XCIX. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VII.

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In a previous contribution of this series [Zilva, 1927, 2] it was shown that the antiscorbutic activity in decitrated lemon juice is associated with a capacity for reducing phenolindophenol to its leuco-base. It was also found that when the juice had been heated in an autoclave at 120° under strictly anaerobic conditions for 1 hour, the antiscorbutic potency and the reducing capacity of the juice disappeared much more quickly on storage than it usually did in a similar juice which had not been previously autoclaved and which was stored under the same conditions. This phenomenon has since been subjected to further investigation in order to throw more light on the subject and some of the results are recorded in this communication.

Experimental.

The method of testing was the same as previously described. Three guinea-pigs weighing 250–300 g. were used for each dose, and doses equivalent to 1·5, 3 and 5 cc. of the original lemon juice were usually employed. The administration of the daily dose began after the animals had subsisted on the basal diet for 14 days. In the case of positive tests the guinea-pigs were chloroformed 60 days after the commencement of the experiment and the degree of scurvy, if present, assessed at the autopsy. With negative preparations, the animals, as is invariably the case on a basal diet of oats, bran and autoclaved milk, succumbed to scurvy within a month.

The instability of the antiscorbutic factor in the absence of the reducing agency.

When phenolindophenol is added to decitrated lemon juice until it is no longer reduced, the solution becomes alkaline owing to the hydrolysis of the indicator. The quick deterioration of the antiscorbutic activity in such solution on storage could, therefore, be ascribed either to accelerated oxidation in alkaline medium [Zilva, 1923] or to the removal of the protective action of the reducing principle. In order to test the second hypothesis it

was consequently necessary to adjust the reaction to neutrality very soon after the addition of the phenolindophenol. This is technically complicated by the fact that the electrometric measurement of the p_H in the adjustment of the reaction is tedious and long, especially as the presence of CO_2 and of carbonate in the medium requires the passing of hydrogen through the electrode for a considerable time before equilibrium is established. On the other hand, the use of p_H indicators in the adjustment of the reaction is complicated by the presence of phenolindophenol in the solution. This difficulty was, however, overcome in the following way. Ordinary decitrated lemon juice was adjusted to p_H 7. Solid phenolindophenol was then added cautiously until the indicator was no longer decolorised, *avoiding the addition of excess*. The p_H of the solution at this stage was approximately 8·4. A citric acid solution was now introduced without delay, drop by drop, until the p_H fell to 7, utilising simultaneously the comparator and capillator methods with phenol red as indicator. With practice, a fair degree of accuracy could thus be obtained. One portion of decitrated lemon juice treated in this way was fed *at once* to the guinea-pigs whilst another portion was stored in the *neutral condition* in the cold room for 24 hours before it was administered to the animals. All the 9 animals which received the stored preparation died of scurvy within a month. The control animals were chloroformed after 43 days, some signs of scurvy being established in the guinea-pigs on the lower doses. Excluding the detrimental action of the indicator on the vitamin *per se* this comparatively rapid inactivation of the decitrated lemon juice on storage can only be ascribed to the disappearance of the reducing activity, since untreated decitrated lemon juice stored under the same conditions at about p_H 7 for 24 hours loses comparatively little of its activity. The control experiment showed that the juice was active before storage. The animals in this set were chloroformed after 43 days as it has already been shown [Zilva, 1927, 2] that this treatment with phenolindophenol does not destroy the potency to any significant extent if tested immediately. The condition of the animals at the time of chloroforming showed that this was also the case in this instance. The post-mortem examination lent further confirmation of the activity of the preparation.

The stability of the antiscorbutic potency of comparatively pure fractions.

If the assumption is justified that in the chemical fractionation of the antiscorbutic factor substances are removed which contribute to the stability of the active principle, pure fractions might be expected to be less stable than the original juice. This was actually found to be the case. An active fraction was prepared from decitrated lemon juice by removing inactive material by clearing the juice with neutral lead acetate at p_H 5·4 and then precipitating the active principle by raising the p_H of the filtrate to 7 [Zilva, 1927, 1][1].

[1] Such preparations are usually active in doses equivalent to 1·5 cc. of the original juice in so far that the guinea-pigs survive on them the test period of 60 days. Occasionally such a degree of activity is not attained but the cause of this variation has not yet been ascertained.

Doses of this fraction equivalent to 5 cc. of the original juice were tested immediately after preparation, after 24 hours' and after 72 hours' storage in the cold room. Four guinea-pigs were used in each set. The animals to which the dose was administered immediately after preparation were chloroformed after 61 days, signs of mild scurvy being established at the post-mortem. Both sets of animals on the stored preparations succumbed to scurvy within a month, *i.e.* the stored doses showed no activity at all. The reducing capacity for phenolindophenol of the active fraction was about one-third of that of the original decitrated lemon juice from which it was prepared. In another experiment a fraction prepared as above was fed immediately after preparation and after 7 days' storage in doses equivalent to 1·5, 3 and 5 cc. of the original juice. In the former case the guinea-pigs survived for 60 days even on the lowest dose, whilst in the latter all the 9 animals, including those receiving the highest dose, succumbed to scurvy within a month. As ordinary decitrated lemon juice of similar antiscorbutic activity does not deteriorate so quickly one may reasonably infer that some substance or substances which have a protective action on the vitamin have been removed in the process of fractionation.

The dialysing properties of the reducing agency.

In view of the fact that, when decitrated lemon juice is dialysed for 3 days in a collodion thimble previously soaked in 92 % alcohol, it loses its antiscorbutic activity, whilst when a thimble which has been soaked in 88 % alcohol is used under the same circumstances comparatively little loss in activity takes place [Zilva and Miura, 1921; Connell and Zilva, 1924], it was of interest to study the behaviour of the reducing agency under these differential conditions of dialysis. Ordinary decitrated lemon juice was, therefore, dialysed as described before in "92 %" and in "83 %" collodion thimbles in the presence of succinic acid for 3 days. The dialysed juice, as well as a control juice kept in a test-tube in the dialysing tank, were tested by daily administration to guinea-pigs for their antiscorbutic activity, whilst the doses were titrated daily with phenolindophenol before being administered to the test animals. An "83 %" collodion thimble was used in the experiment because thimbles with an 88 % alcohol index made from this particular sample of collodion yielded a juice of low activity after dialysis. The juice dialysed in the "83 %" thimble showed some activity in 1·5 cc. doses and full protection for 55 days in the higher doses, as did also the control non-dialysed juice. The decitrated lemon juice dialysed in the "92 %" thimble, as was to be expected, was found to be inactive, even in 5 cc. doses. The phenolindophenol titrations revealed a total loss of the reducing agency in solutions dialysed in the "92 %" thimbles, an approximate loss of more than a half in the solutions dialysed in the "83 %" thimbles, and a loss of less than a quarter in the non-dialysed control. These observations suggest the necessity of revising the interpretation of the results in connection with the dialysing properties

of the antiscorbutic factor. It was previously assumed by the writer and his collaborators that because active solutions when dialysed through membranes with an alcohol index of 92–95 % became inactive, the vitamin had diffused out. Since, as we have seen from the above experiments, the reducing agency diffuses under these conditions, one cannot dismiss the possibility that the active molecule may be larger than was hitherto surmised and that by the diffusion of the reducing agency it becomes inactivated without actually diffusing out of the thimble. The matter requires further investigation.

The effect of acidity on the stability of the antiscorbutic factor in anaerobically autoclaved decitrated lemon juice.

In the previous communication it was shown that when decitrated lemon juice was autoclaved under strictly anaerobic conditions and stored, the antiscorbutic activity deteriorated quickly. The reaction of such solutions during storage was neutral. It was of interest to ascertain whether this deterioration on storage in the treated juice would also take place in an acid medium. The juice was, therefore, autoclaved as previously described, acidified to p_H 3 by the addition of citric acid and stored in the cold room for 7 days, after which time it was tested on the guinea-pigs. Seven out of the nine animals survived the test period of 62 days, showing little scurvy at the autopsy. Of the other two guinea-pigs, one, receiving a 1·5 cc. dose, died after 44 days, and the other, receiving a 3 cc. dose, died after 59 days from an intercurrent disease[1]. The control, non-acidified juice was found, as usual, to be inactive in 3 cc. doses. Experiments with autoclaved preparations stored at p_H 6 and at p_H 6·7 showed that the deterioration on storage was *definitely* retarded even at these hydrogen ion concentrations.

The effect of acidity during the process of autoclaving on the stability of the antiscorbutic factor and of the reducing agency in lemon juice.

Although an acid reaction retards the deterioration of the antiscorbutic activity of autoclaved decitrated lemon juice, it does not prevent, during autoclaving, the change which conduces to the accelerated deterioration of the active juice on storage. This was demonstrated by two experiments. In the first experiment decitrated lemon juice was brought up to p_H 6 by the addition of citric acid and autoclaved as before for 1 hour. After cooling it was adjusted to p_H 7 and kept at this hydrogen ion concentration for 7 days in the cold room before being tested. This stored preparation was found to be inactive even in doses of 5 cc. and had lost almost entirely its capacity for reducing phenolindophenol. In the second experiment freshly expressed lemon juice was autoclaved under strictly anaerobic conditions in the usual way. It was then decitrated, adjusted to p_H 7 and stored in this neutral condition in the cold room for 7 days before feeding. As a control, the autoclaved lemon

[1] These stored juices were found to reduce phenolindophenol but their reducing capacity was greatly diminished.

juice was tested *immediately* after decitration. Decitrated lemon juice autoclaved anaerobically at p_H 7, and administered at once and after 7 days' storage at p_H 7, was also tested at the same time. Although the guinea-pigs dosed on the autoclaved lemon juice immediately after decitration survived for 60 days even on the lowest dose of 1·5 cc., little activity was revealed by this juice when stored for 7 days at p_H 7. There was a slight delay in the death of the guinea-pigs receiving the higher doses, but all the 9 animals in the set died of scurvy within 30–40 days. As usual, decitrated juice autoclaved at p_H 7 and fed at once was found to be active, whilst after 7 days' storage the same juice failed entirely to protect in doses of 3 cc. The autoclaved lemon juice, immediately after decitration, reduced phenolindophenol almost to its full extent. On storage, this reducing capacity deteriorated to a very great extent, *i.e.* some of the stored specimens showed no reduction at all, in others, the titre was reduced to about a fifth or sixth of its original value.

The effect of heating to 143° *on the antiscorbutic factor in decitrated lemon juice.*

In this experiment decitrated lemon juice at p_H 7 was alternately evacuated and washed out with oxygen-free nitrogen three times and eventually autoclaved for 1 hour at 40 lbs. pressure (143°) in the evacuated ampoule. After cooling, the heated juice was tested at once. Of the guinea-pigs on the 1·5 cc. doses, one died after 44 days, another after 47 days and the third was chloroformed after 61 days. All the animals on this dose showed signs of scurvy at the post-mortem examination. Two guinea-pigs on the 3 cc. dose were chloroformed at the end of the test period of 61 days and were found to be free from scurvy. The third animal died of an intercurrent disease after 60 days, showing mild signs of scurvy. A similar result was obtained with the highest dose of 5 cc. Two of the animals were *fully* protected from scurvy for 61 days after which time they were chloroformed, the third animal died of an intercurrent disease after 60 days showing some signs of scurvy at the autopsy. It is, therefore, seen that, in spite of the very drastic treatment, the loss in the antiscorbutic potency of the juice was not great—a loss most probably due not to the thermal degradation of the vitamin but to the presence of very slight traces of oxygen or to other slight imperfections in the technique.

Conclusions.

The experiments dealt with in this communication are recorded with the object of lending further support to the suggestion made in the earlier paper previously quoted that the stability of the antiscorbutic factor in lemon juice is conditioned by the presence of a reducing principle and of a factor, the functioning of which is destroyed by heat. It was then suggested that the reducing property of the solution acted as a "reduction buffer" for the antiscorbutic vitamin. The results obtained in this investigation seem to justify this hypothesis. It is seen that after the reducing properties have been

destroyed the antiscorbutic activity does indeed disappear very much more rapidly. The dependence of the reducing agency, and consequently of the antiscorbutic factor, on the heat-labile factor is confirmed. Although acidity does not prevent the destruction of this last factor by heat, it exercises definite protection of the reducing and of the antiscorbutic principles after anaerobic heating. In fact, the change effected by autoclaving is such that the heated active solution becomes almost as unstable in the neutral zone as an unheated active solution is in the alkaline region—a change which is most probably due to an increased susceptibility to oxidation. We are thus faced with a vague picture of a complex mechanism in which the following points stand out in relief. The antiscorbutic factor, a principle which can withstand such drastic treatment as heating at 143° in the absence of air, becomes very labile in the presence of air, on changing the protective conditions of its natural medium. Experiments on purified fractions reveal that the process of chemical purification is one of the means of removing this protection.

As a mode of expression the writer has been compelled to employ such terms as "reducing agency" and "thermo-labile factor." This needs some qualification. The experimental evidence so far produced does not warrant the assumption of the existence of such substances in a preformed state in the cell and consequently the possibility of the presence of a large active molecule which, on destruction of the tissues of the plant, is degraded or modified and thus rendered less stable, must be borne in mind. This can only be decided by further work.

Summary.

When phenolindophenol is added to decitrated lemon juice until the indicator is no longer reduced and the solution is adjusted immediately to p_H 7, the antiscorbutic activity disappears within 24 hours.

Purified antiscorbutic fractions from lemon juice lose their activity much more rapidly than does decitrated lemon juice of similar activity.

Decitrated lemon juice dialysed in collodion thimbles of a permeability which leaves the solution inactive after 3 days, loses the capacity for reducing phenolindophenol. This reducing capacity is retained to a great extent by the juice when dialysed in thimbles of a permeability which yields an active juice at the end of the dialysis.

Acidity retards the deterioration, on storage, of the antiscorbutic activity in anaerobically autoclaved decitrated lemon juice. On storage at p_H 3 the deteriorating effect of autoclaving is hardly perceptible.

Lemon juice autoclaved anaerobically, even in a very acid medium, deteriorates much more rapidly at p_H 7 on storage than similar solutions which have not been autoclaved.

Comparatively little loss is registered in decitrated lemon juice which has been autoclaved at 40 lbs. pressure (143°) for 1 hour under *strictly* anaerobic conditions.

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