LXXXII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. II.

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This communication describes a number of further experiments in which antiscorbutically active fractions have been obtained from lemon juice. In the previous contribution of this series [Zilva, 1924] it was shown that it was possible to remove the acids and sugar from juice without perceptibly influencing its potency. These substances, as is well known, form the great bulk of the solid matter of the juice. The active residue left after the removal of the acid and sugar usually comprises only about $\frac{1}{30}$th part of the total solids in spite of the fact that it contains the entire activity of the equivalent quantity of lemon juice. This active residue was submitted to further fractionation, utilising various precipitating methods, and the writer is now able to record results which show, that it is possible to remove still more extraneous matter without vitiating the activity of the antiscorbutic principle; in fact preparations have been obtained containing only ·03–·07 % of solids whilst retaining the same activity as the original juice. This was demonstrated by adhering to the practice of testing out every preparation quantitatively.

As was mentioned in the last communication, the sugar can be removed from the decitrated lemon juice by fermentation, leaving an active fraction consisting of an apparently complex mixture of substances and showing an appreciable nitrogen content with a total solids content of 0·3–0·5 %. When this fermented active juice is concentrated to a small volume and treated with absolute alcohol a precipitate is formed. This precipitate is not active, and it can be shown that the entire activity remains in the supernatant fluid. Basic lead acetate also forms a precipitate when added to fermented decitrated lemon juice, but in this case the active principle goes entirely into the precipitate. By combining these two procedures, *i.e.* by precipitating with absolute alcohol first, distilling off the alcohol and adding basic lead acetate to the active aqueous solution, a very potent preparation is obtained on removing the lead from the precipitate.

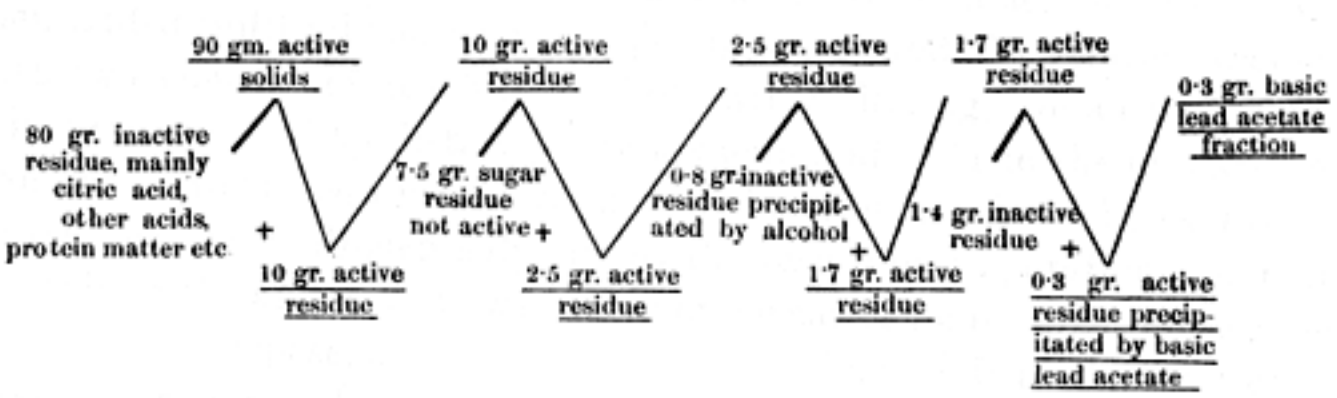
Attempts were made to elaborate a method which would render possible the production of such an active fraction with less labour. In the first place it was found that basic lead acetate brings down the vitamin entirely from decitrated lemon juice before fermentation. In this case, however, some sugar

and other non-active substances are also removed in the precipitate and, therefore, this treatment does not yield as pure a fraction as the one described above. Secondly, it was found that the procedure of decitrating the lemon juice could be modified in a way which conduces to economy in labour and materials and which at the same time yields a juice more free from non-active matter than that obtained by the original method.

When such decitrated juice is precipitated with basic lead acetate and the precipitate freed from the lead, the residue after evaporation taken up in a small volume of water and treated with absolute alcohol in order to remove some inactive matter by precipitation, an active fraction is obtained of considerable purity but still containing sugar.

The purest fraction has been obtained by fermenting juices decitrated by the modified method, and submitting the fermented solutions to alcohol and basic lead acetate precipitations as previously described. The following table gives the scheme of fractionation. Every fraction contains the entire antiscorbutic activity of a litre of lemon juice.

Table I.



The above figures are an approximate average. They vary to some extent with the juice.

Experimental.

All the fractions were tested out for their activity on guinea-pigs. The activity of the ultimate fraction obtained in this investigation was also confirmed on monkeys. The guinea-pigs were kept on the usual scorbutic diet of oats, bran and a limited quantity (40 cc.) of autoclaved milk. The preparations to be tested were administered daily. The dosing commenced after the animals had been about 14 days on the scorbutic diet and was continued in the case of positive tests until two calendar months had elapsed from the time the animals were placed on the diet. Only in two sets was this period reduced to 54 days. All the fractions were freshly prepared either daily or every other day. In the latter case the preparation was acidified and kept in a vacuum over night. Daily doses equivalent to 1·5 cc., 3 cc. and 5 cc. of the original lemon juice were tried in every case. The lowest of these doses may be considered the approximate minimum daily dose of lemon juice capable of protecting a guinea-pig from scurvy for the above period. The animals were

regarded as protected when at the end of the two months only slight signs or no signs of scurvy were found after chloroforming them.

Exp. 1. Fermented decitrated juice was concentrated to about one-sixth of its volume, and about ten times its bulk of absolute alcohol was gradually added. A precipitate was formed which was centrifuged and dissolved in a quantity of water equivalent to the original juice. The alcohol was distilled from the supernatant fluid in a vacuum at 50°. This fraction was also made up to the equivalent volume of the original juice. The solution of the precipitate when tested out for its antiscorbutic activity was found not to be able to prevent or even delay scurvy in guinea-pigs in daily doses of 1·5 cc., 3 cc. and 5 cc. It did not reduce ammoniacal silver nitrate in the cold. The solution from which this precipitate was removed when tested on two animals in each of the above doses was found in every case to prevent the onset of scurvy for two months. This solution reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. As an average four Kjeldahl determinations gave 1·5 cc. $N/50$ NH_3 for an equivalent of 10 cc. of original juice.

Exp. 2. Basic lead acetate was gradually added to fermented decitrated juice until no more precipitate was formed. The solution was then quickly centrifuged and the residue dissolved in acetic acid. Magnesium sulphate was then added until all the lead was precipitated, and the solution was treated with two volumes of absolute alcohol. After filtering, the alcohol and acetic acid were removed by distillation in a vacuum at 50° and the preparation was brought up to the equivalent volume of the original juice. The lead could not be removed by hydrogen sulphide as the resulting preparation was found to be toxic to the animals. It was also not considered advisable to use sulphuric acid for the purpose, as even a small excess of acid left in the solution would have had a harmful effect on the animals, whilst any excess of magnesium sulphate could only, at its worst, act as a purgative. Two pigs were employed on each of the doses in this experiment. The animals on the 1·5 cc. succumbed to scurvy after about 40 days. The 3 cc. and 5 cc. doses protected the animals for two months. The slight loss in the antiscorbutic activity in this experiment was undoubtedly due to imperfect manipulation. This active fraction gives a less intense Pauli reaction, reduces ammoniacal silver nitrate in the cold and decolorises potassium permanganate. As an average four Kjeldahl determinations gave 0·5 cc. $N/50$ NH_3 for an equivalent of 10 cc. of original juice.

The filtrate from the above precipitate was freed from lead as described above. The resulting solution was tested on six guinea-pigs in the usual doses. All the animals succumbed to scurvy in 21–30 days. The entire active principle was, therefore, removed by the precipitate.

Exp. 3. In this experiment the procedures employed in Exps. 1 and 2 were combined, *i.e.* after removing the inactive residue from fermented decitrated juice by alcohol precipitation as described in Exp. 1, the solution was precipitated with basic lead acetate until no further precipitate was obtained. The precipitate was then treated as in Exp. 2. Three animals were used for

each dose. All the nine guinea-pigs were protected from scurvy for two months by the administration of doses of 1·5 cc., 3 cc. and 5 cc. of this fraction. This shows that no loss in activity was incurred by this manipulation. The average solid matter content of this preparation was found to be ·05–·07 %. It reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·3 cc. $N/50$ NH_3. In three phosphorus estimations equivalents of 100 cc. of the original juice gave ·000172, ·000175 and ·000141 g. P. It gave an almost negative Pauli reaction.

Exp. 4. Decitrated lemon juice was treated with basic lead acetate without being previously fermented. An active fraction fairly free from extraneous matter was thus obtained but, as was to be expected, the lead acetate precipitated under these conditions, together with the vitamin, more inactive matter, including sugar in varying quantities, than when the juice was treated as in Exp. 3. The sugar content of this fraction is, however, only about one-tenth of that of the original decitrated juice. Three guinea-pigs receiving 1·5 cc. doses, two, 3 cc. doses and four, 5 cc. doses were protected from scurvy for two months by the administration of this preparation.

Exp. 5. In decitrating the lemon juice of the above experiments the method described on several previous occasions was used. It consisted in neutralising the lemon juice with an excess of chalk, treating the neutralised solution with two or three volumes of absolute alcohol, filtering and removing the alcohol by distillation in a vacuum. This procedure can be advantageously modified. The lemon juice is treated with excess of chalk as before and allowed to remain for 30–60 minutes. This enables salts to deposit. It is then filtered and the filtrate concentrated in a vacuum at 50° to about a fifth of the original volume. A volume of alcohol equal to about half the original volume of juice is gradually added. The precipitate is then filtered off, washed with a little alcohol, and the alcoholic washings are combined with the filtrate, the alcohol distilled off in a vacuum at 50° and the residue made up to the original volume. By this modification not only are alcohol and time saved but an active preparation is obtained from which more of the non-active matter is removed than by the old method. Tested out on six guinea-pigs, it was found that 1·5 cc. was the minimum protective dose for two months.

Exp. 6. Lemon juice decitrated as in Exp. 5 was precipitated with basic lead acetate. The active precipitate was dissolved in acetic acid, treated with magnesium sulphate and two volumes of alcohol and concentrated to a small bulk. It was then treated with excess of alcohol. The gummy precipitate formed by this treatment was centrifuged and the supernatant solution after removing the alcohol by distillation was made up to the original volume. Four guinea-pigs on a dose of 1·5 cc., two on a dose of 3 cc. and three on a dose of 5 cc. were protected from scurvy for 54 days. The preparation contains less non-active matter and sugar than the one obtained in Exp. 4. It reduces ammoniacal silver nitrate in the cold and decolorises potassium permanganate.

As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·5 cc. $N/50$ NH_3. Three phosphorus estimations carried out on an equivalent of 100 cc. gave 0·0000166 g., 0·000049 g., 0·0000543 g. P. It gave a very faint Pauli reaction.

Exp. 7. This fraction was prepared in the same way as that of Exp. 3 with the difference that the decitrated lemon juice from which it was precipitated was prepared as in Exp. 5. A purer fraction was obtained by this method, the total solids of which came to 0·03–0·05 %. As an average of six Kjeldahl determinations an equivalent of 10 cc. gave 0·2 cc. $N/50$ NH_3. Two phosphorus estimations carried out on an equivalent of 100 cc. gave 0·000137 g. and 0·0000909 g. P. It reduced ammoniacal silver nitrate in the cold and decolorised potassium permanganate. It gave a very faint almost negative Pauli reaction. Daily doses of 1·5 cc., 3 cc. and 5 cc. were found to protect guinea-pigs from scurvy for 54 days. This fraction was also tested out curatively on monkeys. The monkeys were first kept on a scorbutic diet of boiled rice, marmite, salts, caseinogen and cod liver oil for about seven weeks, when they developed scurvy. This same diet was continued throughout the experiment.

Protocol.

Monkey A. Developed pseudoparalysis, very well marked ulceration of the gums in the vicinity of upper incisors on 23. iii. 24. Received amount of the preparation dissolved in water equivalent to 100 cc. of the original juice at 1 p.m. on the same day. On 24. iii. 24 improvement in the pseudo-paralysis was observed, but proptosis and orbital haemorrhages had developed since the first symptoms were observed. Received another equivalent of 100 cc. of lemon juice at 10 a.m. on that day. Gradual improvement followed and by 28. iii. 24 the animal had recovered.

Monkey B. Developed very marked proptosis, orbital haemorrhages and pseudoparalysis on 25. iii. 24. Received an equivalent of 100 cc. of lemon juice. Gradual improvement. On 28. iii. 24 received an equivalent of 50 cc. of lemon juice. By 31. iii. 24 all symptoms of scurvy disappeared.

Discussion.

Two points emerge from this investigation which deserve comment. The first is the very low nitrogen and phosphorus content of the purest fractions. The above experiments, however, do not offer enough evidence to show whether these elements are associated with the active principle or not. In the case of the nitrogen averages are given, but it is to be pointed out that cases were met in which nitrogen could not be found at all in the fraction as prepared in Exp. 7. This is rather suggestive of the possibility that the active principle may not contain any nitrogen. The subject is now being followed up.

The second point of interest is that all the active fractions reduce ammoniacal silver nitrate in the cold and decolorise potassium permanganate. This has

been studied in some detail [Connell and Zilva, 1924], and evidence is now available that an absolute parallelism between the reducing substance or substances and the vitamin does not exist.

Summary.

A series of experiments is described in which some very active antiscorbutic fractions have been prepared. The most potent preparation contains only ·03–·07 % of solid matter in solution whilst retaining approximately the entire activity of the original lemon juice. All the active fractions reduce ammoniacal silver nitrate and decolorise potassium permanganate. The purest preparations showed an extremely low nitrogen and phosphorus content.

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REFERENCE.

Connell and Zilva (1924). *Biochem. J.* **18**, 638.
Zilva (1924). *Biochem. J.* **18**, 182.