[From THE BIOCHEMICAL JOURNAL, Vol. XXVI, No. 3, pp. 865-870, 1932]

CV. THE CHEMICAL NATURE OF VITAMIN C.

By JOSEPH LOUIS SVIRBELY[1] and ALBERT SZENT-GYÖRGYI.

From the Institute of Medical Chemistry, University of Szeged, Hungary.

(Received April 25th, 1932.)

Hexuronic acid was discovered [1927] and isolated from plants and the adrenal cortex [1928] by Szent-Györgyi at the Biochemical Laboratory, Cambridge, and was investigated in regard to its function and chemistry [1928, 1930, 1931]. Its chemical configuration has been studied by Haworth, Hirst and Reynolds [1932]. The striking similarity of this substance with vitamin C was evident from the beginning. As far as it is known, its distribution in plants closely follows the distribution of the vitamin. The chemical properties of both substances agree and both are biologically active in minute quantities.

As is known, hexuronic acid has strongly reducing properties and the reducing power of many plant extracts is mainly due to this substance. The close relation between vitamin content and reducing power has been emphasised by Tillmans *et al.* [1932], whose careful work strongly suggested that the reducing power of plant extracts was in part due to the vitamin itself, which was identical with hexuronic acid.

Tillmans and his co-workers claim to have shown that the vitamin content and the reducing capacity run on strictly parallel lines under many conditions. Furthermore they have shown that the reducing factor can be oxidised in two different ways, reversibly and irreversibly. They have succeeded in reversing the oxidation of the reversible type by the application of reducing agents such as H_2S. They observed that by the reversible oxidation the reducing factor becomes very labile and prone to undergo irreversible changes. The vitamin showed a closely analogous behaviour. On treatment with oxidising agents, which produce a reversible oxidation of the reducing factor, the vitamin did not lose its activity, but became very labile. Tillmans and his collaborators were led to the conclusion that the reducing factor and the vitamin were in fact identical.

As is known, hexuronic acid can be oxidised reversibly and irreversibly, the reversible form being readily again reduced by H_2S. The agents reported by Tillmans to produce a reversible change in reducing factor also oxidise hexuronic acid reversibly. Agents, such as molecular oxygen, which produce an irreversible change in the reducing factor, also destroy hexuronic acid.

[1] Holder of an American-Hungarian Exchange Fellowship, 1931-32, from the Institute of International Education, New York.

The strongly reducing properties of active fractions of lemon juice were also emphasised by King and his co-workers [King and Waugh, 1932; Smith and King, 1931; Svirbely and King, 1931].

In a series of his well-known publications also, Zilva [1927, 1928, 1930] has found many parallels in the distribution of vitamin and reducing factor. Zilva, however, partly in collaboration with Connell [1924], was able to show that reducing capacity and antiscorbutic activity can vary independently during the course of fractionation. He succeeded in preparing fractions with a high antiscorbutic activity and no reducing power as well as fractions with a marked reducing capacity and no antiscorbutic activity. From this he justly concluded that vitamin C and the reducing factor could not be identical. Zilva also observed that on oxidising the reducing factor the antiscorbutic activity did not disappear, but the antiscorbutic principle became very labile, in which observation he was corroborated later by Tillmans. Zilva explained this change in stability by a protecting action of the reducing factor.

The reversible oxidation of hexuronic acid admits the possibility that in Zilva's extracts, which were antiscorbutically active but showed no reducing power, the hexuronic acid was present in a reversibly oxidised state. On the other hand it is known that the reducing factor is of a complex nature, as was also lately brought out by Guthrie and Wilcoxon [1932]. One of us (A. Sz.) has spent much time in studying a phenolic substance, found in relatively great quantities in orange juice, which shows a marked reducing power. It shows similarities to the flavonols but also gives reactions different from this group of substances. This substance is also readily carried down by lead acetate. Some years ago it was kindly tested by Zilva for antiscorbutic activity with negative results. The presence of such a reducing substance, not identical with hexuronic acid, might explain the reducing power of fractions which show no antiscorbutic activity. Taking all this into account, the authors think that Zilva's evidence against the identity of hexuronic acid and vitamin C cannot be regarded as conclusive.

Another objection raised recently by Zilva [1932] against the identity of vitamin C and hexuronic acid is based on the magnitude of the protective dose of hexuronic acid. The protective daily dose of lemon juice is 1·5 cc., which would keep growing guinea-pigs entirely free from scurvy. This quantity of lemon juice contains approximately 0·5 mg. of hexuronic acid. The protective dose of this substance would be thus of this magnitude. Zilva points out that with 0·5 mg. of fractions of lemon juice, which were still grossly contaminated, animals were protected against scurvy by himself and other authors. Thus the vitamin must have a higher potency.

The authors are of opinion that this evidence is not conclusive. It is difficult to judge to what extent these fractions were contaminated, and how much hexuronic acid they contained. If, however, 0·5 mg. of hexuronic acid gives full protection against scurvy, it is natural that somewhat smaller doses should have some activity. On the other hand 0·5 mg. of hexuronic acid for

1·5 cc. lemon juice gives only an approximate value, and it is possible that the hexuronic acid content of lemon juice is somewhat lower. Owing to the lack of a specific reaction it is impossible to state at present to what extent the reducing activity of a plant extract is entirely due to hexuronic acid or other substances and only rough estimates can be given.

We think that no circumstantial evidence can have much value either way in a question which can be subject to direct experiment. If it can be shown that the hexuronic acid present in the lemon juice has the same activity as the lemon juice itself, there can be little doubt about the identity of vitamin and hexuronic acid.

The striking similarity of vitamin C and hexuronic acid has been evident ever since the discovery of the acid. The possible identity of both substances has been expressed repeatedly by one of us. The collaboration of the present authors at the Institute of Medical Chemistry of the University of Szeged offered the necessary facilities for the testing of the antiscorbutic activity of hexuronic acid. The experiment was undertaken last autumn with a positive result. The negative controls died with symptoms of severe scurvy, while the animals receiving hexuronic acid were kept alive and free from scurvy in a 56 days' test. Owing however to the small number of animals used and certain deficiencies in the diet, which prevented normal growth, the results of the test were not published. A short account of this test was given in our first preliminary note [1932].

In the test to be reported[1], all shortcomings of the first experiment were remedied. The authors think that the experiment shows clearly that the hexuronic acid present in the lemon juice has the same activity as lemon juice itself, and thus clearly brings out the identity of the two substances[2].

Experimental.

The general procedure used in testing the antiscorbutic activity of hexuronic acid was that recommended by Sherman *et al.* [1922]. In addition to the basal diet, 0·5 cc. of cod-liver oil was given to each test animal every week.

The hexuronic acid used in the test was prepared in crystalline form from ox suprarenal glands 2 years ago at the chemical department of the Mayo Foundation at Rochester. We wish to emphasise the fact that our preparation was derived from animal tissues. If our hexuronic acid had been prepared from lemon juice or other plant sources the objection might arise that the activity was not due to the acid itself but to a contamination by a more potent antiscorbutic substance.

[1] A brief account of this experiment was given in our two preliminary notes in *Nature*.

[2] Simultaneously with our preliminary note King and Waugh [1932] reported that they had obtained crystals from lemon juice, which showed antiscorbutic activity and were apparently similar to a hexuronic acid. Since no chemical analysis was reported the chemical nature of their product is left in doubt. Since no mention was made of weight curves, number of animals, duration of the experiment and *post mortem* examinations, their claim of an antiscorbutic activity, based on "vitality," cannot be judged.

A sufficient amount of the acid was weighed out every 8 days and dissolved in water. Aliquot portions of the solution, adequate for daily feeding, were put in small phials and kept at $-18°$ in an atmosphere of CO_2. The solution was administered to the animals by a pipette. The minimum protective dose of lemon juice is generally regarded as 1·5 cc., and this quantity contains approximately 0·5 mg. of hexuronic acid. In our test, however, 1 mg. of hexuronic acid has been given daily to the animals since, owing to the age of the preparation and the unavoidable exposure to air, some of the acid may have become decomposed.

There was only one disturbing factor in our experiment, which however did not essentially influence the result. Owing to the general economic conditions we were unable to obtain milk powder until the 19th day of the experiment, at which time it was introduced into the diet. It was due to this fact that until that day the animals did not show normal growth, which however was promptly attained as soon as the milk powder was given. This growth was not due to traces of vitamin present, since the skimmed milk powder was heated in a tray in the drying-oven at 110° for 4 hours. It was specially tested in two animals on the 43rd day of the main test (see Table I and Fig. 1).

Table I.

Fed daily	No. of animals	Average survival (90 days' test) days	Average scurvy score*	Average gain in weight g.
1 mg. of hexuronic acid	3	55†	0	41
1 cc. of lemon juice	2	55†	2	81
Basal diet only	9	26	15	−103
1 mg. of hexuronic acid	7	90	0	281
1 cc. of lemon juice	6	90	5	147
Basal diet only	2	30	19	−114

* Highest possible score is 24. † Chloroformed.

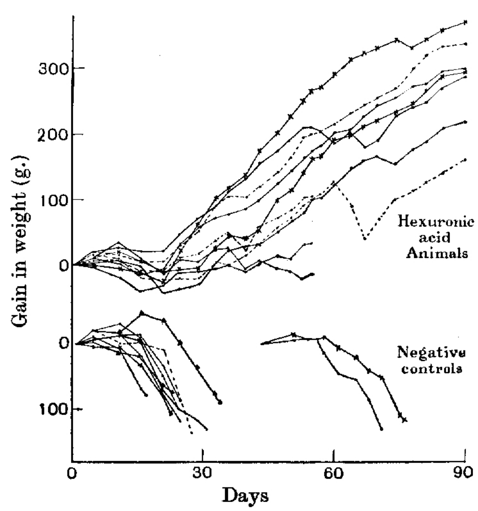

Fig. 1.

The following groups of test animals were used: (1) those receiving 1 mg. of hexuronic acid daily; (2) the positive controls, which received 1 cc. of lemon juice daily; (3) the negative controls, which received the basal diet only.

The results of the test are given in Table I. In Fig. 1 the weight curves of the animals receiving hexuronic acid are contrasted with those of the negative controls; Fig. 2 gives the average weight curves of the three groups of animals used in the test.

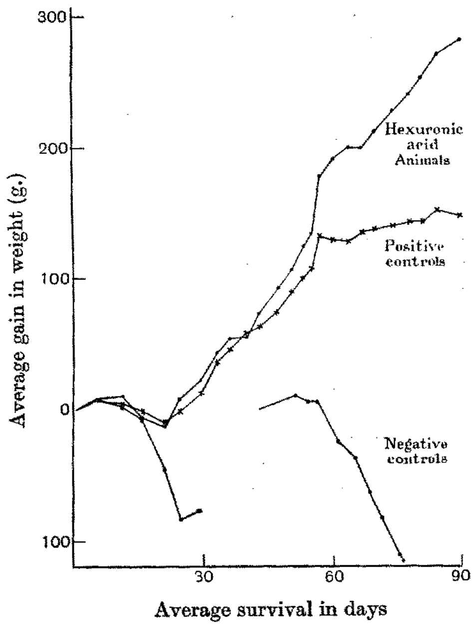

Fig. 2.

As seen from the Table and Figs. all the negative controls died within the period of 20–34 days with the usual loss in weight. At autopsy they all showed symptoms of severe scurvy. The animals receiving hexuronic acid showed normal growth. Three of these animals were killed at the end of 55 days and the rest at the end of 90 days' test period. At autopsy they were found entirely free from symptoms of scurvy. The positive controls receiving 1 cc. lemon juice showed mild scurvy at autopsy.

Summary.

Details of an experiment are described in which hexuronic acid was tested for antiscorbutic activity. The acid was administered in doses comparable with the hexuronic acid content of the protective dose of lemon juice. Given in this quantity (1 mg. daily), hexuronic acid affords complete protection against scurvy in a 90 days' test. Since it is generally assumed that animals kept free

from scurvy for 90 days would remain so indefinitely under identical conditions, it is concluded that vitamin C is a single substance and identical with hexuronic acid.

This research was sponsored by the Ella Sachs Plotz Foundation.

REFERENCES.

Connell and Zilva (1924). *Biochem. J.* **18**, 632.
Guthrie and Wilcoxon (1932). *Contr. from Boyce Thompson Institute*, **4**, 99.
Haworth, Hirst and Reynolds (1932). *Nature*, **129**, 576 (April 16).
King and Waugh (1932). *Science*, **75** (April 1), No. 1944.
Sherman, La Mer and Campbell (1922). *J. Amer. Chem. Soc.* **44**, 165.
Smith and King (1931). *J. Biol. Chem.* **94**, 491.
Svirbely and King (1931). *J. Biol. Chem.* **94**, 483.
—— and Szent-Györgyi (1932). *Nature*, **129**, 576; 690 (April 16, May 7).
Szent-Györgyi (1927). *Nature*, **129**, 782 (May 28).
—— (1928). *Biochem. J.* **22**, 1387.
—— (1930). *Science*, **72**, No. 1857.
—— (1931). *J. Biol. Chem.* **90**, 385.
Tillmans, Hirsch and Dick (1932). *Z. Unters. Lebensmittel*, **63**, 267.
—— —— and Hirsch (1932). *Z. Unters. Lebensmittel*, **63**, 1.
—— —— and Jakisch (1932, 1). *Z. Unters. Lebensmittel*, **63**, 241.
—— —— —— (1932, 2). *Z. Unters. Lebensmittel*, **63**, 276.
—— —— and Siebert (1932). *Z. Unters. Lebensmittel*, **63**, 21.
Zilva (1927). *Biochem. J.* **21**, 689.
—— (1928). *Biochem. J.* **22**, 779.
—— (1930). *Biochem. J.* **24**, 1687.
—— (1932). *Nature*, **129**, 690 (May 7).