LXXXVII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. III.

BY SYLVESTER SOLOMON ZILVA.

From the Biochemical Department, Lister Institute, London.

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IN this communication is brought forward further information in connection with the fractionation of the antiscorbutic factor and the chemical properties of active fractions. It was previously shown [Zilva, 1924] that basic lead acetate precipitated the active principle from lemon juice quantitatively and that very active fractions could be obtained in this way by removing the sugar in the decitrated lemon juice by fermentation previous to precipitation. An endeavour was made, so far without success, to find a precipitating reagent which would have the advantage over basic lead acetate of removing the factor from active solutions accompanied by less extraneous matter. In this unsuccessful quest one reagent, namely, neutral lead acetate, yielded results which are of interest and worth recording. Whilst basic lead acetate precipitates the antiscorbutic factor from decitrated lemon juice quantitatively, the neutral salt does not precipitate it at all. It, however, removes considerable quantities of non-active matter from such solutions and also from the ultimate basic lead acetate fraction described in the preceding communication of this series. This reagent is, therefore, of value in the chemical fractionation of antiscorbutic solutions. Bezssonoff [1922, 1] has also found that it does not remove the vitamin from cabbage juice.

Three experiments were instituted with the object of studying the action of neutral lead acetate on active solutions. In the first experiment decitrated lemon juice was treated with neutral lead acetate until no more precipitate was formed. The solution was then centrifuged and the precipitate dissolved in the least quantity of acetic acid. Magnesium sulphate was added to this solution until all the lead was precipitated, after which treatment two volumes of absolute alcohol were introduced and the alcoholic solution allowed to stand for about an hour. It was then filtered and the filtrate evaporated to a small volume under reduced pressure at 50°. This fraction was finally made up to the original volume of the lemon juice from which it was prepared and tested out for its antiscorbutic potency on guinea-pigs in the usual way. The doses were prepared daily except during the week-ends when 2 days' supply was prepared, acidified with citric acid, exhausted and kept in a vacuum until required for feeding. Thus the chance of deterioration on storage was reduced to a minimum. Doses equivalent to 1.5 cc.,

3 cc. and 5 cc. of the original lemon juice were tested out, three guinea-pigs being employed for each dose. All the animals succumbed to scurvy within about a month.

Before it could be definitely taken that the neutral lead acetate failed to precipitate the vitamin, the presence of the latter in the solution after precipitation had to be demonstrated. With this end in view, in the second experiment, decitrated lemon juice was precipitated with neutral lead acetate, centrifuged and the resulting supernatant solution treated with magnesium sulphate and alcohol in order to remove any excess of lead. After distilling off the alcohol under reduced pressure and making up to the original volume the preparation was tested out for the antiscorbutic vitamin. Doses equivalent to 1.5 cc., 3 cc. and 5 cc. were employed in this test. Three guinea-pigs were used for each dose. The three animals on the lowest dose died of scurvy after 50 days, 34 days and 37 days respectively. Those on the 3 cc. dose died after 42 days, 44 days and 60 days. The animals on the highest dose survived for 60 days after which time they were chloroformed. Two of these animals showed very slight signs of scurvy at the post-mortem examination. The third animal was found normal.

In the third experiment 1500 cc. of decitrated lemon juice were concentrated to 600 cc. and precipitated with neutral lead acetate. The precipitate was centrifuged quickly and the supernatant fluid treated with an excess of basic lead acetate. A precipitate was formed which was centrifuged and dissolved in acetic acid. The acid solution was precipitated with magnesium sulphate and by the subsequent addition of two volumes of absolute alcohol and filtered. The alcohol from the filtrate was removed by distillation under reduced pressure and the volume was eventually made up to 1500 cc. This batch was acidified with citric acid and stored in a vacuum. A sufficient quantity was withdrawn every day for 6 weeks for testing purposes. It was found almost unworkable to prepare daily fresh doses for testing purposes owing to the great labour involved. This preparation, as was to be expected, was found to be active, although a very significant loss in the antiscorbutic potency was recorded. A daily dose equivalent to 2 cc. of lemon juice failed even to delay the onset of scurvy in guinea-pigs whilst on a 3 cc. dose one guinea-pig succumbed to scurvy after 40 days and another animal after 54 days.

The above experiments make it plain that neutral lead acetate does not precipitate the active principle at all and that antiscorbutic solutions precipitated by this reagent retain their activity in the filtrate. Although in the above experiments an appreciable loss was observed after precipitation with neutral lead acetate, there is no doubt that it is brought about by the general manipulation and not by the precipitating agent. Experiments to be described below corroborate this assumption.

The action of neutral and of basic lead acetate on the antiscorbutic factor in another source, namely, swede juice, was next investigated. In this case too the basic salt precipitated the active principle whilst the neutral did not.

In one experiment the root was expressed and the turbid juice thus obtained was treated with neutral lead acetate until no more precipitate was formed. After centrifuging, the excess of the lead salt in the supernatant fluid was removed with magnesium sulphate and alcohol as described before. After distilling off the alcohol, the preparation was made up to its original volume. The dry matter estimated on seven different samples of this preparation varied from 4.4–6.8 %, giving an average of 5.1 %. Daily doses of 3 cc. were given to three guinea-pigs and of 5 cc. to two animals. Owing to the intervention of the Christmas holiday this set of animals was chloroformed 53 days instead of 60 days after the commencement of the experiment. The guinea-pigs which received the 3 cc. dose showed very mild scurvy at the post-mortem examination. The animals receiving the higher dose did not show any signs of scurvy at all. There was no doubt that both sets of animals could have survived the usual 60 days. As the minimum protective dose of unprecipitated swede juice was found to be 3 cc. it is safe to conclude that the neutral lead acetate did not precipitate any of the active principle at all. The experiment also shows that the loss incurred in a similar experiment with lemon juice mentioned above was not due to the precipitating agent.

In another experiment the swede juice was treated with two volumes of alcohol and filtered. After removing the alcohol by distillation at a low pressure the solution was made up with distilled water to its original volume. This treatment removes a good deal of non-active matter which is precipitated by basic lead acetate. After treatment the solution was precipitated with basic lead acetate and the precipitate after centrifuging was taken up in acetic acid. Perfect solution could not be effected in this way. Without filtering, however, the solution was treated with magnesium sulphate and then with alcohol as described before in order to remove the lead; after distilling off the alcohol it was made up to the original volume of the swede juice. The dry matter estimated on seven specimens varied from 1.1–2.8 % with an average of 1.5 %. There was, therefore, a considerable reduction in the total solids as compared with the swede juice preparation of the previous experiment. This was mainly due to the reduction of the sugar content. Five guinea-pigs were used in this experiment, three on a daily dose equivalent to 3 cc. of the original juice and two on a daily dose of 5 cc. All the animals as in the preceding experiment had to be chloroformed after 53 days and none showed any scorbutic signs at the post-mortem examination.

The behaviour of the antiscorbutic factor in swede juice towards these two precipitating reagents is, therefore, the same as in lemon juice.

In a previous communication it was pointed out that the purest fractions showed a very low nitrogen content. An attempt was made to study the chemical nature of the nitrogenous constituents of such fractions. On careful examination they were found to contain both amino and amide nitrogen. The latter is small in amount and escaped detection at first owing to the fact that too little of the preparation was employed in the test. The presence of

amide nitrogen can, however, be demonstrated in the following way. Decitrated lemon juice equivalent to 200 cc. of lemon juice is first precipitated with basic lead acetate, the precipitate suspended in distilled water and decomposed with hydrogen sulphide and the hydrogen sulphide removed. The solution is then precipitated with neutral lead acetate, filtered, and the filtrate treated with hydrogen sulphide, concentrated, precipitated with absolute alcohol and the filtrate eventually made up to 50 cc. To this 5 cc. of concentrated sulphuric acid are added and the mixture is autoclaved at 5–10 lbs. pressure for 2 hours. It is then distilled with 40 cc. of 40 % NaOH and the ammonia received in *N*/50 sulphuric acid. Amide nitrogen equivalent to 1–2 cc. *N*/50 ammonia per 200 cc. of original lemon juice can be demonstrated in every case.

The amino nitrogen was estimated in a Van Slyke micro-apparatus. The following were the quantities of the reagents employed. 2.5 cc. glacial acetic acid, 10 cc. of nitrite solution and 3 cc. of a preparation equivalent to 60 cc. of the original solution obtained as described above. In the blank estimation 3 cc. of distilled water replaced the concentrated preparation. About 80 % of the gas was given off in the first 15 minutes and the entire evolution of the gas was usually complete in 2–4 hours. Quantities of amino nitrogen of the order of 0.5 cc. were thus obtained for the equivalent of 60 cc. of lemon juice.

The sum of the amino and amide nitrogen, however, forms only a part of the total nitrogen as will be seen from the figures obtained from three representative batches. The equivalent of 200 cc. of original juice was used in each estimation.

	Batch I	Batch II	Batch III
	%	%	%
Total nitrogen	0.001386	0.00175	0.00119
Amino „	0.0003693	0.000554	0.0004683
Amide „	0.0001680	0.000196	0.00014
Percentage of amino and amide nitrogen	38.7	42.85	51.11

It was possible to show that the amide group was not indispensable for the antiscorbutic activity. 50 cc. of concentrated lemon juice were treated with 5 cc. of concentrated H_2SO_4 . The solution, after being exhausted under a vacuum pump, was saturated with carbon dioxide and the receptacle filled with that gas. It was then placed in an autoclave, which was brought up to 5–10 lbs. pressure, after which it was cooled down. Preliminary experiments had shown that this treatment was sufficient to remove all the amide nitrogen. Before testing, the solution was neutralised with calcium carbonate and filtered. With the exception of the week-end this preparation was carried out daily for testing purposes. Of the eight guinea-pigs employed in this test the three receiving 1.5 cc. per day succumbed to scurvy in the usual time, of those receiving 3 cc. per day two lived for 46 days and 48 days respectively, whilst one survived for 60 days. The two animals on the 5 cc. dose lived for 60 days, after which time they were chloroformed. One of these animals showed slight, the other no scorbutic signs at the post-mortem. It is thus seen

that although the drastic treatment destroyed some of the activity the solution was markedly active, notwithstanding that all the amide nitrogen had been removed.

It is more difficult to ascertain whether the amino nitrogen has any bearing on the activity. The difficulty of feeding deaminated products without causing any ill-effects to the animals has not yet been overcome. The formation of double compounds with reagents that usually combine with amino acids, amino purines and similar compounds has also met with little success so far and the problem is still under investigation. The study of the chemical nature of the concentrated fractions is being continued. The great obstacle in the way of chemical progress at this stage is the fact that the concentrated fractions are of a syrupy consistency and consequently are not very amenable to separation and identification. By repeated precipitation with alcohol crystals are sometimes obtained.

Bezssonoff in a recent investigation [1925] utilising a scheme of fractionation similar to mine [Zilva, 1924] on cabbage juice, namely, the repeated precipitation by basic lead acetate and alcohol, has also obtained crystals by extracting such a fraction with acetone and found them to be active. The animal tests for activity employed by Bezssonoff are, however, in the author's opinion, entirely unsatisfactory, and in any case the great likelihood must be borne in mind that the activity of a crystalline fraction may not be due to the crystalline substance itself. This is evident from the work done by various investigators on the fractionation of vitamin B.

It is of interest to note that Bezssonoff finds that the above fraction does not give a coloration with the phosphomolybdotungstic acid reagent suggested by him for the detection of the antiscorbutic vitamin. This would not be surprising even if the activity of this fraction were confirmed, since it was found [Connell and Zilva, 1924] that the active concentrated fraction previously obtained by the writer failed to react with the reagent [cf. Bezssonoff, 1922, 2; 1923; Kay and Zilva, 1923; Bezssonoff, 1924].

SUMMARY.

Unlike basic lead acetate, which precipitates the antiscorbutic factor from lemon juice quantitatively, neutral lead acetate does not precipitate it at all.

The behaviour of these two precipitating reagents towards the antiscorbutic factor in swede juice is the same as to that in lemon juice.

Small quantities of amino and amide nitrogen are present in certain concentrated antiscorbutic fractions. They, however, form only a part of the total nitrogen content which is of the order of 1-2 mg. %.

There is no connection between the amide nitrogen and the antiscorbutic activity of lemon juice.

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