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Isolation of D-Glyceric Acid from Cress Seedlings and its Relationship to the Synthesis of L-Ascorbic Acid

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In the preceding paper (Isherwood, Chen & Mapson, 1953) it has been shown that the transformation in cress seedlings and rats of the two common hexoses D-glucose and D-galactose into L-ascorbic acid can be represented in outline by the following series of reactions:

D-glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid

and

D-galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone → L-ascorbic acid

The possibility that the transformation might occur as the result of the breakdown of the hexose into two three-carbon compounds and that the synthesis of L-ascorbic acid might start from these was also considered but was rejected in favour of the scheme given above in which the carbon chain of the hexose is not broken.

The present paper describes for the first time the isolation of the three-carbon compound, D-glyceric acid, from cress seedlings and examines the relationship between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid during the growth of the seedlings. D-Glyceric acid was shown not to be a precursor of L-ascorbic acid though its synthesis is in some way coupled with that of L-ascorbic acid.

EXPERIMENTAL

Methods

Culture of cress seedlings. This has been described previously (Isherwood *et al.* 1953). In most of the preliminary experiments the seedlings were grown in 0·02 M-$(NH_4)_2SO_4$ or water or 0·04 M-$NaHCO_3$ for 96 hr. It was found that the amounts of L-ascorbic acid present at this time in each group of seedlings were approximately 0·2, 0·6 and 0·9 mg./100 seedlings, respectively. After 96 hr. the rate of formation of L-ascorbic acid began to decline. If the seedlings are grown for shorter periods, the rate of L-ascorbic acid synthesis may be somewhat higher, but the seedlings are much smaller and it is difficult to separate the testas from them.

Estimation of L-ascorbic acid and sugars. The methods used have been described previously (Isherwood *et al.* 1953).

Chromatography. In preliminary experiments an extract of germinating cress seedlings, prepared by grinding them with an equal weight of ethanol and centrifuging, was examined in a paper chromatogram using either *n*-butanol:acetic acid:water (4:1:5 by vol. respectively) or

n-propanol: conc. NH_3 (7:3 by vol. respectively) as solvent. The apparatus and procedure were essentially the same as those described by Isherwood & Jermyn (1951) for the sugars. Amino acids were detected by spraying with 0·1% (w/v) ninhydrin in moist butanol and heating at 100° for 10 min. Sugars and acids were detected by spraying with 0·1 M-$AgNO_3$ in 4 N aqueous NH_3 and then heating at 100° for 20 min. Acids were also detected by spraying with 0·1% (w/v) thymol blue in water to which sufficient 0·1 N-NaOH had been added to render the solution deep blue. Phenols and phenolic acids were detected either by examination under ultraviolet light, which caused many of them to fluoresce, or by spraying with 1% (w/v) $FeCl_3$ in water or 8% (w/v) benzenediazosulphonic acid in water.

Examination and estimation of organic acids. The acids were extracted by the method described by Isherwood (1946) using a silica gel column and then separated and estimated by the paper chromatographic method described by Isherwood & Hanes (1953). The phenols and phenolic acids were removed by treating the extract of acids, before examination on a paper chromatogram, with a very slight excess of benzenediazonium sulphate (1% w/v in water) and then $Ba(OH)_2$ to remove the H_2SO_4 present in the solution. The precipitated azo compounds and $BaSO_4$ were removed by centrifuging. The presence of an excess of the diazo reagent was detected by using a solution of 2-naphthol in dilute alkali as an external indicator. The traces of azo compounds still in solution usually had very much higher R_F values than the organic acids or the original phenols, and did not interfere in the subsequent examination on the paper chromatogram. Usually 300 seedlings were taken for each analysis.

Experiments on rats. The rats were housed and fed as described in the previous paper (Isherwood *et al.* 1953). In the few experiments in which the rats were dosed with chloretone by mouth (20 mg. of the drug dissolved in five drops of arachis oil given to each rat every day by a dropping pipette), the urine was collected using toluene as a preservative (no oxalic acid was used). This urine was then examined chromatographically for the presence of compounds such as D-glyceric acid by the method of Isherwood & Hanes (1953).

Preparation of chemicals

D-*Glyceric acid.* This was prepared from a commercial sample of DL-glyceric acid by resolution with quinine (Anderson, 1909). The final material was a colourless syrup. Quinine salt m.p. 178°, $[\alpha]_D^{20} - 116°$ in water (*c*, 1). Calcium salt, $[\alpha]_D^{20} + 10·9°$ in water (*c*, 5). (Found: Ca, 14·0. $(C_3H_5O_4)_2Ca, 2H_2O$ requires Ca, 14·0%.)

D-*Glyceric acid ethyl ester.* This was prepared by heating 10 g. of the free acid in 50 ml. of absolute ethanol at 190° for 4–5 hr. in a sealed glass tube. The ethanolic solution was concentrated and the residual syrup distilled in a high vacuum. The clear, colourless, viscous liquid had $[\alpha]_D^{20}$ approx. +7° in water (*c*, 10) and b.p. 110°/2–4 mm. The DL-ester was prepared in a similar manner.

L-*Gulono-γ-lactone.* This was prepared from D-glucurono-γ-lactone by reduction in slightly alkaline solution with sodium amalgam (Thierfelder, 1891). The lactone had m.p. 180° and $[\alpha]_D^{20} + 55°$ in water (*c*, 1).

D-*Glucurono-γ-lactone.* This was a commercial sample. The m.p. and rotation were identical with the figures given for the pure material.

RESULTS

Preliminary experiments

In a few experiments a crude extract of germinating cress seedlings which had been cultured in water was examined on a paper chromatogram. The assumption was made that the precursors would be at their highest level when synthesis was most active. Examination of the paper chromatograms showed the presence of a very large number of overlapping spots, none of which could be linked with the synthesis of L-ascorbic acid. These early experiments indicated that to obtain any useful information by this method, it was essential to be able to dissociate the synthesis of L-ascorbic acid from the normal growth of the seedlings. Comparative examination of cell saps from seedlings with a high and low rate of synthesis might then reveal which compounds were significant in the synthesis.

The experiments which were carried out subsequently were based on this general idea. The cress seedlings were cultured in different nutrient solutions to change the rate of synthesis of L-ascorbic acid and were then examined as before on a paper chromatogram. Mapson, Cruickshank & Chen (1949) had shown that culture in different nutrient solutions (0·02 M ammonium sulphate, water and 0·04 M sodium bicarbonate) changed the pH of the sap (4·2, 4·6 and 5·2, respectively) and that this influenced the synthesis of L-ascorbic acid (0·2, 0·6 and 0·9 mg./g. at 96 hr.) from the hexose sugars; detailed analysis of the results showed that the rate at which L-ascorbic acid was synthesized could be changed independently of the hexose content of the tissue. The seedlings in all cases grew normally, the respiration and appearance being very similar, so that the synthesis of L-ascorbic acid appeared to concern only a minor part of the metabolism. A preliminary experiment in which a careful comparative examination was made of the sap of seedlings grown as described by Mapson *et al.* (1949) showed that the acids present changed markedly with a change in pH. Sugars and amino acids did not change appreciably. Later experiments in which the organic acids were separated from the other substances present in the crude extract by using the silica gel column method of Isherwood (1946) and were then examined on a paper chromatogram showed that while the total amount of acid present changed with a change in the pH of the cell sap, not all the acids were similarly affected. One acid, later identified as D-glyceric, appeared to vary directly with the amount of L-ascorbic acid present in the original cress seedlings and later experiments were mainly concerned with this acid. Tracings of comparable chromatograms from seedlings grown in 0·02 M ammonium sulphate, water and 0·04 M sodium bicarbonate for 96 hr. are shown

in Fig. 1. The organic acids were extracted from the crude acidified extracts of the cress seedlings by the method of Isherwood (1946) and then examined on a paper chromatogram using *n*-propanol:concentrated ammonia as developing solvent.

It can be seen that the relative sizes of the glyceric acid spots are very roughly proportional to the above-mentioned amounts of L-ascorbic acid in the original seedlings. These paper chromatograms had been sprayed with ammoniacal silver nitrate and the depth of the staining is known to be only very roughly proportional to the amount of glyceric acid present, so that no great reliance can be placed on the accuracy of visual comparisons. However, the observation that there might be a link between glyceric acid and L-ascorbic acid was rendered more likely by a consideration of the behaviour of the other acids. In cress seedlings grown in ammonium sulphate and water the total amount of acid present in the cell sap is virtually the same (equivalent to 11·9 and 12·5 ml. of 0·1N alkali/300 seedlings after 96 hr.) and examination of extracts of the acids from the two groups indicates that, of all the acids present, only glyceric acid appears to change markedly. In seedlings grown in sodium bicarbonate the total amount of acid in the cell sap is larger (equivalent to 16·5 ml. 0·1N alkali/300 seedlings after 96 hr.) and an examination of an extract of the acids indicates that, while malic acid has increased noticeably and possibly accounts for a good deal of the increased acid, all the acids have increased. In spite of this general increase, the proportionality between the amounts of glyceric acid and L-ascorbic acid still persists and it appears that the relation between the two is largely independent of the mechanism regulating the other organic acids.

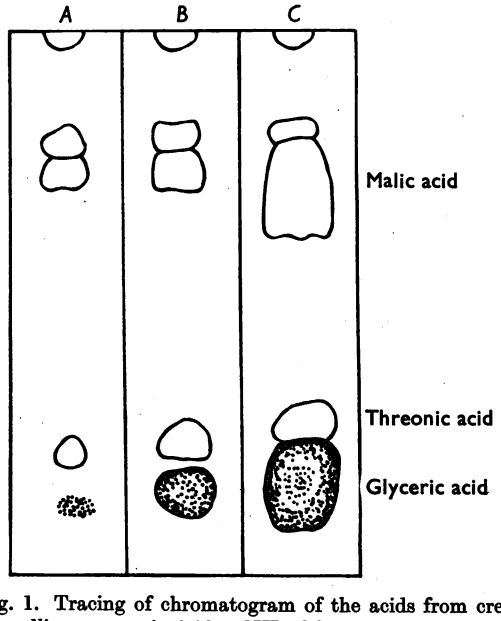

Fig. 1. Tracing of chromatogram of the acids from cress seedlings grown in 0·02M-$(NH_4)_2SO_4$ (*A*), water (*B*), and 0·04M-$NaHCO_3$ (*C*), at 20° in the dark. Paper, no. 1 Whatman. Solvent, *n*-propanol:conc. NH_3 (7:3 by vol.). Papers sprayed with ammoniacal $AgNO_3$ and heated at 100° for 20 min.

In certain experiments the possibility was considered that the glyceric acid might arise from L-ascorbic acid during the process of extraction. L-Ascorbic acid (10 mg.) was added to one-half of the extract from 300 cress seedlings (acidified with sulphuric acid, pH 2·0), the other half being used as a control. Both portions were then treated as described above and the organic acids examined on a paper chromatogram. Comparison of the chromatograms from the two portions showed that the very small spot due to L-threonic acid on the chromatogram of the control portion had been very greatly increased by the addition of the L-ascorbic acid, but that the spots due to glyceric acid were very similar on both chromatograms. The amount of L-ascorbic acid added was about 10 times the normal amount present in the seedlings. It seemed clear that the glyceric acid did not originate from the L-ascorbic acid during extraction.

Isolation of D-*glyceric acid*

Sufficient D-glyceric acid for a chemical examination was isolated by a large-scale repetition of the methods used for the qualitative examination of the acids (Isherwood & Hanes, 1953). The acids were finally separated on a paper chromatogram using *n*-propanol:concentrated ammonia as developing solvent. The appropriate pieces of paper containing the glyceric acid were extracted chromatographically with water and the extracts dried *in vacuo*. About 120 mg. of syrupy ammonium glycerate were obtained from 60 ml. of the original extract prepared from 3000 seedlings which had been grown for 96 hr. in water.

The chromatographic behaviour of the ammonium glycerate was the same as that of an authentic specimen of ammonium D-glycerate either alone or mixed together. *n*-Butanol:acetic acid:water and *n*-propanol:concentrated ammonia were used as solvents. The ammonium glycerate could readily be distinguished from the salts of either glycollic or threonic acid, substances which might be expected in an extract from plant material. On the paper chromatogram it gave a characteristic purple-black colour with the ammoniacal silver nitrate reagent, a colour which was also given by threonic acid but not by glycollic acid. The crude ammonium glycerate had $[\alpha]_D^{20}$ approx. +10° (*c*, 0·24) in dilute ammonia, and $[\alpha]_D^{20}$ +77° (*c*, 0·18) in 6% (w/v) ammonium molybdate. A sample of authentic

ammonium D-glycerate had $[\alpha]_D^{20} + 14°$ (*c*, 0·3) in dilute ammonia and $[\alpha]_D^{20} + 154°$ (*c*, 0·21) in 6% (w/v) ammonium molybdate. The figures for the rotation would suggest that the glyceric acid isolated was mainly D-glyceric acid but that some was present in the racemic form. This conclusion was confirmed by an examination of the calcium salt prepared by the method of Nef, Hedenburg & Glattfeld (1917). This slowly crystallized from aqueous ethanol but was extremely difficult to recrystallize. The impure calcium salt had $[\alpha]_D^{20} + 9°$ in water (*c*, 2·7) and $[\alpha]_D^{20} + 89°$ in 6% (w/v) ammonium molybdate (*c*, 2·1). For a sample of authentic calcium D-glycerate the figures were + 10·1° and + 107°, respectively. The calcium content was 13·5; calc. for $(C_3H_5O_4)_2Ca, 2H_2O$, 14·0%.

Quantitative relationship between D-*glyceric acid and* L-*ascorbic acid in cress seedlings*

The evidence from a preliminary chromatographic examination of the cell sap of seedlings grown in 0·02M ammonium sulphate, water and 0·04M sodium bicarbonate for 96 hr. showed that the relative amounts of D-glyceric and L-ascorbic acids remained roughly the same in spite of culture in different nutrient solutions. The implication was that the formation of D-glyceric acid and the synthesis of L-ascorbic acid were linked together. This has been confirmed by later experiments in which the relation between the two acids was examined on a quantitative basis throughout the growth of the seedling (up to 9–10 days) and at different temperatures. The glyceric acid was measured by the chromatographic method of Isherwood & Hanes (1953) which does not distinguish between the D- and L-isomers. The figure given for glyceric acid therefore represents the total amount in the tissue and though some of it was undoubtedly in the racemic form, the bulk was probably the D form so that the figure for glyceric acid has been assumed to convey a reasonably accurate measure of the D-glyceric acid present. In Fig. 2 the amounts of D-glyceric and L-ascorbic acids are plotted against the time in days from the start of germination for seedlings grown in 0·02M ammonium sulphate, water and 0·04M sodium bicarbonate at 20°.

From the results it is clear that in each group of seedlings the amounts of D-glyceric and L-ascorbic acids present vary in a very similar manner throughout the period of growth. The mean figures for the ratio of D-glyceric acid to L-ascorbic acid in each group of seedlings, 2·2, 4·5 and 7·0, respectively, suggest that the link between the two acids is affected by the pH of the cell sap, 4·2, 4·6 and 5·2, respectively, and that the ratio increases as the sap becomes more alkaline.

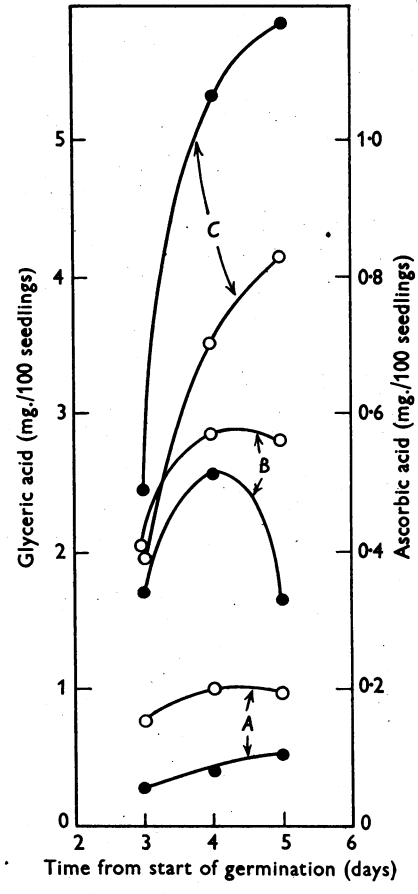

Fig. 2. Relation between D-glyceric and L-ascorbic acids in cress seedlings cultured in 0·02M-$(NH_4)_2SO_4$ (*A*), water (*B*), and 0·04M-$NaHCO_3$ (*C*), at 20°. ●—●, Glyceric acid; ○—○, L-ascorbic acid.

In addition to the effect of the pH of the cell sap on the synthesis of L-ascorbic acid, it is known that changing the temperature at which the seedlings are grown also affects the synthesis. It was therefore of interest to determine whether such a temperature change affected the link between the formation of D-glyceric and L-ascorbic acids; an experiment based on this is described in Fig. 3. The seedlings were cultured in water at 20°, some were left at this temperature and the others were transferred to a temperature of 1°. At 20° the synthesis of L-ascorbic acid proceeds normally, whereas at 1° it appears to be almost completely arrested. Transferring the seedlings back to 20° caused the synthesis to start again at much the same rate as before the change of temperature. The seedlings were analysed for sucrose, reducing sugars, L-ascorbic acid and D-glyceric acid. Sucrose and the reducing sugars were measured because they are known to be

affected by a change in temperature (Chen, 1950). The results show that lowering the temperature arrested both L-ascorbic and D-glyceric acids. The formation of the reducing sugars was partially arrested and the formation of sucrose was stimulated. Transfer back to 20° reversed the changes to the extent that L-ascorbic and D-glyceric acids were again formed, the reducing sugars rose rapidly and sucrose declined to a very low value; the formation of D-glyceric acid closely followed the synthesis of L-ascorbic acid in marked contrast to the behaviour of sucrose and the reducing sugars.

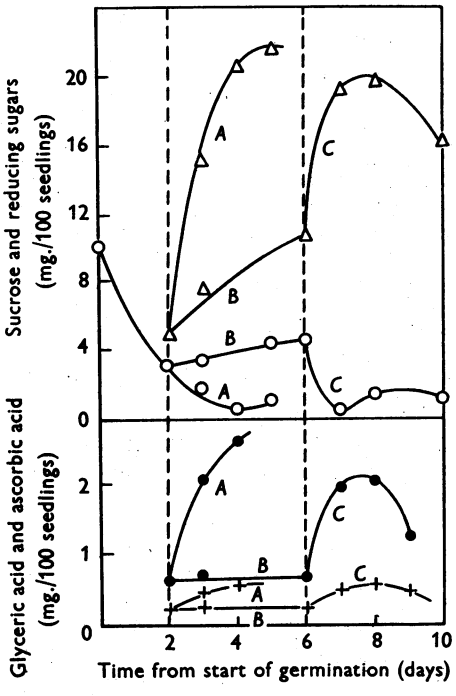

Fig. 3. Relation between D-glyceric acid, L-ascorbic acid, sucrose and reducing sugars in cress seedlings grown in water and subjected to changes of temperature. (*A*) Seedlings at 20°, (*B*) seedlings placed at 1°, (*C*) seedlings which had been at 1° changed to 20°. ○—○, Sucrose; △—△, reducing sugars; ●—●, glyceric acid; +—+, L-ascorbic acid.

Table 1. *Effect of feeding various sugar derivatives to cress seedlings on the formation of glyceric acid*

Substance fed (0·5%, w/v)	Duration of experiment (hr.)	Glyceric acid as % of control grown in water	L-Ascorbic acid as % of control grown in water
L-Ascorbic acid	96	76	630
D-Glucurono-γ-lactone	90	44	124
	114	83	120
	138	59	128
	162	77	150
L-Gulono-γ-lactone	72	12	154
	94	27	141
	120	34	144
	144	28	174

Another method of changing the synthesis of L-ascorbic acid in cress seedlings is to feed D-glucurono- and L-gulono-γ-lactones. These compounds have been shown (Isherwood *et al.* 1953) to increase the formation of L-ascorbic acid and, in the experiments now to be described, the effect on the relationship between glyceric and L-ascorbic acids was examined. The cress seedlings were cultured in 0·5% (w/v) solutions of L-ascorbic acid, and D-glucurono- and L-gulono-γ-lactones, and the formation of D-glyceric and L-ascorbic acids measured. The results are given in Table 1. The amounts of glyceric and L-ascorbic acids are expressed as a percentage of the corresponding amounts in seedlings grown in water. The results show that feeding L-ascorbic acid raised the L-ascorbic acid content of the seedlings until it was over 600% of the control but that the glyceric acid was only affected (80% of control) to a very small extent considering the large increase in L-ascorbic acid. Feeding D-glucurono- and L-gulono-γ-lactones also depressed the formation of glyceric acid (about 70% and 25% of the control value, respectively). The extra amounts of L-ascorbic acid present (30% and 50%, respectively) would seem to be too small to cause such an effect and the only reasonable explanation must be that the formation of D-glyceric acid is in some way coupled with the formation of L-ascorbic acid.

However, D-glyceric acid is not a precursor of L-ascorbic acid. Feeding an 0·5% (w/v) aqueous solution of ethyl DL-glycerate or D-glycerate (the ester was used as a convenient method of introducing glyceric acid into the cell; Beevers, Goldschmidt & Koffler, 1952) to cress seedlings did not increase significantly the formation of L-ascorbic acid, even though the glyceric acid in the sap increased by 20%.

Experiments on rats analogous to those on cress seedlings

The fact that there is a connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid in the cress seedling made it of interest to determine whether any similar relationship existed in animals such as the rat which can synthesize L-ascorbic acid.

The rat is a convenient animal to study because other workers (Longenecker, Fricke & King, 1940; Smythe & King, 1942) have reported that an appreciable stimulation of the synthesis of L-ascorbic acid occurs as the result of the administration of chloretone, the excretion of L-ascorbic acid in the urine rising to several times the normal value. The excretion of other compounds also increases because

there is a marked increase in the excretion of conjugated glucuronides and if glyceric acid was formed in larger amount in the tissues, the excretion of this might rise also.

An experiment in which 20 mg. of chloretone were given to each rat every day showed that while the L-ascorbic acid in the urine rose from 3 in the case of the undosed controls to 20 mg./rat/day, the glyceric acid also rose from 2·6 to 4·6 mg./rat/day. This result suggests that there may be a connexion between the formation of the two compounds in the rat similar to that found in the cress seedling. To test whether D-glyceric acid is a precursor of L-ascorbic acid in the rat, 100 mg. each of ethyl DL- and D-glycerates were injected subcutaneously into rats, and the excretion of L-ascorbic acid in the urine was measured. No changes were observed and it was concluded that D-glyceric acid was not a precursor of L-ascorbic acid.

DISCUSSION

The isolation of D-glyceric acid from cress seedlings, in which it occurs in appreciable amounts under certain conditions (0·5 % of the fresh weight after the seedlings have been cultured for 5 days in 0·04M sodium bicarbonate solution), is interesting not only because the presence of this acid in plant materials has not been recorded previously, but also because it is a three-carbon compound and therefore may be closely connected with the breakdown of the hexoses in the seedlings. In the present study the main interest has been the relationship of the formation of this three-carbon compound to the formation of the six-carbon compound L-ascorbic acid in cress seedlings. Quantitative studies of the synthesis of the two compounds suggests that there is some connexion between them, for the formation of each runs parallel when the seedlings are cultured in different nutrient solutions and when they are subjected to rapid changes of temperature. The nature of the connexion is obscure for it is difficult to reconcile the previous observations with the fact that feeding precursors of L-ascorbic acid such as D-glucurono- and L-gulono-γ-lactones markedly depresses the formation of D-glyceric acid, though the very fact that the synthesis of D-glyceric acid is depressed supports the suggestion that there must be a connexion. In other plant tissues this connexion may be absent. Examination of mature strawberry leaves showed that they contained negligible amounts of glyceric acid ($<0·1$ mg./g.) yet the amount of L-ascorbic acid was several times that of cress seedlings. However, this result may be misleading if no synthesis of either compound was occurring in the leaves, for the connexion is assumed to be between the amounts undergoing synthesis and not between the total concentrations present.

Experiments on rats analogous to those on cress seedlings suggest that a similar connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid also exists in the rat.

It is clear, however, that both in the cress seedling and in the rat, D-glyceric acid is not a precursor of L-ascorbic acid, since feeding the ester of D-glyceric acid to cress seedlings or injecting it into rats gave no increase in the synthesis of L-ascorbic acid.

The origin of the D-glyceric acid is unknown, but two suggestions have been tentatively considered.

(1) The D-glyceric acid is derived from 3-phospho-D-glyceraldehyde by dehydrogenation to 3-phospho-D-glyceric acid and loss of a phosphate group. In this connexion the work of Tewfik & Stumpf (1951) is suggestive. They found that the triose phosphate dehydrogenase only occurs in a limited number of tissues and is probably missing in leaf tissue so that the results obtained on strawberry leaves mentioned above could be explained by the absence of the dehydrogenase.

(2) The glyceric acid is produced by the oxidation of the glycerol, liberated as the fat in the seed breaks down during germination and growth. Rough calculations have shown that the amount of glycerol liberated is of the right order of magnitude. The main interest in this suggestion lies in the analogy between the oxidation of the primary alcohol group of the glycerol to give glyceric acid and that of the glucose (carbon atom 6) to give glucuronic acid as in the scheme described in the previous paper (Isherwood *et al.* 1953).

On the basis of either (1) or (2), the connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid would lie in the fact that both require some common enzyme or coenzyme system.

SUMMARY

1. Cress seedlings cultured for 3–5 days in the dark at 20° were shown by chromatographic methods to contain from 0·05 to 0·5 % of D-glyceric acid/g. fresh weight. Quantitative studies of the amount of D-glyceric and L-ascorbic acids present throughout the period of growth of the seedling (up to 5 days) in various nutrient solutions (0·04M sodium bicarbonate, water and 0·02M ammonium sulphate) have shown that the formation of each runs roughly parallel. This is also true when seedlings cultured in water are subjected to changes in temperature during their growth.

2. Feeding D-glucurono- and L-gulono-γ-lactones, which are known to be precursors of L-ascorbic acid, to cress seedlings definitely depressed the formation of D-glyceric acid.

3. A somewhat similar relationship to that observed in the cress seedling between the formation

of D-glyceric and L-ascorbic acids possibly exists in the rat, for treatment with the drug chloretone causes the rat to excrete in its urine increased amounts of both D-glyceric and L-ascorbic acids.

4. It is clear, however, that D-glyceric acid is not a direct precursor of L-ascorbic acid, since feeding the ethyl ester of D-glyceric acid (the ester was used as a convenient method of introducing D-glyceric acid into the cell) to cress seedlings or injecting it into the rat did not increase the formation of L-ascorbic acid. The connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid remains obscure.

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Biological Synthesis of L-Ascorbic Acid: the Conversion of L-Galactono-γ-lactone into L-Ascorbic Acid by Plant Mitochondria

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Evidence presented by Isherwood, Chen & Mapson (1953) has indicated that the synthesis of L-ascorbic acid *in vivo* in both plants and animals can be represented in outline by one or both of the following sequences:

(1) D-glucose→D-glucurono-γ-lactone→L-gulono-γ-lactone→L-ascorbic acid;

(2) D-galactose→D-galacturonic acid methyl ester→L-galactono-γ-lactone→L-ascorbic acid.

In the present paper an account is given of the enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by extracts of plant tissues. A following paper will deal with the conversion both of L-gulono-γ-lactone and L-galactono-γ-lactone to L-ascorbic acid by extracts from animal tissues.

EXPERIMENTAL

Selection of material. Most plant tissues contain highly active enzymic systems capable of oxidizing ascorbic acid, and this fact had to be taken into account before selecting a tissue in which to demonstrate the synthesis of ascorbic acid. Clearly it is more difficult to study the subject in tissues which are actively oxidizing the vitamin, the use of inhibitors of oxidases, e.g. cyanide, being precluded by the knowledge, from our earlier studies *in vivo*, that this also inhibits the synthesis (Isherwood, Chen & Mapson, unpublished observations). It was desirable to choose a tissue in which the rate of synthesis *in vivo* was known to be rapid and, for this reason, seeds during the early stage of germination were selected. Most of the experiments reported in this paper have been carried out with pea seeds, but sufficient work has been done with mung bean seeds to show that the pea seed is not exceptional.

Pea seedlings contain an active ascorbic acid oxidase, but fortunately for our purpose the oxidative enzyme activity in the very early stage of germination is low, not reaching its full development until some 120 hr. after germination at 25°. The varieties of pea seeds used included Laxton Superb, Kelvedon Wonder and Onward, and differed only in their degree of activity.

Enzyme material—plant tissues. Dry pea seeds were soaked in water for periods of 12–48 hr. at 20°. The soaked seeds (30 g.) were ground up with sand and 40 ml. of medium of the desired composition in a mortar. The brei was centrifuged at approximately 500 *g* for 5 min. and the supernatant solution removed. This solution is referred to in the text as the whole extract and cytoplasmic particles (mitochondria) were separated from it by centrifuging at 10000 *g* for 20 min. Washed mitochondria were prepared by suspending the residue from the first centrifugation in 20 ml. of the medium and recentrifuging, this procedure