LXXVII. SPECIFICITY OF HEXURONIC (ASCORBIC) ACID AS ANTI-SCORBUTIC FACTOR[1].

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ALTHOUGH it has been shown that specimens of hexuronic acid[2] possess anti-scorbutic properties [Svirbely and Szent-Györgyi, 1932, 1; Harris, Mills and Innes, 1932; Zilva, 1932, 2] it has remained to determine whether this activity is an inherent property of the pure substance itself or ascribable to some associated impurity. So often in the past has the vitamin activity of an apparently pure and crystalline material been shown later to be due either to some major admixture (*e.g.* in the case of the original preparation of calciferol) or else to traces of a contaminant (as with vitamin B_1) that the issue was by no means a foregone conclusion. Indeed, from the start Zilva [1932, 1] has opposed the idea that hexuronic acid could be identical with the vitamin, and more recently [1932, 3] has contended that there is in fact no consistent relationship between antiscorbutic activity and reducing capacity—one of the most distinctive properties of hexuronic acid.

The steps leading up to the present position may be summarised as follows. Szent-Györgyi [1928] demonstrated the existence in various vegetable and animal tissues of a naturally occurring reducing substance; he succeeded in isolating it, and named it hexuronic acid (from its supposed analogy with the glycuronic acids). As he pointed out in his first paper on the subject [1928] hexuronic acid appeared to be identical with a reducing substance known to occur in active vitamin C concentrates. Unfortunately however the matter was pursued no further at the time because observations by Zilva seemed to show that the vitamin was independent of the reducing principle. At about the same time, Tillmans, in the course of his work as Public Analyst for Frankfurt, to distinguish between fresh and stale or natural and artificial foods measured their reducing capacity, employing for the purpose the well known oxidation-reduction indicator 2:6-dichlorophenolindophenol. He reported that natural, but not "artificial," lemon juice reduced the indicator [Tillmans, Hirsch and Reinshagen, 1928]; and in further series of papers Tillmans and his collaborators [1932] drew attention to the fact that there existed a considerable degree of parallelism (with some irregularities) between the antiscorbutic activity of foods or vitamin C preparations and their reducing capacity. The conclusion was definitely drawn that "a reducing substance is the 'carrier' of vitamin C activity" [Tillmans, Hirsch and Jackisch, 1932] and in a paper published in March 1932 attention was specifically drawn to Szent-Györgyi's hexuronic acid as having similar pro-

[1] Communicated to the Biochemical Society, February 17th, 1933 [Birch, Harris and Ray, 1933].

[2] Since renamed ascorbic acid.

perties to the reducing substance in question, and being "possibly identical" with it [Tillmans, Hirsch and Dick, 1932]. Shortly after this, in April 1932, a preliminary note by Svirbely and Szent-Györgyi [1932, 1] mentioned that a test was in progress to discover whether hexuronic acid possessed antiscorbutic properties, and a little later [1932, 2] it was recorded that hexuronic acid, when fed at the level of 1 mg. per day had protected guinea-pigs over the usual 90 day test period. (Other levels of dosage were not tried.) Simultaneously with the contribution of Svirbely and Szent-Györgyi, King and Waugh [1932], of the University of Pittsburgh, announced that they had succeeded in isolating vitamin C and that it turned out to have the properties of hexuronic acid. It may perhaps be mentioned here that Szent-Györgyi's colleague Svirbely had but recently been in collaboration with King in some of the later stages of his work on the concentration of the vitamin.

From their evidence that a dose of 1 mg. had protected guinea-pigs for 90 days, Svirbely and Szent-Györgyi [1932, 2] concluded that "vitamin C is a single substance and identical with hexuronic acid." This conclusion was immediately combated by Zilva [1932, 1] who stated that he had himself obtained fractions active in doses of 0·5 mg. (*cf.* 1 mg. for hexuronic acid) which were "evidently grossly contaminated"—a fact which "militated against the contention that hexuronic acid and the antiscorbutic factor are identical." Actually the limited evidence available seemed insufficient to warrant a final decision in either direction.

In favour of the theory that hexuronic acid was identical with the vitamin, King and Waugh [1932] could point to the fact that the final stages of their isolation process led to a product of constant chemical composition. Svirbely and Szent-Györgyi [1932, 1] advanced the argument that the amount of hexuronic acid presumed to be present in orange juice was of the right order to account for its activity. Since, however, the amount of hexuronic acid in orange juice was not known with any certainty, no method for its estimation being in fact yet available (the amount actually present being as we now know about double the amount imagined), and since also the activity of hexuronic acid itself had not yet been determined, the necessity for further work was fully evident. Earlier papers from this laboratory [Harris, Mills and Innes, 1932; Harris and Ray, 1932; 1933; Birch, Harris and Ray, 1933] have given a preliminary account of experiments which definitely supported the theory of the identity of hexuronic acid and vitamin C. The detailed results, together with further supporting evidence, are set out in the present paper.

Experimental.

Our experimental evidence is best presented as a series of separate lines of testimony, as follows. (1) The antiscorbutic activities of several natural sources were in the first place shown to run parallel with their apparent hexuronic acid contents as roughly indicated by the yields of the latter recoverable from them. (2) Specimens of hexuronic acid derived from different sources were next shown to have identical antiscorbutic activities. (3) This activity remained unchanged after various repurification processes. (4) The hexuronic acid contents of a large variety of foodstuffs, as determined by a chemical titration method were shown to account exactly in all cases for their observed activities. (5) Hexuronic acid as measured by the intensity of the absorption band at 265 $m\mu$ likewise accounted for observed activity. (6) In guinea-pigs deprived of vitamin C, the hexuronic acid disappeared from suprarenals or liver coincidently with loss of vitamin C

activity. (7) In dogs or rats on the other hand, examples of species which are able to thrive in the absence of vitamin C, antiscorbutic activity and hexuronic acid remained unimpaired together. (8) The rate of destruction of hexuronic acid by oxidation, heat and alkali was similar to that of antiscorbutic activity. (9) Hexuronic acid was synthesised concurrently with vitamin C activity in the plant.

1. *Correlation between antiscorbutic activity and hexuronic acid recoverable.*

In the first paper on this question from this laboratory [Harris, Mills and Innes, 1932] it was pointed out that on the basis of the hexuronic acid theory the suprarenal gland, although not previously recognised as having any antiscorbutic activity, should be intensely active, since this organ was a better source of hexuronic acid even than such active materials as orange or lemon juice or cabbage. This was found to be the case, and full results have already been published [Harris and Ray, 1932] which showed that ox suprarenal cortex had three times the activity of orange juice, in exact accord with the fact that its apparent hexuronic acid content, as judged by the yield recoverable, was likewise three times as great. As we pointed out, there is a considerable loss of hexuronic acid during the recovery process, but about the same fraction is lost in the two cases. Our conclusions have been confirmed by further investigations, both upon the hexuronic acid content determined chemically and upon the antiscorbutic activity as measured by more than one method. The following figures may be cited in illustration[1].

Hexuronic acid content of ox suprarenal [Birch, Harris and Ray, 1933]	1·85 mg. per g.
Hence calculated "minimum protective dose" (*i.e.* amount containing 0·9 mg.)	0·49 g.
"Minimum protective dose" found (*i.e.* biologically equivalent to 1·5 cc. orange juice)	0·5 g.

2. *Constant activity of hexuronic acid from different sources.*

A number of specimens of hexuronic acid, derived from both an animal and a vegetable source (*viz.* suprarenal and paprika) have been examined for antiscorbutic activity under the same conditions. As is shown by Fig. 1 (typical curative tests), no difference could be detected. Coincident results were obtained also in a series of experiments in which the activity was determined by the alternative tooth-structure technique and by preventive tests. Table I gives results by the former method for hexuronic acid from paprika. A similar preliminary test on hexuronic acid from ox suprarenals has already appeared [Harris, Mills and Innes, 1932], and as shown by Fig. 2, the ratio between the effective doses of orange juice and the two materials is the same: 1 g. of hexuronic acid from suprarenal was equivalent to 1·5 cc. of orange juice in giving almost complete protection in an 11 day test, and 2 g. of hexuronic acid from paprika to 3·0 cc. of orange juice in a 15 day test.

Degree of activity of hexuronic acid. This question has already been opened elsewhere [Harris and Ray, 1932]. Our preliminary results showed uniformly that the amount of hexuronic acid equivalent to 1 cc. of orange juice was greater than 0·33 and less than (or equivalent to) 0·66 mg., the best value being 0·6 mg. Confirmatory experiments (see, *e.g.*, Fig. 2) give values from 0·6 to 0·66 mg. The "minimum protective dose" of hexuronic acid, equivalent to 1·5 cc. of orange

[1] Additional work on the vitamin activity and hexuronic acid content of suprarenals from various species will be published in a later paper.

juice hence works out at 0·9 (or 1·0) mg. This agrees exactly with the amount of hexuronic acid present in orange juice, as determined chemically (see below). At this point we would wish to suggest the adoption of a specimen of hexuronic

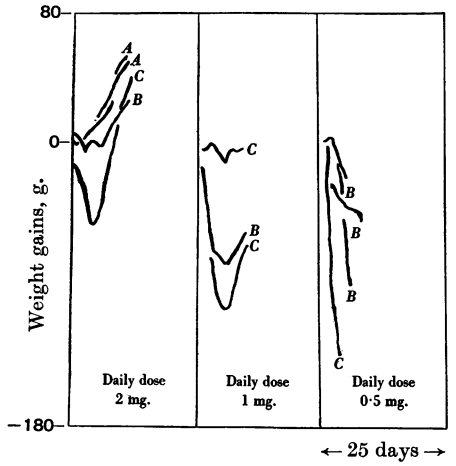

Fig. 1.

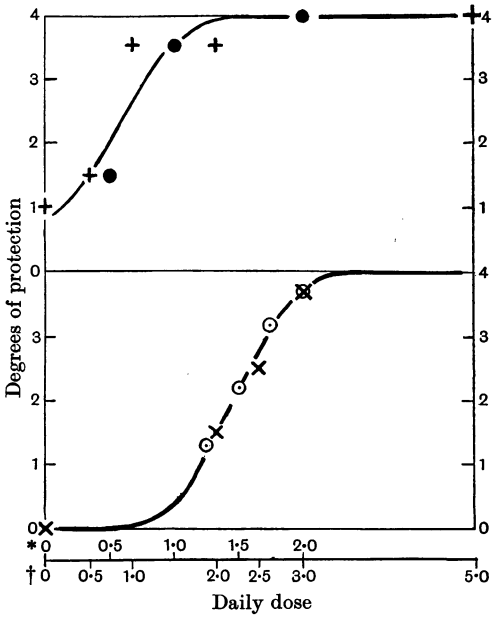

Fig. 2.

Fig. 1. Curative tests on guinea-pigs, showing equal activity of hexuronic acid derived from different sources, or repurified and recrystallised. *A* = Hexuronic acid from suprarenal cortex, crude; *B* = Hexuronic acid from paprika; *C* = Hexuronic acid from paprika, repurified and recrystallised.

Fig. 2. Equivalent activity of hexuronic acid from suprarenal (above) and paprika (below). Comparisons with orange juice as standard of reference. ● Hexuronic acid from suprarenals, + Orange juice, 11 day test; ⊙ Hexuronic acid from paprika, × Orange juice, 15 day test.

* Scale for hexuronic acid, mg.; † Scale for orange juice, cc.

Table I. *Comparison of antiscorbutic activities of hexuronic acid (from paprika) and orange juice by the tooth-structure method.*

Amount of material fed per day	Degree of protection
Hexuronic acid, 2 mg.	4, 3, 4 (av. 3·7)
,, 1·75 mg.	2–3, 3, 4 (av. 3·2)
,, 1·5 mg.	2, 2–3 (av. 2·2)
,, 1·25 mg.	1, 2, 1 (av. 1·3)
Orange juice, 3·0 cc.	4, 3, 4 (av. 3·7)
,, 2·5 cc.	2–3, 2–3, 2–3 (av. 2·5)
,, 2·0 cc.	1–2, 1, 2 (av. 1·5)
,, 0 cc.	0, 0 (av. 0)

acid as international standard of vitamin C activity. The advantage of substituting a definite uniform preparation, which can be preserved dry in a stable condition at constant activity, in place of the present standard (any specimen of lemon juice), would seem obvious.

3. *Constant activity of repurified hexuronic acid.*

We have had in our hands a number of specimens of hexuronic acid, of varying degrees of purity, iodine titrations of which have shown, *e.g.* values from 93 to over 99 %. Our results showed that a given amount of hexuronic acid always had the same activity irrespective of the presence of impurities. We were fortunate enough to be able to examine also a specimen of hexuronic acid which, after having been repurified by Prof. Szent-Györgyi, was further recrystallised at the Birmingham laboratory, and kindly put at our disposal by Dr E. L. Hirst, who described it as being in an extremely high state of purity. Again the activity showed no difference from that of the original specimens (see Fig. 1). This was confirmed in a second set of assays.

4. *Antiscorbutic activities of foods determined from their hexuronic acid contents.*

In an earlier paper [Harris and Ray, 1932] a method was described for estimating hexuronic acid chemically, and it was shown that the hexuronic acid content of orange juice exactly accounted for its antiscorbutic potency. The same result was obtained with several other materials including lemon juice, grape-fruit juice, tomato juice and pineapple juice. The method has since been adapted for use on a micro-scale by our colleague Mr T. W. Birch [Birch, Harris and Ray, 1933] and a large variety of foodstuffs has since been tested, covering the widest possible range of biological activities, with relative values varying from about 4 to 300. From these analyses we have calculated the antiscorbutic activities of the various materials, again on the basis of the relation that 0·6 mg. of hexuronic acid is equal in antiscorbutic potency to 1 cc. of

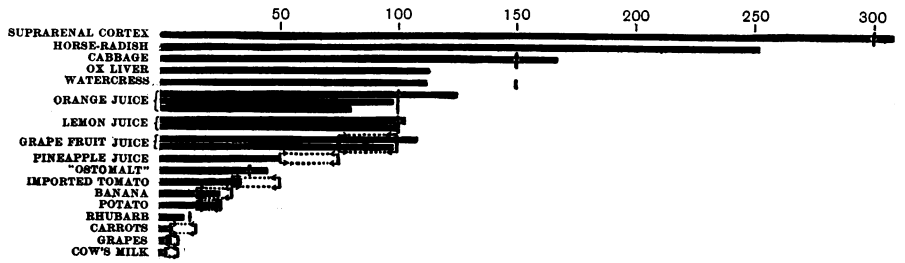

Fig. 3. *Antiscorbutic activities of foods.* Lengths of columns represent activities as calculated from hexuronic acid content, estimated chemically. Cross lines represent values of activities as determined biologically.

orange juice. It is perhaps the most compelling evidence that could be offered for the hexuronic acid theory, that in all cases the vitamin C value thus calculated from the hexuronic acid content agreed with the value as determined biologically. This is shown graphically in Fig. 3. The variation in antiscorbutic potency of different varieties of apples even is quantitatively confirmed from the hexuronic acid content (Table II).

5. *Agreement between ultra-violet absorption at 265* mμ *and antiscorbutic activity.*

The presence of an ultra-violet absorption band at 265 $m\mu$ [Bowden and Snow, 1932] suggested another means of checking the correlation between hexuronic acid and vitamin C activity. Observations by our colleague Dr W. J.

Table II. *Antiscorbutic value of apples.*

	Calculated from hexuronic acid content	Reputed value (determined biologically)
Bramley's Seedling, peel	128	150
Newton Wonder, peel	40	50
Bramley's Seedling, cortex	27	50–100
Newton Wonder, cortex	8	15
Edward VII, cortex	3	<7

Dann showed that in many natural sources it was unfortunately impossible to measure intensity of absorption at 265 $m\mu$ direct owing to the masking of the band by absorption in the same region by other constituents. It is possible however to make use of this criterion in the case of hexuronic acid or vitamin C concentrates, and also in certain classes of food materials; and measurements of absorption intensities upon lemon juice, orange juice, grape-fruit juice, and hexuronic acid itself, by Dr Dann gave values in good accord with our titration results and biological determinations (Table III).

Table III. *Hexuronic acid content.*

	As determined from intensity of absorption at 265 $m\mu$ (W. J. Dann)	As estimated by titration	As calculated from anti-scorbutic activity
Lemon juice	0·86	0·62	0·6
Orange juice	0·66	0·6–0·75	0·6
Grape-fruit juice	0·40	0·6	0·45–0·6

6. *Coincident disappearance of hexuronic acid and vitamin C activity during scurvy in guinea-pigs.*

In earlier papers [Harris and Ray, 1932; 1933] we reported that suprarenals of normal guinea-pigs had intense antiscorbutic activity, and that this activity disappeared with the development of scurvy. This loss of antiscorbutic activity ran parallel with the disappearance of hexuronic acid. This was demonstrated both qualitatively, by means of Szent-Györgyi's silver staining method (carried out by Dr Thomas Moore) [Moore and Ray, 1932] and quantitatively by analyses by the titration method (carried out by Mr T. W. Birch) (see Table IV). A

Table IV. *Effect of vitamin C intake on the hexuronic acid content of suprarenals in guinea-pigs.*

Amount of vitamin C received per day as supplement to scurvy-producing diet (in mg. of hexuronic acid)	Amount of hexuronic acid remaining in suprarenal, after 14 days on diet (in mg. per 1 g. of gland)
0	0·21
1	0·43
2	0·62

similar loss of hexuronic acid occurred from the liver, which again ran parallel with its loss of antiscorbutic activity in scurvy. Our biological estimations showing the loss of antiscorbutic activity have already been published [Harris and Ray, 1933].

7. *Synthesis of hexuronic acid parallel with vitamin C in other species.*

One of us [Harris, 1932] has recently shown that dogs are able to synthesise their own vitamin C when fed for prolonged periods on vitamin C-free diets. This has been confirmed in a further series of tests. Liver from such dogs administered to scorbutic guinea-pigs was found to cure the latter of scurvy (Fig. 4), in pronounced contrast with the effect of liver from guinea-pigs which

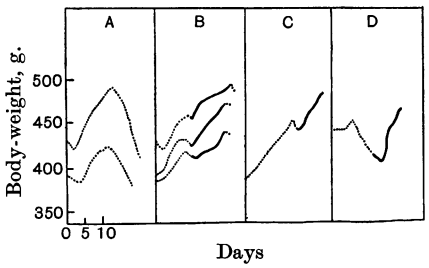

Fig. 4. Guinea-pig feeding tests, demonstrating synthesis of vitamin C by the dog. A, Negative controls; B, Extract of liver from dogs on vitamin C-free diet; C, Extract of liver from dog on complete diet; D, Positive control (5 g. cabbage per diem). ..... Vitamin C-free diet; —— Experimental diet.

had been similarly fed on a vitamin C-free diet. The suprarenals from these dogs were also tested in the same way, and, although not enough material was available for a long test, definite evidence of antiscorbutic activity was again obtained. Similar feeding tests with the suprarenals of rats kept on a vitamin C-free diet will be discussed in a later paper [1]. Chemical tests, both silver staining and estimation by the titration method on the suprarenals or livers of these two species, showed that the maintenance of vitamin C activity was associated with the presence of hexuronic acid, a result which should be compared with the simultaneous loss of activity and hexuronic acid in the guinea-pig.

8. *Destruction of hexuronic acid by oxidation, heat and alkali.*

The data given in Table V show that the conditions which favour the destruction of hexuronic acid are the same as those which are known to determine the destruction of vitamin C activity. That is, destruction is favoured by aeration, by alkalinity and by heat. The actual rate of destruction of hexuronic acid under different conditions of p_H, oxygen access, and temperature agree with what data are available for vitamin C activity. Where oxygen was rigidly excluded hexuronic acid was surprisingly stable to heat even in presence of alkali, which again agrees with the behaviour of the vitamin. Oxidation and not heat or alkalinity *per se* was the determining factor bringing about destruction, although of course such oxidation was accelerated with increased p_H and rise of temperature. There was evidence that when hexuronic acid was boiled in alkaline solution the amount destroyed depended on such "accidental" variables as: (1) the amount of oxygen already dissolved in the solution and (2) the oxygen which gained access to the solution after the boiling had stopped and before the titration could be begun. Under these circumstances it has been found difficult

[1] The suprarenal of the rat appears to be very rich in vitamin C, compared *e.g.* with that of the normal guinea-pig, a fact which may be of significance in view of the circumstance that the rat but not the guinea-pig is able to synthesise the vitamin.

Table V. *Effect of heat, aeration and* p_H *on destruction of hexuronic acid.*

	Percentage of hexuronic acid undestroyed (as determined by titration)
Orange juice, fresh	100
,, boiled 2 hours	34
,, aerated 2 hours	79
,, unheated	100
,, heated on water-bath, 1 hour	60
,, ,, 2 hours	30
Solution of hexuronic acid, at p_H 3·4	100
,, heated ½ hour	82·8
,, heated 1 hour	
Solution of hexuronic acid, at p_H 4·5	100
,, heated 1 hour	16·5
,, heated 2 hours	0
Solution of hexuronic acid, before treatment	100
,, after 15 minutes' aeration in alkali	9
,, after 30 ,,	0

to make direct quantitative comparisons between the exact rates of destruction of hexuronic acid and antiscorbutic potency. There can however be no doubt that there is good agreement both qualitatively, in the sense that the conditions which destroy hexuronic acid are the same as those which inactivate the vitamin, and also quantitatively, in the sense that the rates of destruction can be shown to be at least approximately parallel.

9. *Coincident synthesis of hexuronic acid and antiscorbutic activity by the plant.*

It is well known that in the vegetable kingdom synthesis of vitamin C occurs during germination. We have been able to show that hexuronic acid appears concurrently, as measured by this titration method (Table VI is representative of several experiments).

Table VI. *Synthesis of hexuronic acid during germination.*

	Hexuronic acid content	
	mg. per g.	mg. in single seed or seedling
Seed peas, before germination	0·00	0·00
,, soaked for 24 hrs. (not germinated)	0·08	0·02
,, ,, 48 hrs. (germinated)	0·69	0·21
,, ,, 72 hrs. (germinated)	0·82	0·26
,, ,, 96 hrs. (germinated)	0·86	0·27
Oatmeal, ground	0·00	—
Oats, whole grain	0·11	—
,, after 96 hrs. germination	0·20	—
,, after 120 hrs. germination	0·42	—

Discussion.

The results given in the experimental section may be expressed by the generalisation that a given amount of hexuronic acid invariably possesses the same biological activity. This statement applies, as we have shown, not only to

its distribution throughout nature (over 30 natural sources, with relative hexuronic acid contents ranging from 1 to 300 having been examined), but also to the specimens of hexuronic acid recovered from various sources (and representing only a fraction of the full amount present), to preparations of hexuronic acid repurified through chemical means and then repeatedly recrystallised, to its disappearance and synthesis in the animal and plant organisms, and to its destruction by chemical means. Now, we found full antiscorbutic activity for specimens of hexuronic acid which were at least 99 % pure, judging by our titration curve results [Birch and Harris, 1933], by iodine titration, or by elementary analysis. In other words, therefore, one alternative only remains to our conclusion that hexuronic acid is identical with the vitamin. That is, it would be necessary to assume that wherever hexuronic acid is found or however it is treated it invariably occurs combined with the same unvarying but minute amount (less than 1 %) of some unspecified contaminant—*i.e.* that this hypothetical substance accompanies it throughout nature, and, in unalterable proportion, clings to it throughout the process of recovery, and is synthesised with it, is utilised with it and is destroyed with it. In the absence of any positive evidence in favour of such a supposition (and it would appear unreasonable to interpret so extended an association as a mere matter of coincidence) it seems only logical to conclude that hexuronic acid is itself the vitamin.

Summary.

Experiments have been carried out with the object of ascertaining whether antiscorbutic activity is an inherent property of hexuronic acid itself or due to some associated impurity. The evidence is consistently in favour of the former view, an unvarying and very extensive correlation having been established between hexuronic acid and antiscorbutic activity, as follows:

1. In the first place it was shown that the antiscorbutic activities of several natural sources were approximately proportional to the amounts of hexuronic acid recoverable from them: thus suprarenal cortex (ox), not hitherto recognised as an antiscorbutic, had three times the potency of orange juice.
2. Specimens of hexuronic acid from different sources (suprarenal; paprika) were assayed biologically and found to have identical antiscorbutic activity.
3. Hexuronic acid after repurification processes and recrystallisation always maintained its original activity.
4. The hexuronic acid contents of over 30 different natural sources (with relative hexuronic acid contents varying from 1 to 300) as determined chemically (see following paper) were shown to account quantitatively for their known antiscorbutic activities.
5. In the case of several materials it was also possible to estimate the hexuronic acid content by intensity of absorption at 265 $m\mu$ (carried out by W. J. Dann), and this result was also in agreement with the biological and chemical tests.
6. The rate of destruction of hexuronic acid under varying conditions of aeration, heat and alkali was found to resemble that of antiscorbutic activity.
7. In the guinea-pig the antiscorbutic activity of the suprarenal (or liver) was lost with the development of scurvy and this coincided with the disappearance of hexuronic acid.
8. With other species, the rat or dog, which are able to synthesise their own vitamin when none is provided in the diet, the antiscorbutic activity and the hexuronic acid both remain unaffected.

9. Hexuronic acid is synthesised concurrently with antiscorbutic activity by the plant on germination.

The antiscorbutic activity of hexuronic acid has been determined by several alternative methods (curative, tooth-structure and preventive). The amount equivalent to 1·0 cc. of orange juice is greater than 0·33 mg. and less than (or equal to) 0·66 mg.; best value = 0·6 mg.; or "minimum protective dose" = 0·9 mg. per guinea-pig per day. We recommend its adoption as international standard.

Generous gifts of hexuronic acid from Prof. Szent-Györgyi's laboratory have greatly facilitated this work.

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