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Biological Synthesis of Ascorbic Acid: L-Galactono- γ -lactone Dehydrogenase

BY L. W. MAPSON AND E. BRESLOW*

*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge
and Department of Scientific and Industrial Research*

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The terminal step in the biosynthesis of L-ascorbic acid in peas has been shown to be the oxidation of L-galactono- γ -lactone by an enzyme present in mitochondria (Mapson, Isherwood & Chen, 1954). This enzyme has been solubilized from mitochondria obtained from freshly germinated peas, cabbage leaves and cauliflower florets. Since the enzyme preparations from the mitochondria of the cauliflower florets were found to be more active than those from either peas or cabbage, we have used this material in most of the work reported here. The present paper describes the preparation and partial purification of the enzyme, together with an account of some of its properties.

EXPERIMENTAL

Chemicals

L-Galactono- γ -lactone was prepared by the reduction of D-galacturonic acid by borohydride as follows. D-Galacturonic acid (10 g.) was dissolved in 40 ml. of water and neutralized with NaOH to between pH 8.5 and 9.0. Borohydride was added gradually with stirring at room temperature. Samples were removed and acidified with acetic acid to remove excess of borohydride, and galacturonic acid was tested for by boiling with Fehling's solution. After reduction was complete, the solution was acidified with acetic acid to pH 5.0, a slight excess of barium acetate added, and the precipitate filtered off. Ethanol (2 vol.) was added to the solution and the precipitate was collected. After the precipitate had been washed twice with 60% (v/v) ethanol, barium was removed by Dowex 50 resin, and to the filtrate 1–2 drops of 3N-HCl were added. The solution was concentrated to a syrup and dried *in vacuo*. The lactone was recrystallized from absolute ethanol.

L-Gulono-, D-altrono-, D-glucono- and D-mannono- γ -lactone were prepared as described by Isherwood, Chen & Mapson (1954). Glucurono- γ -lactone, D-gulono- γ -lactone and D-galactono- γ -lactone were purchased from L. Light and Co., Colnbrook, Bucks. Cytochrome *c* was prepared from ox heart by the method of Keilin & Hartree (1937).

Phenazine methosulphate was prepared by the addition of dimethyl sulphate to phenazine dissolved in nitrobenzene (Kehrmann, 1913; Hillemann, 1938). Flavin mononucleotide (FMN) and flavinadenine dinucleotide (FAD) were purchased from the Sigma Chemical Co., U.S.A.

Preparation of the enzyme

Extraction from mitochondria step 1. The florets (1 kg.) of cauliflower after separation from leaves and stalk were ground in a mechanical mortar with 1 l. of a sucrose-phosphate solution (0.4M-sucrose and 0.1M-Na phosphate buffer, pH 7.4). The mortar was cooled to -20° and the sucrose phosphate solution to 0° before grinding, which was carried out as rapidly as possible (usually within 30 min.) at room temperature. The mixture was pressed through muslin and centrifuged at 20 000 g for 20 min. The spun-down mitochondria were washed with 0.1M-phosphate buffer (pH 7.4), recentrifuged at 20 000 g for 20 min., and resuspended in 50–100 ml. of the same buffer. To this suspension was slowly added 10 times the volume of acetone, previously cooled to -20° . After 15 min. the precipitate was collected, the acetone partially removed *in vacuo*, and the residue suspended in 150 ml. of the phosphate buffer (0.1M). The suspension was dialysed against at least two changes of the same buffer for 3 hr. at $+1^{\circ}$ to remove most of the acetone.

Fractionation with ammonium sulphate step 2. The suspension was centrifuged to remove undissolved protein, and the clear solution brought to 40% saturation by the addition of 243 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. of solution. The precipitated protein was removed, and the solution brought to 70% saturation by the further addition of 205 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. The precipitate was collected, dissolved in 20 ml. of 0.01M-Na

* Present address: Department of Biochemistry, New York University College of Medicine, New York.

phosphate buffer (pH 6.8) and dialysed for 2 hr. against the same buffer at 1°.

Absorption and elution from calcium phosphate gel step 3. (At this stage and on all of the subsequent steps operations were carried out at a temperature of 1°.) The solution was treated with calcium phosphate gel (Keilin & Hartree, 1938) (1 mg. of gel/1.5 mg. of protein), left for 15 min. and centrifuged for 5 min. at 1500 g. The gel was washed with phosphate buffer 0.05M (pH 6.8), re-centrifuged, and the enzyme eluted with 0.5M-Na phosphate buffer (pH 7.4). The eluate was dialysed against 0.01M-phosphate buffer (pH 6.8) for 3 hr.

Second fractionation with ammonium sulphate step 4. The solution was brought to 53% saturation by the addition of 336 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. of solution, and after removal of the precipitated protein the solution was brought to 80% saturation [192 g. of $(\text{NH}_4)_2\text{SO}_4$ /l.]; the precipitate was collected and dissolved in 5 ml. of 0.1M-phosphate buffer (pH 6.8). The solution was dialysed for 2 hr. against the same buffer.

Absorption of contaminating protein with calcium phosphate gel step 5. The solution was treated with calcium phosphate gel at 1° (1 mg. of gel/1.5 mg. of protein), left for 15 min. and centrifuged. The gel was discarded and the solution containing the enzyme stored at -3°. This solution should contain not less than 10 mg. of protein/ml.; weaker solutions have been found to be unstable. The amount of enzyme lost and the purification achieved by these operations are summarized in Table 1.

Assay methods

Protein. The protein content of the various enzyme preparations was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Phenazine. The solubilized enzyme was unable to reduce indophenol, ferricyanide or tetrazolium at appreciable

rates. It could, however, reduce phenazine methosulphate, and a spectrophotometric assay has been devised with this dye as hydrogen acceptor. The reaction mixture contained enzyme, L-galactono- γ -lactone, phenazine methosulphate and indophenol, the phenazine after reduction by the lactone in turn reducing 2:6-dichlorophenolindophenol, the course of this latter reduction being followed on an EEL colorimeter with an EEL 607 filter. The reaction was carried out anaerobically to prevent the reoxidation of the reduced phenazine. The Thunberg tubes used were specially constructed with a light-path of 1.2 cm. so as to fit the colorimeter. Because the product of the reaction is the reducing agent, ascorbic acid, 2 moles of indophenol are reduced for every mole of lactone oxidized.

The presence of an indophenol oxidase in the more impure preparations of the enzyme necessitated the use of cyanide during the assay. A blank rate must also be corrected for, the blank being determined by running all assay components with the exception of the enzyme. This blank is the result of some interaction between substrate, dyes and cyanide. With crude preparations of the enzyme, contaminating substances may be present which also reduce the dye. The true blank is then the blank as determined above plus the difference between the blank due to enzyme, cyanide and dye and that due to cyanide and dye alone.

In a Thunberg tube were placed the enzyme, 1 ml. of 0.01M-KCN in 0.1M-phosphate buffer (pH 7.4), 0.5 ml. of L-galactono- γ -lactone (24 mg./ml.) and phosphate buffer to a volume of 5 ml. In the side arm were placed 1 ml. of phenazine methosulphate (1 mg./ml.) in 0.1M-phosphate buffer (pH 7.4) and 0.08 ml. of indophenol dye (1 mg./ml.). (The exact volume of indophenol may be varied slightly to give convenient optical-density values.) The tube was evacuated and flushed with N_2 several times and incubated for 10 min. at 37°. The dye was then tipped in and readings were taken on the colorimeter every 2 min. for 12 min.

Table 1. *Stages in the purification of L-galactono- γ -lactone dehydrogenase*

Stage of purification	Phenazine assay			Cytochrome assay			Cytochrome activity/phenazine activity
	Total units	Recovery (% of original extract)	Specific activity (units/mg. of protein)	Total units	Recovery (% of original extract)	Specific activity (units/mg. of protein)	
Phosphate (0.1M) (pH 7.4) extract of acetone-dried powder (step 1)	985	—	2.7	8900	—	24	9
40–70% saturation $(\text{NH}_4)_2\text{SO}_4$ and dialysed (step 2)	630	64	4.4	7560	85	53	12
Treated with GSH and dialysed for 5 hr.	750	76	5.0	9680	109	65	13
Absorption on $\text{Ca}(\text{PO}_4)_3$ gel and elution (step 3)	515	53	13.2	5850	66	150	11
53–80% saturation $(\text{NH}_4)_2\text{SO}_4$ and dialysed (step 4)	168	17	12	2800	31	200	17
Treated with GSH and dialysed for 5 hr.	254	26	18	2910	33	208	12
Treated with $\text{Ca}(\text{PO}_4)_3$ gel in 0.1M-phosphate (pH 7.4) (step 5)	161	16	16.5	2440	27	250	15
Supernatant treated with GSH and dialysed	270	27	27.5	2950	33	300	11

One unit of enzyme was defined as the amount that decreased the optical density by one scale unit/min. Proportionality between rate of reaction and enzyme concentration was achieved with an error of less than 10% over a tenfold range of enzyme concentration.

Cytochrome c. Reduction of cytochrome *c* was followed by measuring the increase in absorption at 550 m μ in a Hilger Uvispek spectrophotometer at room temperature. In a final volume of 3 ml. of 0.01M-phosphate buffer (pH 7.8) were placed the enzyme, L-galactono- γ -lactone (2 mg./ml.) and cytochrome *c* (1.5 mg./ml.). Under these conditions the rate of reaction was proportional to enzyme concentration and was linear with time over periods of 15 min. The unit of enzyme activity was defined as that amount of enzyme which increased the optical density by 0.001/min. at room temperature (20°).

Formation, isolation and identification of the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid

Formation and isolation. L-Galactono- γ -lactone (2 mg./ml.) was dissolved in 4.5 ml. of 0.1M-phosphate buffer (pH 7.4) to which phenazine (1 mg.) dissolved in 1 ml. and the necessary amount of enzyme in 0.5 ml. of the same buffer were added. After incubation at 35°, 1 ml. samples were removed and mixed with 4 ml. of 5% trichloroacetic acid. Precipitated protein was removed, if necessary, and the solution treated with the 2:4-dinitrophenylhydrazine reagent (Roe & Kuether, 1943). The formation and estimation of the phenylhydrazone was carried out in the manner described by these authors. The hydrazone was isolated from similar mixtures, after incubation with the phenylhydrazine reagent, by centrifuging and recrystallization from either acetic acid or acetone-methanol (1:1, v/v) solution. For comparison the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid was prepared from L-ascorbic acid, after oxidation with bromine, in a similar manner.

Identification. The hydrazones were analysed by paper chromatography with an aqueous solvent containing 10% (v/v) of acetic acid, 10% (v/v) glycerol and 10% (w/v) phenol; both hydrazones ran as a single spot with an R_f of 0.59. By using the upper phase of a mixture composed of *tert.*-amyl alcohol-ethanol-water (50:5:40, by vol.) and developing the chromatogram on paper previously buffered with 0.2M-phosphate (pH 6.25), a similar result was obtained. The hydrazones ran as single spots with an R_f of 0.86.

The absorption spectra of both hydrazones were identical, with absorption maxima at 370 and 530 m μ , and the ratios of the values of the optical densities measured at 530 and 450, 370 and 450, and 370 and 530 for the enzymic and synthetically produced hydrazones were 2.02, 2.25 and 1.11 and 2.05, 2.29 and 1.05 respectively. These values agreed well with those reported by Probst & Schultze (1950) for the same compound.

RESULTS

Electron acceptors

As is shown below, in the absence of a suitable electron acceptor there was no oxidation of L-galactono- γ -lactone by the dehydrogenase. Various

substances were tested for their ability to act as electron acceptors with the solubilized enzyme; these included methylene blue, ferrieyanide, tetrazolium, 2:6-dichlorophenolindophenol, phenazine methosulphate and cytochrome *c*. Only the last two were reduced by the enzyme in the presence of substrate at any significant rate. Reduction of cytochrome *c* may be followed either aerobically or anaerobically since it proceeds equally well under either condition. Reduction of phenazine is more conveniently carried out under anaerobic conditions, so as to avoid the autoxidation of phenazine which would interfere with the reduction of indophenol.

Product of reaction

Ascorbic acid is oxidized by both phenazine and cytochrome *c*; so with both these substances as electron acceptors the ascorbic acid formed in the enzyme reaction is further oxidized under the conditions of the test to dehydroascorbic acid, which at the pH of the reaction mixture is in turn rapidly converted into 2:3-dioxo-L-gulonic acid (Penney & Zilva, 1943). The formation of ascorbic acid has therefore been shown to have occurred by

Fig. 1. Formation of 2:3-dioxo-L-gulonic acid by the enzymic oxidation of L-galactono- γ -lactone with phenazine as electron acceptor. Each solution in a total vol. of 6 ml. contained 4.5 ml. of 0.1M-phosphate buffer (pH 7.4), 6 mg. of L-galactono- γ -lactone, 1 mg. of phenazine methosulphate. To one solution (●) 0.1 ml. of enzyme preparation (specific activity 250) was added, the other (○) serving as control. 1 ml. of reaction mixture was removed at intervals for the estimation of 2:3-dioxo-L-gulonic acid; temp. 37°.

the isolation and identification of the bis-2:4-dinitrophenylhydrazone of 2:3-dioxo-L-gulonic acid. The derivative prepared experimentally by the enzymic oxidation of L-galactono- γ -lactone was shown to be identical with that prepared from L-ascorbic acid after oxidation by bromine by (1) their similar behaviour when chromatographically analysed, (2) their identical absorption spectra and (3) the close agreement between the ratios of the optical densities measured at wavelengths of 530 and 450, 370 and 450, and 370 and 530 m μ ; the last property has been shown to be an excellent criterion of identity and purity of this compound (Probst & Schultze, 1950).

The formation of 2:3-dioxo-L-gulonic acid during the course of the enzymic oxidation of L-galactono- γ -lactone with phenazine (Fig. 1) has been followed by preparing the 2:4-dinitrophenylhydrazone derivative according to the method of Roe & Kuether (1943). Similar results have been obtained with cytochrome *c*; in neither experiment was 2:3-dioxo-L-gulonic acid formed in the absence of an electron acceptor. This procedure as a method of assay is not as satisfactory as either of the methods in which the reduction of phenazine or cytochrome *c*

is followed, owing to the instability of 2:3-dioxo-L-gulonic acid at the pH required for enzyme action (Penney & Zilva, 1943). Borate, which stabilizes 2:3-dioxo-L-gulonic acid, cannot be used, since it also forms complexes with L-galactono- γ -lactone.

Properties

Sulphydryl requirement. The enzyme appears to require sulphydryl groups for its activity, for this was inhibited, in both the phenazine and cytochrome *c* reactions, by reagents which inactivate these groups. *o*-Iodosobenzoate, Cu²⁺ ions and *p*-chloromercuribenzoate (PCMB) all inhibited strongly (50% or more) at concentrations of 0.1 mM. Iodobenzoate, oxidized glutathione and *p*-aminophenyl arsenoxide did not inhibit at a level of 0.1 mM even after 30 min. preincubation with the enzyme. The inhibition by PCMB has been studied in more detail and the results (Table 2) show that inhibition by the mercurial was immediate and was not noticeably increased when the enzyme was incubated with the inhibitor for 30 min. at 0°, although with longer periods of incubation total inactivation was observed. Complete reversal of this inhibition occurred if reduced

Table 2. *Inhibition by p-chloromercuribenzoate and reactivation by glutathione*

Expt.	Treatment	Reduction (% of control)	
		Phenazine	Cytochrome <i>c</i>
1	Enzyme treated with PCMB (0.4 mM) for 30 min. at 0° before test	0	—
2	Enzyme treated with PCMB (0.1 mM) tested immediately	27	—
	Enzyme treated with PCMB (0.1 mM) tested after 10 min. at 0°	34	—
	Enzyme treated with PCMB (0.1 mM) tested after 30 min. at 0°	20	—
	Enzyme treated with PCMB (0.1 mM) tested after 60 min. at 0°	0	—
3	Enzyme treated with PCMB (0.1 mM) for 5 min. at room temperature	0	—
	Enzyme mixed with substrate before addition of PCMB	38	—
	Enzyme mixed with substrate and 0.1 mM-FAD before addition of PCMB	40	—
4	Enzyme treated with PCMB (0.1 mM) tested at once	65	—
	Enzyme treated with PCMB then dialysed, 17 hr. against 0.1 M-phosphate buffer (pH 7.4) at 1°	52	—
	Enzyme treated with PCMB, GSH (10 mM) added, left for 1 hr. at 15°, and dialysed against 0.1 M-phosphate buffer (pH 7.4), for 17 hr. at 1°	120	—
5	Enzyme treated with GSH (10 mM) for 1 hr. at room temperature dialysed against 0.01 M-phosphate buffer (pH 7.4), for 17 hr. at 1°	280	—
	Enzyme as above treated with PCMB (0.1 mM) for 45 min. at room temperature	130	—
	Enzyme after PCMB treatment re-treated with GSH (10 mM) and tested after dialysis for 17 hr. at 1°	200	—
6	Enzyme treated with PCMB (0.1 mM) tested at once	—	70
	Enzyme left 30 min. room temp. with PCMB (0.1 mM) dialysed for 17 hr. at 1° against 0.01 M-phosphate buffer	—	16
	Enzyme similarly treated as above except that GSH (10 mM) was added after 30 min. incubation with PCMB	—	72
	Enzyme similarly treated as above, except that cysteine (10 mM) was added after 30 min. incubation with PCMB	—	61

glutathione (GSH) was added within a short time after the addition of the PCMB; when added 45 min. after PCMB, considerable though not complete reactivation was obtained. In similar experiments it was found that cysteine could replace GSH. The enzyme can be partially protected against inhibition by PCMB by preincubation with the substrate (Table 2), suggesting that an -SH group is concerned with the attachment of substrate to enzyme.

In agreement with these results was the observation that GSH was capable of restoring activity which had been lost during various stages in the purification (Table 1), and what was more interesting was the observation that the enzyme activity, as determined with phenazine, appeared to be more adversely affected than that with cytochrome *c* as electron acceptor. This phenomenon has also been observed during the ageing of enzyme preparations kept at 0°. The activity of the enzyme, as assayed by phenazine, has often been found to decrease appreciably while the activity with cytochrome *c* has decreased to a much smaller extent. When this has happened, treatment with GSH restored the former activity fully, and had a much smaller effect on the latter. In a typical experiment the activity of an enzyme, as assayed against phenazine and cytochrome *c*, decreased by 55 and 20% respectively on ageing; after treatment with GSH the activity was fully restored with phenazine but was not altered with cytochrome *c*. In these experiments, excess of GSH was removed by dialysis to prevent its interfering in the assay.

These changes in the activity of the enzyme against the different electron acceptors, the greater instability of the enzyme with phenazine and the differential effect of GSH in restoring the activity suggest that an -SH group additional to that required for substrate-binding may be necessary for the interaction of phenazine with the enzyme.

Inhibitors. The enzymic reduction of either phenazine or cytochrome *c* in the presence of L-galactono- γ -lactone was unaffected by most metal-binding agents. Cyanide, ethylenediaminetetraacetic acid, pyrophosphate, azide and $\alpha\alpha'$ -dipyridyl in mM concentrations were without effect on enzyme activity. No inhibition with these reagents was observed after a 15 min. preincubation treatment with the enzyme at room temperature. *o*-Phenanthroline (1:10-phenanthroline), however, inhibited the enzyme, and this inhibition was increased by prior incubation with the inhibitor (Table 3). None of the ions Mn^{2+} , Zn^{2+} , Fe^{3+} or MoO_4^{2-} had any effect on enzyme activity. Antimycin *A* in a concentration of 10 $\mu g./ml.$ of reaction solution did not inhibit the enzyme.

Fig. 2. Effect of pH on the activity of the enzyme with phenazine and cytochrome *c* as electron acceptors. O, With phenazine. The reaction mixture was contained in a total vol. of 6 ml. 0.1 M-Phosphate buffer (4.5 ml.) at stated pH, 1 mg. of phenazine, and 12 mg. of L-galactono- γ -lactone; temp. 37°; ●, with cytochrome *c*. The reaction mixture was contained in a total vol. of 3 ml. 0.01 M-Phosphate buffer (2.0 ml.) at stated pH, 1.5 mg. of cytochrome *c* and 6 mg. of L-galactono- γ -lactone; temp. 17°.

Table 3. Inhibition of L-galactono- γ -lactone dehydrogenase by *o*-phenanthroline

Expt.	<i>o</i> -Phenanthroline added (mM)	Treatment	Inhibition (%)	
			Phenazine reaction	Cytochrome reaction
1	0.5	Tested at once	—	16
		Preincubation for 30 min. at room temp.	—	26
	1	Tested at once	—	18
		Preincubation for 15 min. at room temp.	—	35
	2	Tested at once	—	31
		Preincubation for 30 min. at room temp.	—	50
2	1	Preincubation for 60 min. at room temp.	—	55
		Tested at once	35	—
	5	Preincubation for 10 min. at room temp.	35	—
		Preincubation for 10 min. at 37°	57	—
		Preincubation for 30 min. at 37°	100	—

pH and activity. With phosphate buffer the optimum pH for the reaction with the solubilized enzyme was about 7.8–7.9 at 17° with cytochrome *c* as electron acceptor, whereas with phenazine at 37° the optimum was 7.4–7.7 (Fig. 2). With mitochondrial preparations from peas the optimum pH found was about 7.5–7.6 at 37° (Mapson *et al.* 1954).

Substrate-affinity constants. The Michaelis constant for the substrate L-galactono- γ -lactone was determined by the method of Lineweaver & Burk (1934), with phenazine and cytochrome *c* as electron acceptors at 17°; with phenazine at pH 7.4 the constant was 4 mM and with cytochrome *c* at pH 7.8 was 2 mM. These constants are about 10 times that found for the reaction as catalysed by intact mitochondria prepared from pea seedlings (Mapson *et al.* 1954). The reason for this is unknown. One possibility is that during solubilization and extraction from the mitochondria minor changes are produced in the structure of the enzyme which are reflected in a reduced affinity of enzyme for substrate.

Specificity. The solubilized enzyme appears to be specific for L-galactono- γ -lactone. The enzyme as isolated from cauliflower florets failed to catalyse

the reduction of cytochrome *c* in the presence of D-galactono- γ -lactone, D-glucono- γ -lactone, D-glucurono- γ -lactone, D-gulono- γ -lactone, L-gulono- γ -lactone, D-mannono- γ -lactone or L-galactonic acid, when tested at a concentration of 10 mM. In an equivalent concentration D-altrono- γ -lactone reduced cytochrome *c* at a rate less than one-tenth of that observed with L-galactono- γ -lactone.

Further studies with D-galactono- γ -lactone and D-gulono- γ -lactone have shown that these lactones inhibit competitively the oxidation of L-galactono- γ -lactone by the dehydrogenase with cytochrome *c* as electron acceptor. Both lactones inhibited the reaction to about the same extent; there was about a 20% reduction in rate when they were added in twice the molecular concentration (40 mM) to the substrate L-galactono- γ -lactone. Results with D-gulono- γ -lactone are shown in Fig. 3. These results are of interest in the light of the earlier work of Mapson *et al.* (1954) and are discussed below.

Isherwood *et al.* (1954) and Mapson *et al.* (1954) both observed the inhibition of formation of ascorbic acid by DL-glyceraldehyde, the former workers with growing cress seedlings and the latter when studying the conversion of L-galactono- γ -lactone into ascorbic acid by pea mitochondria. These results have now been supplemented by the observation that DL-glyceraldehyde in a concentration of 10 mM and with the substrate in a concentration of 15 mM reduced the enzyme activity by about 30%, an effect of similar magnitude to that obtained by Mapson *et al.* (1954).

Formation of ascorbic acid by cauliflower mitochondria

To determine whether the high specificity of the solubilized enzyme was also a characteristic of the enzyme in the mitochondria, experiments were carried out with mitochondria prepared from the florets by the method previously used for preparing pea mitochondria (Mapson *et al.* 1954). The mitochondrial preparations so obtained contained a very active ascorbic acid-oxidizing system, so that the formation of ascorbic acid from L-galactono- γ -lactone could not be detected unless an enzyme preparation (Mapson, 1953) from dried peas containing glutathione reductase, dehydroascorbic acid reductase and malic and isocitric enzymes, together with triphosphopyridine nucleotide (TPN), glutathione and malate or isocitrate, was added to maintain the ascorbic acid in the reduced form. Under such conditions the formation of ascorbic acid was readily observed (Fig. 4). It was more convenient to use the alternative procedure of following the reaction by determining the rate of formation of 2,3-dioxo-L-gulonic acid, for testing the activity of the different lactones. The results

Fig. 3. Competitive inhibition of L-galactono- γ -dehydrogenase by D-gulono- γ -lactone. The reaction mixture was contained in a total vol. of 3 ml. 0.01 M-Phosphate buffer (pH 7.8) (2 ml.), cytochrome *c* 1.5 mg., L-galactono- γ -lactone and D-gulono- γ -lactone at the concentrations indicated; temp. 17°. D-Gulono- γ -lactone: ●, 7 mg./ml. of reaction mixture; △, 3.5 mg./ml.; ○, 1.75 mg./ml. *V* = units of enzyme activity. *S* = concn. of L-galactono- γ -lactone (mg./ml. of reaction solution).

Table 4. Formation of 2:3-dioxo-L-gulonic acid from certain aldonic acid lactones by cauliflower or pea mitochondria

Source	Lactone added (mg./ml. of reaction mixture)	2:3-Dioxo-L-gulonic acid formed (μ g./ ml. of reaction mixture/hr.)	Relative rate (L-galactono- γ - lactone = 100)
Cauliflower florets	L-Galactono- γ -lactone	184	100
	L-Gulono- γ -lactone	0	0
	D-Altrono- γ -lactone	28	15
	D-Arabetono- γ -lactone	14	7
Cauliflower florets	L-Galactono- γ -lactone	88	100
	D-Mannono- γ -lactone	0	0
	D-Gulono- γ -lactone	0	0
	D-Galactono- γ -lactone	0	0
Fresh green peas	L-Galactono- γ -lactone	320	100
	D-Altrono- γ -lactone	192	60
	D-Arabetono- γ -lactone	200	63
Cotyledons from 5-day- germinated pea seeds	L-Galactono- γ -lactone	135	100
	D-Altrono- γ -lactone	95	70
	D-Arabetono- γ -lactone	90	67

Fig. 4. Formation of ascorbic acid by cauliflower mitochondria. Mitochondria were suspended in 0.4M-sucrose, 0.1M-phosphate (pH 7.4), containing glutathione reductase and dehydroascorbic acid-reductase enzyme systems from peas (see text). In addition, TPN (60 μ g./ml.), DL-malate (1 mg./ml.) and GSH (1 mg./ml.) were added at zero time; temp. 37°. $\downarrow$ denotes addition of L-galactono- γ -lactone (1 mg./ml.).

(Table 4) showed that the mitochondrial preparations behaved similarly to the solubilized enzyme; there was the same high specificity towards the lactones, L-galactono- γ -lactone being the only one which was oxidized at a high rate. D-Altrono- γ -lactone and D-arabetono- γ -lactone were oxidized only slowly and the other lactones tested, including L-gulono- γ -lactone, were not oxidized. There was no oxidation of L-galactonic acid.

When these experiments were repeated with mitochondria prepared from fresh green peas, or

from the cotyledons of 5-day-germinated pea seeds, both D-altrono- and D-arabetono- γ -lactones were oxidized at about 60 % of the rate of L-galactono- γ -lactone. A similar result to this was obtained in experiments with the solubilized enzyme from green peas, where, with cytochrome *c* as acceptor, both lactones were oxidized at about half the rate of L-galactono- γ -lactone. It seems probable that the enzyme as it occurs in cauliflower and peas differs in degree in its specificity.

Prosthetic group

There is no evidence that either diphosphopyridine nucleotide (DPN) or TPN participates in the reaction. Neither DPN nor TPN increased the rate at which either phenazine or cytochrome *c* was reduced, nor were the coenzymes reduced (as determined by the increase in absorption at 340 m μ) in the presence of enzyme and substrate.

Evidence was obtained which suggested that a flavin group is involved in the reaction with cytochrome *c*. The enzymic reduction of cytochrome *c* was strongly inhibited by either Atebrin or riboflavin, known flavoprotein inhibitors (Haas, 1944; Wright & Sabine, 1944; Hellerman, Lindsay & Bovarnick, 1946) at concentrations of 0.1-1.0 mM (Table 5). Of the two inhibitors, riboflavin was the most active, a finding in agreement with the results of Singer & Kearney (1950) on the flavo-enzyme L-amino acid oxidase. Equally in agreement with their results was our failure to diminish the inhibitory effect of Atebrin (3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)acridine dihydrochloride or riboflavin by the addition of flavinadenine dinucleotide (FAD) or flavin mononucleotide (FMN).

In contrast with these results was the observation that the reaction with phenazine was not

inhibited by either Atebrin or riboflavin, although acriflavine, especially if incubated with the enzyme before test, caused some inhibition (Table 5). This inhibition was reduced with FAD, although FAD did not stimulate the activity of the unincubated enzyme. The decrease of inhibition with FAD appeared to be due, however, to a protective effect on the enzyme during incubation at 37° rather than to any reversal of the inhibition.

Attempts to resolve the enzyme reversibly into apoenzyme and prosthetic group have so far met with little success. The enzyme was very sensitive to changes in pH; when the pH of the solution was decreased to 5.0 or below, in the presence or absence of ammonium sulphate, almost complete inactivation occurred within a period of 5 min. at 0°. This inactivation could not be reversed by the addition of FAD, FMN or a boiled extract of the enzyme. Attempts were also made to remove a prosthetic group by less drastic acid treatment, e.g. by dialysis against sodium acetate-acetic acid buffers (0.01M) at a pH of 4.5-5.0 for periods of 2-17 hr. at 0°, or by precipitating the enzyme within this pH range with ammonium sulphate. In all these attempts no appreciable reactivation was obtained with either FAD or FMN.

Absorption spectrum. The absorption spectrum of the most highly purified preparations so far obtained (specific activity 300-340 cytochrome units) showed evidence of a flavin component with maxima at 375 and 455 m μ (Fig. 5). These absorption bands disappeared on the addition of L-

Fig. 5. Absorption spectra of an enzyme preparation (specific activity 298) before and after the addition of L-galactono- γ -lactone (1 mg./ml.). —, Oxidized; ---, reduced.

Table 5. *Effect of Atebrin, riboflavin and acriflavine on enzyme activity*

Expt.	Inhibitor	Concn. (mm)	Treatment	Activity (% of control)	
				Phenazine reduction	Cytochrome c reduction
1	Atebrin	0.1	Tested after addition	100	85
		1	Tested after addition	93	41
		1	Tested after addition	100	35
		2.5	Tested after addition	—	11
		2.5 + FAD 0.01	Tested after addition	—	11
		2.5 + FAD 0.1	Tested after addition	—	13
		6	Incubated with enzyme for 10 min. at 37° before test	96	—
		6	Incubated with enzyme for 60 min. at 37° before test	93	—
2	Atebrin	1	Tested after addition	—	56
		1 + FMN 1	Tested after addition	—	55
3	Riboflavin	1	Tested after addition	100	—
		2	Incubated with enzyme for 10 min. at 37°	97	—
		0.1	Tested after addition	—	56
		0.2	Tested after addition	—	44, 38
		0.2 + FAD 0.1	Tested after addition	—	48
		0.5	Tested after addition	—	34
4	Riboflavin	0.1	Tested after addition	—	66
		0.1 + FMN 1	Tested after addition	—	76
		0.1 + FMN 10	Tested after addition	—	60
5	Acriflavine	0.1	Incubated with enzyme for 10 min. at 37° before test	63	—
		1	Incubated with enzyme for 20 min. at 37° before test	50	—
		3	Incubated with enzyme for 15 min. at 37° before test	39	—
		3 + FAD 1	Incubated with enzyme for 15 min. at 37° before test	79	—
		1	Tested immediately	—	66

galactono- γ -lactone, and the difference spectrum obtained was typical of many flavo-protein enzymes. The oxidized spectrum showed an additional absorption band in the Soret region with a peak at 407 m μ , which on reduction with the lactone increased and the maximum shifted to 412 m μ . When dithionite was added this absorption band was still further intensified, and in addition two bands at 518–520 m μ and at 550–555 m μ were observed. This suggests that a haem compound may be present as an additional component (cf. Mahler, Mackler, Green & Bock, 1954).

Identification of flavin. The most active enzyme extracts so far obtained showed a weak, yellowish fluorescence in ultraviolet light, which was increased three- to four-fold after acidification with trichloroacetic acid and neutralization. Estimation of riboflavin by the lactobacillus test (Clegg, Kodicek & Mistry, 1952) was positive and showed, in the latter stages of purification, that the proportion of flavin to protein rose as the specific activity increased. With the purest preparations so far obtained (specific activity 300) a mole of flavin was associated with about 800 kg. of protein, which would indicate either that the content of flavin in the enzyme is much lower than is usually found in flavoproteins, or, what is more likely, that much inert protein is still present. We hope to continue the purification when larger amounts of enzyme become available.

An analysis of the flavin component of the enzyme has been carried out by paper chromatography. After the enzyme had been dialysed against distilled water for 3 hr. at 0°, the flavin was split from the holoenzyme either with acid at pH 2.5 or by heat-denaturation. The solution, centrifuged to remove protein, was concentrated *in vacuo* to dryness, and the residue extracted with 50% ethanol to remove the flavin. This procedure was found to be essential in order to free the small amount of flavin present from inorganic salts. The ethanol extract was then chromatographed with two solvents. With either a solvent mixture of butanol-acetic acid-water (4:1:5, by vol.), or a mixture of 5% (w/v) disodium hydrogen phosphate and 3-methylbutan-1-ol (Carter, 1950) and flavin-adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin as markers, the flavin component of the enzyme was resolved into two spots, one with the same R_F as FMN (R_F 0.1 in the first solvent and 0.5 in the second) and the other with an R_F corresponding to riboflavin (R_F 0.3 in the first solvent and 0.3 in the second). No FAD could be detected. These results were confirmed by using a mixed chromatographic analysis, whereby the addition of FMN or riboflavin to the flavins obtained from the enzyme gave a chromatogram

identical with that obtained from the flavins of the enzyme alone.

The presence of FMN after splitting was confirmed by a positive reaction with the FMN-dependent apoenzyme of TPNH cytochrome *c* reductase prepared from yeast by the method of Haas, Horecker & Hogness (1940) and the absence of FAD was likewise confirmed by the inability of the split flavins to reactivate the apoenzyme of D-amino acid oxidase (Huennekens & Felton, 1957).

Fluorimetric assay of the two flavins by the method described by Burch (1957) showed that FMN was present in a concentration three times that of riboflavin. At present it is not clear whether the riboflavin found is an impurity associated with the enzyme (though this seems unlikely in view of the steps used in its purification), or whether it originated from a breakdown of the flavin prosthetic group during the splitting from the holoenzyme, similar to that described by Mahler, Sarkar, Vernon & Alberty (1952) for DPN cytochrome *c* reductase.

Phosphate and activity

The phenazine-reducing system did not exhibit maximal activity except in the presence of phosphate. It is not certain whether the requirement for phosphate is absolute, as even preparations dialysed to reduce phosphate may contain enough to account for the residual activity. The results (Table 6) indicate that maximal activity was attained at a level of approximately 0.1M-phosphate. When either veronal or 2-amino-2-hydroxy-methylpropane-1:3-diol (tris) was used as buffer, the activity depended on the presence of phosphate, but the maximal activity obtained with the added phosphate was less than that of the phosphate alone. This may be due to an inhibitory effect of these buffers in high concentrations, although they were not inhibitory at mM levels.

Pyrophosphate and arsenate, among other salts tested, were even more effective than phosphate, especially when added in the presence of tris buffer; sodium chloride was without effect.

The stimulative effect of phosphate on the flavo-enzymes, xanthine and aldehyde oxidases, in their reactions with certain electron acceptors, has been described by Mackler, Mahler & Green (1954) and by Mahler *et al.* (1954), and a similar effect to that reported here (of phosphate on the enzymic reduction of phenazine by succinic dehydrogenase) has been observed by Singer, Kearney & Zastrow (1955).

Inhibition of reduction of cytochrome c by anions. In contrast with the results with phenazine the reduction of cytochrome *c* was inhibited by raising the concentration of phosphate. This inhibition was, however, not specific for phosphate, for

Table 6. *Stimulative effect of phosphate and other salts on the reduction of phenazine*

Arsenate, chloride, phosphate and pyrophosphate were added as sodium salts neutralized to pH 7.4 where necessary before addition.

Expt.	Buffer	Concn. (M)	Salt	Concn. added (M)	Enzyme activity (phenazine units)
1.	Phosphate	0.01	—	Nil	1.20
		0.03	—	Nil	1.95
		0.04	—	Nil	2.35
		0.10	—	Nil	3.25
		0.13	—	Nil	3.55
		0.16	—	Nil	3.15
2. Enzyme (3 ml.) dialysed overnight against 2 l. of 0.1 M-tris buffer (pH 7.4)	Tris	0.08 (pH 7.4)	Phosphate	Nil	0.65
		0.08		0.046	1.05
		0.08		0.06	1.25
		0.08		0.08	2.05
		0.08		0.125	2.00
		0.08		0.133	2.05
		—		0.09	2.85
		—		0.15	2.85
3.	Veronal	0.05 (pH 7.4)	Phosphate	0.008	0.60
		0.05		0.10	1.20
		—		0.10	1.95
4.	Tris	0.05 (pH 7.4)	—	Nil	0.50
		0.05	Phosphate	0.1	1.30
		0.05	Pyrophosphate	0.1	2.60
		0.05	Arsenate	0.1	2.00
		0.05	Chloride	0.1	0.20
		—	Phosphate	0.1	1.80
		—	Pyrophosphate	0.1	2.20

Fig. 6. Competitive nature of the inhibition by phosphate on the enzymic reduction of cytochrome *c*. Each solution contained enzyme, L-galactono- γ -lactone (2 mg./ml.) with varying amounts of cytochrome *c* and phosphate (pH 7.8); temp. 17°. Phosphate: ●, 0.025 M; ▲, 0.015 M; ○, 0.01 M. V = units of enzyme activity. S = concn. of cytochrome *c* (mg./ml. of reaction solution).

inhibition in varying degrees was observed with many anions. The inhibitory effect of several sodium salts was tested at a level of 0.02 M on the reaction with cytochrome *c* in the presence of

0.01 M-phosphate buffer (pH 7.8). The results showed that the inhibition (52 %) due to SO_4^{2-} ions was greatest, followed in descending order by NO_3^- (43 %), F^- (30 %), Cl^- (24 %), Br^- (21 %) and AcO^- ions (8 %). Sucrose, an un-ionized solute, had no inhibitory effect in concentrations as high as 0.4 M.

The inhibitory effect of anions on the activity of certain flavin enzymes has been noted by Theorell & Nygaard (1954) for the 'old yellow enzyme' and by Walaas & Walaas (1956) for D-amino acid oxidase, and pronounced effects of anions on the activity of DPN- and TPN-linked enzymes such as alcohol dehydrogenase and glucose 6-phosphate dehydrogenase have also been described (Theorell, Nygaard & Bonnichsen, 1954; Rutter & Rolander, 1955). The former workers found that the anions inhibit by increasing the dissociation of coenzyme from apoenzyme, thereby reducing velocity of the enzyme reaction. Lineweaver-Burk plots of $1/V$ against $1/S$, where S was equal to the concentration of cytochrome *c* at three different levels of phosphate, indicated that inhibition by phosphate was competitive (Fig. 6), and similar results were obtained with sodium chloride. This reversal of the inhibition by cytochrome *c* suggests that the anions affect the binding of this compound to the enzyme. It is of course possible that the anions may also affect the dissociation of coenzyme from apoenzyme.

DISCUSSION

The solubilization and isolation of L-galactono- γ -lactone dehydrogenase from a plant mitochondrial preparation adds further to our knowledge of the mechanism whereby L-ascorbic acid is synthesized *in vivo*. Of special interest in this respect was our observation of the high specificity of the enzyme. The inability of the enzyme to oxidize the closely related L-gulono- γ -lactone would indicate an enzymic specificity for the configuration of the groups on C (2) and C (3) of the aldonic acid lactone; in L-galactono- γ -lactone these are L and D respectively. In agreement with these results were the observations that the enzyme failed to catalyse the oxidation of D-mannono-, D-glucono-, D-galactono- or D-gulono- γ -lactone, in which the configuration on C (2) and C (3) is also different from that of L-galactono- γ -lactone, and in addition the configuration on C (5) is D, not L. D-Altrono- γ -lactone, which has the same configuration of the groups on C (2) and C (3) as L-galactono- γ -lactone but the opposite configuration on C (5), was oxidized only very slowly; this would indicate that the configuration of the groups on C (5) influences the rate of oxidation of the lactone.

The fact that essentially similar results to these were obtained with cauliflower mitochondria makes it improbable that during extraction and purification any changes had occurred in the enzyme protein which had altered its specificity. These results may be compared with those obtained in the earlier work with cress seedlings (Isherwood *et al.* 1954) and with pea mitochondria (Mapson *et al.* 1954). With growing cress seedlings L-galactono- and D-altrono- γ -lactone were converted into an ascorbic acid to about the same extent, the only difference being that L-ascorbic acid was formed with the first lactone and D-araboascorbic acid with the second. Under the same conditions L-gulono- γ -lactone as a precursor of L-ascorbic acid was only about one-tenth as effective as L-galactono- γ -lactone. With pea mitochondria D-altrono- γ -lactone was converted into an ascorbic acid at about one-half, and L-gulono- γ -lactone at only one-twenty-fifth, of the rate of conversion of L-galactono- γ -lactone.

The additional results given in this paper indicating a higher specificity of the enzyme from cauliflower as compared with peas suggests that the differences are due to real differences in the specificity of the enzymes in the different plants. It is unlikely that the results can be explained by the removal of waldenase-like enzymes during extraction and purification of the solubilized enzymes, which catalyse the interconversion of the lactones. Whatever may be the true explanation, it seems clear that with all plant enzymes so far examined

L-galactono- γ -lactone is pre-eminent as a precursor of L-ascorbic acid.

Hassan & Lehninger (1956) reported that mitochondrial preparations from rat liver were able to form ascorbic acid from the free acid as easily as from the lactone. Neither in this work nor in the earlier work with pea mitochondria (Mapson *et al.* 1954) have we observed the formation of ascorbic acid from L-galactonic acid.

The nature of the electron path from the lactone to phenazine and cytochrome *c* cannot be determined until further purification of the enzyme is accomplished. Preliminary evidence, however, indicates that the paths to these two electron-acceptors are not identical. Inhibition of the cytochrome *c* reaction by Atebrin, acriflavine and riboflavin suggests that flavin is involved. The changes in the spectrum accompanying the reduction of the enzyme by substrate further support the theory that the enzyme is a flavoprotein. The reduction of phenazine, on the other hand, is insensitive to Atebrin or riboflavin, and it is thus conceivable that the phenazine reaction proceeds without flavin's being involved.

The requirement of L-galactono- γ -lactone dehydrogenase for inorganic phosphate for the reduction of phenazine is similar to the requirement of succinic dehydrogenase for phosphate (Singer *et al.* 1955). Another similarity between the two enzymes is the insensitivity of both to most metal-binding agents with the exception of o-phenanthroline (Singer, Kearney & Massey, 1956) and it may be that L-galactono- γ -lactone dehydrogenase is also a ferroflavoprotein. Singer *et al.* (1956) assumed that, with succinic dehydrogenase, electrons were transferred from succinate to flavin to iron and then to phenazine. Such a scheme is clearly inapplicable to the reduction of phenazine by L-galactono- γ -lactone dehydrogenase in view of the insensitivity of the reaction to Atebrin and riboflavin. In both *Bacterium tularense* (Wadkins & Mills, 1956) and in *Tetrahymena* (Eichel, 1956) it has been found that the reduction of phenazine by succinic dehydrogenase is Atebrin-insensitive, whereas the succinoxidase reaction is readily inhibited. Assuming the presence of flavin in the microbial dehydrogenases as in beef-heart dehydrogenase, it seems that in these, as well as in L-galactono- γ -lactone dehydrogenase, flavin may not be the initial electron acceptor but that electrons are transferred from substrate to a primary acceptor (conceivably metal) and then either to flavin or to phenazine. A scheme cannot be excluded, however, in which phenazine is positioned in an electron chain between a primary Atebrin-insensitive flavin component and a flavin which is Atebrin-sensitive. In any event, it appears likely that L-galactono- γ -lactone dehydrogenase

will fall into that class of flavin enzymes in which the active sites involve electron transport between different prosthetic groups on the same enzyme surface.

SUMMARY

1. An enzyme oxidizing L-galactono- γ -lactone to L-ascorbic acid has been extracted from cauliflower mitochondria and purified 12-fold from an acetone-dried powder.

2. Only phenazine methosulphate and cytochrome *c* of the various electron acceptors tested were reduced at a significant rate by the enzyme in the presence of substrate.

3. Phosphate stimulated the enzymic reduction of phenazine, but phosphate and other anions inhibited the corresponding reduction of cytochrome *c*.

4. The enzyme requires thiol groups for its activity; it was inhibited strongly by certain thiol inactivators and this inhibition was reversed by glutathione.

5. The enzyme was not inhibited by cyanide, ethylenediaminetetra-acetic acid, pyrophosphate, azide or α -dipyridyl, but was inhibited by *o*-phenanthroline.

6. Evidence of the flavin nature of the prosthetic group of the enzyme was obtained from the absorption spectrum, the presence of flavin in the most active preparations and the inhibition of the enzyme by riboflavin and Atebrin.

7. The substrate specificity of the enzyme was high; of the several aldonic acid lactones tested, L-galactono- γ -lactone was the only one oxidized at a high rate, D-altrono- γ -lactone was oxidized only very slowly and none of the others were oxidized.

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