The authors wish to express their cordial thanks to Mr R. Venkatraman, Superintendent, Fisheries Technological Station, Kozhikode, Madras, for supplying the sample of oil which made the present work possible.

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The Nature of the Effect of Ammonium Sulphate on the Biosynthesis of Ascorbic Acid in Plants

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(Received 9 August 1955)

Ever since Ray (1934) showed that excised pea embryos could synthesize ascorbic acid on a semi-solid medium containing hexoses, it has been known that ascorbic acid may be formed from carbohydrate sources. However, the first direct evidence for the hypothesis was provided by Jackel, Mosbach, Burns & King (1950), who demonstrated a conversion of uniformly labelled [^{14}C]glucose into uniformly labelled ascorbic acid in the rat. Previous work based essentially on the increased excretion of ascorbic acid when various precursors were administered had not been conclusive, owing to the well-known stimulatory effect of various substances, some of which could not, by their very nature, be precursors (Longenecker, Musulin, Tully & King, 1939). The work of Jackel

et al. (1950) provided convincing evidence that glucose served as a precursor for this vitamin.

An analogous position is found when plants are considered. Vitamins of the B group and a number of amino acids, as well as intermediates of the citric acid cycle, have all been found to stimulate ascorbic acid synthesis (Bharani, Shah & Sreenivasan, 1953). Hence it was desirable to obtain direct evidence for the conversion of glucose into ascorbic acid in plants as well, by means of an isotopic technique.

That glucose is the most likely precursor has been shown by the work of Isherwood, Chen & Mapson (1954). Their work has indicated that glucose may be converted into ascorbic acid according to the sequence shown in Fig. 1. The

Fig. 1. Conversion of glucose into ascorbic acid.

hypothesis is based mainly on the increase in ascorbic acid synthesis produced by the intermediates of this scheme in germinating seedlings. The work of Horowitz & King (1954) and the well-known similarity of the synthetic mechanisms in plants and animals also lend strong support to such an hypothesis.

The biosynthesis of ascorbic acid in plants is known to be affected by various salts, and the evidence obtained from the studies carried out by various workers has been generally conflicting and difficult to interpret. The work of Mapson and others (Mapson & Cruickshank, 1943; Mapson, Cruickshank & Chen, 1949) has thrown much light on this question. They showed that the effect of a salt such as ammonium sulphate, chloride or succinate was dependent on the nature of the anion combined with the NH_4^+ radical. If this anion was not utilizable by the plant, as with other ions, e.g. Cl^- , SO_4^{2-} , there was a marked depressant effect, which was related to the pH changes induced by the particular salt in the cell sap. On the basis of their results, they suggested that the salts probably acted by reducing the efficiency of conversion of a 'hexose precursor' into ascorbic acid.

However, other explanations have also been suggested by various workers (Nightingale, 1948; Åberg & Ekdahl, 1948). In view of the existing situation, the effect of ammonium sulphate—a typical inhibitory salt—has been studied in detail. The studies presented herein show conclusively that this salt does inhibit the conversion of glucose into ascorbic acid, and that the step inhibited is most probably the conversion of glucose into glucuronic acid.

EXPERIMENTAL

In the present investigation, healthy green gram seeds (*Phaseolus radiatus*) were used. Germination of the seeds was done under aseptic conditions, the seedlings being cultured in sterile, 9 cm. Petri dishes on disks of Whatman no. 1 filter paper. The germinations were all carried out at room temp., which varied between 26° and 30°. The procedure adopted in germinations was as follows.

The seeds (2 g., accurately weighed) were sterilized by standing in 0.1% HgCl_2 solution for 2–3 min. and then washing off the HgCl_2 thoroughly with sterile, glass-distilled water. These seeds were then transferred to a sterile Petri dish, containing the medium, in the vicinity of a Bunsen flame. The Petri dishes, as well as the culture media, were sterilized by autoclaving at 15 lb. pressure for 15 min., then dried overnight at 100° and allowed to cool. All transfers of solutions into the dishes were afterwards done in the neighbourhood of a Bunsen flame, under aseptic conditions. Commonly 10 ml. of glass-distilled water was added to each dish, and the compounds whose effects were to be investigated were dissolved in this.

In radioactive experiments [^{14}C]glucose was added as

a sterile solution having a total count of the order of $10^6/\text{min}$. The glucose thereby included in the culture medium was approximately 0.2 mg.

Except where ascorbic acid was included in the medium, when it was dissolved in sterile glass-distilled water, and a definite amount of this solution was added to the rest of the culture medium which had been separately sterilized, all solutions were sterilized by autoclaving at 15 lb. pressure for 15 min.

Estimation of ascorbic acid. The total ascorbic acid of seedlings was estimated by the method of Roe & Kuether (1943) after they had been ground up with 4% metaphosphoric acid, centrifuged and made up to volume. These values were checked by titration against dichlorophenolindophenol in the usual manner.

Isolation and measurement of radioactive ascorbic acid. For isolation of radioactive ascorbic acid, the germinated seedlings were washed thoroughly with excess of glass-distilled water and ground up with 5% oxalic acid (A.R.). The extract was centrifuged, ascorbic acid estimated with indophenol, and enough carrier was added to bring the total content to 100 mg. This extract was treated with solid neutral lead acetate till there was no more precipitation, and the last traces of lead acetate were removed with freshly prepared saturated oxalic acid solution, added drop by drop. The extract was then centrifuged and the ascorbic acid in the supernatant adsorbed on an Amberlite IR-4B column prepared according to Jackel, Mosbach & King (1951). The method adopted for elution of ascorbic acid, its isolation as the osazone with 2,4-dinitrophenylhydrazine and subsequent purification of the osazone by recrystallization from boiling absolute ethanol-acetone (1:1, by vol.) to constant specific activity, was similar to that of Jackel *et al.* (1951).

Radioactive measurements were carried out with the aid of a Tracerlab flowgas counter (Tracerlab Inc., Boston, Mass.) connected to an autoscaler. At the counting rates employed, the counting was accurate to within $\pm 1.5\%$. The osazone was counted in steel planchets, after dispersion in acetone and drying to a thin layer. Whenever comparisons of radioactive conversions were made, identical amounts (10 mg.) of osazone were counted, so that the results were strictly comparable.

RESULTS

Phaseolus seeds were germinated in presence of uniformly labelled [^{14}C]glucose, with and without the addition of ammonium sulphate, and the extent of conversion of glucose under the two conditions was studied. Ammonium sulphate was chosen as typical of salts which inhibit ascorbic acid synthesis as shown by the earlier work of Mapson *et al.* (1949). The solution added to the seeds was so adjusted that the entire amount was quantitatively absorbed during the 72 hr. germination period, and this amount of solution was determined by a preliminary experiment. Such a procedure was found necessary because the seedlings treated with ammonium sulphate absorbed slightly less than untreated ones for the same period of germination. The results presented in Table 1 show that [^{14}C]glucose is converted into

ascorbic acid by the seedlings, and that the percentage conversion is of the same order as that found in the rat by Jackel *et al.* (1950) and Horowitz & King (1952). These latter workers had found that during a 24 hr. period rats could convert between 0.3 and 0.8 % of [^{14}C]glucose into ascorbic acid. Apparently both animals and normal plants utilize glucose to approximately similar extents for synthesis of vitamin C. Further, the results obtained show that this conversion in *Phaseolus* seeds is definitely inhibited by ammonium sulphate, as

Table 1. *Effect of ammonium sulphate on the conversion of [^{14}C]glucose into ascorbic acid in Phaseolus seedlings*

Seeds (2 g.) germinated for 72 hr. Radioactivity expressed in counts/min.

	Seeds grown with	
	[^{14}C]Glucose alone	[^{14}C]Glucose + 50 mg. of ammonium sulphate
Ascorbic acid content (mg.)	2.06	1.06
Radioactivity in glucose supplied	3.347×10^6	2.757×10^6
Radioactivity in ascorbic acid synthesized	2.545×10^4	1.067×10^4
Conversion of glucose (%)	0.7603	0.3870

amounts of ammonium sulphate added in (c) and (d) were also equal. At the end of 24, 48 and 72 hr., the total ascorbic acid content of the seedlings was determined. Where ascorbic acid was supplied, 5 mg. was added as a freshly prepared solution in sterile glass-distilled water, once every 24 hr. The results presented in Table 2 show that germinating seedlings can take up and retain ascorbic acid when it is supplied under both conditions. The difference in ascorbic acid content between seedlings germinated as in (c) and (d) is the same, within limits of experimental error, as the corresponding difference between (a) and (b), showing that the presence of ammonium sulphate makes no difference in the net amount of ascorbic acid present under these conditions.

Since ammonium succinate has been shown by Mapson *et al.* (1949) to have little effect on the synthesis of ascorbic acid in germinating seedlings, the experiment was repeated with ammonium succinate replacing ammonium sulphate. The results are shown in Table 3.

The differences in ascorbic acid content given in Tables 2 and 3 show the same pattern, although in the one case the salt used is inhibitory and in the other it is not inhibitory. Thus it is apparent from these results that ammonium sulphate inhibits only ascorbic acid synthesis and not its catabolism, and that it inhibits the synthesis of ascorbic acid

Table 2. *Effect of ammonium sulphate on the uptake and metabolism of ascorbic acid in Phaseolus seedlings*

Seeds (2 g.) in 10 ml. of culture medium; ascorbic acid values in mg./100 g. of seeds.

Germination details	24 hr.		48 hr.		72 hr.	
	Ascorbic acid	Diff.	Ascorbic acid	Diff.	Ascorbic acid	Diff.
(a) Control	65.0	35.0	75.0	90.0	122.5	147.5
(b) Ascorbic acid supplied (5 mg. once every 24 hr.)	100.0		165.0		270.0	
(c) $(\text{NH}_4)_2\text{SO}_4$ (50 mg.)	37.5	37.5	50.0	105.0	60.0	145.0
(d) $(\text{NH}_4)_2\text{SO}_4$ as in (c) + ascorbic acid as in (b)	75.0		155.0		205.0	

shown by the correspondence between the ascorbic acid values as determined by analysis and the percentage incorporation of radioactivity under the two conditions.

To study whether the presence of ammonium sulphate in the culture medium was leading to an increased catabolism of ascorbic acid as compared to untreated seedlings, the following investigations were carried out. *Phaseolus* seeds were germinated under four different conditions: (a) with water alone, (b) with ascorbic acid, (c) with ammonium sulphate, and (d) with ammonium sulphate and ascorbic acid. Identical amounts of ascorbic acid were added to (b) and (d) and the

Table 3. *Effect of ammonium succinate on uptake and metabolism of ascorbic acid in Phaseolus seeds*

Seeds (2 g.) germinated for 72 hr.

Germination details	Ascorbic acid (mg./100 g. of seeds)	Diff.
Control	140.0	100.0
Ascorbic acid (5 mg. every 24 hr.)	240.0	
Ammonium succinate (55 mg.)	125.0	105.0
Ammonium succinate (55 mg.) + ascorbic acid as above	230.0	

from glucose. Since Isherwood *et al.* (1954) have recently investigated some of the intermediate stages in the conversion of glucose into ascorbic acid, it was thought worth while to study the influence of ammonium sulphate on the utilization of glucurono- γ -lactone by the seedlings. For this purpose, *Phaseolus* seeds were germinated with ammonium sulphate and with ammonium sulphate + glucurone, and the ascorbic acid synthesis under these conditions was compared with the corresponding synthesis in absence of the salt. Since it was found that the addition of glucurone at the beginning of the experiment tended to inhibit the germination, this compound was added after the seedlings had grown for 24 hr. The results in Table 4 indicate that glucurone causes the same increase in ascorbic acid in the seedlings, irrespective of the presence or absence of ammonium sulphate in the medium. The utilization of glucurone is therefore unaffected by the salt.

Table 4. *Effect of ammonium sulphate on the utilization of glucurone by Phaseolus seedlings*

Seeds (2 g.) germinated for 72 hr.		
Germination details	Ascorbic acid (mg./100 g. of seeds)	Diff.
Water only	120.0	90.0
Glucurone (50 mg.)	210.0	
(NH ₄) ₂ SO ₄ (50 mg.)	67.5	92.5
(NH ₄) ₂ SO ₄ (50 mg.) + glucurone (50 mg.)	160.0	

DISCUSSION

As was pointed out above, the work of Mapson & Cruickshank (1943) and Mapson *et al.* (1949) strongly suggested that the inhibitory effect of a salt such as ammonium sulphate was probably on the efficiency of conversion of a 'hexose precursor' into ascorbic acid. Evidence presented in the present paper shows that the precursor is glucose.

Alternative explanations of the salt effect are the higher rate of respiration induced by the nitrogen supply (Nightingale, 1948), and a depressed availability of 'photosynthetic products available for synthesis' by stimulation of other competitive processes (Åberg & Ekdahl, 1948). But it will be obvious from the present study that the first explanation would imply a more rapid destruction of ascorbic acid treated with ammonium sulphate, whereas the second implies that less glucose is made available for synthesis. The results presented in Tables 2 and 3 show that neither a depressant salt, such as ammonium sulphate, nor one that has little effect, such as ammonium succinate, affects the uptake and accumulation of ascorbic acid by the plant. If the ascorbic acid

absorbed by the plant from the medium is subject to the same metabolic processes as the ascorbic acid synthesized by the plant—an assumption that is justified in view of the normal physiological range of concentrations of ascorbic acid employed—and if an altered metabolism were to be taking place in presence of the inhibitory salt, one would expect the difference between (c) and (d) in Table 2 to be less than the corresponding difference between (a) and (b). But since this is not so, it is obvious that ammonium sulphate has an adverse effect only on the biosynthesis and not on the catabolism of ascorbic acid.

The results in Tables 1 and 4 taken together are particularly interesting since they reveal, for the first time, that ammonium sulphate, at a level which reduces the synthesis of ascorbic acid by as much as 50 %, does not, however, affect the conversion of glucurono- γ -lactone into ascorbic acid. The entire inhibitory influence appears to centre round the conversion of glucose rather than that of glucurone. The results show that, of the various steps in the sequence

only step A is inhibited by ammonium sulphate.

Mapson *et al.* (1949) have shown that the presence of a salt such as ammonium sulphate increases the acidity of the cell sap, and it seems likely that under these conditions the enzyme system involved in the conversion of glucose into glucuronic acid is adversely affected.

SUMMARY

1. The conversion ratio of [¹⁴C]glucose into ascorbic acid is of the same order in *Phaseolus* seeds as that established by earlier workers for rats.

2. Ammonium sulphate inhibits the conversion of uniformly labelled [¹⁴C]glucose into ascorbic acid. This inhibition accounts quantitatively for the observed decrease in ascorbic acid content of seedlings grown in the presence of the inhibitory salt.

3. Ammonium sulphate has no effect on the catabolism of ascorbic acid. The lowered ascorbic acid content found in the seedlings grown with this salt is attributable only to a decreased synthesis.

4. Ammonium sulphate inhibits only the utilization of glucose and not that of glucurono- γ -lactone in the *in vivo* synthesis of ascorbic acid in *Phaseolus* seedlings.

We wish to thank Corn Products Co. Ltd., New York, U.S.A., for their generous gift of the glucurone used in the above investigation. Thanks are also due to the University of Madras for the award of a research studentship to one of us (K.S.S.).

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The Estimation of 1-Monoglyceride

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(Received 14 September 1955)

Pohle, Mehlenbacher & Cook (1945) first reported a method for the estimation of 1-monoglyceride (1-MG) based on the oxidation of the vicinal hydroxyl groups of the glycerol part of the molecule by periodic acid. The procedure was modified by Pohle & Mehlenbacher (1950), who used a chloroform-acetic acid mixture as a medium for the oxidation which was allowed to continue for 20 min. The residual periodate was estimated by adding iodide and titrating with thiosulphate solution.

Kummerow & Daubert (1950) found that this method indicated the presence of small amounts of 1-MG in various natural fats, but that fatty acid derived from fats also showed an apparent 1-MG content. Mattson, Benedict, Martin & Beck (1952) recorded values for 1-MG of up to 1.5% with pure tri- and di-glycerides.

Borgström (1954) found that lead tetraacetate, which has oxidizing properties similar to those of periodic acid, gave erroneous values for 1-MG with samples containing unsaturated fatty acids. He attributed the high values to the reduction of the tetraacetate by partially oxidized double bonds in the unsaturated fatty acids.

We have confirmed the observations of Kummerow & Daubert (1950), and in addition have found that the apparent 1-MG content increases with the time during which periodic acid is allowed to act, up to 24 hr.

We have developed an alternative method for the estimation of 1-MG, based on the fact that oxidative splitting of the vicinal hydroxyl groups of the glycerol part of the molecule yields formaldehyde, whereas oxidation of any double bond (other than a terminal one) in the fatty acid part of the

molecule would not give formaldehyde. This principle has been applied to the estimation of adrenocortical steroids by Corcoran & Page (1948) and Daughaday, Jaffe & Williams (1948). Our procedure is on a somewhat larger scale than that of Corcoran & Page (1948), the main points of difference being: (1) the replacement of acetic by propionic acid in the oxidation mixture; (2) the removal of chloroform by aeration under reduced pressure; (3) the use of steam in addition to ordinary distillation to remove the formaldehyde.

EXPERIMENTAL

Apparatus

A modified all-glass semi-micro-Kjeldahl apparatus (Fig. 1) is used for distillation of formaldehyde formed. The flask used is of 50 ml. capacity.

Reagents

Periodic acid solution. Periodic acid (0.5 g.) (British Drug Houses Ltd.) dissolved in 10 ml. of distilled water is used. The solution is stable for some weeks if kept in the dark.

Each batch of periodic acid is assayed by adding an excess of glycerol (>12 mg.) to 1 ml. of periodic acid solution and standing for 15 min. Dilute acid (5 ml. of $\text{N-H}_2\text{SO}_4$) and KI solution (5 ml., 10%, w/v) are then added and the I_2 liberated is titrated with 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$ solution. The titration is repeated with the glycerol omitted. A difference in titration values of 1 ml. of 0.1 N- $\text{Na}_2\text{S}_2\text{O}_3$ is equivalent to 9.6 mg. of periodic acid.

Propionic acid. Sodium propionate (250 g.) (British Drug Houses Ltd.) is dissolved in 200 ml. of water, 20 ml. of 2.5 N-NaOH added and then KMnO_4 solution (about 10%, w/v) until a purple tinge remains on shaking. The mixture is heated to precipitate oxides of manganese and