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A STUDY OF THE PREPARATION AND PROPERTIES OF VITAMIN C FRACTIONS FROM LEMON JUICE[1]

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Vitamin C fractions from lemon juice have been studied by Zilva and co-workers,[2] and Bezssonov has investigated the antiscorbutic vitamin as obtained from such sources as cabbage juice and potatoes.[3] However, very little is known as yet regarding the constitution of Vitamin C. It is probably the least stable of the vitamins and accordingly has been studied very little from a chemical point of view. This investigation of the preparation and properties of antiscorbutic fractions from lemon juice has been carried out in cognizance of the fact that such a study might furnish further information leading toward the isolation of Vitamin C.

Experimental

The general procedure used in testing the various fractions was the method which has been described by Sherman and co-workers.[4]

The guinea pigs used for testing the preparations were fed daily doses which were known to be equivalent to a certain volume of original lemon juice. The test period used was generally fifty-six days.

In order to avoid inactivation of the vitamin, all laboratory operations were carried out rapidly, under nitrogen gas, at relatively low temperatures, and in acid solution if possible. Fresh preparations were made each week. All fractions were stored under nitrogen in tightly stoppered bottles kept in a refrigerator. The liquids fed were always water solutions, free from toxic substances such as alcohols, ethers, heavy metals or appreciable amounts of acids.

Preparation of Vitamin C Fractions

Preparation I.—Lemon juice was decitrated with an excess of neutral lead acetate solution, the lead being removed from the remaining solution

[1] This paper is based upon a thesis presented to the Graduate School of the University of Pittsburgh by Mr. H. L. Sipple in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] S. S. Zilva, *Biochem. J.*, **22**, 779 (1928); *ibid.*, **21**, 354, 689 (1927); *ibid.*, **19**, 589 (1925); *ibid.*, **18**, 182, 632 (1924); *ibid.*, **17**, 410 (1923); *ibid.*, **16**, 42 (1922); S. J. B. Connell and S. S. Zilva, *ibid.*, **18**, 638, 641 (1924); A. Harden and S. S. Zilva, *ibid.*, **12**, 93 (1918).

[3] N. Bezssonov, *ibid.*, **17**, 420 (1921); *Compt. rend.*, **186**, 259 (1928); *ibid.*, **180**, 970 (1925); *Bull. soc. chim. biol.*, **9**, 578 (1927).

[4] H. C. Sherman, "Chemistry of Food and Nutrition," **1926**, The Macmillan Co., New York, 3d ed., p. 424; H. C. Sherman, V. K. La Mer and H. L. Campbell, THIS JOURNAL, **44**, 165 (1922).

by precipitation with 10% phosphoric acid solution. After concentration *in vacuo* below 50°, the liquid was treated with two volumes of alcohol and filtered. The alcohol was removed from the filtrate by vacuum evaporation, the residual concentrate diluted with distilled water to one-half the equivalent volume of lemon juice, and fed to guinea pigs. Table I clearly indicates that no appreciable loss in Vitamin C content occurred in this treatment.

TABLE I

TEST DATA ON PREPARATION I

Lemon juice equivalent fed daily, cc.	Number of animals	Average survival (56-day test)	Average scurvy score	Average gain in weight, g.
Lemon juice, 1.5	2	56	0	151
Decitrated lemon juice, 1.5	2	56	0	169
Decitrated lemon juice, 3.0	2	56	0	155
0	2	26	15	−68

Preparation II.—Decitrated lemon juice containing an excess of lead acetate was treated with dilute ammonium hydroxide until the P_H was brought to 7.4–7.6 (phenol red). The yellow precipitate which formed was quickly centrifuged and the supernatant liquid decanted. The precipitate was dissolved in dilute acetic acid and dilute ammonium hydroxide added until the solution was brought to a P_H of 7.4–7.6. The yellow precipitate was again centrifuged and dissolved in dilute acetic acid. The lead was removed from all fractions by precipitation with 10% phosphoric acid solution.

The results of feeding the various fractions of Preparation II appear in Table II. Both the first and second precipitates retain the vitamin with but slight loss in potency. Reprecipitation can evidently be employed as a further step in the purification since the first precipitate, as fed, contained 6.72 mg. of total solids per cc. of lemon juice equivalent while the second precipitate fraction contained 4.45 mg. of solids per cc. of lemon juice equivalent.

TABLE II

TEST DATA ON PREPARATION II

Lemon juice equivalent fed daily, cc.	Number of animals	Average survival (56-day test)	Average scurvy score	Average gain in weight, g.
Filtrate from first active ppt., 1.5	3	26	21	−83
Filtrate from second active ppt., 1.5	3	23	18	−87
First act. ppt., 1.5	2	56	tr.	10
First act. ppt., 3.0	2	56	0	113
Second act. ppt., 1.5	3	56	8	12
Second act. ppt., 3.0	2	56	tr.	50
Lemon juice, 1.5	2	56	0	189
0	2	22	19	−72

Preparation III.—Continuing the purification of Preparation II, an aqueous solution of the material from the second active precipitate (lead

free) was treated with two successive portions of *n*-butyl alcohol, the alcohol being drawn off each time. The butyl alcohol removed practically all of the yellow coloring matter from the aqueous solution, leaving the vitamin in the water phase (Table III), showing that Vitamin C is not closely associated with the bulk of the yellow coloring matter, and that the vitamin is much more soluble in water than in *n*-butyl alcohol. This does not necessarily indicate that Vitamin C is insoluble in *n*-butyl alcohol.

TABLE III

TEST DATA ON PREPARATION III

Lemon juice equivalent fed daily, cc.	Number of animals	Average survival (56-day test)	Average scurvy score	Average gain in weight, g.
Lemon juice, 1.0	2	56	0	178
Aqueous fraction, 1.0	3	56	7	41
Aqueous fraction, 2.0	3	56	tr.	70
Butyl alc. fraction, 1	5	30	19	−127
Butyl alc. fraction, 2	5	29	18	−112
0	2	27	17	−97

Preparation IV.—The solid material obtained from an active aqueous fraction prepared as in Preparation III was dissolved in 98% alcohol and the solution treated with one volume of peroxide-free absolute ethyl ether. The white crystalline precipitate (principally ammonium salts) which formed was centrifuged and the supernatant yellow liquid decanted. Table IV shows that in this preparation the vitamin remained in the liquid phase.

TABLE IV

TEST DATA ON PREPARATION IV

Lemon juice equivalent fed daily, cc.	Number of animals	Average survival (49-day test)	Average scurvy score	Average gain in weight, g.
Positive controls, second act. ppt., 1	2	49	tr.	83
Solid phase, 2	5	25	18	−75
Liquid phase, 2	6	49	0	90
Both phases, 2 cc. each	5	49	0	100
0	3	20	15	−69

Upon analysis the ether–alcohol liquid was found to contain 0.56 mg. of total solids per cc. of equivalent lemon juice. The ash was inappreciable. Reducing substances (Munson–Walker method) calculated as glucose gave a value of 0.45 mg. per cc. The ammonium salts present were found, by steam distillation from alkaline solution, to be 0.045 mg. per cc. expressed as nitrogen. The total nitrogen as determined by the micro-Kjeldahl method was 0.145 mg. of N per cc. The acidity of the total solids was equivalent to 0.39 cc. of 0.01 *N* NaOH per cc. The ferric chloride test for phenolic bodies was negative. Ammoniacal silver nitrate was reduced slightly in the cold. An osazone reaction indicated glucosazone by its crystal form. A mildly positive carbylamine reaction was obtained, which might have been due to the interaction of sugars and ammonium salts present.

Barium Acetate and Sodium Hydroxide as Precipitating Reagents.—The precipitation of the vitamin by lead at a P_H of 7.4–7.6 seems to be a more or less specific adsorption phenomenon and not dependent upon the hydrogen-ion concentration alone. This was indicated by the results of substituting barium acetate for lead acetate or sodium hydroxide for ammonium hydroxide in making vitamin precipitations.

When barium acetate was substituted for neutral lead acetate in the precipitation procedure, the procedure being unchanged otherwise, a yellow precipitate formed at the usual P_H (7.4–7.6) but it did not remove the antiscorbutic factor from solution. The filtrate proved to be nearly as active as the original lemon juice.

In another experiment dilute sodium hydroxide solution was substituted for ammonium hydroxide in making the basic lead acetate precipitation, care being taken to make the precipitation in the customary P_H range of 7.4–7.6. It was found that the antiscorbutic material was not precipitated and that the treatment caused some inactivation of the vitamin.

Extraction with Absolute Ether.—The highly purified active alcohol–ether preparation of Vitamin C described above was evaporated to dryness *in vacuo*, and the residue extracted with absolute ether. The vitamin was not extracted from the solid material by the absolute ether, but a significant amount of amorphous material was removed by the ether, which would not redissolve in water. This left the total solids of the active fraction equivalent to 0.28 mg. per cc. of lemon juice.

Conclusions and Summary

Lemon juice has been decitrated by the addition of an excess of neutral lead acetate, and a lead complex containing practically all of the Vitamin C has been precipitated from the resulting liquid at a P_H of 7.4–7.6 by the addition of dilute ammonium hydroxide. This active complex has been reprecipitated, effecting a decrease in the total solids without appreciable loss of the vitamin. Barium acetate or sodium hydroxide could not be substituted for lead acetate or ammonium hydroxide in the precipitation of the vitamin. Absolute ethyl ether precipitated white crystalline inactive material, principally ammonium salts, from alcoholic solution of a highly purified vitamin preparation without precipitating the vitamin. Absolute ethyl ether did not extract Vitamin C from the solid material obtained by evaporation of the active alcohol–ether fraction. The total solids per cc. of lemon juice has been reduced from approximately 100 mg. in the original juice to 0.28 mg. for the final active preparations. The solid material in the final active fraction was chiefly carbohydrate in nature. The removal of inactive material has been accomplished without a great loss of the original Vitamin C content.

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