

## XXVI. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. I.

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It has been shown [Zilva, 1923] that on treating decitrated lemon juice with alkali, inactivation of the antiscorbatic factor is brought about in the presence of air with a simultaneous increase in the hydrogen ion concentration of the solution. In the absence of air this treatment with alkali does not influence either the activity or the reaction to any perceptible degree. The increase in the hydrogen ion concentration was attributed to the probable formation of acid from the sugar in the solution. It was also pointed out at the time that the destruction of the vitamin proceeded at a much quicker rate than the formation of the acid, an observation which excluded the probability of a direct connection between the two changes. The results did not, however, exclude the possibility of a connection between the formation of an intermediate compound in the production of the acid and the inactivation of the vitamin. The investigation was, therefore, followed up on these lines with the ultimate object of obtaining more information on the chemical character of the antiscorbatic factor.

It was desirable at this stage to ascertain whether the sugar present in active decitrated lemon juice has any bearing on its antiscorbatic activity and the obvious task was, therefore, to remove the sugar and see whether the potency was destroyed. The removal of the sugar from the active solution without actually impairing the rather unstable antiscorbatic factor was found to be associated with certain difficulties. Owing, however, to the recently ascertained facts concerning the conditions of the inactivation of the antiscorbatic vitamin it was possible to devise a biochemical method of removing the sugar without perceptibly influencing the antiscorbatic activity, namely, by fermenting the sugar with yeast in an atmosphere of carbon dioxide.

By this method it is possible to eliminate almost the entire sugar content without perceptibly influencing the activity of the solution. This observation disposes of the idea that any connection exists between the oxidation of the sugar and the inactivation of the antiscorbatic factor in decitrated lemon juice. Furthermore, evidence can now be produced showing that the antiscorbatic factor in the fermented decitrated lemon juice behaves in alkaline

solution in the presence of air similarly to that in unfermented decitrated solutions.

The elimination of the sugar from the decitrated lemon juice without influencing its activity limits further our field for the chemical investigation of the potent fraction. We may now exclude from the total solids of lemon juice the organic acids which form the bulk of the solids and the sugar as having no connection with the activity. The average dry matter content of decitrated lemon juice is about 1 % (calculated on the original volume of the lemon juice) although samples with a content as high as 1.7 % of solids are occasionally observed. When the sugar which is shown to be invert sugar is deducted from this figure it leaves us with an active fraction containing less than half of the dry matter of the decitrated juice. This has a nitrogen content varying from 0.004 %–0.014 %. The residual active fraction consists mostly if not almost entirely of nitrogenous substances. Although the investigation of the chemical character of these nitrogenous substances is not yet complete a certain amount of preliminary information has been obtained at this stage and is given in another part of this communication.

#### EXPERIMENTAL.

On preparing an osazone from decitrated lemon juice, glucosazone is obtained. Microscopical examination of the crystals shows that no other osazone is present. The decitrated lemon juice also gives a very marked Selivanoff reaction. The solution is laevorotatory and reduces Fehling's solution. In assuming that the sugar present is entirely composed of invert sugar and calculating accordingly, the sugar content from the reduction values obtained by Bertrand's method, a specific rotation is obtained which approximates that of the supposed sugar. The following specific rotations were observed in five typical experiments:  $-19^{\circ}$ ,  $-17.5^{\circ}$ ,  $-16.5^{\circ}$ ,  $-22.9^{\circ}$ ,  $-16.5^{\circ}$ , giving an average  $(\alpha) \frac{D}{18} - 18.5^{\circ}$ . The theoretical value for invert sugar is  $-20.1^{\circ}$ . These facts show that the sugar in decitrated lemon juice consists mostly, if not entirely, of invert sugar.

In order to remove the sugar by fermentation precautions were taken to ensure an acid medium and the absence of air. In the early experiments the acidity was regulated by the addition of succinic acid. It was found later, however, that fermentation in an atmosphere of carbon dioxide gave equally good results. The following procedure was adopted. To 50 cc. of decitrated lemon juice 0.5 g. of succinic acid and 1 g. of washed brewer's yeast were added. Carbon dioxide was then passed for about 15 minutes, and the flasks closed with a rubber stopper containing a mercury seal. The mixture was incubated for about 18 hours at  $25^{\circ}$  at the end of which time it was filtered through a Berkefeld filter. The solution thus obtained was ready for feeding purposes and had a hydrogen ion concentration of about  $p_H 3$ . The yeast used in these experiments was obtained fresh from the brewery on the day

when the fermentation was carried out. It was pressed free of wort, washed and centrifuged once with tap and once with distilled water and filtered on a Buchner funnel, until the residue became a doughy mass. The solutions were fermented every other day and were administered on the two following days, being stored during the interval in the absence of air at room temperature.

To control the above fermented solution, decitrated lemon juice containing the same quantity of succinic acid was incubated for the same time at 25° without yeast, and another batch of juice of the same acidity was treated with the same quantity of yeast as the above only for 15 minutes, filtered after that time through a Berkefeld filter and incubated at 25° for 18 hours. This last experiment was instituted with the purpose of eliminating any error that might have been produced by adsorption by the yeast. The reduction of the solutions before and after treatment was determined in all the batches by Bertrand's method. It was found that in all the controls there was hardly any change in the reducing power of the solutions whilst in the fermented solution almost the entire reducing sugar was removed by the treatment. (About 0.2 cc. of Bertrand  $\text{KMnO}_4$  required for 2 cc. of fermented solution.)

The three batches were tested out for their antiscorbutic content on guinea-pigs. As will be seen from Table I the decitrated lemon juice from which the invert sugar has been removed by fermentation showed as good an activity as that of the control batches.

Table I.

| Treatment of the decitrated lemon juice | Daily dose in cc. | No. of days animal lived | Cause of death                  | Degree of scurvy |
|---|-------------------|--------------------------|---------------------------------|------------------|
| Fermented ... ..                        | 1.5               | 60                       | Chloroformed                    | +                |
|   | 1.5               | 57                       | Cause unknown, not scurvy       | +                |
|   | 3                 | 60                       | Chloroformed                    | Nil              |
|   | 3                 | 60                       | "                               | +                |
|   | 5                 | 60                       | "                               | Nil              |
|   | 5                 | 60                       | "                               | "                |
| Incubated without yeast ...             | 1.5               | 43                       | Cause of death not acute scurvy | ++               |
|   | 3                 | 60                       | Chloroformed                    | +                |
|   | 5                 | 57                       | Cause of death not scurvy       | Nil              |
| Treated with yeast for 15 mins          | 1.5               | 50                       | Scurvy                          | +++              |
|   | 3                 | 60                       | Chloroformed                    | +                |
|   | 5                 | 60                       | "                               | Nil              |

A similar retention of the antiscorbutic potency by fermented decitrated lemon juice saturated with  $\text{CO}_2$  in the absence of air was shown by the following experiment. The juice was treated with the same quantity of yeast under the same conditions as in the previous set of experiments only no succinic acid was added. After fermentation and filtration through a Berkefeld filter the preparations were fed to guinea-pigs in daily doses of 1.5 cc., 3 cc. and 5 cc. All the animals (two on each dose) survived the period of two months after which time they were chloroformed. With the exception of those which received the daily dose of 1.5 cc., which showed very slight signs of scurvy, all the animals were found to be normal. These results are identical with those

obtained with unfermented decitrated lemon juice and, therefore, the antiscorbutic is not influenced to any marked degree by the removal of the invert sugar by fermentation.

It was further found that the activity of fermented decitrated solutions is influenced by reaction and the presence of air as in the case of the unfermented solutions, namely, that in the absence of air  $N/20$  alkali had no marked deteriorating action on the antiscorbutic potency whilst a solution of the same reaction had an inactivating influence when no precaution was taken to exclude air. The technique employed in these experiments was the same as that already described [Zilva, 1923]. A daily dose of 5 cc. of the fermented solution made  $N/20$  alkaline and kept under laboratory conditions for 24 hours failed even to delay the onset of scurvy in guinea-pigs whilst a dose of 1.5 cc. of such a preparation kept for the same time under strictly anaerobic conditions maintained the animals for 56 days.

The above experiments bring us a step further in the investigation of the active fraction of lemon juice and afford the possibility of obtaining an antiscorbutic more free from impurities than any potent substance so far described. As already mentioned, indications which are so far available seem to show that the active fraction is a complex mixture of substances, the better part of which are of a nitrogenous character. Fermented decitrated juices give negative biuret, sulphur and tryptophan tests. A very faint murexide reaction which is masked by a brown coloration is generally obtained. Precipitates are obtained with mercuric sulphate, and basic lead acetate. Phosphotungstic acid occasionally, but not invariably, gives a very slight precipitate. Millon's reagent gives a precipitate, which is soluble in excess of the reagent. The solution reduces ammoniacal silver nitrate and gives a marked Pauli reaction. No amide nitrogen can be detected. Very little extraneous matter seems to be introduced by solution from the yeast and most of the above reactions can also be obtained in the unfermented decitrated lemon juice. It is hoped that a detailed investigation of this fraction will afford further information on the chemical nature of the antiscorbutic vitamin.

#### SUMMARY.

A great part of the solids of decitrated lemon juice consists of invert sugar. It is possible to ferment the sugar and thus reduce the solids without appreciably altering the antiscorbutic activity of the solution. The remaining solids seem to consist of substances mainly of a nitrogenous character. Some chemical characteristics of the active fraction are given.

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#### REFERENCE.

Zilva (1923). *Biochem. J.* 17, 410.