Synthesis of L-Ascorbic Acid in Plants and Animals

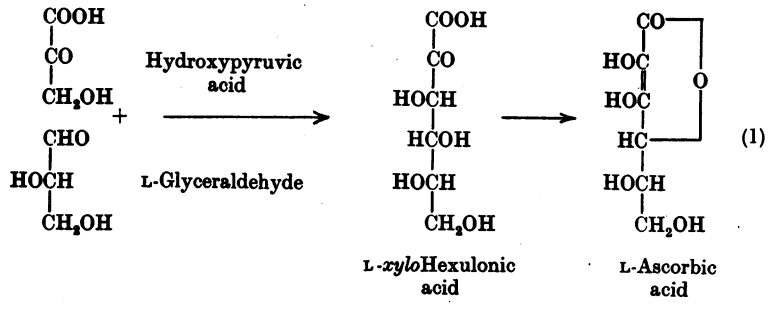
By F. A. ISHERWOOD, Y. T. CHEN and L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(*Received* 13 *May* 1953)

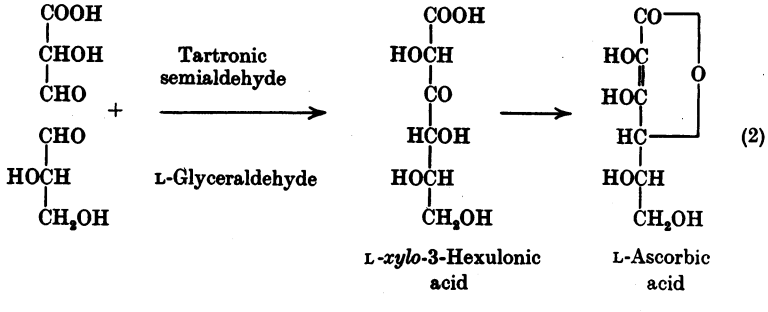
The chemical synthesis of L-ascorbic acid from D-galactose by Haworth & Hirst (1933) forms an outstanding achievement in the chemistry of the carbohydrates, and has frequently stimulated speculation as to whether the biological synthesis of L-ascorbic acid proceeds along similar lines. The very common occurrence of sugars in both plants and animals, and the fact that L-ascorbic acid is closely related to a sugar, L-gulose, has tended to give support to this idea. The idea has not hitherto been supported by any extensive body of facts, although there is a strong suggestion from earlier work that hexose sugars provide the carbon skeleton of the molecule. A number of experiments have been described in the literature which suggest that the carbon skeleton of L-ascorbic acid in both plants (Ray, 1934) and animals (Ruffo & Tartaglione, 1948; Jackel, Mosbach, Burns & King, 1950) is formed from a hexose sugar, though none of them definitely identified any one sugar as the precursor. In the case of plants, some workers (Ray, 1934; Tadokoro & Nisida, 1940; De & Barai, 1949) have claimed that D-mannose is the precursor, whilst others (Sugawara, 1941; Ahmad, Qureshi, Babbar & Sawhney, 1946; Itô & Mizuno, 1948) have suggested that a number of sugars can act as precursors. Such conflicting claims are not unexpected in view of the large variety of plant materials which have been examined. The hexose sugars and probably a number of other compounds such as sorbitol and inositol are interconvertible, but to different extents in different plants, so that the success obtained by feeding any of these compounds may well depend on whether the plant can convert them into the actual precursor of L-ascorbic acid. It is perhaps significant that with every kind of plant material examined D-glucose has caused the formation of L-ascorbic acid, whereas the other sugars, D-mannose, D-galactose and L-sorbose, have in a number of experiments given negative results (Itô & Mizuno, 1948; Devyatnin, 1950; Mapson, Cruickshank & Chen, 1949). In the case of animals the work of Jackel *et al.* (1950) has shown that a direct link exists between D-glucose and L-ascorbic acid though the evidence does not eliminate the possibility that D-glucose may be converted into D-mannose or D-fructose before being transformed into L-ascorbic acid; feeding D-[^{14}C]glucose labelled uniformly in all positions to chloretone-treated rats gave L-ascorbic acid which was also labelled uniformly in all positions. Mosbach and co-workers suggest that the carbon chain of the D-glucose is not broken before being converted to L-ascorbic acid or, if so, the fragments are recombined without any major differential dilution effect. No clue is given in any of the above work as to the series of changes by which the D-glucose is transformed into L-ascorbic acid.

The observation that there is a close link between a hexose sugar, which may be D-glucose, and L-ascorbic acid has stimulated speculation as to the method by which the transformation from one to the other is effected, particularly the means by which a member of the D-series of sugars is changed to a member of the L-series. The investigations to be described in this and subsequent communications are directed towards establishing the nature of the hexose precursor and elucidating the mechanism by which the hexose is transformed into L-ascorbic acid; and the present paper describes evidence in support of a suggested mechanism for the transformation of two common hexoses, D-glucose and D-galactose, into L-ascorbic acid by a series of simple reactions which could reasonably be imagined as occurring in biological systems. Various possible sequences of reactions have been examined in which the main emphasis has been on changes in configuration necessary for the sugar to be transformed into L-ascorbic acid and in which no attempt has been made to portray the sugars or sugar acids in their cyclic forms, even though in practice the sugar acids were fed or injected as lactones in order to facilitate their entry into the cell. In every sequence of reactions the assumption has been made that the starting sugar is D-glucose though, in the brief discussion which follows, an indication has been given as to whether D-galactose or D-mannose could replace D-glucose.

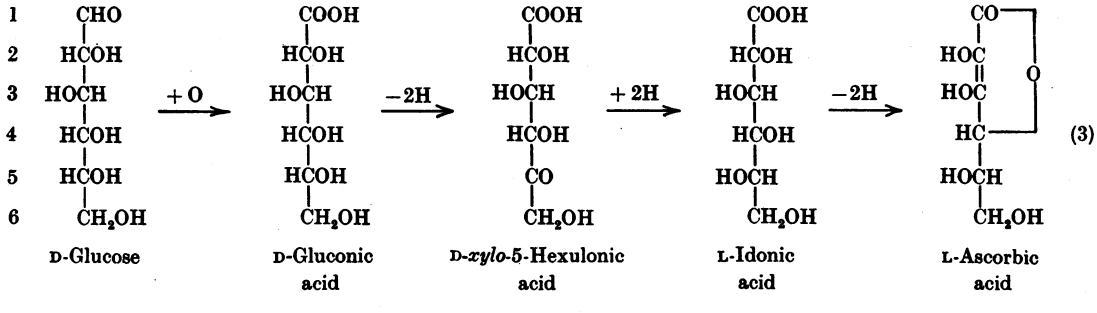
The schemes described represent only a few of the many which on chemical grounds can be suggested for the transformation, but they serve to focus attention on the type of compounds which might be precursors of L-ascorbic acid. For convenience in presentation they have been grouped under two main headings.



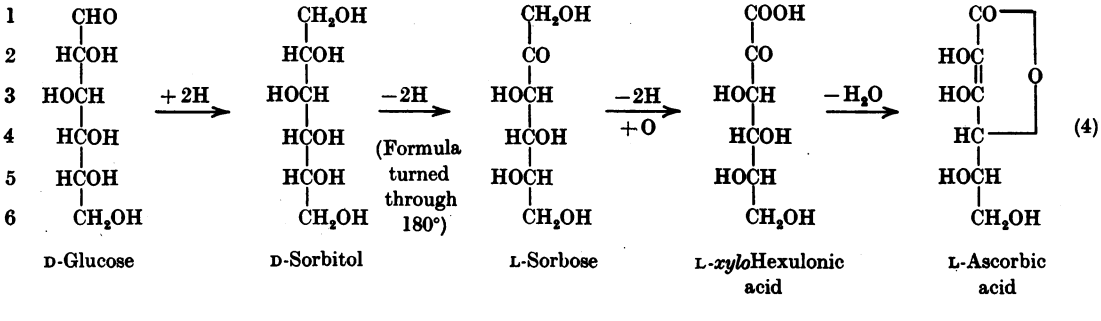
(1)



(2)



(3)



(4)

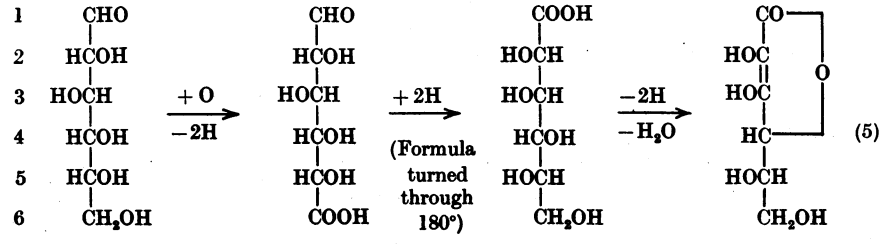
Indirect conversion. Indirect conversion, in which the carbon chain is broken and then reformed, involves the breakdown of the D-glucose via fructose diphosphate to the triose phosphates and the conversion of these into other three-carbon compounds, which then combine to give L-ascorbic acid. Assuming that this occurs, two types of condensation reaction can be suggested.

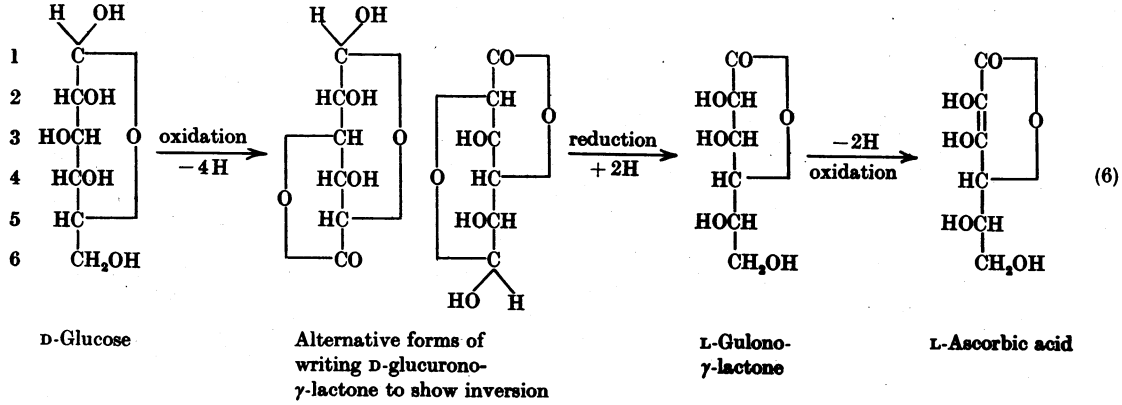
The first (1) is based on an aldol condensation between L-glyceraldehyde and hydroxypyruvic acid (Hough & Jones, 1951, 1952) catalysed by an enzyme similar to the aldolase of animal tissues (Meyerhof, Lohmann & Schuster, 1936) and plant tissues (Stumpf, 1948).

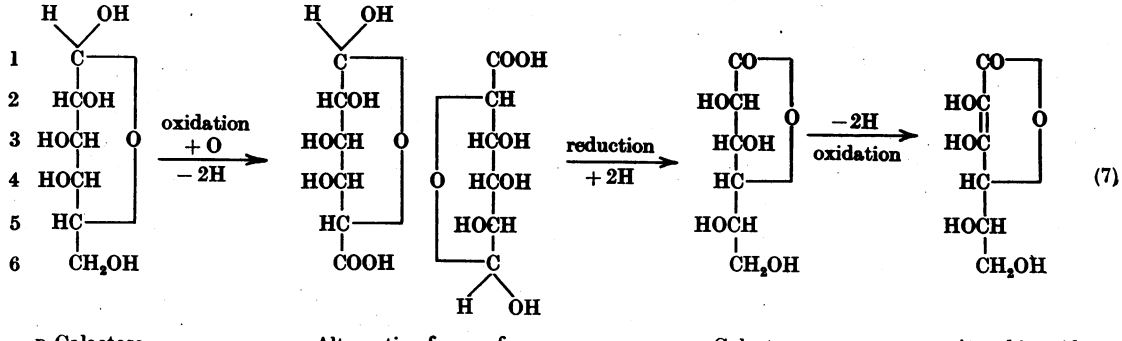
The second type of condensation (2) is an acyloin reaction between tartronic semialdehyde and L-

glyceraldehyde. L-*xylo*-3-Hexulonic acid (3-keto-L-gulonic acid) is known to be in tautomeric equilibrium with L-ascorbic acid, the equilibrium being very much in favour of the L-ascorbic acid (Cox, Hirst & Reynolds, 1932).

Some support for the general idea of indirect conversion came from the observation, which is described at length in another paper, that the formation of the three-carbon compound D-glyceric acid ran parallel with the formation of L-ascorbic acid in germinating cress seedlings and in rats. However, D-glyceric acid does not appear to be a precursor, for feeding the ethyl esters of D- or DL-glyceric acid to cress seedlings or injecting the compounds into rats did not produce any increase in the synthesis of L-ascorbic acid. It seems possible that the parallel formation of D-glyceric acid and L-ascorbic acid is due to a common cell environment rather than to a direct link between the two compounds and may therefore be misleading.







Direct conversion. The observation of Jackel *et al.* (1950) made it seem possible that the conversion of hexose into ascorbic acid takes place without rupture of the carbon chain. This must involve either the inversion of the configuration of the groups on carbon atom 5, or what amounts to an inversion of all the asymmetric carbons as written by transferring in effect the aldehyde or potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain. Thus the reduction of $C_{(1)}$ of an aldohexose to —CH_2OH and the oxidation of $C_{(6)}$ to an aldehyde or potential aldehyde would mean that if the formula is now written according to the usual convention (being turned through 180° so as to bring the new aldehyde group at the top) the stereochemical designation of each hydroxyl group (D or L) is necessarily reversed. In the case of D-glucose the hydroxyl group on the new $C_{(5)}$ (old $C_{(2)}$) is now in the correct orientation for L-ascorbic acid.

The inversion of the groups attached to carbon atom 5 could occur through the intermediate formation of a 5-oxo compound, for example as in (3). In such a scheme the configuration of the groups on carbon atoms 2 and 3 is immaterial because in L-ascorbic acid they are not optically active. The configuration of the hydroxyl on carbon atom 4 must be D. This is true of D-mannose but not D-galactose.

The possibility of transferring the potential aldehyde group from carbon atom 1 to the terminal atom of the carbon chain has been thoroughly investigated in the present study. One scheme which illustrates the idea (4) is very similar in outline to the sequence of reactions used for the commercial manufacture of L-ascorbic acid. The configuration of the hydroxyls on carbon atoms 2 and 3 must be D and L, respectively. This is true of D-galactose but not D-mannose.

A further scheme of the same general type is (5). Support for this came from the observation of Smythe & King (1942) that treatment of rats with various drugs (e.g. chloretone) increased the excretion of both D-glucuronic acid (as glucuronide) and L-ascorbic acid. D-Galactose but not D-mannose could replace the D-glucose.

This scheme appears to be fundamentally the correct one. It can be written more conveniently in terms of the derivatives which were actually used to test its applicability to rats and cress seedlings. Scheme 5 for glucose and galactose can then be written as (6) and (7), respectively, which illuminates, in a striking manner, the crucial inversion from the D- to L-configuration by a series of plausible biochemical reactions.

The chief purpose of this paper is to present evidence that the reaction pathways of these last two schemes actually exist in plant and animal tissues. Briefly, the evidence consists of the demonstration that of a large number of closely related sugar derivatives, four sugar acid derivatives, L-gulono-γ-lactone, L-galactono-γ-lactone, D-glucurono-γ-lactone and D-galacturonic acid methyl ester, when fed to germinating cress seedlings or injected into rats, produced a very marked increase in the synthesis of L-ascorbic acid. These compounds are precisely those which should have an effect if the reactions in the above schemes (6) and (7) described the path by which L-ascorbic acid is synthesized; and moreover, no other closely related compound gives rise to L-ascorbic acid.

METHODS

The possibility that the above schemes outline the pathway by which L-ascorbic acid is synthesized from D-glucose has been examined by feeding various compounds postulated as intermediates in schemes 1–5 to cress seedlings, or injecting them into rats, and by comparing the formation of L-ascorbic acid with that in untreated controls. In the case of rats the tissues are normally saturated with L-ascorbic acid so that they excrete in their urine a small approximately constant amount which can be used as a measure of the synthesis of L-ascorbic acid in the intact animal. Any increase in the synthesis of L-ascorbic acid in the tissues is immediately reflected in the urinary excretion. The experiments on cress seedlings and on rats will be described separately.

Before discussing the significance of the results obtained, it is important to emphasize that certain of the compounds fed were simple derivatives of those originally postulated in schemes 1–5. These derivatives were chosen because they would penetrate into the cell, whereas those originally suggested would not do so, and yet once inside the cell would decompose to give the postulated intermediate. In the present study it was considered essential to make sure that the compound had at least penetrated into the seedling itself. Even then, the possibility remained that it might be unable to reach the active centres of synthesis, but for the present study the assumption was accepted that if the compound was present in the sap, it was available for conversion into L-ascorbic acid. Our experience was that organic compounds (Simon & Beevers, 1951) which were ionized in solution penetrated into the cress seedlings little if at all, whereas compounds which were un-ionized in solution, such as esters, lactones and sugars, penetrated readily (Beevers, Goldschmidt & Koffler, 1952). Acids such as galacturonic were not absorbed, but if they were fed as the methyl or ethyl esters, these penetrated into the seedling and then hydrolysed to give the free acid in the sap. α-Hydroxy acids such as galacturonic can be very conveniently handled in this way because their esters usually hydrolyse fairly rapidly in aqueous solution. Sugar acids such as D-gluconic, D-mannonic, L-gulonic and L-galactonic were fed as the γ-lactones. These were chosen because they were internal esters and were therefore free from the criticism that a 'foreign' alcohol was being introduced into the cell which might affect the synthesis of L-ascorbic acid; in aqueous solution they hydrolyse very slowly and might therefore be expected to penetrate into the cell in the same way as the esters.

Preparation of chemicals

Many of the chemicals used in this study were not obtainable commercially and had to be synthesized. Details are given in Table 1.

Each of the compounds described was examined on a paper chromatogram using as solvent either the water-poor phase from a two-phase mixture of *n*-butanol:glacial acetic acid:water (4:1:5 by vol., respectively) or a single phase solvent *n*-propanol plus concentrated ammonia (7:3 by vol. respectively). In the latter solvent the lactones were decomposed and moved as the ammonium salts of the free acids. In all cases except that of L-gluconic acid the compounds were free from other sugar derivatives. Some of the γ-lactones tended to decompose even with the acid solvent to give small amounts of the free acid and the δ-lactone, but these were readily identified.

Analytical technique

Many of the experiments which will be described later depended upon the detection, and in some cases the quantitative estimation, of minor constituents of the cell sap. For some, chemical methods were available, but for many the only method was the examination of the cell sap on a paper chromatogram. A full description of the apparatus and the general conditions of working in order to obtain the maximum resolution of the solutes on the paper chromatogram has been given in an earlier study on the separation of the sugars from plant extracts by Isherwood & Jermyn (1951). In the present paper only the modifications necessary for the separation of the particular compounds under investigation will be described. In many cases the quantitative chromatographic methods supplemented the results obtained by older chemical methods. Analysis of the urine

Table 1. *Preparation and properties of sugar acid derivatives*

(All but one are γ-lactones. Rotations were in water unless specified otherwise and were at room temperature.)

Substance (γ-lactone)	Method of preparation	References	M.p. (°) (uncorr.)	$[\alpha]_D$ (°) (*c* in parentheses)	Notes
D-Allono	From D-ribose (cyanohydrin)	(*a*)	130	−6 (3·5)	—
D-Altrono	From D-ribose (cyanohydrin)	(*a*)	Syrup	+35 (2·7)	Free acid prepared from Ca salt by addition of HCl; $[\alpha]_D$ +8 (4)
D-Gulono	From D-xylose (cyanohydrin)	(*b*)	180	−56 (1)	—
D-Idono	From D-xylose (cyanohydrin)	(*b*) (*c*) (*d*)	Syrup	−53 (10)	Brucine salt of acid, m.p. 182°. Dibenzal derivative, m.p. 204°
D-Mannono	From D-mannose by oxidation	(*e*)	150–152	+51·5 (1)	—
D-Glucono	From D-glucose by oxidation	(*e*) (*f*)	133–136	+68 (5)	—
D-Galactono	From D-galactose by oxidation	(*e*)	132–133	−73 (5)	—
D-Talono	From D-galactonic acid by epimerization	(*g*)	130	−33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ +17 (5)
L-Gulono	From D-glucuronic acid by reduction with Na/Hg	(*h*)	180	+55 (1)	—
L-Idono	From L-gulonic acid by epimerization	(*c*)	Syrup	+50 (5)	Brucine salt of acid, m.p. 190–192; $[\alpha]_D$ −17 (5)
L-Galactono	From D-galacturonic acid by reduction with Na/Hg	(*h*)	132	+75 (5)	—
L-Talono	From L-galactonic acid by epimerization	(*g*)	130	+33 (2)	Free acid, m.p. 125°; $[\alpha]_D$ −17 (5)
L-Mannono	From L-arabinose (cyanohydrin)	(*b*)	150	−51 (5)	—
L-Glucono	From L-arabinose (cyanohydrin)	(*b*)	—	—	Crude syrup after removal of bulk of L-mannono-γ-lactone
D-Mannurono	From alginic acid	(*i*)	138	+92 (3)	—
D-Galacturonic acid methyl ester	From D-galacturonic acid	(*j*)	145	+94 (2, MeOH)	—

References: (*a*) Phelps & Bates (1934); (*b*) Hudson, Hartley & Purves (1934); (*c*) Fischer & Fay (1895); (*d*) Micheel & Kraft (1933); (*e*) Hudson & Isbell (1929); (*f*) Isbell & Frush (1933); (*g*) Glatthaar & Reichstein (1938); (*h*) Thierfelder (1891); (*i*) Frush & Isbell (1946); (*j*) Jansen & Jang (1946).

of the rat was effected by essentially the same methods as those used for the cress seedling.

The analysis consisted mainly in determinations of L-ascorbic acid, sugars and organic acids such as D-glyceric acid.

L-Ascorbic acid was estimated chemically by oxidation with 2:6-dichlorophenol indophenol, the procedure adopted being essentially that described by Harris & Olliver (1942). The quantitative chromatographic method described by Chen, Isherwood & Mapson (1953) will distinguish between D-*arabo*ascorbic acid and L-ascorbic acid and was used particularly in connexion with experiments in which various sugar acid derivatives were fed to cress seedlings or injected into rats.

Fermentable and non-fermentable sugars were estimated by the method of Mapson, Cruickshank & Chen (1949) or by the chromatographic method of Jermyn & Isherwood (1949). The accuracy of the latter method has been much improved by a modification to the copper reagent used for estimating the separated sugar. Wager (private communication) has found that by carrying out the oxidation of the sugar in the absence of oxygen, the precision of the results with small amounts of sugar is very much increased.

In the qualitative examination of the cell sap of the cress seedling, the amount of acid present was judged visually from the size of the spot on the paper chromatogram using solvents recommended by Isherwood & Hanes (1953). This was particularly useful in the examination of cress seedlings which had been fed with various sugar-acid derivatives, when it was essential to know whether the acid had penetrated into the seedling and whether it had noticeably affected any of the other constituents. Further details of the qualitative examination of the cress seedlings will be given later in the description of the separate experiments.

Culture of cress seedlings

The procedure used varied slightly as the work progressed but the preferred method was as follows.

About 300 seeds were placed on a piece of fine Nylon fabric (area about 60 cm.^{2}) which covered the bottom of a white porcelain jar. Underneath the Nylon fabric was a piece of filter paper of about the same size. Sufficient of the appropriate solution was then added so that the seeds were just wetted. Nylon fabric was chosen because it is not absorptive, and if necessary it can be lifted out of the jar with the seedlings still in position, fresh solution and filter paper introduced, and the Nylon replaced in its original position. This is particularly useful when the aqueous solution slowly decomposes as with the sugar acid lactones and esters and it is essential to feed the seedlings with fresh solution each day. The seeds were germinated in the dark at a temperature of 20° and the jars were covered to prevent evaporation.

For analysis the seedlings were usually washed to remove any adherent solution and then the testas removed. The subsequent treatment depended upon whether they were to be analysed for L-ascorbic acid or whether the constituents of the cell sap were to be examined on a paper chromatogram. For the latter they were frozen at −20° and held at this temperature until required.

Treatment of rats with various compounds

Piebald male rats of varying ages but usually about 140 g. in weight were given a basal diet containing casein 20, sucrose 60, arachis oil 15, and salt mixture 5%, with an additional 10% of dried yeast. Each animal received weekly supplements of vitamins A and D by the administration of two drops of halibut-liver oil (=1500 i.u. of A and D/rat/week, respectively). Vitamin E was given as 1 mg. of (±)-α-tocopherol acetate. The rats were housed in metabolism cages in which all metal parts coming into contact with urine were waxed. The urine was collected in beakers containing 10 ml. of 10% (w/v) oxalic acid to avoid any decomposition of the L-ascorbic acid. The rats were rested until their urinary excretion of L-ascorbic acid was steady and then they were injected subcutaneously with 0·5 ml. of an aqueous solution of the compound to be tested. Commonly 100 mg. of the organic compound was injected.

RESULTS

Experiments with cress seedlings. The effect on the synthesis of L-*ascorbic acid of feeding various sugar derivatives*

In the experiments described in Table 2, compounds which were postulated as intermediates in various possible sequences of reactions linking glucose with L-ascorbic acid were fed to cress seedlings and the formation of L-ascorbic acid in these seedlings compared with that in seedlings grown in water under identical conditions of temperature and humidity; a fresh control experiment on seedlings grown in water was made for each compound.

The results described in Table 2 show that feeding the γ-lactone of D-gluconic acid (scheme 3), L-sorbose and D-sorbitol (scheme 4) and DL-glyceraldehyde (schemes 1 and 2) did not cause any marked increase in the synthesis of L-ascorbic acid as compared with that in seedlings grown in water. Experiments with D-xylose and L-arabinose were included to show the results obtained when compounds were fed which were neither metabolized by the seedlings to any extent, nor likely to be precursors of L-ascorbic acid. These experiments formed a very useful standard by which to judge the other experiments. Thus it would appear that the synthesis of L-ascorbic acid does not proceed through any of the compounds mentioned above, though it must be emphasized that if phosphorylated derivatives of the compounds were the actual intermediates, then the compounds fed to the seedlings would have to be phosphorylated inside the cell before becoming available for conversion into L-ascorbic acid. This possibility would tend to make any conclusions which might be derived from these feeding experiments rather tentative. However, the striking results obtained by feeding under analogous conditions the γ-lactones of D-glucuronic and L-gulonic acids (scheme 5) diminish the importance of this criticism. These compounds definitely increased the synthesis of L-ascorbic acid even though the cell sap became more acid. The growth and appearance of the seedlings were practically indistinguishable from those grown in water.

Table 2. *Synthesis of* L-*ascorbic acid in cress seedlings grown in solutions of various sugar derivatives*

Sugar derivative	Duration of experiment (hr.)	Weight of 100 seedlings (g.)	L-Ascorbic acid as % of control grown in water	Examination* of cell sap
D-Glucono-γ-lactone (0·5%)	72	1·10	61	pH 4·56 C
	96	1·20	43	4·44
	120	1·60	60	
DL-Glyceraldehyde (0·1%)	48	1·0	82	
	72	1·73	87	
	96	2·51	89	C
	120	2·96	95	
	144	3·18	83	
D-Glyceraldehyde (0·1%)	72	1·0	93	
	96	1·4	108	C
	120	1·8	103	
D-Sorbitol (1%)	51	1·03	96	
	72	1·51	98	
	96	1·88	108	C
	120	2·08	102	
	144	2·44	113	
L-Sorbose (1%)	51	1·0	75	
	72	1·38	88	
	96	1·89	103	U
	120	2·14	100	
	144	2·21	103	
D-Glucurono-γ-lactone (1%)	72	1·00	97	
	92	1·30	125	
	118	1·80	127	C
	142	1·90	131	
	163	1·90	149	
L-Gulono-γ-lactone (1%)	72	1·30	152	pH 4·6 C
	96	1·60	139	4·59
	120	1·80	144	4·41
	142	2·10	137	
D-Xylose (1%)	72	1·0	74	
	96	—	100	U
	140	—	114	
	170	1·90	112	
L-Arabinose (1%)	72	0·96	82	
	96	—	100	U
	140	—	108	
	170	2·35	106	

* U, the cell sap contained an appreciable amount of the sugar derivatives as measured by the rise in the unfermentable sugars. C, examination on a paper chromatogram showed that the cell sap contained an appreciable quantity of the sugar derivative.

Feeding L-*xylo*hexulonic ester also appeared to increase the L-ascorbic acid content but this was later found to be due to the spontaneous conversion of the ester into L-ascorbic acid in aqueous solution, especially in the presence of alkaline buffers (pH 6–8). The L-ascorbic acid was readily absorbed by the seedlings and it thus appears likely that the observed increase was a result of a non-enzymic conversion of the ester into L-ascorbic acid. This experiment, which is not described in Table 2, therefore does not provide unequivocal evidence that the ester is a precursor of L-ascorbic acid in the seedling. In connexion with this discovery, it is interesting to note that it has been claimed that the ester has anti-scorbutic properties (Reichstein & Demole, 1936).

The experiment in which DL-glyceraldehyde was fed deserves closer study. Large concentrations of DL-glyceraldehyde inhibit growth but at the concentration employed in the experiment (0·1%, w/v) described in Table 2, growth was practically unaffected (weight 3·2 g./100 seedlings at 144 days as compared with 2·7–3·4 for seedlings grown in water). The L-ascorbic acid content, however, was definitely lower. This appears to be due to the presence of L-glyceraldehyde, for feeding D-glyceraldehyde in the same concentration did not affect the synthesis of L-ascorbic acid.

The interpretation of feeding experiments with the potentially acid compounds was sometimes complicated by the effect of the compounds on the pH of the cell sap apart from the actual synthesis of

the L-ascorbic acid. An example of this can be quoted from the results in Table 2. D-Glucono-γ-lactone (0·5 %, w/v, in water) fed to cress seedlings clearly depressed the formation of L-ascorbic acid (50 %) because the amount in the sap was sufficient (estimated from paper chromatograms to be about 0·5 % at the sixth day) to make it definitely more acid (pH 4·4) than that of seedlings grown in water (pH 4·8). A rough calculation of the amount by which the synthesis of L-ascorbic is depressed by a change in the pH of the sap can be made from the experiments described by Mapson *et al.* (1949). They showed that the synthesis of L-ascorbic acid in cress seedlings was definitely related to the pH of the sap and was depressed when the sap became more acid. From the figures given in their paper, the change in the pH of the cell sap (4·8–4·4) in the experiment with D-glucono-γ-lactone would be expected to depress the synthesis by about 50 %. The observed depression would therefore appear to be almost entirely related to the change in pH of the cell sap, and it seems unlikely that the compound has any specific inhibitory effect on the synthesis.

The results described in Table 2 suggest strongly that the synthesis of L-ascorbic acid from D-glucose proceeds by a series of reactions similar to those outlined in scheme 5. Later experiments have been concerned with confirming the special position of D-glucurono-γ-lactone and L-gulono-γ-lactone in the synthesis and in establishing the specificity of the various reactions involved.

Examination of the specificity of the reactions involved in the conversion of hexoses to L-*ascorbic acid*

Cress seedlings. Two reactions outlined in scheme 5 have been examined in detail. Various compounds closely related to D-glucuronic and L-gulonic acids have been fed to cress seedlings and the L-ascorbic acid content compared with that of seedlings grown in water. The results of these experiments have indicated that two hexoses may act as precursors of L-ascorbic acid, namely D-glucose and D-galactose, the sequence of reactions connecting them with L-ascorbic acid being very similar. This was to be expected on theoretical grounds since the configuration of these sugars is such that they can be transferred from the D- to the L-series of sugars by transposing the terminal groups of the molecule as outlined in scheme 5, and the L sugar derivatives produced have the right configuration for a precursor of L-ascorbic acid. This fact was briefly mentioned when scheme 5 was discussed earlier in this paper.

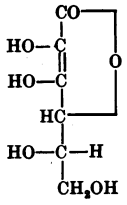
The group of compounds closely related to L-gulonic acid comprised all the known aldohexonic acids. In the feeding experiments, however, the γ-lactones of these were used and the configurations of these, written according to the usual cyclic modification of the Fischer convention, are shown in Fig. 1.

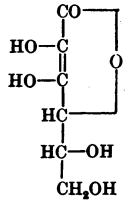
Only two compounds related to D-glucuronic acid (D-glucurono-γ-lactone was actually used) were fed to cress seedlings, namely D-mannurono-γ-lactone and D-galacturonic acid methyl ester. In Fig. 1 these have been drawn in such a way as to emphasize that they have a similar structural relationship to D-mannono- and L-galactono-γ-lactones, respectively, as D-glucurono-γ-lactone has to L-gulono-γ-lactone in scheme 5.

The formulae in Fig. 1 have been grouped so as to bring out as clearly as possible the relationship of the various lactones to L-ascorbic acid. Each horizontal line contains four lactones which have the same configuration as far as the carbon atoms 4 and 5 are concerned but differ between themselves as to the disposition of the groups on carbon atoms 2 and 3. No information can be obtained from the configuration of L-ascorbic acid as to the disposition of the groups on carbon atoms 2 and 3 of its precursor. In Fig. 1 the first line contains lactones which have the same configuration as L-ascorbic acid, the second line lactones in which the configuration of carbon atom 5 is opposite to that of L-ascorbic acid, and the third and fourth lines lactones which are enantiomorphs (mirror images) of the corresponding lactones in the first and second lines respectively.

The results of feeding the various sugar derivatives to cress seedlings are shown in Table 3. It is clear that L-gulono-, L-galactono-, D-altrono-, D-glucurono-γ-lactones and D-galacturonic acid methyl ester markedly increase the formation of an ascorbic-acid-like substance. This is particularly obvious if the results with these sugar derivatives are compared with those obtained with inactive lactones of very similar structure, e.g. D-gulono-, D-galactono- and D-mannurono-γ-lactones. There seems no doubt that all these lactones penetrated into the seedlings (the cell sap was examined on a paper chromatogram) and the difference in behaviour appears to be due to the specificity of the enzymes involved in the conversion to L-ascorbic acid.

The ascorbic-acid-like compound produced by feeding the cress seedlings with L-gulono-, L-galactono- and D-altrono-γ-lactones was separated from the other constituents of the cell sap and examined on a paper chromatogram by the method of Chen *et al.* (1953). Tracings of chromatograms from seedlings which had been fed with L-galactono- and D-altrono-γ-lactones are shown in Fig. 2. (The chromatogram from seedlings fed with L-gulono-γ-lactone has not been included because apart from the very much weaker concentration of the ascorbic-acid-like compound in the sap, the picture was essentially the same as for the L-galactono-γ-


L-Ascorbic acid


D-*arabo*Ascorbic acid

Types I and IV were γ-lactones of aldonic acids, Types V and VI were uronic acid derivatives with the same basic configuration as Types I and II, respectively.

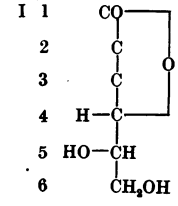
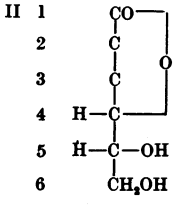
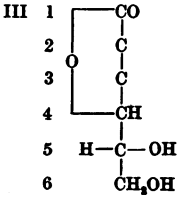
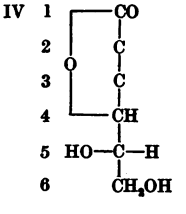
Type (same configuration of groups on $C_{(4)}$ and $C_{(5)}$)	Configuration of groups on $C_{(2)}$ and $C_{(3)}$: HO—C—H / HO—C—H	H—C—OH / HO—C—H	H—C—OH / H—C—OH	HO—C—H / H—C—OH
I 	L-GULONO*	L-Idono	L-Talono	L-GALACTONO*
II 	D-*Mannono** (rat)	D-Glucono	D-Allono	D-*Altrono** (cress)
III 	D-Gulono	D-Idono	D-Talono	D-Galactono
IV 	L-Mannono	L-Glucono	L-Allono	L-Altrono (not examined)
V As I but with —CHO at $C_{(6)}$ instead of —CH_2OH	D-GLUCURONO-* γ-LACTONE	—	—	D-GALACTURONIC ACID METHYL ESTER
VI As II but with —CHO at $C_{(6)}$ instead of —CH_2OH	D-Mannurono-γ-lactone	—	—	—

Fig. 1. Sugar acid derivatives fed to cress seedlings.

* Compounds in capital letters give L-ascorbic acid, whereas compounds in *italics* give D-*arabo*ascorbic acid, in the cress seedling and in the rat as shown.

Table 3. *Effect of feeding various sugar acid derivatives to cress seedlings on the formation of* L-*ascorbic acid*

(All solutions were put in after 48 hr. germination in water and changed every day. At the time of the first ascorbic acid determination the cell sap was, in every case, examined for the substance fed.)

Sugar derivative fed to seedlings*	Duration of experiment (hr.)	Apparent ascorbic acid as % of that in seedlings grown in water
D-Glucuronolactone	72	97
	96	105
	120	117
	144	123
D-Galacturonic acid methyl ester	72	144
	96	140
	140	172
	164	171
D-Mannuronolactone	96	72
	120	58
	148	57
L-Gulonolactone	72	95
	96	137
	120	146
	144	148
L-Idonolactone	72	103
	96	62
	120	46
L-Talonolactone	72	83
	96	84
	120	69
	144	75
L-Galactonolactone	72	320
	96	256
	120	357
	144	482
D-Mannonolactone	72	102
	96	85
	120	77
	144	79
D-Gluconolactone	72	59
	96	47
	120	55
D-Allonolactone	72	97
	96	77
	120	78
	144	61
D-Altronolactone	72	387 †
	96	552 †
	120	580 †
D-Gulonolactone	72	70
	96	34
	120	27
D-Idonolactone	72	97
	96	56
	120	57
D-Talonolactone	72	127
	96	92
	120	78
	144	77
D-Galactonolactone	72	55
	96	60
L-Mannonolactone	72	76
	96	41
	120	43
L-Gluconolactone	96	64

* All 0·5% (w/v) except D-glucurono- and L-gulono-lactones (1%). † D-*arabo*Ascorbic acid produced.

lactone). Comparison of these chromatograms indicated that feeding L-galactono-γ-lactone increased the L-ascorbic acid content of the seedlings, and feeding D-altrono-γ-lactone increased the D-*arabo*ascorbic acid content.

The identification of these compounds as L-ascorbic acid and D-*arabo*ascorbic acid is admittedly not conclusive because the enantiomorphic forms would behave similarly on the chromatogram. However, if the configurations of the lactones which act as precursors in the cress seedling are taken into consideration, this uncertainty is removed; L-gulono- and L-galactono-γ-lactones can produce only L-ascorbic acid, and D-altrono-γ-lactone, D-*arabo*ascorbic acid.

Before discussing the results with cress seedlings, the comparable experiments with rats will be described.

Rats. The results of a number of the experiments on rats are shown in Fig. 3. Most of the experiments in which the titration figure for urine remained unchanged have not been described. The same series of sugar-acid γ-lactones were examined as in the case of cress seedlings.

From Fig. 3 it is clear that L-gulono-, L-galactono, D-mannono and D-glucurono-γ-lactones and D-galacturonic acid methyl ester increase the titration figure. Examination of the urine showed that D-mannono-γ-lactone gave rise to D-*arabo*ascorbic acid, whereas the others all gave L-ascorbic acid. The chromatograms were very similar to those shown previously in Fig. 2 for cress seedlings. It was interesting to note that neither D-altrono-γ-lactone, which might give D-*arabo*ascorbic acid, nor L-idono- and L-talono-γ-lactones, which are very similar in structure to L-gulono- and L-galactono-γ-lactones, respectively, produced any increase in the titration figure. D-Mannurono-γ-lactone which by analogy with D-glucurono-γ-lactone in scheme 5 should give D-mannono-γ-lactone on reduction

does not give D-*arabo*ascorbic acid. D-Mannono-γ-lactone has been shown previously to give D-*arabo*-ascorbic acid. It seems that the enzyme system which converts the uronic acid to the corresponding aldonic acid cannot convert D-mannurono-γ-lactone into D-mannono-γ-lactone.

The four sugar-acid derivatives which have been shown to act as precursors of L-ascorbic acid in cress seedlings and rats can be regarded as intermediates in two hypothetical series of reactions linking the D-hexoses with L-ascorbic acid. The first of these links D-glucose, D-glucuronic acid and L-gulonic acid to L-ascorbic acid and the second, which is very similar to the first, links D-galactose, D-galacturonic acid and L-galactonic acid to L-ascorbic acid. The only difference between the intermediates in the two series of reactions is the disposition of the groups on carbon atom 4 (carbon atom 3 if based on L-ascorbic acid), those in the D-glucose series having the hydroxyl on the right-hand side (formula written with the aldehyde group at the top) and those in the D-galactose series having it on the left. Although the experimental evidence did not allow a decision to be made as to whether D-glucose or D-galactose was the precursor, it was clear that the enzymes catalysing the series of reactions in the animal were not exactly the same in the rat as those in the plant, because their behaviour towards lactones which might form D-*arabo*ascorbic acid was markedly different. Feeding D-altrono-γ-lactone to cress seedlings gave D-*arabo*ascorbic acid but injected into rats had no effect, whereas feeding D-mannono-γ-lactone to cress seedlings had no effect but injected into rats gave D-*arabo*ascorbic acid. A possible explanation for these observations is based on the similarity in structure of L-galactono- and D-altrono-γ-lactones, and L-gulono- and D-mannono-γ-lactones, respectively (see Fig. 1). Each pair of lactones has the same ring structure and differs only in the disposition of the groups in carbon atom 5. It would appear that the enzyme in cress seedlings which converts either L-gulono- or L-galactono-γ-lactones into L-ascorbic acid, reacts more readily with the L-galactono-γ-lactone ring structure, whereas the corresponding enzyme in the rat reacts more readily with the L-gulono-γ-lactone ring structure. This specificity is only shown when an 'unnatural' lactone is present as substrate.

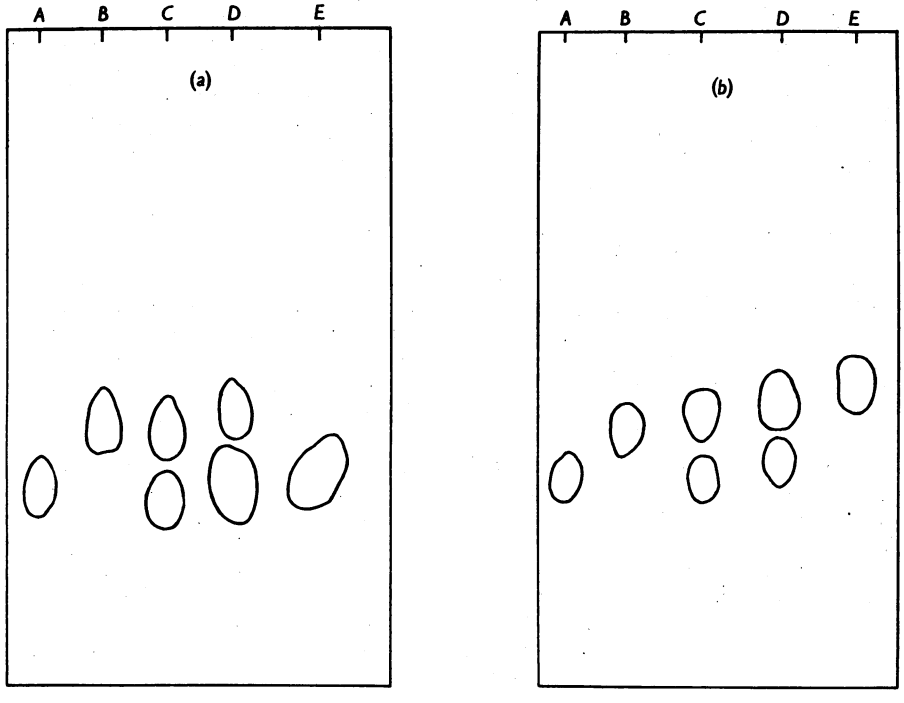


Fig. 2. Tracings of chromatograms showing the compounds present in the sap of cress seedlings which reduce 2:6-dichlorophenol indophenol. The cress seedlings were cultured either in a 0·5% (w/v) aqueous solution of D-altrono-γ-lactone (*a*), or in a 0·5% (w/v) solution of L-galactono-γ-lactone (*b*), for 5 days. Paper, Whatman no. 1. Solvent, phenol: water: oxalic acid. For comparison solutions (0·2%, w/v) of D-*arabo*ascorbic and L-ascorbic acids were spotted on the 'starting line' as shown. *A*, D-*arabo*ascorbic acid; *B*, L-ascorbic acid; *C*, L-ascorbic and D-*arabo*ascorbic acids; *D*, extract of seedlings, L-ascorbic and D-*arabo*ascorbic acids; *E*, extract of seedlings.

The substrate specificity of the enzyme catalysing the conversion of the aldonic acid γ-lactones to ascorbic-acid-like compounds deserves mention, because of sixteen possible precursors only four were active as substrates, two giving L-ascorbic acid. From the results described in Table 3 it is clear that the enzyme requires the disposition of groups on carbon atom 2 to be such that the hydroxyl is on the left-hand side of the molecule. L-Idono- and L-talono-γ-lactones, which are very similar to L-gulono- and L-galactono-γ-lactones, respectively, were without activity. These first two lactones have the hydroxyl on carbon atom 2 on the right-hand side.

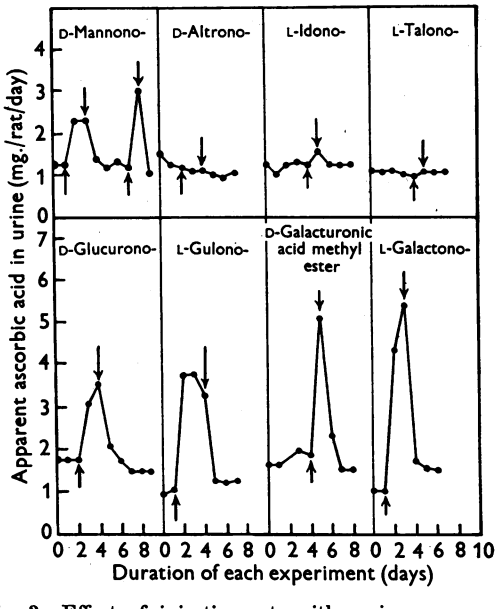


Fig. 3. Effect of injecting rats with various sugar acid derivatives on the excretion of ascorbic acid in the urine. The compounds were γ-lactones in all but one case. ↑ Marks the day the rats were first injected. ↓ Marks the day after which the rats were no longer injected.

The disposition of the groups on carbon atom 3 is not so important for both L-gulono and L-galactono-γ-lactones are active. In these the disposition of the groups on carbon atom 3 is opposite.

It seems essential for the ring to be on the right-hand side of the molecule (hydroxyl on carbon atom 4 on right-hand side), for only lactones which had the ring on the right-hand side were active as substrates.

The efficiency of the conversion of the aldonic acid lactone to L-ascorbic acid is difficult to estimate because it is probable that in the rat a high proportion of the lactone is excreted unchanged and in the cress seedling is converted to the free acid. L-Ascorbic acid (100 mg.) injected into the rat was excreted to the extent of about 60% and by analogy it is possible that a similar proportion of the sugar-acid lactones may be excreted unchanged. The observed ratio between the L-ascorbic acid in the urine and the lactone injected was approximately 3:100 in most of the experiments, though in one experiment when L-galactono-γ-lactone was injected as an aqueous suspension, the ratio was higher, 8:100. This suggests that the efficiency of the conversion might be higher if the lactone was fed continuously so as to maintain a low concentration in the blood, rather than injected all at once. In the case of cress seedlings the efficiency of the conversion has been estimated very roughly to be of the same order of magnitude. The main source of error lies in the fact that the amount of the lactone in the cell sap has been estimated visually from the size of the spot on a paper chromatogram.

CONCLUSIONS

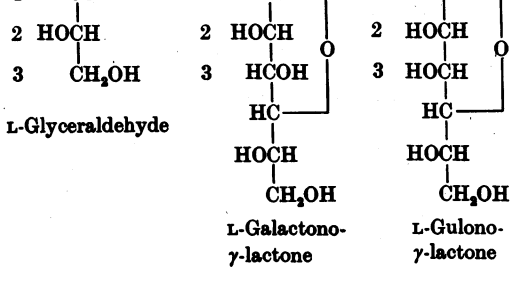
The foregoing experiments constitute a preliminary survey of the series of reactions which convert a hexose, which may be D-glucose or D-galactose, into L-ascorbic acid in both cress seedlings and the rat. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. The significance of this observation is very much increased by the fact that the other sugar-acid derivatives with closely related configurations such as L-idono- and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid. The four compounds described above have previously in this paper been postulated as intermediates in a hypothetical series of reactions (scheme 5) linking D-glucose (and D-galactose) with L-ascorbic acid, and the fact that these compounds can be transformed into L-ascorbic acid lends considerable support to this hypothesis.

It is possible to explain the conversion of these compounds into L-ascorbic acid by a series of reactions other than those described in scheme 5. In scheme 5 the carbon chain of the hexose was not broken but it is easy to imagine a series of reactions in which the carbon chain was broken down to C_2, C_3 or C_4 fragments and then resynthesized to give L-ascorbic acid. The experiments in the present study do not provide a rigid proof that the carbon chain of the hexose molecule remains unbroken but the earlier observations of Jackel *et al.* (1950) that feeding D-glucose uniformly labelled with ^{14}C to chloretone-treated rats gave L-ascorbic acid which was also uniformly labelled, also suggested that the carbon chain was not broken.

A critical test would be to feed D-glucose which was labelled only in one position along the carbon chain (carbon atom 1 or 6 would be convenient) to rats and then examine the distribution of the labelled carbon along the carbon chain of the resultant L-ascorbic acid. If the chain remained unbroken, the labelled carbon would only occur in the corresponding position along the carbon chain of the L-ascorbic acid. Recently two experiments of this type have been described, the results of which strongly support the reaction chain described in scheme 5. Administration of D-[1-^{14}C]glucose to chloretone-stimulated rats resulted in the urinary excretion of ascorbic acid containing ^{14}C chiefly in position 6 (Horowitz, Doerschuk & King, 1952). Similarly, administration of D-[6-^{14}C]glucose gave ascorbic acid containing ^{14}C in position 1 (Horowitz & King, 1953). The D-glucose must have been converted to ascorbic acid without breaking the carbon chain and a complete inversion of the configuration must have occurred.

In the present study two series of reactions (schemes 1 and 2) based on the condensation of L-glyceraldehyde with appropriate three-carbon compounds to give L-ascorbic acid were tested by feeding DL- and D-glyceraldehyde to cress seedlings. It was found that the formation of L-ascorbic acid was unaffected by feeding D-glyceraldehyde but was definitely depressed by the DL-glyceraldehyde. From these results it seemed unlikely that L-glyceraldehyde was concerned in the synthesis of L-ascorbic acid. The reduced formation of L-ascorbic acid by feeding DL-glyceraldehyde was interesting because L-glyceraldehyde (L-sorbose 1-phosphate is the active inhibitor, Lardy, Wiebelhaus & Mann, 1950) is known to inhibit the hexokinase enzyme present in animals and plants which catalyses the formation of D-glucose 6-phosphate from D-glucose.

If this were true of the hexokinase present in cress it would suggest that glucose 6-phosphate is concerned in the chain of reactions from D-glucose to L-ascorbic acid. However, another explanation of the inhibition can be suggested based on the similarity of the configuration of L-glyceraldehyde and the first three carbon atoms of L-gulono- and L-galactono-γ-lactones.



The resemblance is even closer than the formulae suggest because the carbonyl group at C-1 of the lactones has characteristic carbonyl properties which are absent when it exists as part of a carboxyl ion; presumably resonance in the group  masks the true carbonyl properties in this case. The supposition is that the L-glyceraldehyde having a similar structure competes with the γ-lactones for the enzyme system which catalyses their oxidation to L-ascorbic acid.

The specificity of the enzymes catalysing the transformation of an aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L configuration, and that on carbon atom 4 the D configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone only in cress seedlings and D-mannono-γ-lactone only in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in the cress seedling reacts only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid), whereas the enzyme in the rat reacts only with compounds similar to L-gulonic acid (D-mannonic acid). In the case of L-gulonic and L-galactonic acids, which as lactones give rise to L-ascorbic acid in both the rat and cress seedlings, it is conceivable that the two compounds can be converted into each other as in the enzymic conversion of D-galactose 1-phosphate to D-glucose 1-phosphate by a direct inversion of the groups at carbon atom 4 (Leloir, 1951; Topper & Stetten, 1951). The configuration of L-gulose and L-galactose closely corresponds to that of D-glucose and D-galactose, respectively (cf. scheme 5).

The specificity of the enzymes which catalyse the conversion of a uronic acid presumably via the related aldonic acid (scheme 5) to an ascorbic acid-like compound, has been tested in one case only. D-Mannuronic acid-γ-lactone on reduction gives D-mannonic acid, which (as the lactone) had earlier been shown to give D-*arabo*ascorbic acid in the rat. Injection of D-mannurono-γ-lactone into the rat did not affect the 2:6-dichlorophenol indophenol titration figure, so that it appeared that the enzyme was incapable of reducing D-mannuronic acid to D-mannonic acid. This is in marked contrast to D-glucurono-γ-lactone and D-galacturonic acid methyl ester which definitely are converted into L-ascorbic acid. The only difference between D-mannuronic acid and D-glucuronic acid is in the configuration of the groups on carbon atom 2.

The discovery that the conversion of D-glucose to L-ascorbic acid probably proceeds along a pathway similar to that described in scheme 5 raises the important question as to what difference there is between animals such as guinea pigs and man,

which can suffer from scurvy and are incapable of synthesizing L-ascorbic acid, and those, such as the rat, which do not suffer from scurvy. It appears that in all the animals the synthesis can proceed as far as D-glucuronic acid; even in man, normal urine contains 150–200 mg. of D-glucuronic acid per day, and when compounds such as menthol are administered the excretion of conjugated glucuronides is increased many times. The obstacle to the conversion of D-glucose into L-ascorbic acid would therefore appear to lie between D-glucuronic acid and L-ascorbic acid. In a later paper it is hoped to consider this question in more detail.

The discovery of a series of reactions which describe in broad outline the pathway by which the hexose sugars are converted into L-ascorbic acid is important because it opens up the subject for rapid advances on the enzymic side, though it is possible that the reactions involved in the transformation may not be so simple as in the above scheme. The experimental evidence does not allow us to determine the exact ring structure of the sugar derivatives which are the precursors of L-ascorbic acid, for in the solution in the cell fluids several different forms of the sugar derivatives will be present. In addition, no attention has yet been given to phosphorylated derivatives because at present there is no experimental evidence which definitely indicates that phosphate is concerned, but the intermediates in the transformation may well be phosphorylated analogues of the sugar acid compounds described above.

The fact that the ring structure of the γ-lactones of L-gulonic and L-galactonic acids is similar to that of L-ascorbic acid suggests that the intermediate may be a γ-lactone. However, the ready conversion of the methyl ester of D-galacturonic acid (no lactone ring is present) into L-ascorbic acid and the fact that the γ-lactones are formed very slowly at room temperature in the absence of catalysts suggests that the active intermediate may be a derivative of the sugar acid other than the lactone.

SUMMARY

1. Four compounds, L-gulono-, L-galactono- and D-glucurono-γ-lactones and D-galacturonic acid methyl ester, when fed to cress seedlings or injected into rats, have been shown to be transformed into L-ascorbic acid. A number of other sugar acid lactones with closely related configurations such as L-idono and L-talono-γ-lactones are inactive, except that two lactones, D-mannono-γ-lactone in the rat and D-altrono-γ-lactone in the cress seedling, are converted into D-*arabo*ascorbic acid.

2. The four compounds described above have been postulated as intermediates in a hypothetical series of reactions linking D-glucose (and in a similar series D-galactose) with L-ascorbic acid. The series of reactions in outline are:

D-glucose → D-glucuronic acid → L-gulonic acid → L-ascorbic acid

and

D-galactose → D-galacturonic acid → L-galactonic acid → L-ascorbic acid.

3. The specificity of the enzymes catalysing the transformation of the aldonic acid to L-ascorbic acid requires that the hydroxyls on carbon atoms 2 and 5 (compared to L-ascorbic acid) shall have the L-configuration and that on carbon atom 4 the D-configuration. An additional degree of specificity is implied by the fact that D-altrono-γ-lactone in cress seedlings and D-mannono-γ-lactone in the rat are converted into D-*arabo*ascorbic acid. This would suggest that the enzyme in cress seedlings will react only with compounds which have a configuration for the first four carbon atoms similar to L-galactonic acid (D-altronic acid) whereas the enzyme in the rat will react only with those which have a configuration similar to L-gulonic acid (D-mannonic acid).

4. A survey of a number of possible mechanisms by which the hexoses might be transformed into L-ascorbic acid has shown that those which involve L-glyceraldehyde, L-sorbose, D-sorbitol and D-gluconic acid are not present in cress seedlings or the rat.

We are indebted to Dr Dorothy M. Needham, F.R.S., of the School of Biochemistry, Cambridge, for the gift of a sample of D-glyceraldehyde. Mr F. C. Barrett has assisted with many of the preparations of the sugar lactones and the chromatographic isolation of D-glyceric acid. The work described in this communication forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

REFERENCES

Ahmad, B., Qureshi, A. A., Babbar, I. & Sawhney, P. C. (1946). *Ann. Biochem.* **6**, 29.
Beevers, H., Goldschmidt, E. P. & Koffler, H. (1952). *Arch. Biochem. Biophys.* **39**, 236.
Chen, Y. T., Isherwood, F. A. & Mapson, L. W. (1953). *Biochem. J.* **55**, 821.
Cox, E. G., Hirst, E. L. & Reynolds, R. J. W. (1932). *Nature, Lond.*, **130**, 888.
De, H. N. & Barai, S. C. (1949). *Indian J. med. Sci.* **37**, 101.
Devyatnin, V. A. (1950). *Biochemistry, Leningr.* **15**, 325.
Fischer, E. & Fay, I. W. (1895). *Ber. dtsch. chem. Ges.* **28**, 1975.
Frush, H. L. & Isbel, H. S. (1946). *J. Res. nat. Bur. Stand.* **37**, 321.
Glatthaar, C. & Reichstein, T. (1938). *Helv. chim. acta*, **21**, 3.
Harris, L. J. & Olliver, M. (1942). *Biochem. J*, **36**, 155.
Haworth, W. N. & Hirst, E. L. (1933). *Chem. & Ind.* **52**, 645.
Horowitz, H. H., Doerschuk, A. P. & King, C. G. (1952). *J. biol. Chem.* **199**, 193.

Horowitz, H. H. & King, C. G. (1953). *J. biol. Chem.* **200**, 125.
Hough, L. & Jones, J. K. N. (1951). *Nature, Lond.*, **167**, 180.
Hough, L. & Jones, J. K. N. (1952). *J. chem. Soc.* p. 4052.
Hudson, C. S., Hartley, O. & Purves, C. B. (1934). *J. Amer. chem. Soc.* **56**, 1248.
Hudson, C. S. & Isbell, H. S. (1929). *J. Amer. chem. Soc.* **51**, 2225.
Isbell, H. S. & Frush, H. L. (1933). *J. Res. nat. Bur. Stand.* **11**, 649.
Isherwood, F. A. & Hanes, C. S. (1953). *Biochem. J.* **55**, 824.
Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
Itô, N. & Mizuno, T. (1950). *Chem. Abstr.* **44**, 4084*a*.
Jackel, S. S., Mosbach, E. H. Burns, J. J. & King, C. G. (1950). *J. biol. Chem.* **186**, 569.
Jansen, E. F. & Jang, R. (1946). *J. Amer. chem. Soc.* **68**, 1475.
Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
Lardy, H. A., Wiebelhaus, V. D. & Mann, K. M. (1950). *J. biol. Chem.* **187**, 325.
Leloir, L. F. (1951). *Arch. Biochem. Biophys.* **33**, 186.
Mapson, L. W., Cruickshank, E. M. & Chen, Y. T. (1949). *Biochem. J.* **45**, 171.
Meyerhof, O., Lohmann, K. & Schuster, P. (1936). *Biochem. Z.* **286**, 301.
Micheel, F. & Kraft, K. (1933). *Hoppe-Seyl. Z.* **222**, 235.
Phelps, F. P. & Bates, F. J. (1934). *J. Amer. chem. Soc.* **56**, 1250.
Ray, S. N. (1934). *Biochem. J.* **28**, 996.
Reichstein, T. & Demole, V. (1936). *Festschrift für E. C. Barell*, Basel: F. Hoffman-La Roche & Co. Ltd., p. 107.
Ruffo, A. & Tartaglione, T. (1948). *Quad. Nutr.* **10**, 283.
Simon, E. W. & Beevers, H. (1951). *Science*, **114**, 124.
Smythe, C. V. & King, C. G. (1942). *J. biol. Chem.* **142**, 529.
Stumpf, P. K. (1948). *J. biol. Chem.* **176**, 233.
Sugawara, T. (1941). *Jap. J. Bot.* **11**, 147.
Tadokoro, T. & Nisida, (1940). *J. agric. chem. Soc. Japan*, **16**, 501.
Thierfelder, H. (1891). *Hoppe-Seyl. Z.* **15**, 71.
Topper, Y. J. & Stetten, D. jun. (1951). *J. biol. Chem.* **193**, 148.

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

BY F. A. ISHERWOOD, Y. T. CHEN AND L. W. MAPSON
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research

(*Received* 13 *May* 1953)

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