; the other one died of an intercurrent disease. All these animals, however, showed signs of scurvy at the autopsy after being chloroformed. The guinea-pigs on the higher doses all survived the test period and only in a few cases very slight indications of scurvy were found *post mortem*. Acidified decitrated lemon juice treated as above and tested out in precisely the same way showed a somewhat smaller loss in the antiscorbutic potency. The control animals receiving the untreated decitrated lemon juice were, of course, fully protected on all the doses. I am indebted to Miss S. M. L. Snelus, F.I.C. for assisting me in the preparation and the testing of these juices.

The behaviour of the antiscorbutic factor and the reducing agency on storage after being heated under anaerobic conditions.

Decitrated lemon juice treated as described in the preceding section showed very little change in its reducing capacity when titrated with phenolindophenol. Experiments with such preparations after storage yielded interesting results. When crude decitrated lemon juice is stored in the cold room for a week there is comparatively little deterioration either in its antiscorbutic activity or in its reducing capacity. Even the minimum daily dose of 1·5 cc. is practically capable of protecting a young guinea-pig from scurvy for 60 days. In the case of the reducing agency there is a loss of about 30 % during this period. When, however, crude decitrated lemon juice at p_H 7 is heated anaerobically under the conditions described above and then stored for a week the loss in both principles is almost complete. Thus three guinea-pigs on a daily dose of 1·5 cc. of the stored preparation succumbed to scurvy within 4 weeks; two animals on a 3 cc. dose behaved similarly, and two animals on a 5 cc. dose had to be chloroformed after 38 days, scurvy being established at the autopsy. The control guinea-pigs receiving the same autoclaved preparation before storing were alive on all the doses after 38 days. The reducing agency

also disappeared almost entirely during the period of storage. A second experiment confirmed the above observation. These results afford one more illustration of the parallel behaviour of the reducing agency and the antiscorbutic factor.

Conclusion.

One of the main results which emerges from this investigation is that the reducing capacity of antiscorbutic solutions from lemon juice as measured by the reduction of phenolindophenol is not directly associated with the activity. This is revealed by the facts, first, that inactive fractions derived by precipitating decitrated lemon juice with neutral lead acetate at p_H 5·4 show greater reducing properties than the active fraction derived from the same source, and second, that by fully destroying the reducing capacity by the addition of phenolindophenol in excess, the antiscorbutic activity of decitrated lemon juice remains almost intact, if tested immediately after treatment. In other words, the antiscorbutic factor, if one views it as a definite oxidisable substance, is of a lower reducing intensity than the reducing agency. It is therefore not surprising that any treatment such as aeration, storage, etc., which destroys the antiscorbutic activity also destroys the reducing power of the solution. Does this reducing property of the lemon juice fulfil a protective function in the sense of a "reduction buffer" or is its presence merely accidental? Admittedly the evidence produced in this communication does not offer a conclusive answer. The study of the antiscorbutic solutions from other sources and of the behaviour on storage of active solutions after treatment with phenolindophenol would go a long way towards elucidating this. Preliminary work on the latter problem shows that there is actually a rapid inactivation of the antiscorbutic potency under these circumstances, but as the results are complicated by other factors the final decision must at present be deferred.

No doubt can, however, be entertained about the result obtained in connection with the behaviour of heated decitrated lemon juice on storage. Definite evidence is produced showing that by heating the solution it was modified in such a way as to render it less stable as regards its antiscorbutic potency. The actual character of this modification is still under investigation and it is hoped that information will be forthcoming which will throw more light on the subject. It is significant that in this case also the reducing agency disappears with the deterioration of the antiscorbutic activity. It would almost appear as if the stability of the antiscorbutic factor depends on a chain of reactions which are kept in equilibrium in the living cell and that on damaging the cell the equilibrium is disturbed and the individual links are progressively damaged with the ultimate inactivation of the vitamin. The presence of some impurities would, according to this view, be a necessary condition for establishing the antiscorbutic potency, since the removal of such stabilising substances would inactivate the vitamin before the preparation

could be tested. The author has been faced by many disconcerting results in the chemical purification of the vitamin which could be explained on these lines. This hypothesis leads to another conception. So far the antiscorbutic factor has been studied mainly from the aspect of a principle the presence of which is necessary in the diet of certain animals in order to prevent the derangement of unknown physiological functions eventually leading to scurvy. Has it a function in the plant? Is it present there as a necessary link in the metabolic cycle? The author has shown that by certain manipulations it is possible to purify the antiscorbutic factor in lemon juice to a very great extent, but the issue is gradually being narrowed down and the direct application of chemical methods is becoming less effective. The results of this investigation suggest that the frontal attack on the problem must now be supported by thrusts in other directions.

SUMMARY.

(1) Decitrated lemon juice and active fractions derived from that source reduce phenolindophenol to its leuco-compound. In this way the reducing capacity of such solutions can be quantitatively determined.

(2) If insufficient of the indicator to destroy the reducing property of such solutions be added, the reduced compound is re-oxidised in the air and is further reduced by the solution. This alternate reduction and oxidation proceeds until the reducing power of the medium is destroyed.

(3) The reducing agency, like the antiscorbutic factor, is destroyed in alkaline medium in the presence of air, on aerating the active solution and on storage. On fractionating decitrated lemon juice it is, however, found in as high quantities in inactive as in active fractions.

(4) On adding phenolindophenol to decitrated lemon juice until the indicator is no longer reduced and testing the treated solution *immediately*, no very appreciable loss in the antiscorbutic activity is observed.

(5) On heating decitrated lemon juice in a neutral or acid medium in an autoclave at a pressure of one atmosphere for one hour, no very appreciable destruction of the antiscorbutic activity or of the reducing capacity of the solution takes place. On storing, however, both functions deteriorate very much more quickly than in untreated decitrated lemon juice.

(6) It is suggested that the stability of the antiscorbutic factor possibly depends on a chain of reactions, which are kept in equilibrium in the living cell.

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