

CXXXII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. VIII.

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Report to the Medical Research Council.

(Received October 31st, 1929.)

RECENT contributions to this investigation [Zilva, 1927, 1928] dealt with observations bearing on the stability of the antiscorbutic factor in lemon juice. Two phenomena were recorded in this connection; (a) the presence of a reducing principle, the destruction of which preceded the inactivation of the vitamin, and (b) the modification in the stability of the antiscorbutic activity of decitrated lemon juice when stored in neutral medium after having been autoclaved under strictly anaerobic conditions for 1 hour. This latter change was provisionally attributed to the destruction of a "thermolabile factor" in the process of autoclaving, as a suitable working hypothesis. A number of experiments have been carried out in this direction and as would be expected in the case of such a complex subject, the significance of many of the results cannot be fully interpreted in the light of our present knowledge. Some results, however, have yielded information which is considered of sufficient interest to be recorded, especially as they point to the possibility that the effects of the autoclaving may not be due to the destruction of a stabilising factor but to the formation of a substance or substances which conduce to the acceleration of the destruction of the antiscorbutic factor.

The effect of preliminary heating at temperatures lower than 100° on the stability of the antiscorbutic factor in decitrated lemon juice.

On the assumption that the modification in the autoclaved decitrated lemon juice was due to the destruction of a "thermolabile factor" it was of prime interest to ascertain the effect of heating at lower temperatures in order to establish whether a thermolabile enzyme was involved. With this end in view, the activity of stored solutions of ordinary decitrated lemon juice, previously heated at 55–58° and 80–85° respectively, for 1 hour under anaerobic conditions, was studied.

The actual procedure was as follows. 50 cc. of ordinary decitrated lemon juice adjusted to p_H 7 were placed in an ampoule of about 170 cc. capacity. The vessel was then exhausted under a vacuum pump (1 mm.), until the dissolved gases were no longer evolved. This was followed by the admission of pure

nitrogen and the evacuation and filling with the nitrogen were carried out three times, leaving the ampoule finally filled with nitrogen. After the last admission of nitrogen, a small glass S-tube containing a mercury seal was attached to the ampoule which was then immersed in the water-bath adjusted to the required temperature and kept constant by means of a thermostat. Five or ten minutes were allowed for the adjustment of the temperature before the actual commencement of the heating was timed. The nitrogen used was prepared by the action of ammonia on copper turnings and passed through a train of bottles containing sulphuric acid, sodium hydrosulphite and sodium hydroxide. There was little change in p_{H} after the heating. The solutions, which were prepared daily, were adjusted if necessary to p_{H} 7 and kept at this reaction during storage in air for 7 days in the cold room. The biological tests were carried out as described by the author elsewhere.

The first set of experiments made it plain that this preliminary anaerobic heating at 55° or 85° did not bring about the same modification in the stability of the antiscorbutic activity as autoclaving had done. It was, however, observed that active decitrated lemon juice heated at these comparatively low temperatures showed a greater vitamin loss before storing than was previously observed in anaerobically autoclaved juice. This unexpected observation made it advisable to repeat the experiment. Consequently five sets of experiments in which the active solution was heated at temperatures of 55 – 58° and four sets in which temperatures of 80 – 85° were employed were carried out at different times of the year between June, 1927, and January, 1929, thus affording the opportunity of using lemons of different origin and at different stages of senescence. The results obtained on the whole confirmed those observed in the preliminary experiments. Only in one set in which the juice was heated at 55° was there a suggestion of a higher deterioration of the activity on storing. As 140 guinea-pigs were used in this group of experiments it is not proposed to give the details of all the tests. Fig. 1, however, depicts a representative experiment. It is representative in so far that it shows how the same juice after different treatments behaved on storage. It is seen that the stored autoclaved batches, as has been observed on numerous occasions before, showed hardly any antiscorbutic activity at all in the doses tested, whilst the batches that had been heated at the lower temperature did not deteriorate proportionately more than the unheated juice on storage. It must, however, be pointed out that the higher destruction in the antiscorbutic activity of the solutions heated at 55 – 58° and 80 – 85° was more marked in the other sets of experiments and that it has not been traced so far to any fault in the technique.

All the heated and control batches were titrated daily with phenolindophenol before and after storage in order to ascertain the reducibility of the solutions. The destruction of the "reducing principle" on storage in the solutions which were heated at 58° or 85° was of the same order as that of the unheated solutions, whilst in the autoclaved juices the destruction was almost complete after 7 days' storage.

These experiments show that, whatever the modification in the stability of the antiscorbutic activity brought about by autoclaving may be due to, the destruction of a thermolabile enzyme or of a thermolabile link in an enzyme system is not the cause of it.

The effect of the addition of decitrated juice inactivated by aeration on the stability of autoclaved decitrated juice.

It was next of interest to ascertain whether the addition of decitrated lemon juice previously inactivated by aeration at ordinary temperature [Zilva, 1922] could replace the hypothetical "thermolabile factor." To a decitrated lemon juice autoclaved anaerobically, an equal volume of the same juice previously aerated at room temperature for 7-8 hours, which was found by a parallel control test to be antiscorbutically inactive, was added. On testing the equivalents of the usual doses (three animals on a dose), it was found that this addition in no way influenced the deterioration of the antiscorbutic activity of the autoclaved solution.

Attempts to fractionate the supposed "thermolabile factor."

Two fractions were employed. One was the antiscorbutically inactive precipitate obtained by the treatment of ordinary decitrated lemon juice with alcohol and the other was the neutral lead acetate fraction from the same source. In the first case decitrated lemon juice was concentrated to about one-tenth of its original volume. On addition to this concentrated solution of a large excess of alcohol, a precipitate which was soluble in water was formed. After filtration on a Büchner funnel, the filter paper and precipitate were introduced into anaerobically autoclaved decitrated lemon juice and the precipitate dissolved. A precipitate equivalent to two volumes of the decitrated juice was introduced into each volume of the autoclaved solution. These solutions were prepared daily and stored as before for 7 days in the cold room at p_H 7 before testing. Besides this preparation, the redissolved (in water) precipitate and the autoclaved decitrated lemon juice not containing the fraction were tested as controls. The precipitate was, as usual, found to be inactive and the addition of it to the autoclaved solution did not in any way compensate for the modification in the stability of the antiscorbutic activity produced by autoclaving.

The investigation of the compensating property of the neutral lead acetate fraction was complicated by the fact that in this instance the fraction retained some antiscorbutic activity. It has been pointed out already [Zilva, 1928] that the fraction precipitated from decitrated lemon juice by lead acetate between p_H 5 and 7 does not always contain most of the antiscorbutic activity, as found by the author in his earlier experiments. In fact, during the last two years, this fractionation has been carried out many times and a disturbing number of fractions of low activity has been obtained. It is evident that some still unknown factor, possibly a co-adsorbent, regulates this fractional pre-

precipitation of the antiscorbutic factor by lead acetate. Although the cause has not yet been satisfactorily ascertained, one observation has been made which has a bearing on it, namely, that sometimes—not often in the author's experience—considerable activity is found in the neutral lead acetate fraction, *i.e.* the fraction of p_H below 5.4–5.2. In this experiment the neutral lead fraction which was tested as a control showed some antiscorbutic activity and consequently the autoclaved decitrated juice, to which this fraction was added, was found to be rather more active after 7 days' storage than a similar solution to which it was not added. The results of this imperfect experiment did not, however, suggest that the addition of the fraction compensated for the modification in stability produced by the autoclaving. In view of subsequent developments of the problem, it was not considered expedient to repeat this experiment.

Changes produced in decitrated lemon juice by autoclaving.

While studying the effect of various temperatures on the "thermolabile factor," attention was naturally focussed on the enzyme activity of the heated solutions. It was found that decitrated lemon juice possessed the property of oxidising *p*-phenylenediamine after a certain lag when a suitable quantity of hydrogen peroxide was added. This activity was retained, somewhat weakened, after autoclaving. Other sources of the antiscorbutic factor, such as swede juice, cabbage juice, or tomato juice, behaved similarly in this respect. In lemon juice it was found that the peroxidase activity persisted after the vitamin in it was partially or totally destroyed, thus aerated, stored autoclaved solutions and solutions which were inactivated by being made alkaline retained this enzymic activity. The enzyme was also precipitated by lead acetate partly at p_H 5 and partly at p_H 7.

Another observation made in this connection was that whilst the property of reducing phenolindophenol by active solutions was somewhat reduced by autoclaving under anaerobic conditions, the capacity for decolorising iodine was greatly increased. This increase in the capacity for reducing iodine does not take place when the solution is heated anaerobically at 58° or 85°. As an example illustrating this, the following may be quoted. A sample of decitrated lemon juice 5 cc. of which decolorised 20 cc. of 0.02 % phenolindophenol and 26.5 cc. *N*/1000 iodine quickly and approximately a further 20 cc. of the iodine slowly, after being autoclaved anaerobically, decolorised 15 cc. of phenolindophenol, and 30 cc. of the iodine quickly, after which the rate of decoloration of the iodine became definitely slower and a further 60 cc. of iodine (approximately, end-point not sharp) had to be added before the decoloration ceased.

These observations taken in conjunction with the failure so far to demonstrate the presence of a thermolabile stabilising factor in antiscorbutic solutions directed the writer's view to a possible alternative explanation of the modification in the stability of the antiscorbutic activity produced by autoclaving,

namely, the formation of a substance or substances capable of accelerating the mechanism of inactivation of the vitamin in air.

Experimental evidence so far obtained supports this view. Thus, if equal volumes of autoclaved and unheated decitrated lemon juice are mixed and stored at p_H 7 for 7 days, the capacity for reducing phenolindophenol disappears almost to the same extent as it does in autoclaved juice. This disappearance, as pointed out in a previous communication, is much slower in unheated juice. Fig. 2 gives a graphic representation of this phenomenon. The disappearance of the "reducing principle," which is assumed to have a protective action on the vitamin, has so far been found without exception by the writer to be an indication of the inactivation of the vitamin. A preliminary biological test shows that an acceleration in the destruction of the antiscorbutic principle is indeed brought about by the addition of autoclaved to unheated decitrated lemon juice to about the same extent as by autoclaving.

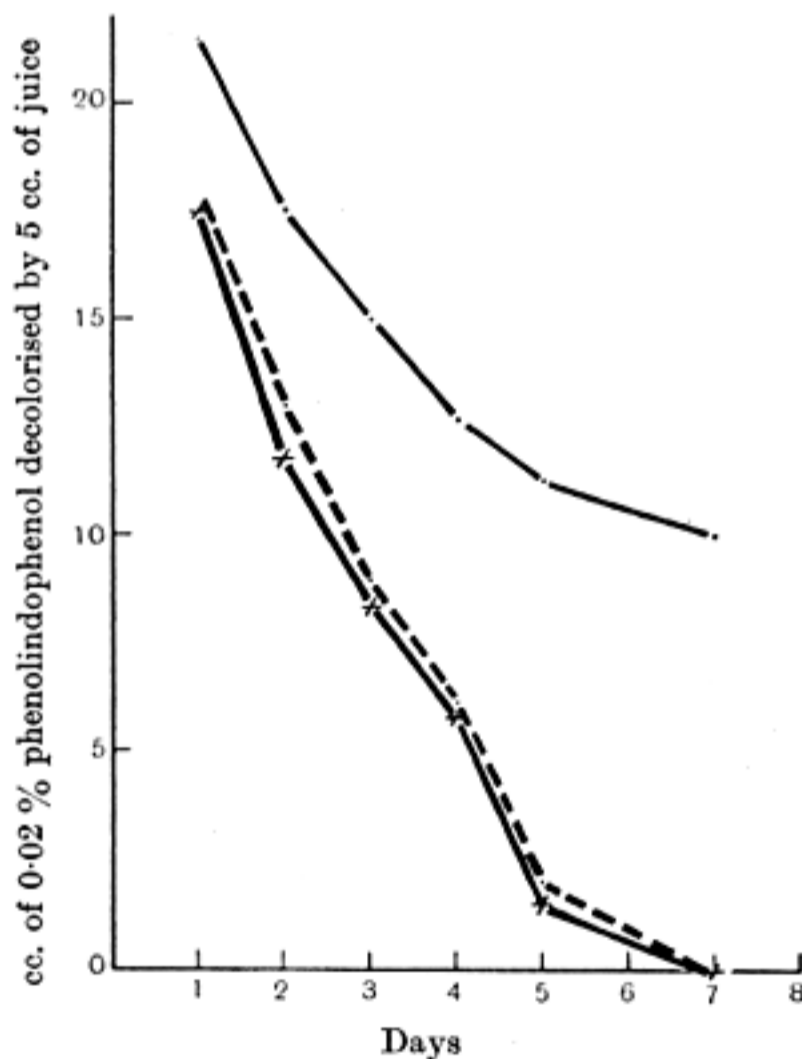


Fig. 2.
 — Unheated juice. - - - Autoclaved juice.
 — x — Autoclaved + unheated juice.

Another experiment also lent support to the above view. Since the formation of a substance which decolorises iodine suggests the likelihood of its being phenolic in character, the influence of the addition of quinol to unheated decitrated juice was studied. In this case, too, there was a quicker disappearance of the "reducing principle". For instance, 5 cc. of a decitrated lemon juice decolorised 23 cc. of 0.02 % phenolindophenol solution. On storage, this titre fell to 14.0 cc., whilst the stored autoclaved juice of the same batch and the unheated juice to which quinol was added (0.12 %) fell to 3 cc. and 2 cc.

respectively. Preliminary experiments show that also in this case the addition decreases the stability of the antiscorbutic activity of the juice to the same extent as autoclaving.

Whether there be a connection or not between the thermostable peroxidase and the increased power of iodine reduction produced by autoclaving, the possibility of such a connection obviously cannot be dismissed until it is proved to be otherwise. The above experimental evidence, in any case, strengthens the view that the modification in the antiscorbutic stability of decitrated lemon juice by autoclaving is produced rather by the formation of a substance or substances conducing to the destruction of the vitamin than by the destruction of a "thermolabile stabilising factor," as originally assumed. This view is being borne in mind by the writer in his general study of the antiscorbutic factor.

SUMMARY.

1. There is no modification in the stability of the antiscorbutic activity of decitrated lemon juice previously heated for 1 hour under anaerobic conditions at 55–58° or at 80–85° as there is when the juice is autoclaved.

2. The addition of decitrated lemon juice, in which the vitamin has been inactivated by aeration at ordinary temperature to autoclaved juice, does not compensate for the modification in stability, nor does the addition of the fraction obtained from decitrated lemon juice by precipitation with alcohol or with neutral lead acetate.

3. Decitrated lemon juice, cabbage juice, tomato juice and swede juice contain a thermolabile peroxidase.

4. Autoclaving decitrated lemon juice under anaerobic conditions diminishes its capacity for reducing phenolindophenol to some extent, but increases its capacity for decolorising iodine. This increased capacity for decolorising iodine is not observed after the decitrated lemon juice has been heated anaerobically at 58° or 85°.

5. The addition of autoclaved decitrated juice to unheated juice accelerates the destruction of the "reducing principle" and of the antiscorbutic activity in it on storage at p_{H} 7. The addition of quinol has a similar effect.

6. It is suggested that the modification in antiscorbutic stability of decitrated lemon juice produced by autoclaving may possibly be due to the formation of a substance or substances conducing to the deterioration of the vitamin rather than to the inactivation of a thermolabile stabilising factor.

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