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Biological Synthesis of Ascorbic Acid: the Conversion of Derivatives of D-Galacturonic Acid into L-Ascorbic Acid by Plant Extracts

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It was suggested by Isherwood, Chen & Mapson (1953, 1954) that the synthesis of L-ascorbic acid *in vivo* can be represented in outline as proceeding by one or both of the following routes:

In route (2), the third stage in the synthesis, namely the conversion of the lactone of L-galactonic acid into L-ascorbic acid, has been shown to occur *in vitro* in the presence of enzymes contained within mitochondria prepared from plant tissues (Mapson, Isherwood & Chen, 1954). The corresponding reaction in route (1), namely the conversion of L-gulonono- γ -lactone into L-ascorbic acid, was catalysed only to a negligible extent by the same extracts, and it is only recently that we have been able to prepare sufficiently active enzyme preparations for this reaction to be detected. The greater facility with which the last reaction in route (2) as compared with that in route (1) proceeded in the pea extracts led us to concentrate on the enzymic reaction involved in route (2) to the exclusion of (1).

In the present work, the second stage in the synthesis, namely the enzymic conversion *in vitro* of derivatives of D-galacturonic acid into derivatives of L-galactonic acid has been investigated and the enzyme mechanism associated with the reduction of the uronic acid characterized.

METHODS

Enzyme preparations

These were prepared from pea seeds which had been soaked for 24 hr. in water at 20° and germinated for a further 24 hr. at the same temperature. To reduce bacterial infection, the

dry pea seeds were immersed in 1% (w/v) HgCl₂ for 1 min. and then thoroughly rinsed in sterile water. The partly germinated peas (30 g.) were ground up with a mixture of sand and 40 ml. of a solution of sucrose (0.4M), phosphate 0.1M, pH 7.4, and 4 mM-Mg²⁺ at 0°. The brei was centrifuged at 500 g for 5 min. and the supernatant removed. This supernatant, designated as homogenate, was further separated by centrifuging at 20 000 g for 20 min. into mitochondria and soluble fraction, the latter containing the enzymes present in the soluble part of the cytoplasm.

In some experiments these preparations were fractionated further by precipitation with acetone at a low temperature, as described by Askonas (1951), or by fractionation with (NH₄)₂SO₄. The enzyme was precipitated by acetone between 30 and 70% (v/v), and by (NH₄)₂SO₄ in a concentration between 50 and 70% at neutral pH.

Chemicals

Methyl D-galacturonate (MDG) and the γ -lactones of L-galactonic, D-glucuronic and D-mannuronic acids were prepared as described by Isherwood *et al.* (1954), and the amide of galacturonic acid was prepared by the method of Isbell & Frush (1950). MDG labelled with ¹⁴C in the methyl group was prepared by a micro-adaptation of the usual method, with [¹⁴C]methanol. When this preparation was used with the pea enzyme, the derivatives formed were examined on a paper chromatogram by means of a Geiger counter.

n-Propyl D-galacturonate was prepared under conditions similar to those used for the methyl ester, substituting *n*-propanol for methanol. Owing to the relative insolubility of galacturonic acid in *n*-propanol the yields were small, and the crude material was not purified. Chromatographic examination of the syrup showed that it consisted, to the extent of at least 85%, of a fast-running compound, presumably the *n*-propyl ester, which on saponification with alkali gave only D-galacturonic acid.

The lactone of galactaric acid was prepared by dissolving 30 g. of the free acid in 2 l. of water, boiling for 20–30 min. until a clear solution resulted, and then evaporating the solution over a free flame until the volume was reduced to

300 ml. The precipitated galactaric acid was filtered off (about 8 g. was recovered) and the filtrate evaporated *in vacuo* (bath temp. 40°) to a thin syrup. A little galactaric acid crystallized out and was filtered off. The syrup was treated with acetone, filtered from any galactaric acid which was precipitated, and the filtrate concentrated *in vacuo* to a thick clear syrup. This syrup crystallized on standing, yielding galactaric acid monolactone, m.p. 128°. Titrated with 0.1N-NaOH at 0°, 0.191 g. of lactone consumed 9.7 ml. of alkali and at 100°, 19.1 ml. (Found: C, 37.4; H, 4.3. Calc. for monolactone of galactaric acid, $C_6H_8O_7$, 10.0 ml. and 20.0 ml. of alkali; C, 37.6; H, 4.2%.)

The solutions of DL-isocitrate were prepared from commercial samples of the lactone by refluxing with the equivalent amount of N-NaOH for 30 min.

The solutions of DL-malate were prepared from commercial samples of the acid recrystallized once from water.

The diphosphopyridine nucleotide (DPN) was purchased from L. Light and Co., Colnbrook, Bucks, and was stated to contain not less than 90% of DPN. It was free from triphosphopyridine nucleotide (enzymic test of Mapson & Goddard, 1951).

Triphosphopyridine nucleotide (TPN) was either purchased from Sigma Chemical Corporation, U.S.A. (contained 65–80% of TPN) or prepared by a slight modification of the method of LePage & Mueller (1949). Instead of direct extraction of the liver with boiling water, it was first minced directly into five times its volume of acetone. The solid matter was filtered off, dried, ground and extracted with boiling water containing 0.5% of nicotinamide. The extracted nucleotides were then purified by adsorption on charcoal as described by LePage & Mueller. The product contained 40–60% of TPN. There was no difference in the results observed when these different preparations of TPN were used.

Paper chromatography

The apparatus and procedure were essentially the same as those described by Isherwood *et al.* (1954). Sugars and acids were detected by spraying with 0.1M-AgNO₃ in 4N aqueous NH₃ and then heating at 100° for 10 min. and those derivatives containing an aldehyde group were detected by spraying with aniline oxalate (Partridge, 1949). Esters and lactones were detected as the corresponding coloured Fe³⁺ hydroxamic acids by the method of Abdel-Akher & Smith (1951).

The sugar acid derivatives were separated with either *n*-butanol-acetic acid-water (3:1:1, by vol.) or *n*-butanol-ethanol-water (5:1:4, by vol.). The *R_F* values of methyl D-galacturonate and the free acid, and of the γ -lactone and free acid of L-galactonic acid, were 0.59, 0.33, 0.59, 0.37 and 0.28, 0.10, 0.34, 0.12, respectively, in the two solvents.

The examination of a particular enzyme digest varied slightly, according to the buffers and other compounds present, but a typical procedure was as follows:

The enzyme digests were acidified with H₂SO₄ to pH 2.0, centrifuged and then evaporated *in vacuo* (bath temp. 40°) to a thin syrup. Absolute ethanol (20 vol.) was added, the mixture centrifuged and the supernatant evaporated *in vacuo* to dryness. The residue was taken up in the minimum quantity of water and examined on a paper chromatogram. In certain experiments the sugar derivatives were eluted from this chromatogram and re-examined with a different solvent.

Estimation of galactonic acid derivatives formed from MDG by enzyme digests

It will be shown later that the enzyme responsible for the formation of the galactonic acid derivative is confined to the soluble part of the cytoplasm. Hence, when this enzyme had been inactivated by heating for 2 min. at 100°, the amount of the galactonic acid derivative which had been formed could be estimated by measuring the amount of ascorbic acid produced from it on the addition of mitochondria.

Washed mitochondria from peas germinated for 2 days were prepared as described by Mapson *et al.* (1954). The washed mitochondria were suspended in 0.4M sucrose-0.1M phosphate, pH 7.4, 1 ml. of which solution contained the mitochondria from 7 to 8 g. of germinated peas. A portion (1 ml.) of this suspension was added to 4 ml. of the test solution and the formation of ascorbic acid, determined by titration against 2,6-dichlorophenolindophenol, was followed over a period of 2–3 hr. at 37°, the maximum amount of ascorbic acid produced being recorded. With L-galactono- γ -lactone under such experimental conditions conversion into L-ascorbic acid was approximately 60% (Mapson *et al.* 1954). Until the galactonic acid derivative formed in the experiments to be reported here has been finally characterized, it is difficult to say how accurate such a means of estimation is. For this reason, in the data recorded in the illustrations we have not attempted to introduce any correction for loss during conversion into ascorbic acid. When other substances have been added to the test solutions, the effect of these on this method of estimating the galactonic acid derivative has been separately ascertained.

RESULTS

Conversion of MDG into L-ascorbic acid by enzyme preparations from peas

When MDG was added to sucrose-phosphate extracts prepared from soaked and germinated peas (homogenate), the formation of a substance reducing 2,6-dichlorophenolindophenol was observed. This substance was identified by the chromatographic procedures of Chen, Isherwood & Mapson (1953) as L-ascorbic acid. D-Galacturonic acid was not converted into L-ascorbic acid under similar circumstances. The ability of the extract to convert methyl D-galacturonate into L-ascorbic acid was subsequently found to be dependent on the presence of both mitochondrial and soluble-enzyme fractions, for on the addition of the methyl ester to either fraction separately, no formation of ascorbic acid could be observed. The synthesis of L-ascorbic acid could, however, be restored if washed mitochondria were added to the soluble fraction, showing that no essential component had been destroyed during separation of the two fractions (Fig. 1a).

Subsequent experiments, the results of which are illustrated in Fig. 1b, showed that the soluble fraction contained a heat-labile component which was necessary for the formation of L-ascorbic acid from MDG. No formation of L-ascorbic acid from

Fig. 1. Dependence of synthesis of L-ascorbic acid from methyl D-galacturonate (MDG) on separate enzymes contained in the soluble fraction of the cytoplasm and in the mitochondria. MDG was added at 3 mg./ml. to the following fractions prepared from peas and the solutions were incubated at 37°. Ascorbic acid was determined on portions as described under Methods. (a) x, Homogenate; O, mitochondria; ●, soluble fraction; Δ, mitochondria added to soluble fraction. (b) x, Mitochondria + soluble fraction; O, mitochondria + soluble fraction which had been inactivated by heating at 100° for 2 min.; Δ, soluble fraction incubated for 1 hr. with MDG, heated and mitochondria added at ↓; □, soluble fraction incubated for 1 hr. without MDG, mitochondria added at ↓; ●, soluble fraction incubated for 1 hr. with MDG, then mitochondria, previously inactivated by heat, added.

Fig. 2. Conversion of MDG, by enzymes present in the soluble fraction, into a compound which in turn is converted into L-ascorbic acid by mitochondria. (a) Portions of the soluble-enzyme fraction were incubated with MDG (3 mg./ml.) at 37° for 1, 1.75 and 3.5 hr., heated and mitochondria added at ↓. (b) The maximum amount of ascorbic acid formed in (a), plotted against time.

the methyl ester could be detected in the presence of washed mitochondria and a soluble fraction which had previously been heated to 100° for 2 min., although L-galactono-γ-lactone was readily converted into L-ascorbic acid by this extract, showing that the activity of the mitochondria had not been altered.

The position was made still clearer by further experiments in which MDG was incubated with the soluble fraction for varying periods of time at 37°. Enzymic activity was arrested by adjusting the pH to 5.5, heating at 100° for 2 min. and removing the coagulated protein by centrifuging. The pH of the supernatant was brought to pH 7.4, and after the addition of washed mitochondria the solution was re-incubated at 37°. Samples of the solution were then taken at intervals for the estimation of L-ascorbic acid. The results showed that L-ascorbic acid was rapidly formed on the addition of mitochondria (Fig. 2a) and that the amount formed increased with increase in time of incubation of the methyl ester with the soluble fraction (Fig. 2b).

Identification of a derivative of L-galactonic acid as an intermediate in the conversion of MDG into L-ascorbic acid

Examination on paper chromatograms of digests containing MDG and the soluble fraction of the extract from soaked peas revealed the presence of a derivative of galactonic acid. To obtain a complete separation of the various compounds present the extract was examined with the *n*-butanol-acetic acid-water solvent, and the fast-running material re-examined with *n*-butanol-ethanol-water as solvent. This chromatogram revealed the presence of a galactonic acid derivative having the same R_f value as L-galactono-γ-lactone (Fig. 3). Control experiments showed that this substance was absent if the enzymes in the extract were first inactivated by boiling before the addition of the ester. In further experiments an approximate quantitative assay of the amount of the galactonic acid derivative formed in the enzyme digest was made by comparing the size and intensity of the spot on the chromatogram with a similar chromatogram prepared from a heat-inactivated enzyme, to which both the methyl ester and an amount of L-galactono-γ-lactone had been added in concentrations equal to those estimated to have been formed in the enzyme digest. This estimate was made by determining, on a separate portion of the enzyme digest, the L-ascorbic acid formed on the addition of mitochondria. The results showed that the amount of galactonic acid formed during the reaction could account for the amount of L-ascorbic acid subsequently formed on the addition of mitochondria.

Fig. 3. Chromatogram of the galactonic acid derivative formed enzymically from MDG.

Further experiments showed that the substance formed from the methyl ester behaved very similarly to L-galactono- γ -lactone in its stability in solutions of pH varying from 6.0 to 8.0. MDG was added to the soluble-enzyme fraction and incubated at 37° for 3 hr. to allow for maximum conversion into the galactonic acid derivative. After inactivating the enzyme by heat or by bringing the pH to 4.0, the pH values of portions were adjusted to 6.2, 6.9 and 7.5, the separate solutions placed at 37°, and the concentration of the galactonic acid derivative in the solution after varying periods of time was measured by determining the amount of L-ascorbic acid formed on the addition of mitochondria. The rates of decomposition of the galactonic acid derivative formed from the methyl ester, and of L-galactono- γ -lactone, showed that both reactions were kinetically of the first order, and the values of k (rate constants) identical within the limits of error inherent in the method of estimation. Furthermore, as Fig. 4 shows, the effect of pH on the values of k was very similar in both cases.

With [methyl- ^{14}C]MDG the galactonic acid derivative isolated on the chromatogram showed no radioactivity, indicating that this substance could not be the methyl ester of L-galactonic acid. As already shown, the galactonic acid derivative is rapidly converted into L-ascorbic acid on the addition of mitochondria, and this fact indicates that the intermediate is a derivative of L-galactonic acid, since derivatives of D-galactonic acid are not so converted (Mapson *et al.* 1954). These results strongly suggest that the L-galactono- γ -lactone is a product of the reduction of the methyl ester; they do not, of course, exclude the possibility that methyl L-galactonate may be formed first and then rapidly converted into the lactone.

The results of these and the earlier experiments show that the formation of L-ascorbic acid from methyl D-galacturonate occurs in two stages. The first stage, that of the reduction of the methyl ester to a derivative of galactonic acid, is catalysed by

Fig. 4. Effect of pH on the rate of decomposition of the galactonic acid derivative formed from MDG (●), and L-galactono- γ -lactone (x). For details see the text.

an enzyme present in the soluble fraction but apparently absent from the mitochondria. The word 'apparently' is used here because it is conceivable that the methyl ester may not be able to reach the active enzyme centre within the mitochondria, but the galactonic acid derivative formed from it may. This is unlikely, however, in view of our previous experience of the ease of penetration of sugar lactones and esters into cells (Isherwood *et al.* 1954). The second stage in the synthesis takes place in the mitochondria and consists of the enzymic oxidation of the galactonic acid compound into L-ascorbic acid.

The reducing system

Having established that the reaction in the soluble fraction was concerned with the reduction of the methyl ester of uronic acid, the next phase in our

investigation was to study the enzyme system catalysing the reduction. The reduction of many compounds in biological systems is mediated through the agency of either DPN or TPN, and we therefore carried out experiments to see whether these coenzymes were involved.

An attempt was made, in the first instance, to separate the reducing system into its components. This was achieved by dialysis of the soluble fraction against 0.025M phosphate buffer, pH 7.4; after 48 hr. the catalytic activity of the dialysed extract was reduced to zero. This loss of activity was not due to inactivation of enzyme, for it could be restored by adding heat-stable components present in the undialysed extract. The nature of these components was revealed in the following experiments. The presence of appreciable quantities of malic and isocitric acids in the undialysed extract was confirmed by chromatographic analysis (cf. Mapson & Goddard, 1951) of the acid fraction separated from the extract by means of ion-

exchange resins according to the method described by Partridge (1952). However, the addition of these acids alone to the dialysed enzyme preparation did not initiate the reaction, but if they were added in addition to TPN, reduction of the methyl D-galacturonate occurred (Fig. 5). DPN could not be substituted for TPN, and the negative response with the former coenzyme was not due to the absence of DPN-linked dehydrogenases or of their substrates. Active formic and alcohol dehydrogenases specific for DPN and malic and isocitric enzymes specific for TPN were known to be present in extracts from ungerminated peas (Mapson & Goddard, 1951), and their presence in these extracts was confirmed. The dialysed extract also contained an active aconitase enzyme, since both citrate and *cis*-aconitate, but not *trans*-aconitate, could be substituted for isocitrate.

Enzymic oxidation of reduced triphosphopyridine nucleotide (TPNH) by derivatives of D-galacturonic, D-glucuronic and D-mannuronic acids

The results of these experiments made it seem likely that the reduction of MDG occurred at the expense of TPNH, and suggested the presence of an enzyme capable of catalysing a reaction between derivatives of galacturonic acid and reduced TPN. This has been confirmed with a purified enzyme preparation which made it possible to follow the oxidation of TPNH spectrophotometrically (decrease in absorption at 340 m μ .) on the addition of the methyl ester.

The enzyme preparation used for these experiments was prepared by extracting 30 g. of peas germinated for 2 days with 40 ml. of 25 mM phosphate, pH 7.4, centrifuging at 20000 g for 20 min., and dialysing the supernatant against the same buffer for 24-48 hr. at 1°. The dialysed enzyme solution was further purified by removal of some protein, which was precipitated at pH 5.0-5.3 with dilute acetic acid, care being taken to keep the solution cold. This procedure was essential, not only to clarify the solution but to remove an enzyme protein which catalysed the oxidation of TPNH in the presence of oxygen. The nature of this enzyme has not been investigated but may be similar to that found by Conn, Kraemer, Pei-Nan Liu & Vennesland (1952). After centrifuging for 5 min. at 20000 g the pH of the clarified enzyme solution was adjusted to 6.5.

The enzyme preparation contained active isocitric and malic enzymes, and the reduction of added TPN could be effected simply by the addition of either isocitrate or malate. A portion (1 ml.) of this enzyme solution was added to 2 ml. of 0.1M phosphate buffer, pH 7.4, containing 4 mM Mg²⁺ in a 1 cm. spectrophotometer cell; a similar solution was used as a blank. TPN (0.35 mg./ml.) was added to one cell and malate or isocitrate to both. These latter compounds were added only in sufficient concentration (80% of the theoretical) to effect a

Fig. 5. TPN as coenzyme in the enzymic reduction of MDG by an enzyme in the soluble fraction of the cytoplasm of peas. ○, Complete system containing enzyme + TPN + malate + MDG; ●, enzyme + TPN + MDG (↓ malate added to a portion); □, enzyme + malate + MDG (↓ TPN added to a portion); △, enzyme + MDG (↓ malate + TPN added to a portion). The enzyme consisted of the soluble fraction dialysed against 0.1M phosphate, pH 7.4, for 48 hr. at 0°. The various substances used were added to give a final concn. of TPN of 10 μ g./ml.; MDG, 3 mg./ml.; malate, 2 mg./ml.; 4 mM-Mg²⁺ was present in all solutions; temp. 37°. The reduction product was estimated as ascorbic acid (see Methods).

partial reduction of the coenzyme. The reduction of TPN was followed by observing the increase in absorption at 340 $m\mu$. After the reduction had ceased, the methyl ester was added (3 mg./ml.) and the oxidation of the TPNH followed by observing the decrease in optical density, a reaction which could be reversed by the addition of further malate or isocitrate.

The results of two experiments of this type are illustrated in Fig. 6, and show the effect of MDG under aerobic and anaerobic conditions. It will be noticed that under aerobic conditions a small but steady oxidation of TPNH, due to the presence of a residual amount of the TPN oxidase referred to above, occurred before the addition of the methyl ester, but that this reaction was prevented in the absence of oxygen.

Similar results were obtained when the *n*-propyl ester was substituted for the methyl derivative, the rate of oxidation of TPNH with the former ester being only slightly slower than that with the latter. The other derivative of galacturonic acid tested was the amide; this, however, was not reduced. The γ -lactones of the two other naturally occurring uronic acids, D-glucuronic and D-mannuronic acids, were reduced, but at slower rates than methyl D-galacturonate; the reaction rate with D-mannurono- γ -lactone was only half, and with D-glucurono- γ -lactone only slightly greater than a third, of the rate observed with the esters of galacturonic acid.

In these experiments it was observed that the rate of reduction of TPN by either the malic or isocitric enzyme systems was several times greater than the enzymic oxidation of TPNH by the methyl

Fig. 6. Oxidation of TPNH by MDG, catalysed by an enzyme from the soluble fraction, at pH 6.9 and 17°. TPN (0.35 mg./ml.) and isocitrate (0.1 mg./ml.) were added at zero time; MDG (3 mg./ml.) and isocitrate (0.1 mg./ml.) were added later; $\uparrow$, MDG; $\downarrow$, isocitrate. (a) In air, (b) in nitrogen. For details of estimation see Results section.

ester, so that under conditions in which either malate or isocitrate is in excess of that required for the complete reduction of the ester, we would anticipate that the concentration of the reduced coenzyme would remain constant throughout the reaction. This was shown to be so in experiments similar to those described above, in which isocitrate was added in excess. Under these conditions there was no significant change in concentration of TPNH after the addition of the methyl ester. These results were found to be of importance in the kinetic experiments which are dealt with later in this paper.

Substrate-velocity and pH relations

The relation between concentration of substrate and rate of reaction of MDG is shown in Fig. 7a. The high Michaelis constant K_m of 10^{-2} indicates a low affinity of the enzyme for this substrate, and suggests that the methyl ester may not be the naturally occurring precursor.

The effect of pH on the enzymic reaction between the methyl ester and TPNH was measured spectrophotometrically, and the results obtained are shown in Fig. 7b. The optimum pH for the reaction appears to be in the region of pH 7.0–7.5. Similar results to these were obtained when catalytic amounts of TPN with the isocitric enzyme and its substrate were used to regenerate TPNH.

Fig. 7. Effect of concentration of substrate and of pH on the rate of reduction of MDG by an enzyme present in the soluble fraction of the cytoplasm from peas. (a) Velocity/substrate concn. relation, (b) velocity/pH relation. $\circ$, Reaction rates at 17° determined by spectrophotometric measurement of the oxidation of TPNH (see text). $\bullet$, Reaction rates at 37° determined by the use of isocitric enzyme system with TPN and measuring the reduction product by converting it into ascorbic acid with mitochondria.

Inhibitors

The effect of a number of inhibitors on the activity of the enzyme was determined. In these experiments the enzyme was prepared by the method described above, and its activity likewise determined by measuring spectrophotometrically the rate of oxidation of TPNH on addition of MDG. The substances tested for their ability to inhibit were added to the enzyme solution in 0.1M phosphate, pH 7.0, at 20° and the assay was made after 15 min. There was little or no inhibition with fluoride, arsenite, arsenate, azide, maleate, iodobenzoate or ethylenediaminetetraacetic acid when these were used in millimolar concentration, or with 0.2 mM dinitrophenol. At millimolar concentration a slight inhibition (18%) was observed with iodoacetate, and a somewhat larger one (25%) with KCN.

Progress of the reaction

MDG is only converted into the L-galactonic acid derivative, even with enzyme extracts of high activity, to an extent of about 10%, even after

allowing for the fact that about 40% of the galactonic acid derivative formed was decomposed under our experimental conditions before it could be converted into ascorbic acid by mitochondria. Examination of the progress curves during the first 3–4 hr. (Figs. 8, 9) show that the cause of this poor conversion was due to the slowing down of the reaction after about 1.5 hr. at 37°. The early termination of the reaction may be thus due to one or more of several causes. These include (1) exhaustion or destruction of substrates or coenzyme, (2) destruction or inhibition of enzymes concerned, (3) instability of reduction product, and (4) attainment of chemical equilibrium.

Consideration was first given to the possibility that an impurity in the methyl ester was the substrate actually being converted. The most likely impurity was the methyl glucoside, which is formed to the extent of 8% during the esterification of galacturonic acid by the method of Jansen & Jang (1948). The possibility was ruled out on finding that the glycoside itself gave negative results, and that recrystallization of the methyl ester gave results in which the percentage conversion was slightly higher rather than lower as would be expected if the real substrate was an impurity.

Fig. 8. Effect of altering the concn. of MDG on the progress of the conversion into L-galactono- γ -lactone (measured after conversion into ascorbic acid) at 37°. O, Initial concn. of MDG at zero time, 12 mg./ml. of digest. $\times$, Initial concn. of MDG, 3 mg./ml.; $\downarrow$ $\bullet$ denotes addition of further amounts of MDG, 9 mg./ml. The digest consisted of the soluble fraction from peas, exhaustively dialysed against 0.1M phosphate, pH 7.4, together with DL-isocitrate (4 mg./ml.), TPN (10 μ g./ml.) and Mg^{2+} , 4 mM.

Fig. 9. A comparison of the experimental-progress curve of the enzymic reduction of MDG to L-galactono- γ -lactone with a theoretical curve based on the assumption of consecutive first-order reactions for this and the succeeding conversion into ascorbic acid (see text). X, Experimental curve; Y, theoretical curve. Experimental curve obtained by following the enzymic production of a galactonic acid derivative from MDG, the concentration of this derivative being estimated by oxidizing it to ascorbic acid with mitochondria (see Methods). Enzyme system composed of dialysed soluble fraction from peas with malate (4 mg./ml.) and TPN (10 μ g./ml.) in 2.5 mM phosphate buffer, pH 7.4, at 37°.

It seems clear that the reaction does not cease because of destruction of either malate or *isocitrate* enzymes, their substrates or the coenzyme TPN. This was shown by adding these substances, either separately or collectively, to an enzyme digest in which the reaction had ceased. The addition of more malate, *isocitrate* or TPN did not prevent the fall off in the reaction. Active *isocitrate* dehydrogenase preparations from heart muscle (Grafflin & Ochoa, 1950), added with or without extra TPN, also gave negative results. Moreover, in enzyme digests which had been incubated for 4 hr. at 37° an active *isocitric* enzyme could be demonstrated by observing the reduction of added TPN spectrophotometrically.

The slowing down of the reaction could not be attributed to the disappearance of the methyl ester. Chromatograms of extracts of enzyme digests taken at times when the reaction had ceased showed that appreciable quantities of the ester were still present. Moreover, if after the reaction had slowed down the concentration of MDG was increased by an amount equal to that originally added, no significant stimulation was observed; only when MDG was added at an earlier stage was any positive effect observed, and this could be attributed simply to the effect of an increased concentration of the substrate on the velocity of the reaction (Fig. 8). These data likewise indicate that the reaction does not slow down because of the attainment of equilibrium between the methyl ester and its reduction product.

The following experiments were carried out to test whether the enzyme catalysing the transfer of electrons from TPNH to the methyl ester was being inactivated during the course of the reaction. The enzyme could be incubated at 37° for 3 hr. before the addition of any of the reactants, without its activity being diminished. The same was true if the enzyme preparation was incubated at 37° for 3 hr. either with malate or TPN or with the methyl ester. It seems clear that the enzyme was not inactivated by any one of the components of the system, and it became necessary to determine if the enzyme was inhibited by products formed during the reaction. Certainly the enzyme was not irreversibly inactivated during the reaction, for when this had ceased and the extract was dialysed overnight to remove soluble constituents, the activity of the enzyme when retested was as great as that observed originally. Nevertheless, the results illustrated in Fig. 8 are most simply interpreted on the basis that some inactivation of the enzyme occurs during the course of the reaction. This explanation is further supported by results of experiments in which it was found that, after the formation of the galactonic acid derivative stopped, addition of fresh enzyme, either in the form of an acetone-dried powder or prepared as a concentrate after fractionation with $(\text{NH}_4)_2\text{SO}_4$,

led to a resumption of the reaction for a period of about 1 hr.; the reaction slowed down again subsequently.

Identification of the inhibitor with products which might be formed during the reaction has so far met with little success. No inhibition of the enzyme was detected in the presence of galacturonic acid, the methyl glycoside of the acid, methanol, galactono- γ -lactone, or galactonic acid, when these were added at a concentration equivalent to that of the methyl ester. In addition, it was shown that ascorbic acid itself did not influence the reduction of the methyl ester. Nor could the products formed by the malic or *isocitric* enzyme systems be responsible, for if these enzymes were allowed to react [e.g. by reducing added oxidized glutathione in the presence of glutathione reductase] before the addition of the methyl ester, the rate and extent of the reduction of the ester were not affected.

Galactaric acid and its lactone, which might be produced by the oxidation of MDG in the extract, both inhibited the enzyme (30% reduction in rate at 2.5 mM concentration), but this inhibition was not increased by previous incubation with the enzyme, as would have been expected if these substances were formed during the reaction and were the agents responsible.

A factor which does operate to limit the concentration of the galactonic acid derivative formed is its instability. We have already shown that the effect of pH on the stability of this derivative was very similar to that of L-galactono- γ -lactone. Whilst a change in pH from 6.5 to 7.5 has no large effect on the rate of reduction of the methyl ester, it has a marked influence on the rate of breakdown of the reduced product, with the result that the maximal amount of this substance formed is much less at pH 7.5–8.0 than at neutral pH. In the system under investigation we have:

where k_1 and k_2 are the appropriate rate constants.

As noted earlier, under the conditions of these experiments, the concentration of TPNH remains constant throughout the reaction. Provided, in addition, that the activity of the enzyme catalysing the reduction of the methyl ester remains unimpaired reaction (3) will be kinetically of the first order. The decomposition of the galactonic acid derivative formed also appears to be kinetically first order as was shown earlier. For a system of consecutive first-order reactions,

the concentration of [B] (galactonic acid derivative) as a function of time (t) is given by (see Daniels, 1948):

$$[B] = \frac{k_1 A_0}{2(k_2 - k_1)} (e^{-k_1 t} - e^{-k_2 t}). \quad (6)$$

The value of k_2 was calculated from the experimental data described earlier, on the rate of decomposition of L-galactono- γ -lactone. Under similar experimental conditions k_1 was evaluated by inserting into eqn. (6) the experimental values for B taken from the early stages of the experimental curve. With these values of k_1 and k_2 a curve was constructed representing the theoretical amounts of the galactonic acid derivative which one might expect to be formed, if the reaction obeyed eqn. (5). Comparison of the theoretical curve (Y) with the experimental curve (X) (Fig. 9) clearly indicates that other factors, besides the unstable nature of the reaction product, are responsible for the poor conversion.

Influence of mitochondria on the progress of the reaction

Observations, susceptible of several interpretations, were made on the effect on the reaction of adding mitochondria. If mitochondria were added to the enzyme digest after the reaction had ceased, the reaction was restarted, as evidenced by the greater amount of ascorbic acid formed compared with a similar enzyme digest which had been inactivated by heat before the addition of mitochondria. This action of the mitochondria could not be interpreted as being due to the supply of co-enzymes or of other heat-stable factors, since the addition of extracts made from mitochondria had no effect.

The effect of the mitochondria was abolished in the absence of oxygen or in its presence if azide and succinate were added, circumstances in which the conversion of galactonic acid derivatives into L-ascorbic acid are prevented by these particles (Mapson *et al.* 1954). However, if the sole action of the mitochondria was to convert the intermediates into L-ascorbic acid, thus preventing the breakdown of the former, the rate of the reaction stimulated by the addition of these particles should be equivalent to the rate of breakdown of the intermediates. In fact, the mitochondria increased the rate of reaction to a value at least four times that which would be expected from this cause alone.

The conversion of MDG into ascorbic acid in the presence of both soluble and particulate fractions proceeds smoothly for a period of 3–4 hr. at 37°, thereafter the rate of the reaction decreases fairly rapidly. The cause of this has not been investigated, but it seems probable that it is connected with changes taking place within the mitochondria.

DISCUSSION

We have shown in this paper that certain derivatives of galacturonic acid are reduced to derivatives of galactonic acid by an enzyme system present in peas. The reaction has features which require further investigation. Because we had shown previously that derivatives of galactonic acid, but not the free acid, were converted into ascorbic acid (Mapson *et al.* 1954) we were not surprised to find that the enzyme would not catalyse the reduction of free galacturonic acid, but we did anticipate that simple derivatives of galacturonic acid, in which the ionization of the carboxyl group had been suppressed, would be reduced. The reduction of the methyl and n -propyl esters of galacturonic acid and of the lactones of glucuronic and mannuronic acid appeared to support this hypothesis. However, the inability of the enzyme to catalyse the reduction of the amide of galacturonic acid seems to indicate that the nature of the group attached to the carboxyl is not unimportant.

The greater rate of reduction of derivatives of the parent sugar D-galactose, compared with derivatives from D-glucose, is in line with the results obtained with the aldonic acids (Mapson *et al.* 1954) and makes it more probable that derivatives of D-galactose rather than D-glucose are the natural precursors of L-ascorbic acid in plants.

The other findings, for which we have as yet no precise explanation, are the premature termination of the reaction and the role of the mitochondria in preventing this. We have examined a number of possibilities and our results indicate that, of these, inactivation of the enzyme during the reaction appears to be the chief factor, though so far we have not been able to find the cause. The low affinity of this enzyme for the methyl ester makes it probable that this simple derivative of galacturonic acid is not its natural substrate.

SUMMARY

1. The enzymic reduction of derivatives of D-galacturonic acid to derivatives of L-galactonic acid has been demonstrated in peas. The enzyme catalyses a reaction between reduced triphosphopyridine nucleotide and esters of D-galacturonic acid; the free acid is not reduced.

2. In peas which have been germinated for 2–3 days, the enzyme is located in the soluble part of the cytoplasm, and is absent from the cytoplasmic particles (mitochondria). The formation of L-ascorbic acid from derivatives of D-galacturonic acid thus requires the presence of this enzyme, and the enzyme system within the mitochondria which converts derivatives of L-galactonic acid into L-ascorbic acid.

3. Evidence from chromatographic and kinetic studies suggests that a product of the reduction of methyl D-galacturonate is L-galactono- γ -lactone,

although the prior formation of an unstable intermediate is not excluded.

4. The poor conversion of esters of D-galacturonic acid into compounds of L-galactonic acid appears to be due mainly to the reversible inhibition of the enzyme during the course of the reaction, and to a lesser extent to the instability of the galactonic acid derivative formed. The substance responsible for the inhibition of the enzyme has not been identified.

5. The low affinity of the enzyme for methyl D-galacturonate (K_m , 10^{-2} M) suggests that this is not the naturally occurring substrate.

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Further Studies on the Reactions of Disulphides with Sodium Sulphite

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In a previous paper (Cecil & McPhee, 1955*b*) the reactions of oxidized glutathione, cystine and some cystine derivatives were described. It was found that SO_3^{2-} ions react according to the equation

but that HSO_3^- ions do not react. Since the reaction with R.S^- is reversible (Footner & Smiles, 1925) the equilibrium

has also to be taken into account. It was found that the negatively charged disulphides react much more slowly than do those with no net charge. Thus with oxidized glutathione and cystine there is an optimum pH at which the observed rate of reaction is maximal. Below the optimum the observed rate falls off because of decreasing SO_3^{2-} concentration, and above it because of increasing negative charge on the disulphide molecule due to dissociation of the amino groups.

This paper describes similar studies on a number of other compounds, including homocystine and two positively charged compounds, bis-(β -aminoethyl)

disulphide (cystamine) and bis-(γ -aminopropyl) disulphide (homocystamine). It has been found, as was expected from the previous results, that the positively charged compounds react much more rapidly than those with no net charge. Homocystine and its derivatives react more slowly than the corresponding cystine derivatives, but otherwise show the same general behaviour. The application of this work to the disulphide groups of proteins is discussed.

The theoretical descriptions of disulphide-sulphite reaction and equilibrium mixtures have been given in detail (Cecil & McPhee, 1955*b*), and the same notation will be used here.

EXPERIMENTAL

Reagents

Cystamine. A sample of the dihydrochloride was kindly supplied by Horlicks Ltd., Slough, Bucks. (Found: Cl, 30.7; $\text{C}_4\text{H}_{14}\text{N}_2\text{S}_2\text{Cl}_2$ requires Cl, 31.6%). Standard solutions of the dinitrate were made by adding the calculated amount of AgNO_3 to solutions of the hydrochloride in 0.1N- HNO_3 , filtering off the AgCl and making the filtrates up to the required volumes.

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