CXXXIX. ON THE NATURE OF THE PRECURSOR OF THE VITAMIN C IN THE VEGETABLE KINGDOM.

I. VITAMIN C IN THE GROWING PEA SEEDLING.

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In an earlier paper [Harris and Ray, 1933], it was shown that ascorbic acid appears in pea seeds as soon as germination begins. Since then the rate of production of ascorbic acid in the seedlings of such pea seeds has been examined, and the result is embodied in Table I. For the estimation of the acid, the

Table I.

Period of germination	Wt. of 20 seedlings g.	Amount of ascorbic acid mg./g. seedlings
12 hours (seeds kept soaked in water)	0·18	0·00
48 hours (germinated)	0·29	0·23
60 ,, ,,	0·60	0·43
90 ,, ,,	1·70	0·50
115 ,, ,,	2·60	0·50
140 ,, ,,	2·50	0·48
170 ,, ,,	4·2	0·49
12 days ,,	4·8	0·49

micro-chemical method of Birch *et al.* [1933] was used. Yellow-pea seeds were kept in sand slightly moistened with water, and after various periods of germination were taken out and washed with water to remove the adhering sand, and the cotyledons were excised away. The separated portions (here termed the seedlings) were dried between two filter-papers and ground up with a little sand and sufficient 20 % trichloroacetic acid to give a final concentration of the latter of about 5 %. The extracts were made up to a suitable volume and filtered, and the filtrates were titrated against a standard solution of 2 : 6-dichlorophenolindophenol. As a rule, 20 seedlings were used for each estimation.

As will be noticed, the amount of ascorbic acid present per g. of the wet seedling tissue increases within a few hours from 0 to a constant value which is not affected by any further growth. This is represented graphically in Fig. 1.

It may, however, be remarked that though the young seedlings are quite rich in vitamin C, the whole of the vitamin content is not confined to them. Judged from separate titration results on the seedlings and the cotyledons, the latter have been found to account for more than 5/6 of the total reducing power. It is doubtful, however, whether the whole of the reducing power of the cotyledons is due to ascorbic acid. It is known that a few substances like cysteine reduce the dye under the conditions of the experiment. As extracts made from the cotyledons show a marked nitroprusside reaction, and as Van Eekelen *et al.* [1934] have

shown that part of the reducing material in the cotyledons is precipitated by mercuric acetate, it seems probable that some free cysteine may be present in them. Johnson [1933] has also found that extracts from germinating peas show much less antiscorbutic value than was to be expected from the titration results. On the other hand extracts from seedlings alone show no nitroprusside reaction,

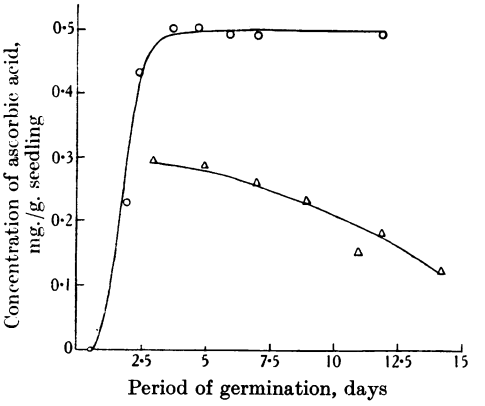

Fig. 1. Curve showing rate of production of ascorbic acid during various periods of germination or cultivation.

o, seedlings grown naturally with the cotyledons.
Δ, seedlings grown artificially on a medium containing fructose.

and they reduce the indicator solutions as rapidly as those of pure ascorbic acid solutions. These findings confirm those of Johnson, who also believes that the reducing power of seedlings is due to ascorbic acid alone.

Nature of the precursor of vitamin C in plants.

Though the synthesis of vitamin C during germination has been known for a long time, no attempt seems to have been made to ascertain the nature of the substance or substances from which it is formed. In their classical work Brown and Morris [1890] had shown that embryos of barley, excised out of the endosperm and cultivated upon nutrient solution, grew steadily at the expense of the carbohydrates or other organic nutrient substances present in the solution. It was thought that by utilising this method, *i.e.* by growing the embryo seedlings of peas on nutrient solutions containing various organic compounds and examining in the different samples whether ascorbic acid is formed or not, some information about the nature of the precursor might be obtained. Experiments in these directions have been successful and some very interesting results have been obtained. These will be discussed below.

Experimental.

The mode of experiment usually adopted was to place the excised embryo seedlings on a suitable semi-solid medium. It is essential that the embryo seedlings should rest on the medium and not be immersed in it, as in the absence of air growth and production of ascorbic acid do not take place.

It was found that a 10 % gelatin mixture constitutes an ideal medium for the cultivation of the seedlings. Gelatin itself is not assimilable by the young

plant and the 10 % solution forms a suitable semi-solid gel, whose viscosity is sufficient to prevent the embryo seedlings from sinking in it, but not great enough to stop the radicles from piercing it and absorbing the proper nutrient substances. In order to provide sufficient nitrogen and other elements necessary for a growing plant, the gelatin was dissolved in the proper quantities of the following solution (Knopp's solution):

Calcium nitrate	0·8 g.
Potassium nitrate	0·2 g.
Potassium dihydrogen phosphate ...	0·2 g.
$MgSO_4,7H_2O$	0·2 g.
Ferric phosphate	Trace
Water	1000 ml.

The various organic substances to be tested were dissolved in this solution in a concentration of 5 %.

At first, great difficulty was experienced in keeping the medium free from bacterial contamination. If so infected, the seedlings failed to grow and little or no ascorbic acid could be detected in them. The following procedure was, however, found to give satisfactory results, and in most cases no appearance of bacterial life or signs of fermentation could be seen for the experimental period of 7 days.

1 g. of purified gelatin is dissolved in 10 ml. of the Knopp's solution containing 0·5 g. of the experimental compound. While still hot and liquid, the gelatin mixture is transferred to a 25 ml. wide-mouthed flask, fitted with a cotton-wool stopper. The flask with the mixture is then sterilised by steaming for half an hour on 3 successive days. It is then left aside for the gelatin to set. In certain cases where it was feared that the drastic steaming process might hydrolyse or decompose the experimental organic substance, the latter was not added at first, but after the gelatin mixture (8 ml.) had been sterilised the compound was dissolved in a small amount (2 ml.) of sterilised Knopp's solution and was added to the gelatin mixture before it had set. Such cases are marked in Table II with an asterisk.

Before the embryo seedlings are excised, the pea seeds are soaked in sterile distilled water for 6 hours. The seed-coats are then removed and with a little pressure the two cotyledons can be separated easily and the embryo seedling taken out by means of a pair of forceps with bent heads. The seedlings are then washed with several changes of sterilised distilled water, and finally placed on the solidified nutrient gelatin. These operations of washing and transference should be carried out as far as practicable in a dust-free room.

The seedlings after suitable periods of cultivation (usually 7 days unless otherwise mentioned) are taken out and suspended in warm water (37°) to remove any adhering gelatin. They are then dried between two filter-papers and extracted as usual with sand and trichloroacetic acid. The ascorbic acid in the extracts is estimated by titration against the standard 2 : 6-dichlorophenol-indophenol solution. The results are given in Table II.

From the data (Table II) it may be seen that while little reducing substance is produced in the embryo seedling when kept at about 20° on 10 % nutrient gelatin alone, a considerable amount appears when certain substances are introduced into the medium. Of these, hexoses seem to function best. The relative ascorbic acid-producing power of the individual hexoses seem to be more or less the same, mannose being, however, an exception, the seedlings being able to generate much more ascorbic acid from mannose than from any of the other three hexoses

Table II. *Results after 7 days' cultivation.*

Nature of the medium	Wt. of 20 seedlings	Amount of ascorbic acid, mg./g. wet seedling tissue
10 % gelatin solution alone	0·49	0·049
,, ,, ,,	0·46	0·050
,, ,, +glucose	0·87	0·194
,, ,, ,,	0·40	0·250
,, ,, ,,	0·80	0·225
,, ,, +fructose	0·80	0·300
,, ,, ,,	1·03	0·281
,, ,, ,, *	0·93	0·264
,, ,, +galactose	0·48	0·237
,, ,, ,,	0·85	0·168
,, ,, ,, *	0·55	0·201
,, ,, +mannose	0·37	0·540
,, ,, ,,	0·36	0·691
,, ,, +sucrose	0·98	0·275
,, ,, ,,	0·55	0·320
,, ,, ,, *	0·60	0·310
,, ,, +lactose	0·60	0·130
,, ,, +maltose	1·15	0·208
,, ,, +arabinose	0·63	0·0
,, ,, ,, *	0·50	0·0
,, ,, +xylose	0·74	0·120 (?)
,, ,, ,, *	0·63	0·027
,, ,, ,, *	0·60	0·054
,, ,, +starch	0·57	0·061
,, ,, +dextrin	0·78	0·078
,, ,, +mannitol	0·43	0·039
,, ,, +sorbitol	0·43	0·0
,, ,, +inositol	0·50	0·054
,, ,, +sodium pyruvate	0·15	0·0
,, ,, +methylglyoxal	0·15	0·0
,, ,, +sodium glutamate	0·20	0·0
,, ,, +glycerol	0·92	0·079
,, ,, +sodium lactate	0·18	0·0
,, ,, +sodium glycerophosphate*	0·20	0·0

* Substance added to gelatin afterwards (see p. 998).

examined. Disaccharides come next in the list but here it is possible that ascorbic acid is not derived from the disaccharides themselves but from the products of their enzymic hydrolysis. Polysaccharides seem to yield a small, though definite, amount of ascorbic acid, but here again it is doubtful whether they themselves or secondary products are responsible. Pentoses yield consistently negative results; the high value obtained in one case from xylose is probably due not to xylose but to the decomposition products arising during the drastic process of long steaming. Of various other compounds examined only glycerol seems to have a little power while all the rest either gave absolutely negative values or values equal to that given by control seedlings grown on nutrient gelatin alone.

It seems very probable, therefore, that ascorbic acid may be produced by the growing embryo from the hexoses present in the seeds. On the other hand, the concentration of ascorbic acid in the seedlings germinated on hexose media is rather low (0·2–0·3 mg. as compared with 0·5 in seedlings grown with their natural cotyledons); mannose, however, is an exception, the concentration of ascorbic acid being similar to that found under natural conditions. These rather low values may mean that the real precursor is perhaps a substance different from the hexoses. If such a substance exists, however, it is quite evident that its addition is not essential for the production of ascorbic acid and that embryo seedlings are well equipped with enzyme systems to convert ordinary hexoses into this hypothetical substance. About the course of the conversion of hexoses

into ascorbic acid nothing is known. Attempts to grow the seedlings on media containing some of the well-known carbohydrate breakdown products have failed (see Table II). The presence of these substances in the nutrient gelatin seems to inhibit growth, and no trace of ascorbic acid can be detected in the seedlings. Similarly all efforts to elucidate the nature of the enzyme system responsible for this synthesis have been unsuccessful. Attempts to produce ascorbic acid from sugar solutions by means of minced or crushed embryo seedling tissue have only given negative results.

Another interesting thing to be observed from Table II is that there seems to be no direct relation between extent of growth and production of ascorbic acid. Thus the seedlings which are grown on mannose show very little growth, whereas they are exceptionally rich in ascorbic acid. On the other hand, arabinose, xylose, dextrin, all seem to favour growth, but the production of ascorbic acid in these cases is either nil or very low. Substances like fructose seem to favour both growth and production of ascorbic acid.

Antiscorbutic activity of seedlings grown on nutrient gelatin.

Though the chemical determination of ascorbic acid is fairly specific it has certain limitations and may give high values in certain cases [Birch *et al.*, 1933; Harris, 1933]. In view of this it was thought best to check the chemical estimation against direct feeding tests. The biological determination gave results in complete agreement with the indophenol titration. The seedlings used for this purpose

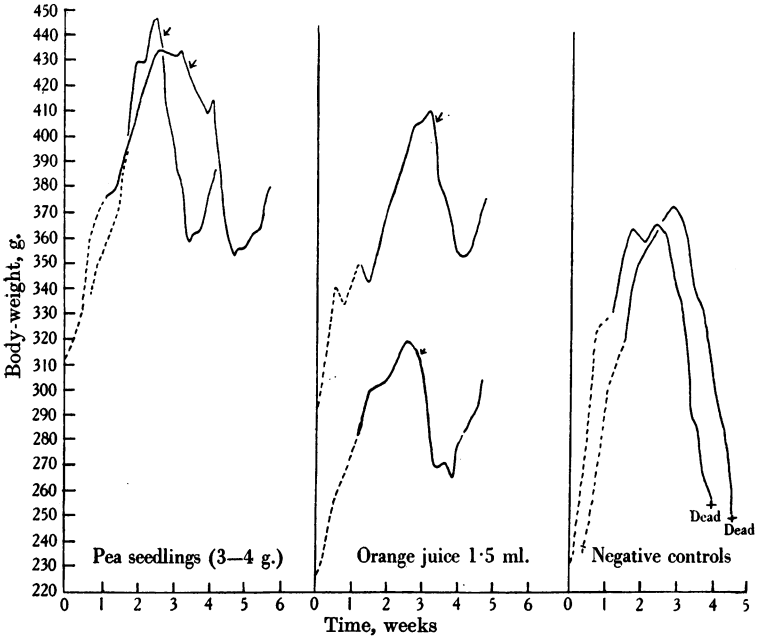

Fig. 2. Weight curves of guinea-pigs, comparing degrees of cure of scurvy with doses of pea seedling and orange-juice.

- - - - -, preliminary complete diet.
———, vitamin C-free diet.
↓, experimental dosing commenced.
+, animal died.

were grown for 7–10 days on nutrient gelatin containing fructose. As it was practicable to grow only a limited amount of these only one level of dosage could be employed. Two guinea-pigs were used for this test and the technique of assay was the curative method described in a previous paper [Harris and Ray, 1932]. A daily dose of 3–4 g. (ascorbic acid content as determined by indophenol titration being 0·75–0·9 mg.) was given to each of the animals. A control group of two guinea-pigs received 1·5 ml. of orange-juice (ascorbic acid content determined chemically about 0·9 mg.) and negative controls were kept on the basal diet alone. The individual growth curves are given in Fig. 2.

3–4 g. of the seedling were found to have nearly the same biological activity as 1·5 ml. of orange-juice. This result corresponds within the limits of experimental error with that calculated from the chemical titration. This shows that the reducing power of the seedling is due solely to the ascorbic acid.

Rate of production of ascorbic acid in seedlings grown on nutrient gelatin containing fructose.

It has already been remarked that using different substances as substrate, no correlation can be detected between growth and synthesis of ascorbic acid. It seemed of interest to examine whether any such correlation exists when the seedlings are cultivated for various periods on media containing one and the same substance. For this purpose, embryo seedlings were grown on media containing fructose (5 %) as the substrate. In order to avoid formation of secondary products during the steaming process, the fructose dissolved in a little sterilised Knopp's solution was added to the gelatin mixture after the latter had been steamed. A large number of flasks each containing 25 embryo seedlings were used and after the proper time of cultivation these were opened, 20 of the best seedlings being taken out for every day's estimation. The result is given in Table III.

Table III.

Period of cultivation days	Wt. of 20 seedlings g.	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
3	0·58	0·170	0·293
5	0·71	0·200	0·282
	0·63	0·180	0·285
7	0·93	0·246	0·264
9	1·35	0·318	0·235
11	1·10	0·165 (?)	0·150 (?)
12	1·20	0·216	0·180
14	1·40	0·177	0·127

It will be noticed that as a rule the greater the growth, the less was the concentration of the ascorbic acid formed, but no simple relation between the two appeared to exist; on the other hand, the concentrations when plotted against the periods of cultivation were found to form a continuous curve (see Fig. 1). The shape of the curve shows that ascorbic acid was produced in great quantities during the first 3 or 4 days of cultivation; afterwards however the rate of formation fell and became zero after about 10 days. After that period some of the ascorbic acid seemed to disappear. On the other hand, the rate of growth continued unchanged and as a consequence the concentration steadily fell. This behaviour is entirely different from that seen in seedlings grown with their cotyledons, the concentration of ascorbic acid rises in these conditions to a constant value.

Correlation between concentration of substrate and production of ascorbic acid.

A further lack of correlation between growth and the synthesis of ascorbic acid was observed when the production of ascorbic acid in seedlings grown for 7 days on media containing varied amounts of fructose was measured (Table IV).

Table IV.

Percentage concentration of sugar solution	Wt. of 20 seedlings	Total amount of ascorbic acid, mg. in the 20 seedlings	Ascorbic acid, mg./g. seedling
1·0	1·2	0·100	0·083
2·5	1·4	0·217	0·155
5·0	0·93	0·246	0·264
7·5	0·80	0·210	0·263
10·0	0·60	0·156	0·260

It is at once apparent that the rate of growth and the rate of production of ascorbic acid follow two different lines, the optimum concentration of the substrate for growth being about 2·5 %, while for the production of ascorbic acid it is much higher, about 5 %. Further the two rates plotted against the concentration form two different types of curves emphasising thereby that the two phenomena are quite different and possibly independent of each other (see Fig. 3).

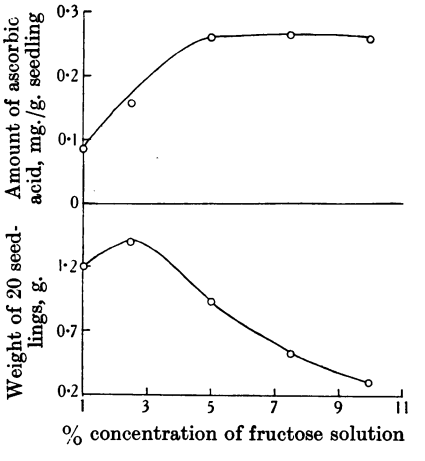

Fig. 3. Relation of rate of production of ascorbic acid by pea seedlings (upper curve) and rate of growth (lower curve) to the concentration of the substrate in the medium on which the seedlings are grown.

Cultivation of seedlings on media containing a mixture of substances.

From Table II it was seen that while some substances favour growth, others seem conducive to the production of ascorbic acid. Attempts were made to combine the two properties, *i.e.* to have good growth together with large production of ascorbic acid. For this embryo seedlings were grown for 7 days on media containing a mixture of two substances—the one favouring growth and the other production of ascorbic acid. As however the results in Table V show, these experiments have not been successful. The volume of medium was 10 ml.

Table V.

Nature of medium	Wt. of 20 seedlings, g.	Concentration of ascorbic acid, mg./g. seedling
0·1 g. fructose + 0·5 g. mannose	0·27	0·596
0·5 g. arabinose + 0·5 g. mannose	0·15	0·553

The presence of mannose seems to inhibit growth altogether.

SUMMARY.

1. The rate of production of ascorbic acid in pea seedlings germinated naturally has been estimated. The amount formed was found to rise for 2 days and then reach a constant concentration of 0·5 mg. of the acid per g. of the wet weight of the seedlings.

2. Embryo seedlings from peas were excised from the cotyledons and grown on nutrient gelatin. Seedlings cultivated on such a medium, containing hexoses, were found to have synthesised a large amount of ascorbic acid. Of the four hexoses examined, mannose gave the highest figure, 0·5 mg. of ascorbic acid per g. of seedling. Disaccharides also gave moderately high figures but all other substances examined either gave very low or absolutely negative values. It is suggested that hexoses may serve as precursors of vitamin C in germinating seeds.

3. The rate of production of ascorbic acid in the cultivated excised seedlings bore no direct relation to the rate of growth, *e.g.* seedlings grown on mannose showed very little growth but had a high concentration of ascorbic acid while arabinose and xylose seemed to favour growth but production of ascorbic acid was nil.

4. The concentration of ascorbic acid present per g. in the excised seedlings fell steadily during prolonged periods of cultivation.

5. The concentration of ascorbic acid formed was approximately proportional to the concentration of the substrate up to a certain optimum value, after which it remained constant.

6. Unsuccessful attempts were made to produce good growth combined with large production of ascorbic acid by cultivating excised seedlings on a medium containing a mixture of two substances—one of them found to favour growth and the other production of ascorbic acid.

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