CXXXIV. THE DETERMINATION OF THE VITAMIN C VALUE OF ASCORBIC ACID.

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RECENT work by Svirbely and Szent-Györgyi [1932; 1933] has shown that the substance originally supposed to be hexuronic acid and later renamed "ascorbic acid" [Haworth, Hirst and Reynolds, 1932; Cox, Hirst and Reynolds, 1932; Szent-Györgyi and Haworth, 1933; Hirst, Percival and Smith, 1933] has high antiscorbutic activity, a daily dose of 1 mg. being sufficient to protect young guinea-pigs from the onset of scurvy. Zilva [1932] and Harris *et al.* [1932] have examined samples of ascorbic acid which they received from Prof. Szent-Györgyi. Zilva showed that a daily dose of 1 mg. was sufficient to protect young guinea-pigs from scurvy for 55 days. Although his animals were beginning to lose weight at the end of the experiment, *post mortem* examination failed to reveal any signs of disease. Since, by definition, 0.1 cc. lemon juice contains 1 international unit of vitamin C and since 1.5 cc. lemon juice is generally accepted as the minimum dose which will afford complete protection from scurvy in all guinea-pigs, it may be concluded from Zilva's results that 1 mg. ascorbic acid contains at least 15 international units of vitamin C. Harris, Mills and Innes [1932] examined their sample of ascorbic acid from Prof. Szent-Györgyi by Höjer's method as described by Key and Elphick [1931] and also by a curative growth test. They showed that the antiscorbutic activity of 2 mg. ascorbic acid was equal to or slightly greater than that of 2 cc. orange juice. The potency of orange juice was found in this laboratory to be approximately 8–10 units vitamin C per cc. Assuming that the potency of Harris's orange juice was equal to our average, then his ascorbic acid would contain 8–10 or slightly more units vitamin C per mg. This sample of ascorbic acid had been stored for some time and the authors suggest that some deterioration may have taken place. Later work by Birch, Harris and Ray [1933] on ascorbic acid from different sources showed that 1 cc. orange juice was equivalent to 0.6 mg. ascorbic acid, from which the potency of the acid appears to be 13–16 units vitamin C per mg. It should be noted, however, that these calculations assume the constancy of the potency of orange juice, and it is easily conceivable that the results do not indicate differences in potency of different samples of ascorbic acid, but differences in potency of different samples of orange juice.

We received a sample of ascorbic acid from Prof. Szent-Györgyi on January 12th, 1933, and began its examination on January 18th. Three separate experiments were performed in order to determine its potency as accurately as possible. The technique was the modified Höjer's method described by Key and Elphick [1931].

Exp. 1. On January 16th, 1933, about 100 mg. ascorbic acid were removed from the stock and the tube containing the remainder of the sample was placed in a test-tube which was immediately exhausted, filled with nitrogen and sealed. Twelve small tubes were drawn out ready for sealing and 8 mg. ascorbic acid (from the 100 mg. portion) were transferred to each. These small tubes were immediately filled with nitrogen and sealed. They were stored at 0–5° until required.

Each day one tube containing 8 mg. ascorbic acid was opened, and its contents were dissolved in water so that 1 mg. acid was present in 4 cc. solution. Some guinea-pigs were given 0.15 mg. and others 0.3 mg. ascorbic acid daily. The doses were given from a graduated pipette as 0.6 and 1.2 cc. of solution respectively, double doses being given on Saturdays. 0.75 cc. fresh lemon juice (not decitrated) was tested simultaneously on another group of guinea-pigs. The degree of protection from scurvy afforded by each dose was estimated by reference to the scheme of Key and Elphick in which 0 represents no protection and 4 complete protection. Table I shows that 0.15 mg. ascorbic acid did not produce any measurable protection, but 0.3 mg. produced slight protection, the average figure for this dose being 0.9 and the corresponding abscissa 0.26 on the curve used in this laboratory relating response to dose of vitamin C. The results given by 0.75 cc. lemon juice were 1.6 for the average degree of protection and 0.88 for the corresponding abscissa. Thus the ratio of the antiscorbutic potencies of 0.3 mg. ascorbic acid and 0.75 cc. lemon juice $\frac{0.26}{0.88} = 0.3$, or 1 mg. ascorbic acid contains approximately 7.5 units vitamin C.

This result is lower than that obtained by other workers (see above).

Exp. 2. Since the doses of ascorbic acid given in the last experiment gave very little protection from scurvy, a dose of 1 mg. daily was examined.

The stock of ascorbic acid was re-opened on February 17th, 120 mg. were removed and the remainder was then sealed in a tube, filled with nitrogen as before. Ten tubes each containing 8 mg. acid and 2 tubes each containing 16 mg. acid were prepared as in *Exp. 1*. The contents of 1 tube were dissolved in water each day so that 1 mg. was contained in 2.5 cc. solution (double strength on Saturdays). The dose of lemon juice tested simultaneously was 1.25 cc. daily.

The average degree of protection produced by 1 mg. ascorbic acid was 1.9 with 1.14 as the corresponding abscissa (see Table I). The average degree of protection produced by 1.25 cc. lemon juice was 3.2 with 2.30 as the corresponding

Table I.

Date of beginning of experiment	Daily dose	No. of guinea-pigs	Degree of protection from scurvy shown by each guinea-pig (scale 0 for no protection to 4 for complete protection)	Average degree of protection from scurvy for each dose	Corresponding abscissa on the curve relating average degree of protection to dose of vitamin C given	Potency of ascorbic acid obtained by comparison with lemon juice tested simultaneously (units per mg.)
<i>Exp. 1</i>	0.15 mg. ascorbic acid	5	0.5 0 1.0 0.5 1.0	0.6	0	*
18. i. 33	0.3 mg. ascorbic acid	5	0.5 1.0 0.5 1.5 1.0	0.9	0.26	7.5
	0.75 cc. lemon juice	5	1.5 2.0 2.5 2.0 0	1.6	0.88	
<i>Exp. 2</i>	No dose	5	0.5 0.5 1.0 0.5 1.0	0.7	0.08	
17. ii. 33	1.0 mg. ascorbic acid	5	1.0 0.5 3.0 3.5 1.5	1.9	1.14	6.2
	1.25 cc. lemon juice	5	1.5 2.5 4.0 4.0 4.0	3.2	2.30	
<i>Exp. 3</i>	No dose	4	1.0 2.0 1.0 1.0	1.25	0.56	
30. iii. 33	0.75 mg. ascorbic acid	5	3.0 2.5 2.0 2.0 3.0	2.5	1.68	10 } Average
	2.0 mg. ascorbic acid	5	4.0 4.0 4.0 4.0 4.0	4.0	3	>7 } >8.5
	1.0 cc. lemon juice	5	4.0 0.5 4.0 3.5 3.5	3.1	2.21	

Average value for the potency of ascorbic acid from 3 experiments = 7.4 units per mg.

* Dose too low—no measurable protection.

abscissa. Thus the ratio of the potencies of 1 mg. ascorbic acid and 1.25 cc. lemon juice = $\frac{1.14}{2.30} = 0.5$, or 1 mg. ascorbic acid contains 6.2 units vitamin C. This result, therefore, agrees quite well with that of Exp. 1.

Exp. 3. In the last two experiments three doses of ascorbic acid had been examined, but none had produced complete protection from scurvy. In this experiment, therefore, a dose of 2 mg. daily was given to each of 5 guinea-pigs and this dose was found to be sufficient to protect all of them completely. At the same time 5 other guinea-pigs were each given 0.75 mg. ascorbic acid daily in order to obtain another point on the curve relating response to dose of acid.

This test was begun on March 30th. Sealed tubes, each containing sufficient ascorbic acid for 1 day's doses, were prepared as before. A daily dose of 1 cc. fresh lemon juice was tested simultaneously. The results showed that the ratio of the potencies of 0.75 mg. ascorbic acid and 1.0 cc. lemon juice = $\frac{1.68}{2.21} = 0.76$. Thus 1 mg. ascorbic acid contains 10 units vitamin C. Also the ratio of the potencies of 2 mg. ascorbic acid and 1.0 cc. lemon juice = $\frac{3}{2.21} = 1.4$. Thus, 1 mg. ascorbic acid contains 7 units vitamin C. This value may be lower than the true value since 2 mg. acid produced complete protection and possibly a lower dose would also have produced complete protection. From this experiment, the average value for the potency of the ascorbic acid is, therefore, at least 8.5 units vitamin C per mg.

The results from all three experiments are shown in Table I. The average of all the values found for the potency of the ascorbic acid was 7.4 international units of vitamin C per mg.

Curve of response to doses of ascorbic acid.

The results were used in constructing the curve in Fig. 1 in which the average degree of protection produced by a dose of ascorbic acid was plotted against that dose.

All the guinea-pigs were bred on the same farm. They were given bran, oats and green food *ad lib.* in the laboratory for at least 24 hours before receiving the scorbutic diet.

Fig. 1. Curve of response to graded doses of ascorbic acid compared with curve of response to graded doses of orange juice. Continuous line: curve of response to graded doses of ascorbic acid. Dotted line: curve of response to graded doses of orange juice.

It was recognised that the condition of the animals or any number of unknown factors might be affecting the result so that one experiment could not

be directly compared with another, but it was obvious that increasing doses had on the whole produced increasing responses and it seemed probable that the short interval of 3 months between the beginning of Exp. 1 and the end of Exp. 3 had not appreciably changed the conditions. In Fig. 1 the best straight line was drawn through the points representing the four lowest doses of ascorbic acid. Since 2 mg. acid daily produced complete healing this point was not included when constructing the line. Its true value might be represented by some figure higher than 4 on the scale of protection. The fact that the points do not lie very near to this line is probably largely accounted for by variations in the whole group of guinea-pigs from time to time. The curve in general use in these laboratories for determining the relation between degree of protection and dose of vitamin C was constructed from the results of graded doses of orange juice, all tested at the same time. Fig. 1 shows that the curve for ascorbic acid is almost coincident with that for orange juice; thus probably the same factor has been measured in the two substances.

Variations in the responses of guinea-pigs in different experiments.

In Fig. 2 the doses of ascorbic acid and the doses of lemon juice were plotted against the average degree of protection for each dose and the points on the graph obtained in each experiment were joined by straight lines. In Exp. 2 all

Fig. 2. Variations in the response of guinea-pigs at different times. Exp. 1, Jan., 1933; Exp. 2, Feb., 1933; Exp. 3, April, 1933.

the responses were higher than in Exp. 1 and in Exp. 3 they were still higher. As it is highly improbable that the ascorbic acid could increase in potency, these results seem to indicate either that the lemon juice of the different experiments has differed in potency, or that the sensitivity of the animals has differed in the different experiments. Both of these possibilities are contrary to Silva's experience, who bases all his tests on the assumption that a given dose of lemon juice always produces the same result in his guinea-pigs, and therefore the guinea-pigs do not vary in sensitivity nor does lemon juice vary in potency. We shall not, however, attach great importance to our indications of the variations of lemon juice and guinea-pigs until we have collected sufficient data to work out the probable error of the test; without this information we should not be justified in drawing conclusions from relatively small numbers of animals and experiments.

DISCUSSION.

On the assumption that lemon juice contains a constant amount of vitamin C the ascorbic acid used in the experiments described above appeared to have about one-half the antiscorbutic potency of most of the samples examined by other workers. It was possible that some of the activity of our sample had been lost during storage and at the end of the last experiment Mr J. C. Gage kindly carried out titrations with iodine to test this possibility. His results showed that the sample contained 97·3 % ascorbic acid ($C_6H_8O_6$). There could, therefore, have been very little oxidation of the sample. That there was no loss of antiscorbutic potency was also shown by the fact that the results obtained by direct comparison with lemon juice showed no decrease in activity of the acid from the first to the third experiment. It is interesting to note that our results are in agreement with those first published by Harris *et al.* [1932] when they examined the sample of ascorbic acid which they had received from Prof. Szent-Györgyi. As already stated, their figure for the antiscorbutic potency of the acid would be about 8–10 units per mg. The average result from our experiments is 7·4 units per mg., which is in good agreement with Harris's figure. Harris *et al.* suggested that some deterioration had taken place in their sample during storage because apparently it had less activity than Szent-Györgyi had thought. So far as we know, no direct comparison has been made between Szent-Györgyi's sample and any other sample of ascorbic acid.

The experiments here described indicate that the ascorbic acid stored in a nitrogen-filled tube at 0–5° had not lost activity during the 3 months which elapsed from the beginning of the first to the end of the third experiment. It would be necessary, however, to examine its stability over a much longer period before this substance could be recommended for adoption as a standard of reference for the estimation of vitamin C. The ease with which it can be handled and dissolved in water and the readiness with which the animals take the solution are factors in favour of its use as a standard if its stability can be established.

SUMMARY.

A sample of ascorbic acid from Prof. Szent-Györgyi was examined for its antiscorbutic potency and found to contain 7·4 international units of vitamin C per mg. This is approximately one-half the activity claimed by Prof. Szent-Györgyi, if it is assumed that lemon juice has a constant potency. There is no conclusive evidence that it has, and we think it more likely that the variation occurred in the lemon juice than in the ascorbic acid.

Five different doses ranging from 0·15 to 2·0 mg. were tested at three different times. The curve of response to the doses was found to be almost identical with the curve of response to graded doses of orange juice drawn up in this laboratory about 2 years ago.

That guinea-pigs change from time to time in sensitiveness was shown by the fact that the curve of response was in a slightly different position for each experiment. Therefore, it is necessary to use a dose of some standard in every experiment.

Certain results indicated that there may be a variation in the vitamin C potency of lemon juice, showing that lemon juice may not be a satisfactory standard of reference.

An interval of 3 months between tests is obviously not great enough to allow conclusions to be drawn as to the stability of ascorbic acid, but certainly no loss

of potency was detected in the sample of ascorbic acid with which we worked during that period. The evidence supports the suggestion of Birch, Harris and Ray [1933] that ascorbic acid is a better standard for vitamin C than lemon juice.

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