XL. THE CHEMICAL NATURE OF VITAMIN C.

By JOSEPH LOUIS SVIRBELY[1] and ALBERT SZENT-GYÖRGYI.
From the Institute of Medical Chemistry, The University, Szeged, Hungary.

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The object of this paper is to show that the antiscorbutic activity of our "hexuronic acid" preparations is due to the acid itself and not to a contamination by some more potent substance. The authors think that they have demonstrated this and that the experiments presented give definite evidence for the identity of the acid and vitamin C. In accordance with the latest results of chemical analysis [Karrer *et al.*, 1933; Cox *et al.*, 1932] "hexuronic acid" will henceforth be called *ascorbic acid* [Szent-Györgyi and Haworth, 1933].

Preparation of ascorbic acid from paprika.

Progress in our work was dependent on the possibility of obtaining larger quantities of ascorbic acid. Unfortunately, adrenal glands, the only material heretofore suitable for large scale preparation, were not available in the necessary quantity. All our efforts to prepare ascorbic acid on the large scale from tomatoes, cabbages or oranges failed.

In a quest for more suitable material, we have found that local varieties of paprika (Hungarian red pepper, *Capsicum annuum*) showed a strikingly high reducing power. 1 cc. of the juice of the fresh and ripe fruit reduces in acid solution on the average 2·4 cc. of 0·01 *N* iodine or the equivalent quantity of dibromophenolindophenol. This would correspond to about 2 mg. of ascorbic acid if this substance were solely responsible for the reduction.

In animal experiments paprika juice shows a strong antiscorbutic activity. With 0·25 or 0·5 cc. of the juice, guinea-pigs were kept practically free from scurvy in a 53-day experiment (see Table II). Throughout this experiment the same juice was used, which was kept *in vacuo* at 0°. This brings out the fact, that paprika juice not only contains relatively large amounts of the antiscorbutic factor, but also contains it in a relatively stable condition, suitable for chemical work.

50 kg. of paprika, freed from the core, were minced in a meat grinder and 1750 g. of barium acetate in the form of a hot saturated solution were added. After standing for an hour the pulp was pressed out in a fruit press, 40 litres of juice being obtained. In absence of air this juice is stable for a period of 2 to 3 weeks. In the decanted clear liquid, 5 % of normal lead acetate is dissolved and the solution made slightly alkaline to bromothymol blue with ammonia. The precipitate is sharply separated on the centrifuge, suspended in very little water and 25 % sulphuric acid added till the fluid colours thymol blue slightly red. The lead sulphate is removed by centrifuging and 10 % of barium acetate is dissolved in the liquid. The inactive precipitate is removed on the centrifuge.

[1] Holder of an American-Hungarian Exchange Fellowship (1931–33) from the Institute of International Education, New York.

A sufficient quantity of lead acetate (5–15 %) is dissolved in the liquid, which ought to give no more precipitate at p_H 8·5 on the addition of excess of lead. The fluid is made alkaline to bromothymol blue, the precipitate sharply separated on the centrifuge, and treated as above. After the first lead treatment the volume is about 8000 cc., after the second about 1500–2000 cc. The total reducing power drops to one-half of the original value.

Ammonia is added till the fluid no longer affects thymol blue. The solution is distilled *in vacuo* with a low inside temperature (20–30°) to a syrupy consistency (200–300 cc.). About double the volume of methyl alcohol is added with constant stirring and the relatively inactive precipitate separated on a Büchner funnel. Then an equal volume of acetone is added and the fluid decanted from the resinous precipitate. The precipitate is extracted with a little methyl alcohol to which acetone is again added.

The combined extracts are concentrated to a syrupy consistency *in vacuo* to a volume of about 150 cc. 100 cc. of methyl alcohol are added and subsequently 500 cc. of acetone. The fluid is decanted from the precipitate which is extracted again with a small quantity of methyl alcohol. The second extract is treated with acetone and the fluids are combined. To this combined fluid, an equal volume of freshly distilled anhydrous ethyl ether is added. After decantation, the syrupy residue is extracted with methyl alcohol and the extract treated with acetone and ether. The combined fluids are concentrated *in vacuo* to a syrup. This syrup is transferred into a large flat-bottomed crystallisation dish and placed over sulphuric acid *in vacuo* till the next day. Granulated NaOH is placed in the same desiccator. On drying, most of the ascorbic acid separates in large well-formed crystals, which are freed from the resinous mother-liquor by washing with small amounts of methyl alcohol. The crystals, collected on a Büchner funnel, are washed with acetone and dried *in vacuo*. The entire preparation up to this stage takes on the average 3 days. The mother-liquor is treated once more with excess of acetone and ether as above and a second crystallisation effected.

The product consists of slightly yellow, well-formed crystals, melting at 187–189°; yield: 6·5 g. per 10 l. of juice, of which amount 5–6 g. separate on the first crystallisation. In animal experiments (see Table II) 0·5 mg. of the substance protects guinea-pigs against scurvy in a 65 day test period.

Recrystallisation. The crystals are dissolved with slight heating in a mixture of methyl alcohol (3 parts) and dioxan (2 parts), 5 cc. being used for every g. of the acid. The solution is distilled *in vacuo* to a small volume and left in the ice-box overnight. The crystals are washed with acetone and dried *in vacuo* over sulphuric acid. More crystals can be obtained from the mother-liquor on further concentration. The recrystallisation can be effected with very little loss.

The product consists of white, well-formed crystals, which melt sharply at 192°; $[\alpha]_D^{20°} = +24°$. Reduction equivalent weight is 88, so that the molecular weight is 176. The product thus corresponds to a pure preparation of ascorbic acid.

By this method about 450 g. of recrystallised ascorbic acid have been prepared in our laboratory.

Monoacetone-ascorbic acid.

If a crystalline preparation of a substance is found active in vitamin work, the question arises, whether the activity is due to the substance itself or to its contaminations. Even repeated recrystallisation of the substance does not

decide the question, since the contaminating substances might lend themselves to the formation of mixed crystals of constant composition. Definite evidence can be obtained by the preparation of a derivative of the substance in question. If the derivative is recrystallised and the original substance recovered and found active, little doubt is left about its identity with the active substance. There is very little chance that the contaminating substances should fit into the crystalline structure of the derivative in the same way as with the original substance.

In collaboration with Vargha [1932] we set out in search of a suitable derivative. Acetyl and benzoyl derivatives could not be crystallised. An almost ideally suitable derivative was found in the monoacetone derivative.

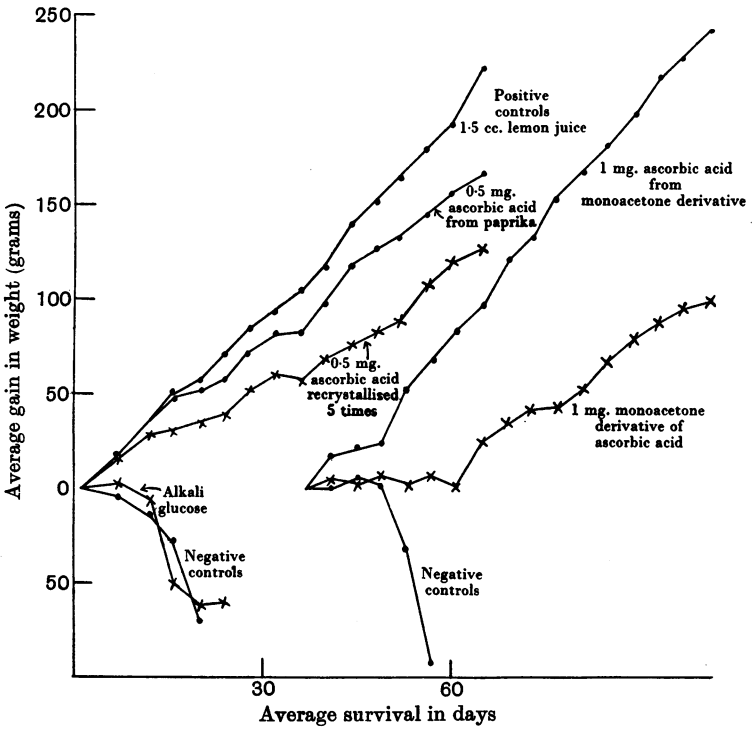

Fig. 1.

20 g. of ascorbic acid were shaken in 500 cc. of acetone in the presence of 50 g. of anhydrous copper sulphate for 24 hours. After filtration, the fluid was evaporated *in vacuo* to one-third of its volume. On addition of a double volume of light petroleum the monoacetone derivative of ascorbic acid crystallised out in large well-formed colourless prisms. These were dissolved in acetone and recrystallised by the addition of light petroleum; 70 % of the theoretical yield was obtained.

The acetone derivative is slowly decomposed in cold, readily in hot watery solution. If a fresh watery solution is quickly dried *in vacuo* at low temperature, the acetone derivative is recovered in crystals. If, however, the watery solution is heated for 15 mins. on the water-bath and then evaporated, ascorbic acid is obtained in crystalline form and can be identified by all its physical and chemical constants.

The acetone derivative was kept *in vacuo*, solutions being made immediately before feeding to the animals. The ascorbic acid recovered in crystalline form from the acetone derivative was administered in an analogous solution.

The results of this experiment can be seen in Fig. 1 and Table II. It will be noted that the ascorbic acid recovered from the acetone derivative is fully active, while the acetone derivative itself shows but a moderate activity which might be due to the acetone derivative itself or to its partial hydrolysis.

Recrystallisation experiment.

In order to obtain additional evidence for the activity of ascorbic acid, our original preparation, obtained from adrenal glands, was recrystallised five times using the original procedure, *i.e.* adding light petroleum to an anhydrous solution of ascorbic acid, using methyl alcohol as the primary solvent. After each crystallisation, the crystals were washed with acetone and dried[1].

The original M.P. was 184° and rose in the course of the recrystallisations to 187° showing that no entirely pure preparations can be obtained by this method. The crystals at the end of the fifth recrystallisation were well-formed but were slightly yellow.

For reasons outlined above, we do not attach too much value to such an experiment. The preparation obtained after five subsequent recrystallisations was found to be protective against scurvy in doses of 0·5 mg. in a 65 day test period (see Table II).

The ascorbic acid content of the adrenal gland.

If ascorbic acid is a vitamin we have to expect that animals liable to scurvy are unable to build up this substance. It has been shown in a previous communication by one of us (A. Sz.) that the adrenal cortex contains in the normal animal relatively large quantities of ascorbic acid. It seemed to be of interest to find out the behaviour of the ascorbic acid content of the gland on a vitamin C-free diet.

The ascorbic acid of the adrenal glands was estimated in the following manner. The adrenal glands of freshly killed guinea-pigs were weighed, then minced with sand in a small mortar. The pulp was suspended in 4 cc. of 1 % trichloroacetic acid and centrifuged. The fluid was titrated with dibromophenol-indophenol (0·1 %), which had been standardised against a known solution of ascorbic acid. The first permanent faint pink colour was regarded as the end-point, correction being made for the blank. Glutathione does not reduce the dye under the experimental conditions.

The results are given in Table I. As will be seen, the glands of animals which had been fed in addition to the basal diet on very liberal amounts of spinach for 10 days contain high amounts of the reducing substance. If the amount of spinach is restricted to about half a handful on alternate days, the values are much lower. The average value for the former group is 0·9 mg. ascorbic acid per g. of the adrenal gland, while the latter group shows an average of 0·2 mg.

Kept on a vitamin C-free diet for 20 days, the average value dropped to 0·03 mg. per g. This drop is already pronounced on the ninth day with an average value of 0·09 mg. per g.

[1] We express our gratitude to Prof. E. C. Kendall, Director of the Department of Chemistry of the Mayo Clinic at Rochester, Minn., for the supply of ascorbic acid from adrenal glands, which he was kind enough to send to us.

Table I.

	Weight of adrenals mg.	Final weight of animals g.	Ascorbic acid mg./g. gland
Very liberal diet of spinach	176	365	1·08
	490	730	0·8
	196	485	1·0
	234	305	0·6
	130	250	1·0
Restricted diet of spinach	265	490	0·2
	280	545	0·22
	290	480	0·2
	220	400	0·15
9 days on basal diet only	560	800	0·06
	195	240	0·08
	402	490	0·09
	174	320	0·15
	163	265	0·15
	627	330	0·08
	468	290	0·03
20 days on basal diet only	500	475	0·03
	580	645	0·03
	450	560	0·03
	473	525	0·03
	272	473	0·03
	273	160	0·03
1·5 cc. lemon juice	320	495	0·11
	228	415	0·12
	219	480	0·17
0·5 mg. ascorbic acid from paprika	440	515	0·09
	310	465	0·08
	327	545	0·10
1 mg. ascorbic acid from acetone derivative	290	585	0·11
	299	460	0·18
	231	495	0·15
	214	490	0·17

The positive controls which received 1·5 cc. lemon juice (minimum dose giving full protection against scurvy) show a fairly low value of 0·13 mg. per g. The animals receiving 0·5 mg. ascorbic acid from paprika juice (corresponding with 0·8 cc. lemon juice) had 0·09 mg. per g. The animals receiving daily 1 mg. ascorbic acid recovered from the acetone derivative showed 0·15 mg. per g. of gland.

We wish to emphasise the fact, that these results suggest that there is a wide limit between health and scurvy and that animals fed on restricted amounts of the vitamin, though not showing signs of scurvy, are greatly depleted of their vitamin store.

Similar results have been obtained lately by Moore and Ray [1932] who observed the disappearance of the silver reduction in the gland on vitamin-free diet.

Alkaline glucose solutions.

It is known that glucose treated with alkali at high temperatures gives rise to the formation of strongly reducing substances, with a similar reducing power to ascorbic acid.

10 % glucose solution was treated at 100° with *N* NaOH for 30 secs. The solution, after neutralisation, strongly reduced silver nitrate and iodine in acid

solution at room temperature. If the reduction had been due to the formation of ascorbic acid, 10 % of the glucose had been transformed into this substance.

1 cc. of the solution was fed daily to guinea-pigs. The animals, as seen in Fig. 1, lost weight in a similar way to the negative controls and died with symptoms of severe scurvy. Thus no ascorbic acid is formed on the treatment of glucose with alkali.

Experimental.

The general procedure used in testing the antiscorbutic activity of the different substances was that recommended by Sherman *et al.* [1922]. Instead of the customary 90 day period, a 65 day test period was chosen since definite results are also obtained by the shorter test period. The animals placed on the various tests varied in weight from 200 to 350 g., care being taken to ensure an equal distribution in regard to weight.

The solutions of the substances to be tested were administered daily by means of a graduated pipette.

No difficulty was encountered in feeding the paprika juice to the animals, who took it eagerly. The p_H of the juice as estimated by indicators was about 4.

Table II.

Fed daily	No. of animals	Average survival (65 days' test) days	Average scurvy score*	Average gain in weight g.
Basal diet only	3	19	13	-98
1·5 cc. lemon juice	3	65	0	222
0·125 cc. paprika juice	2	53†	7	-70
0·25 cc. ,,	3	53†	3	33
0·5 cc. ,,	3	53†	1	87
1 mg. monoacetone-ascorbic acid	4	65	1	100
1 mg. ascorbic acid from acetone derivative	5	65	0	243
0·5 mg. ascorbic acid from paprika	6	65	0	167
0·5 mg. ascorbic acid recryst. 5 times	4	65	1	129
Basal diet only	3	21	13	-83
1 cc. alkali glucose	6	19	17	-62

* Highest possible score is 24.
† Chloroformed owing to depletion of supply of paprika juice.

Summary.

It is shown that paprika (*Capsicum annuum*) contains ascorbic acid in relatively large quantities and under conditions which make its isolation fairly simple. The method of preparation is described.

The monoacetone derivative of ascorbic acid was prepared. It is shown that this substance is moderately active as an antiscorbutic. The ascorbic acid recovered from the monoacetone derivative is fully active. This is regarded as definite evidence concerning the identity of ascorbic acid and vitamin C.

It is shown that ascorbic acid (from adrenal glands) recrystallised five times retains its activity.

Ascorbic acid readily disappears from the adrenal glands of animals on a vitamin C-free diet.

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REFERENCES.

Cox, Hirst and Reynolds (1932). *Nature*, **130**, 888.
Karrer, Salomon, Schöpp and Morf (1933). *Vierteljahrssch. Naturforsch. Ges. Zürich*, **78**, 9.
Moore and Ray (1932). *Nature*, **130**, 997.
Sherman, La Mer and Campbell (1922). *J. Amer. Chem. Soc.* **44**, 165.
Szent-Györgyi and Haworth (1933). *Nature*, **131**, 24.
Vargha (1932). *Nature*, **130**, 847.