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Biological Synthesis of L-Ascorbic Acid in Animal Tissues: Conversion of D-Glucuronolactone and L-Gulonolactone into L-Ascorbic acid

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The synthesis of L-ascorbic acid in animal tissues is believed (Horowitz & King, 1953*a*, *b*; Isherwood, Chen & Mapson, 1954; Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957, 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960*a*) to follow the sequence:

$$\text{D-Glucuronolactone} \xrightarrow{\text{reduction}} \text{L-gulonolactone} \xrightarrow{\text{oxidation}} \text{L-ascorbic acid.}$$

The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid has previously been reported to be located in the microsomal fractions of the liver homogenates of the rat and goat, and of kidney homogenates of the chick (Burns *et al.* 1956; Chatterjee *et al.* 1958*b*, *c*; Roy & Guha, 1958). The synthesis from D-glucuronolactone, irrespective of the species studied, is greatly stimulated in the presence of sodium or potassium cyanide but the conversion of L-gulonolactone into L-ascorbic acid does not require the presence of cyanide and is, in fact, inhibited by it (Chatterjee *et al.* 1958*c*). The function of cyanide therefore lies apparently in the reduction of D-glucuronolactone to L-gulonolactone and it probably acts by activating the thiol enzyme involved in this reduction (Chatterjee *et al.* 1958*c*).

The rate of conversion of D-glucuronolactone into L-ascorbic acid by the microsomes from any of the species mentioned above is maximum with 50 mM-cyanide, the rate of synthesis decreasing with decreasing cyanide concentration. With rat-liver and chick-kidney microsomes no appreciable synthesis is observed with 5 mM-potassium cyanide even when the time of incubation is prolonged from 1·5 to 3·0 hr. It has, however, been observed that the synthesis by rat-liver microsomes takes place also in the presence of 5 mM or even a lower concentration of cyanide if reduced diphosphopyridine nucleotide, reduced triphosphopyridine nucleotide, glutathione or a chelating agent such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl, 8-hydroxyquinoline or the boiled rat-liver supernatant is added to the system (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1958*a*). Substances such as reduced di- and tri-phosphopyridine nucleotide and glutathione probably function by stimulating the action of cyanide in reducing the thiol-enzyme for the conversion of D-glucuronolactone into L-gulonolactone, which constitutes the first phase of the reaction. The metal-binding agents such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline, and the boiled supernatant have been reported previously (Chatterjee *et al.* 1958*a*; Chatterjee *et al.* 1960*a*) to enhance the conversion of L-gulonolactone into L-ascorbic acid. It is likely therefore that these agents promote the formation of L-ascorbic acid from D-glucuronolactone by stimulating the second phase of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid.

Goat-liver microsomes behave somewhat differently from rat-liver and chick-kidney microsomes. Although the maximum conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes takes place with 50 mM-KCN, appreciable synthesis is also observed with 5 mM or an even lower concentration of cyanide if the time of incubation is prolonged from 1·5 hr. to 3·0 hr. This synthesis by the goat-liver microsomes is not further augmented by any of the aforesaid agents.

The lactone forms of the precursors, D-glucuronolactone and L-gulonolactone, have been found to be specifically active in the microsomal synthesis of

ascorbic acid; the sodium salts of the corresponding free acids are not acted on (Chatterjee *et al.* 1958*b*, *c*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*, *b*; Chatterjee *et al.* 1960*a*). Our observation on the specificity of the lactone structure has recently been confirmed by Kanfer, Burns & Ashwell (1959) as well as by Bublitz & Lehninger (1959). The latter have now withdrawn the former claim (Grollman & Lehninger, 1957) that it was the free acid and not the lactone that was specifically acted on in the biosynthesis of L-ascorbic acid.

It has already been reported that the oxidative conversion of L-gulonolactone into L-ascorbic acid by liver microsomes of the rat and goat is an aerobic process (Chatterjee *et al.* 1958*b*, *c*). It had been supposed by some workers (Grollman & Lehninger, 1957) that the intermediate was 3-keto-L-gulonic acid. We had, however, considered for several reasons that 2-keto-L-gulonolactone (L-*xylo*hexulonolactone) was the likely intermediate (Chatterjee *et al.* 1960*a*). Recently the products of oxidation have actually been identified as L-*xylo*hexulonolactone and hydrogen peroxide (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*c*; Chatterjee, Ghosh, Ghosh & Guha, 1959*d*). The possibility of L-*xylo*hexulonolactone being the intermediate in the oxidation of L-gulonolactone to L-ascorbic acid has also been suggested by Kanfer *et al.* (1959).

This paper describes a detailed investigation, covering some of the aspects mentioned above, into the mechanism of conversion of D-glucuronolactone and of L-gulonolactone into L-ascorbic acid by the liver microsomes of the rat and goat and the kidney microsomes of the chick as well as by soluble-enzyme preparations obtained from these microsomes.

METHODS

Preparation of nuclei, cell debris and mitochondria. The nuclei and cell-debris fraction was sedimented at 500 *g* and the mitochondrial fraction was sedimented at 8500 *g*, by the method of Schneider & Hogeboom (1950). Both the fractions were washed twice with iso-osmotic sucrose solution.

Preparation of microsomes and soluble supernatant. The microsomes and the soluble supernatant were prepared by the method described by Chatterjee *et al.* (1960*a*).

Preparation of a soluble enzyme. Goat-liver microsomes (10 ml., equivalent to 320 mg. of protein) were added during 2 min. to a solution (5 ml.) of sodium deoxycholate (250 mg.) in sodium phosphate buffer (0·2 M, pH 7·4) with constant stirring. After addition, the mixture was shaken vigorously for another 1–2 min., diluted to 35 ml. with 0·2 M-phosphate buffer and centrifuged at 100 000 *g* for 40 min. in a Spinco ultracentrifuge. The residue was discarded and the protein fraction of the supernatant precipitated at 30% saturation of ammonium sulphate (pH 7·0) was collected and dissolved in 0·1 M-phosphate buffer (10 ml.), pH 7·2. The enzyme solution was then dialysed for 1 hr. against 30 vol. of sodium phosphate buffer (0·01 M, pH 7·2) with constant stirring. After dialysis the enzyme solution was centrifuged at 10 000 *g* for 15 min. and the sedimented protein fraction was discarded. The supernatant (10–12 mg. of protein/ml.) containing the active enzyme was stored at −20°. The temperature throughout the procedure was kept at 0–2°.

Rat-liver microsomes as well as chick-kidney microsomes gave active soluble enzyme preparations by the same treatment except that in these cases the amount of deoxycholate used was half that used with goat-liver microsomes. This was done because the rat-liver microsomes and chick-kidney microsomes, obtained from a given weight of the tissues, were found to contain approximately half the amount of the protein in goat-liver microsomes obtained from the same weight of tissue, and the amount of deoxycholate needed was dependent on the protein content of the microsomes. The activity of the soluble-enzyme preparation from rat-liver microsomes or from chick-kidney microsomes was almost equal to that from goat-liver microsomes on the protein basis and was only about one half on the basis of the weight of the fresh tissues.

The boiled supernatant was prepared by the method already described (Chatterjee *et al.* 1960*a*).

Incubation media. Unless otherwise mentioned, the test system contains 20 mM-sodium phosphate buffer (pH 7·2), 10 mM-D-glucuronolactone, 0·25 ml. of the microsomal dispersion (equivalent to 250 mg. of wet liver or kidney), KCN (50 mM or less as indicated in the text). Total volume was 2·5 ml., incubated at 37° in air for 1·5 hr.

When L-gulonolactone (5 mM) was used as the substrate, cyanide was omitted from the incubation media.

Estimation of ascorbic acid. Ascorbic acid was identified and estimated by the methods described by Chatterjee *et al.* (1960*a*). In the presence of potassium ferricyanide ascorbic acid was estimated by Roe & Kuether's (1943) method and in the presence of cyanide Roe & Kuether's method was used after removal of cyanide by acidification with HCl (M) and evaporation to dryness under suction at 40° and taking up the residue with a known volume of water.

Identification and estimation of D-*glucuronolactone and* L-*gulonolactone.* D-Glucuronolactone and L-gulonolactone were identified by paper chromatography and estimated by an adaptation of the hydroxamic acid procedure described before (Chatterjee *et al.* 1960*a*).

Estimation of protein. Protein was estimated by the method already described (Chatterjee *et al.* 1960*a*).

Estimation of Hg^{2+} *ion.* Hg^{2+} ion was estimated colorimetrically with dithizone (Feigl, 1954). Hg^{2+} ion present in the samples of adenosine triphosphate was estimated by adding, with vigorous shaking, 1 ml. of a solution of 5 mg. of dithizone in 100 ml. of carbon tetrachloride to 1 ml. of a solution of adenosine triphosphate (25–50 mg.) that had been heated on a boiling-water bath with a drop of concentrated HCl for about a minute. The mixture was then diluted to 4 ml. with absolute ethanol and the colour produced was compared with that obtained with a standard solution of $HgCl_2$ treated similarly.

Preparation of the o-*phenylenediamine derivative of* L-*xylohexulonate.* L-*xylo*Hexulonolactone formed as an intermediate in the enzymic oxidation of L-gulonolactone to L-ascorbic acid was trapped in the system as L-*xylo*hexulonate. The *o*-phenylenediamine derivative of L-*xylo*hexulonate was then prepared as follows (Lanning & Cohen, 1951).

The soluble-enzyme preparation (2 ml.) was incubated at 37° in air for 30 min. with 10 mg. of L-gulonolactone in the presence of 50 mM-sodium phosphate buffer, pH 7·9, and 4 mM-KCN, the total volume being 2·5 ml. Potassium cyanide was added to prevent the oxidation of the synthesized ascorbic acid to the corresponding dehydro form, because the latter has also been found to give a condensation reaction with *o*-phenylenediamine which might interfere with the identification of the ketogulonate. It may be mentioned that the ene-diol form of ascorbic acid has not been found to give such condensation reaction under similar conditions. After incubation the mixture was deproteinized with 0·25 ml. of 30% metaphosphoric acid and filtered. The filtrate (1 ml.) was neutralized with 10% NaOH and to this was added 0·1 ml. of a solution of 1·5% *o*-phenylenediamine in 0·25 M-HCl. The mixture was heated on a boiling-water bath for 30 min. The *o*-phenylenediamine derivative thus produced was identified by the methods described below.

Identification of the o-phenylenediamine derivative of L-xylo*hexulonate.* (*a*) Paper-chromatographic analysis. A portion (50 μl., equivalent to approx. 20–25 μg. of the L-*xylo*hexulonate) of the *o*-phenylenediamine derivative prepared as stated above was applied on paper (Whatman no. 1) for chromatographic separation with butan-1-ol–formic acid (1:1) as well as phenol–water (9:1) as the irrigating solvents. A parallel run was given with a portion of the *o*-phenylenediamine derivative prepared from an authentic sample of L-*xylo*hexulonate. After the run was over (4–6 hr.) the paper was air-dried and the spots were located by their typical blue fluorescence in the ultraviolet light. No spot could be detected by incubating L-gulonolactone with a boiled enzyme preparation. The R_F values with the butanol and the phenol solvents were 0·94 and 0·90 respectively. The spot obtained with the *o*-phenylenediamine derivative of dehydroascorbic acid (R_F 0·89, butanol solvent) was distinguished by a typical yellow fluorescence.

(*b*) Absorption-spectra analysis. The absorption-spectra analysis of the *o*-phenylenediamine derivative of the biosynthesized L-*xylo*hexulonate was carried out according to the method of Lanning & Cohen (1951) with a Beckman model DU spectrophotometer. About 25 μl. of the compound (equivalent to approx. 10–12 μg. of L-*xylo*hexulonate) was added to a 1 cm. cuvette containing glass-distilled water and the absorption spectra were analysed over the region 325–360 mμ, an authentic sample of *o*-phenylenediamine derivative of L-*xylo*hexulonate being kept in the reference cell.

Identification and estimation of hydrogen peroxide. Hydrogen peroxide formed as a product of microsomal oxidation of L-gulonolactone to L-ascorbic acid has been identified and estimated indirectly by the coupled oxidation of methanol to formaldehyde in the presence of catalase, formaldehyde being estimated by a modified method of Keilin & Hartree (1945).

MATERIALS

D-Glucuronolactone and sodium D-glucuronate were purchased from the California Foundation for Biochemical Research, California.

L-Gulonolactone, sodium L-gulonate and L-*xylo*hexulonate were prepared by the methods described before (Chatterjee *et al.* 1960*a*).

Adenosine triphosphate (ATP, crystalline), diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and reduced diphosphopyridine nucleotide (DPNH) were obtained from the Schwarz Laboratories Inc. Reduced triphosphopyridine nucleotide (TPNH) was generated *in situ* from TPN (2 mM), glucose 6-phosphate (5 mM) and yeast glucose 6-phosphate dehydrogenase (1·5 units). Antimycin A was obtained through the courtesy of Kyowa Fermentation Ind. Co. Ltd., Tokyo, Japan.

RESULTS

Intracellular localization of the enzyme system converting D-*glucuronolactone into* L-*ascorbic acid.* The relative enzymic activities of the different subcellular fractions for the conversion of D-glucuronolactone into L-ascorbic acid are given in Table 1. The results (Table 1) show that the entire activity of the homogenates from the livers of the rat and goat and from the kidney of the chick is located in the microsomal fractions. The observed activity of the mitochondria is probably due to their contamination by microsomes since it has been found that the activity of the mitochondria decreases with washing.

Effect of cyanide on the rate of microsomal conversion of D-*glucuronolactone into* L-*ascorbic acid.* The rate of conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes has been found to be maximum with 50 mM-KCN, decreasing with decreasing cyanide concentration, and is not appreciable with 5 mM-KCN. But if the time of incubation is prolonged some synthesis is observed even with 5 mM-KCN (Fig. 1). Chick-kidney microsomes behave similarly. It has, however, been found that with goat-liver microsomes the conversion of D-glucuronolactone into L-ascorbic acid in the presence of 5 mM-KCN approaches that obtained with 50 mM-KCN if the time of incubation is prolonged (Fig. 1).

Effect of some other factors in the presence of cyanide on the microsomal conversion of D-*glucurono-*

Table 1. *Conversion of* D-*glucuronolactone into* L-*ascorbic acid by different subcellular fractions in the presence of* 50 mM-*potassium cyanide*

Each fraction represents 250 mg. of original liver or kidney. Other conditions were as stated in the Methods section.

Fraction	Ascorbic acid synthesized (μmole)		
	Goat liver	Rat liver	Chick kidney
Homogenate	0·55	0·35	0·40
Nuclei, cell debris	0	0	0
Mitochondria	0·10	0·06	0·06
Microsomes	1·20	0·6	0·65
Soluble supernatant	0	0	0

lactone into L-*ascorbic acid.* It has been found that though no appreciable synthesis of L-ascorbic acid from D-glucuronolactone by the rat-liver microsomes occurs in the presence of 5 mM-KCN, a marked synthesis takes place if DPNH or TPNH is added to the system (Table 2). The reduced pyridine nucleotides can be more efficiently replaced by some metal-binding agents, namely sodium pyrophosphate, $\alpha\alpha'$-dipyridyl, 8-hydroxyquinoline or by reduced glutathione (GSH). Addition of DPNH, TPNH or GSH to a system containing any of these chelating agents does not further enhance the synthesis. Some synthesis is also observed with sodium citrate, ATP or ADP, but not with adenosine monophosphate (AMP). Nicotinamide and antimycin A were ineffective. The results are given in Table 2. None of the aforesaid factors can, however, promote the synthesis in the presence of 50 mM-KCN. In the absence of cyanide no synthesis is observed in any of these cases. The behaviour of chick-kidney microsomes is similar. But none of the agents which stimulated the synthesis by rat-liver and chick-kidney microsomes has any influence on the synthesis by goat-liver microsomes.

Effect of boiled supernatant on the conversion of D-*glucuronolactone into* L-*ascorbic acid by the microsomes.* It has been observed that in a system containing 5 mM-KCN and a chelating agent, the chelating agent can be completely replaced by the boiled rat-liver supernatant for the conversion of D-glucuronolactone into L-ascorbic acid. The boiled supernatant from rat liver is not, however, specific; that obtained from goat liver, chick kidney and even guinea-pig liver is also equally active (Table 3). Like the chelating agent the boiled supernatant obtained from any of these species cannot, however, further enhance the synthesis stimulated by 50 mM-KCN, whereas in the absence of cyanide it is ineffective. Chick-kidney microsomes behave in a similar way to rat-liver microsomes in the presence of the boiled supernatant, which, however, has no influence on the synthesis by goat-liver microsomes.

Some properties of the boiled supernatant. The activity of the boiled supernatant is almost entirely lost if it is dialysed for 72 hr. against 30 vol. of 10 mM-sodium phosphate buffer, pH 7·2, treated with 4 % trichloroacetic acid, shaken with activated charcoal or heated at 100° with N-HCl for 30 min. or with N-NaOH for 10 min. The activity of the

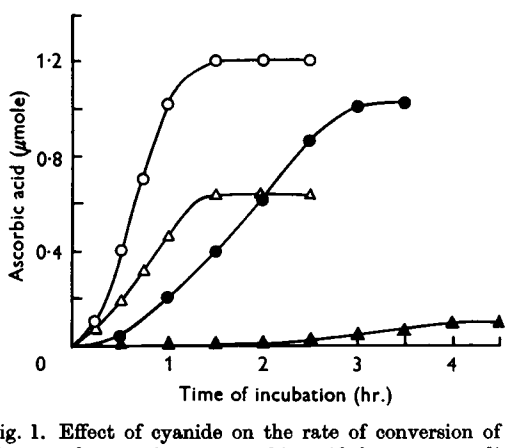


Fig. 1. Effect of cyanide on the rate of conversion of D-glucuronolactone into L-ascorbic acid by: ○, goat-liver microsomes with 50 mM-KCN; ●, goat-liver microsomes with 5 mM-KCN; △, rat-liver microsomes with 50 mM-KCN; ▲, rat-liver microsomes with 5 mM-KCN.

Table 2. *Effect of reduced pyridine nucleotides, chelating agents and some other factors on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by rat-liver microsomes*

Test system: where DPNH or TPNH was used, nicotinamide (30 mM) was added; incubation was for 3 hr. Other conditions were as stated in the Methods section. ADP, Adenosine diphosphate.

KCN (mM)	Factors	Concn. used (mM)	Ascorbic acid synthesized (μmole)
None	None	—	0
50	None	—	0·65
50	DPNH	1	0·66
50	Sodium pyrophosphate	5	0·67
5	None	—	0·02
5	TPNH	1	0·30
5	DPNH	1	0·25
5	Sodium pyrophosphate	5	0·75
5	$\alpha\alpha'$-Dipyridyl	2	0·70
5	8-Hydroxyquinoline	0·5	0·80
5	GSH	10	0·50
5	Sodium citrate	10	0·22
5	ATP	5	0·25
5	ADP	5	0·20
5	AMP	5	0·01
5	Antimycin A	1	0·01
5	Nicotinamide	30	0·02

Table 3. *Effect of boiled supernatant obtained from different species on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by rat-liver microsomes in the presence of* 5 mM-*potassium cyanide*

Test system: boiled supernatant, equivalent to 100 mg. of wet tissue; incubation was for 3 hr. Other conditions were as stated in the Methods section.

Source of boiled supernatant	Ascorbic acid synthesized (μmole)
None	Nil
Rat liver	0·80
Goat liver	0·50
Chick kidney	0·75
Guinea-pig liver	0·80

boiled supernatant is retained in the supernatant fraction after precipitating the protein with $Zn(OH)_2$ or by bringing down the pH to 3 with HCl. Attempts to separate the active factor present in the boiled supernatant by paper chromatography or paper electrophoresis have not given any clear-cut results.

Effect of L-*gulonolactone on the microsomal conversion of* D-*glucuronolactone into* L-*ascorbic acid.* It has been observed that though goat-liver microsomes do not convert D-glucuronolactone into L-ascorbic acid in the absence of cyanide, they do so when L-gulonolactone is added to the system (Tables 4 and 5). With an optimum concentration of L-gulonolactone (5 mM) the synthesis becomes maximum with an optimum concentration of D-glucuronolactone (10 mM), decreasing with the decreasing concentration of D-glucuronolactone (Table 4). Again with an optimum concentration of D-glucuronolactone (10 mM) the synthesis depends upon the concentration of L-gulonolactone present in the system, being maximum with the optimum concentration of L-gulonolactone (5 mM) and decreasing with its decreasing concentration (Table 5). The microsomes from rat liver or chick kidney behave similarly. The effect of L-gulonolactone on the conversion of D-glucuronolactone into L-ascorbic acid has been found to be similar to that obtained with KCN (Tables 4 and 5).

Effect of some electron acceptors on the conversion of D-*glucuronolactone into* L-*ascorbic acid by the rat-liver microsomes.* The effect of some electron acceptors on the synthesis of ascorbic acid has been studied. It has been found that although oxidized glutathione and methylene blue are ineffective, potassium ferricyanide at mM concentration completely inhibits the conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes in the presence of 5 mM-KCN and sodium pyrophosphate. Potassium ferricyanide, however, has no influence on the conversion of L-gulonolactone into L-ascorbic acid (Table 6).

Effect of adenosine triphosphate on the synthesis of ascorbic acid by rat-liver microsomes. Adenosine triphosphate has been found to stimulate the conversion of D-glucuronolactone into L-ascorbic acid by the rat-liver microsomes in the presence of 5 mM-KCN (Table 2). It may be mentioned that in previous experiments (Chatterjee *et al.* 1958*c*) ATP was found to be inhibitory to the synthesis of ascorbic acid by rat- and goat-liver microsomes. But in subsequent investigations the observed inhibition by ATP was found to be due to the presence of Hg^{2+} ions (3 μg./mg.) in the sample of ATP used (Schwarz Laboratories Inc.; lot no. 5501, 1955) and not due to the ATP itself (Chatterjee *et al.* 1958*a*). Hg^{2+} ion has already been reported to be inhibitory to the synthesis of ascorbic acid (Chatterjee *et al.* 1958*c*, 1960*a*). Fig. 2 shows that the amount of Hg^{2+} ion present in the sample of ATP used can account for the observed inhibition. This shows, incidentally, that the biosynthetic reaction is very sensitive, even to traces of Hg^{2+} ion.

Effect of soluble supernatant on the relative conversion of D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid by the microsomes.* The microsomes in the presence of cyanide can convert D-glucuronolactone but not sodium glucuronate

Table 4. *Effects of* L-*gulonolactone and of potassium cyanide on the synthesis of* L-*ascorbic acid by goat-liver microsomes from different concentrations of* D-*glucuronolactone*

Concentrations of L-gulonolactone and potassium cyanide used were 5 and 50 mM respectively. Incubation was for 3 hr. The values of ascorbic acid given below with L-gulonolactone represent those obtained after deducting the value of ascorbic acid (1·40 μmole) obtained with L-gulonolactone alone.

D-Glucuronolactone (μmoles)	Ascorbic acid synthesized (μmole)	
	With L-gulonolactone	With KCN
50	1·20	1·10
25 (optimum)	1·30	1·20
12	0·62	0·55
6	0·35	0·30
3	0·18	0·16

Table 5. *Relative effects of different concentrations of* L-*gulonolactone and of potassium cyanide on the conversion of* D-*glucuronolactone into* L-*ascorbic acid by goat-liver microsomes*

Concentration of D-glucuronolactone used was 10 mM. Other conditions were the same as stated in Table 4.

KCN (μmoles)	Ascorbic acid synthesized (μmole)	L-Gulonolactone (μmoles)	Ascorbic acid (μmoles) synthesized from: L-Gulonolactone	L-Gulonolactone plus D-glucurono-lactone
250	1·15	25	1·40	2·65
125	1·20	12	1·40	2·65
62	0·58	6	0·75	1·40
31	0·31	3	0·40	0·75
15	0·15	1·5	0·20	0·37

into L-ascorbic acid (Chatterjee *et al.* 1958*b*). The soluble supernatant, irrespective of species, when added to the system inhibits the synthesis from D-glucuronolactone (Fig. 3). The inhibitory factor in the supernatant has been traced to a lactone-splitting enzyme which hydrolyses D-glucurono-lactone to D-glucuronic acid (identified by paper chromatography as the lactone after non-enzymic lactonization in the presence of HCl), the latter being not acted on by the microsomal enzyme. In the absence of cyanide, however, both the lactone and the sodium salt of the free acid are converted, though to a small extent, into L-ascorbic acid, provided that the soluble supernatant and TPNH are present along with the microsomes (Table 8). This synthesis in the presence of the soluble super-natant and TPNH is not much influenced by sub-strate concentration nor by the concentration of the soluble supernatant. On increasing the concen-

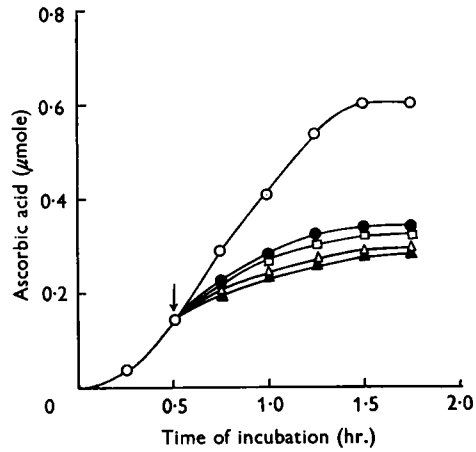


Fig. 3. Effect of soluble supernatants from organs of different species on the rate of conversion of D-glucurono-lactone into L-ascorbic acid by rat-liver microsomes: ○, rat-liver microsomes without additions; ●, after addition of goat-liver supernatant; □, after addition of chick-kidney supernatant; △, after addition of guinea-pig-liver supernatant; ▲, after addition of rat-liver supernatant. The arrow indicates addition of 0·5 ml. of the supernatant.

Table 6. *Effect of some electron acceptors on the synthesis of* L-*ascorbic acid by rat-liver microsomes*

The system contains 5 mM-sodium pyrophosphate and with D-glucuronolactone 5 mM-KCN was also present. Concentrations of potassium ferricyanide, methylene blue and oxidized glutathione were 1 mM, 1 μM and 2 mM respectively. Incubation was for 3 hr.

Electron acceptor	Ascorbic acid synthesized (μmole) D-Glucurono-lactone	L-Gulono-lactone
None	0·80	1·30
Potassium ferricyanide	Nil	1·28
Methylene blue	0·80	1·30
Oxidized glutathione	0·78	1·25

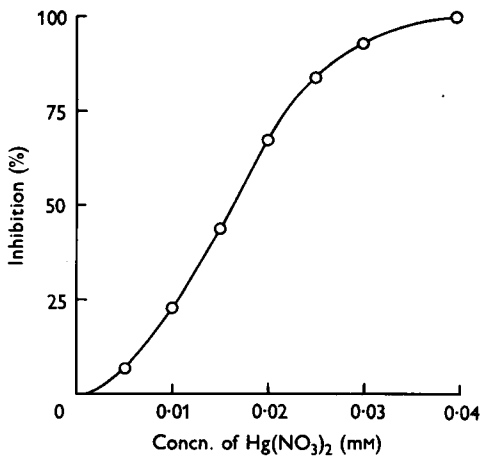


Fig. 2. Effect of Hg^{2+} ions on the synthesis of L-ascorbic acid from D-glucuronolactone by rat-liver microsomes. KCN (50 mM) was added 10 min. after the addition of $Hg(NO_3)_2$.

Table 7. *Effect of varying concentrations of sub-strate, soluble supernatant and microsomes on the synthesis of* L-*ascorbic acid from* D-*glucuronolactone and sodium glucuronate*

Test system: TPNH, mM; nicotinamide, 30 mM. (*a*), Rat-liver microsomes, 0·25 ml.; rat-liver supernatant, 0·5 ml.; (*b*), microsomes, 0·25 ml.; D-glucuronolactone, 10 mM; sodium glucuronate, 10 mM; (*c*), supernatant, 0·5 ml. and substrate as in (*b*). Incubation was for 2 hr. at 37° in air.

		Ascorbic acid synthesized (μmole) D-Glucurono-lactone	Sodium glucuronate
Concn. of substrate (mM)			
(*a*)	None	0	0
	2	0·05	0·06
	4	0·15	0·16
	10	0·15	0·16
	25	0·10	0·14
Concn. of supernatant (ml.)			
(*b*)	None	0	0
	0·10	0·02	0·03
	0·25	0·08	0·10
	0·50	0·16	0·16
	1·00	0·17	0·18
	1·50	0·16	0·17
Concn. of microsomes (ml.)			
(*c*)	None	0	0
	0·10	0·08	0·10
	0·25	0·16	0·15
	0·50	0·23	0·24
	1·00	0·30	0·31
	1·50	0·36	0·37

Table 8. *Relative effects of soluble supernatant, reduced triphosphopyridine nucleotide and potassium cyanide on the conversion of* D-*glucuronolactone and sodium* D-*glucuronate into* L-*ascorbic acid by rat-liver microsomes*

Test system: microsomes and soluble supernatant (rat liver), 0·25 ml. and 0·5 ml. respectively; TPNH, mM; nicotinamide, 30 mM; KCN, 50 mM, where used. Incubation was for 2 hr. Other conditions were as stated in Table 7.

System	Ascorbic acid synthesized (μmole)	
	D-Glucoronolactone	Sodium glucuronate
Microsomes	0	0
Microsomes plus supernatant	0	0
Microsomes plus supernatant plus TPNH	0·16	0·15
Microsomes plus supernatant plus TPNH plus KCN	0·35	0
Microsomes plus supernatant plus KCN	0·36	0
Microsomes plus KCN	0·62	0

Table 9. *Effect of different concentrations of potassium cyanide on the relative conversion of* D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid by microsomes and soluble supernatant from rat liver*

Conditions were as stated in Table 8.

Concn. of KCN (mM)	Ascorbic acid synthesized (μmole)	
	D-Glucurono-lactone	Sodium glucuronate
0	0·16	0·16
1	0·15	0·16
2	0·16	0·15
5	0·17	0
50	0·35	0

tration of the microsomes, however, the synthesis increases to a small extent (Table 7). When 50 mM-KCN is added to a system containing the microsomes, the soluble supernatant and TPNH, the synthesis from D-glucuronolactone is significantly enhanced and the requirement for TPNH no longer exists. Omission of the soluble supernatant further enhances the synthesis. On the other hand cyanide at such a high concentration (50 mM) completely inhibits the synthesis from sodium glucuronate (Table 8). The effects of different concentrations of cyanide on the relative conversion of D-glucuronolactone and sodium glucuronate in the presence of rat-liver microsomes, rat-liver soluble supernatant and TPNH are shown in Table 9. It may be mentioned that the microsomes or the supernatant obtained from a homogenate prepared with an iso-osmotic KCl solution have been found to behave in a similar way to those prepared with an iso-osmotic sucrose solution. Microsomes and the soluble supernatant from goat liver and chick kidney behave similarly to those from rat liver in the conversion of D-glucuronolactone or sodium glucuronate into L-ascorbic acid.

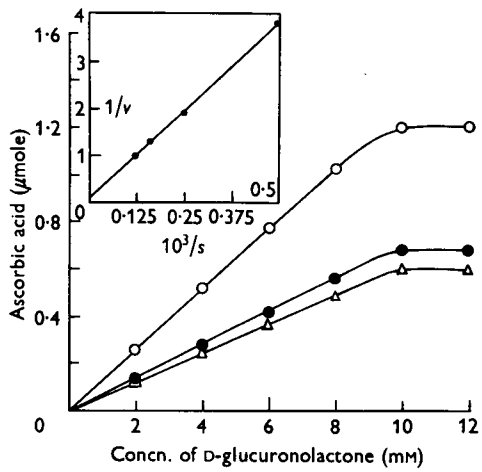


Fig. 4. Effect of varying concentrations of D-glucuronolactone on the synthesis of L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: soluble enzyme, 0·4 ml.; KCN, 50 mM; incubation was for 1 hr. Inset figure represents $1/v$–$1/s$ for D-glucuronolactone with the soluble-enzyme preparation from goat-liver microsomes, where v is μmoles of ascorbic acid formed/hr. and s is D-glucuronolactone in moles/l.

Some properties of the soluble-enzyme preparations obtained from the livers of rat and goat and the kidney of the chick. The effect of variation of the concentration of D-glucuronolactone on the synthesis of L-ascorbic acid by the soluble-enzyme preparations from the goat, rat and chick is shown in Fig. 4. The Michaelis constant for D-glucuronolactone with the goat-liver enzyme, as calculated from the overall reaction, is 93 mM. The Michaelis constants for L-gulonolactone with the soluble-enzyme preparations from the goat, rat and chick, as calculated from Fig. 5, are 10·1 mM, 19 mM and 12 mM respectively. With either D-glucuronolactone or L-gulonolactone as the substrate the

synthesis is proportional to the enzyme concentration (Figs. 6, 7).

In contrast to the great stability of the microsomes, the activity of the soluble-enzyme preparation from goat liver falls sharply on storage at $-15°$ and is completely lost after 6–7 days, and that obtained from the rat liver or the chick kidney is stable only for 1–2 days. As reported for microsomes (Chatterjee *et al.* 1958*c*, 1960*a*), the activity of the soluble-enzyme preparation from goat liver is strongly inhibited by *p*-chloromercuribenzoate, the inhibition being reversed by reduced glutathione. Soluble-enzyme preparations from rat liver and chick kidney behave similarly.

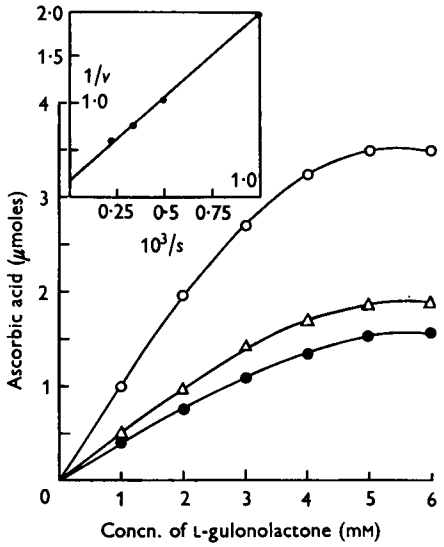


Fig. 5. Effect of varying concentrations of L-gulonolactone on the synthesis of L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: soluble enzyme, 0·4 ml.; incubation was for 1 hr. Other conditions were as stated in the text. Inset figure represents $1/v$–$1/s$ for L-gulonolactone with soluble-enzyme preparation from goat-liver microsomes, where v is μmoles of ascorbic acid formed/hr. and s is L-gulonolactone in moles/l.

Mechanism of oxidation of L-*gulonolactone by the soluble-enzyme preparations from the rat, goat and chick.* During the study of the oxidation of L-gulonolactone by the soluble-enzyme preparations from the rat, goat and chick with the manometric technique, it has been observed that the molar ratio of ascorbic acid formed to oxygen uptake in the presence of added catalase is approximately 2 (Table 10). In the absence of added catalase, this ratio (1·6–1·70) approaches 2 owing apparently to the presence of catalase activity in the enzyme preparation itself. When methanol is added along with the catalase, it will be observed (Table 10) that for 1 mole of ascorbic acid formed 1 mole of oxygen is consumed, with the consequent formation of approximately 1 mole of formaldehyde. The results indicate that in the absence of methanol the hydrogen peroxide formed during the oxidation of L-gulonolactone is decomposed by the catalase and the net oxygen uptake becomes 0·5 mole of oxygen/mole of ascorbic acid formed, as would be expected. In the presence of methanol, however, the hydrogen peroxide is utilized in the oxidation of methanol, producing formaldehyde, and thus the net oxygen uptake becomes 1 mole of oxygen/mole of ascorbic acid formed. With a soluble-enzyme preparation from guinea-pig liver, no oxygen uptake was observed under similar conditions.

Effect of cyanide on the oxidative formation of L-*ascorbic acid by the soluble-enzyme preparation from goat-liver microsomes.* Results showing the effect of cyanide on the oxygen consumption by L-gulonolactone and D-glucuronolactone in the presence of

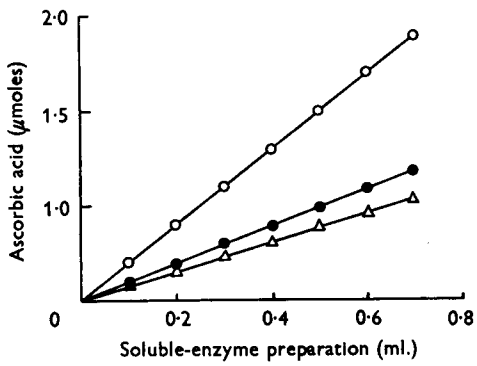


Fig. 6. Effect of enzyme concentration on the conversion of D-glucuronolactone into L-ascorbic acid by soluble-enzyme preparations from goat (○), chick (●) and rat (△). Test system: D-glucuronolactone, 10 mM; KCN, 50 mM; incubation was for 1 hr.

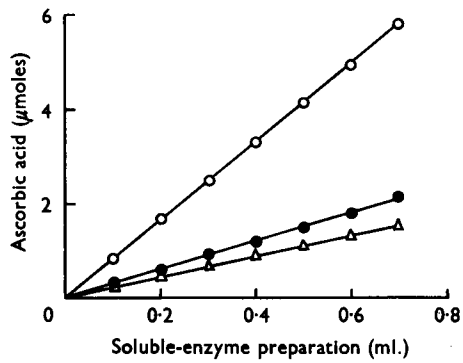


Fig. 7. Effect of enzyme concentration on the conversion of L-gulonolactone into L-ascorbic acid by soluble-enzyme preparations from goat (○), rat (●) and chick (△). Test system: L-gulonolactone, 5 mM; incubation was for 1 hr.

Table 10. *Oxidation of* L-*gulonolactone by soluble-enzyme preparations from goat, rat and chick*

Methanol and catalase, where used, were 20 mM and 0·015 mg. of haematin equivalent respectively; enzyme preparation was 5 mg. of protein equivalent. Total volume was 2·7 ml. L-Gulonolactone was added from the side arm of the Warburg flask. Incubation was at 37° for 45 min. with an equilibration period of 10 min.

Source of enzyme	Addition	Ascorbic acid formed (μmole) / Uptake of O_2 (μmole)	Formaldehyde (μmole)
Goat liver	L-Gulonolactone	1·70	—
	L-Gulonolactone plus catalase	1·98	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·90
	Catalase plus methanol	—	0·01
Rat liver	L-Gulonolactone	1·60	—
	L-Gulonolactone plus catalase	1·95	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·85
	Catalase plus methanol	—	0·01
Chick kidney	L-Gulonolactone plus catalase	1·85	—
	L-Gulonolactone plus catalase plus methanol	0·98	0·80
	Catalase plus methanol	—	0·01
Guinea-pig liver	L-Gulonolactone plus catalase	0	—
	L-Gulonolactone plus catalase plus methanol	0	0·01
	Catalase plus methanol	—	0·01

Table 11. *Effect of cyanide on the oxygen consumption by* L-*gulonolactone and* D-*glucuronolactone in the presence of a soluble-enzyme preparation from goat liver*

Test system: D-glucuronolactone, 10 mM; L-gulonolactone, 5 mM; enzyme preparation, 0·25 ml.; other conditions were as stated in Table 10.

Substrate	KCN (mM)	Oxygen consumed (μmoles)	Ascorbic acid formed (μmole)
L-Gulonolactone	0	0·98	1·90
L-Gulonolactone	2	1·02	1·85
L-Gulonolactone	50	2·20	0·85
D-Glucuronolactone	0	0·01	0
D-Glucuronolactone	2	0·02	0
D-Glucuronolactone	50	2·04	1·12

Table 12. *Effect of* pH *on the oxidative conversion of* L-*gulonolactone into* L-*ascorbic acid by a soluble-enzyme preparation from goat liver*

Test system: soluble-enzyme preparation, 0·25 ml.; L-gulonolactone, 5 mM; sodium phosphate buffer, 20 mM. Other conditions were as stated in Table 10.

Substrate	pH	Oxygen consumed (μmole)	Ascorbic acid formed (μmole)
L-Gulonolactone	7·0	0·98	1·7
	7·3	0·98	1·6
	7·6	0·95	1·2
	7·9	0·90	0·8

the soluble enzyme preparation from goat-liver microsomes are given in Table 11. Table 11 shows that though a high concentration of cyanide (50 mM) is inhibitory to the conversion of L-gulonolactone into L-ascorbic acid, the oxidation of L-gulonolactone to L-*xylo*hexulonolactone (the identification of which is described in the following section) is not hampered. Similar results have been obtained with microsomes from rat liver and chick kidney. The inhibition in the conversion of L-gulonolactone into L-ascorbic acid has been found to decrease with a decreasing cyanide concentration and no inhibition is observed with 2 mM-KCN. This indicates that the inhibition by cyanide is due probably to the formation of a cyanohydrin derivative of the keto-intermediate, thus arresting its spontaneous conversion into L-ascorbic acid. The amount of oxygen consumed with 50 mM-KCN is about twice that consumed in the absence of cyanide, owing apparently to the inhibition of the catalase activity of the enzyme preparation by cyanide. Table 11 also shows that with D-glucuronolactone there is no oxygen consumption in the absence of cyanide, which indicates that probably no L-gulonolactone is formed under this condition.

Product of oxidation of L-*gulonolactone by the soluble-enzyme preparations from goat, rat and chick.* The immediate product of oxidation of L-gulonolactone by the soluble-enzyme preparations from the livers of the goat and rat and the kidney of the chick has been found to be L-*xylo*hexulonolactone

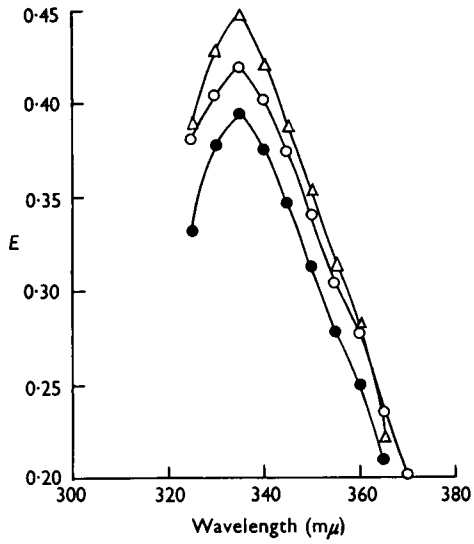


Fig. 8. Absorption-spectra analysis of the *o*-phenylenediamine derivative of L-*xylo*hexulonate. Each cuvette (1 cm.) contains *o*-phenylenediamine derivative of approximately 10–12 μg. of sodium L-*xylo*hexulonate in glass-distilled water. Total volume was 3 ml. △, *o*-Phenylenediamine derivative of an authentic sample of L-*xylo*hexulonate; ●, *o*-phenylenediamine derivative of the synthesized L-*xylo*hexulonate; ○, mixture of the authentic and the synthesized samples.

identified as the *o*-phenylenediamine derivative of L-*xylo*hexulonic acid. L-*xylo*Hexulonolactone but not the corresponding free acid is spontaneously converted into L-ascorbic acid. Hence L-*xylo*-hexulonolactone was made to accumulate in the system as sodium L-*xylo*hexulonate by competitive hydrolysis of the lactone keeping the pH of the incubation medium high. At this high pH (7·9) the oxidation of L-gulonolactone was not at all hampered (Table 12), but the relative amount of ascorbic acid formed was small, indicating that the intermediate was trapped in the system. The intermediate thus obtained could, however, be converted into L-ascorbic acid (identified as described in the Methods section) by non-enzymic lactonization in the presence of HCl. The trapped intermediate was converted into the *o*-phenylenediamine derivative, the preparation of which has been described in the Methods section. The *o*-phenylenediamine derivative thus formed was found to be identical with that prepared from an authentic sample of sodium L-*xylo*hexulonate by its typical blue fluorescence in the ultraviolet region and by its paper-chromatographic analysis as described in the Methods section. The identity of the quinoxaline derivative was further confirmed by the measurement of absorption spectra over the region 325–360 mμ. An absorption maximum at 335 mμ with 1·51 as the ratio of the extinctions at 330 and 360 mμ, characteristic of the quinoxaline derivative of an authentic sample of sodium L-*xylo*hexulonate, was obtained (Fig. 8). It may be mentioned that the condensation product of dehydroascorbic acid with *o*-phenylenediamine gives a yellow fluorescence and shows no absorption in the region 325–360 mμ.

DISCUSSION

Site of the enzyme system

The enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid has been found to be located entirely in the microsomal fraction of liver homogenates of the rat and goat and of kidney homogenates of the chick (Table 1). The synthesis by the homogenate is significantly lower than that by the microsomes. This is due to the fact that the soluble supernatant in the homogenate contains the lactonase (as shown in this paper and by Chatterjee *et al.* 1958*c*, 1960*a*), which hydrolyses D-glucuronolactone and L-gulonolactone into the corresponding free acids, the latter being not acted on by the microsomes for the synthesis of L-ascorbic acid.

Effect of cyanide and some other factors on the biosynthesis

Synthesis by rat-liver and chick-kidney microsomes. The conversion of D-glucuronolactone into L-ascorbic acid by the microsomes presumably follows the sequence:

$$\text{D-Glucuronolactone} \xrightarrow{\text{reduction}} \text{L-gulonolactone} \xrightarrow{\text{oxidation}} \text{L-ascorbic acid.}$$

The synthesis from D-glucuronolactone is greatly stimulated in the presence of potassium cyanide in all the species studied. Maximum conversion of D-glucuronolactone into L-ascorbic acid is obtained with a concentration of cyanide of the order of 50 mM, decreasing with a decreasing cyanide concentration in all the species (Fig. 1). With rat-liver and chick-kidney microsomes no appreciable conversion is noted with 5 mM-potassium cyanide. At this concentration of cyanide the synthesis could, however, be stimulated by DPNH, TPNH or reduced GSH (Table 2). DPNH, TPNH and GSH can be replaced more efficiently by a metal-binding agent such as sodium pyrophosphate, αα′-dipyridyl or 8-hydroxyquinoline, or by the boiled supernatant obtained from livers of the rat, goat, guinea pig or the kidney of the chick (Tables 2 and 3). The possibility that cyanide, chelating agent or the

boiled supernatant promotes the synthesis by inhibiting the microsomal lactonase, which hydrolyses D-glucuronolactone to D-glucuronic acid (Chatterjee *et al.* 1958*c* and this paper; Winkelman & Lehninger, 1958; Yamada, 1959), and by thus increasing the availability of the lactone form does not exist since none of these agents has been found in this Laboratory to have any influence on the uronolactonase activity.

Since no cyanide is needed for the conversion of L-gulonolactone into L-ascorbic acid, the function of cyanide lies apparently in the reduction of D-glucuronolactone to L-gulonolactone, which constitutes the first stage of the reaction. The microsomal-enzyme system catalysing the reduction of D-glucuronolactone to L-gulonolactone probably contains some thiol groups, which are activated by cyanide (Chatterjee *et al.* 1958*c*). The rate of reduction of D-glucuronolactone is presumably dependent on the rate of activation of the thiol groups, the latter being dependent on the concentration of the cyanide present in the system, being maximum with 50 mM-potassium cyanide. When, however, the cyanide concentration becomes as low as 5 mM, probably the activation of the thiol enzyme in rat-liver and chick-kidney microsomes is not sufficient to produce L-gulonolactone in the concentration necessary for the production of a detectable amount of L-ascorbic acid. It is likely that the addition of DPNH, TPNH or GSH to such a system stimulates the function of cyanide in promoting the first stage of the reaction, with the consequent formation of an appreciable quantity of L-gulonolactone leading to the synthesis of L-ascorbic acid. However, the formation of L-ascorbic acid in this system can be further enhanced even in the absence of DPNH, TPNH or GSH if any of the chelating agents such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl and 8-hydroxyquinoline or the boiled supernatant is added (Tables 2 and 3). Since these agents have been found to promote the conversion of L-gulonolactone into L-ascorbic acid (Chatterjee *et al.* 1960*a*), it would appear that they enhance the overall reaction from D-glucuronolactone by stimulating the second stage of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid. This is supported by the observation in this Laboratory that with a concentration of L-gulonolactone as low as 1 mM, no appreciable synthesis of L-ascorbic acid is brought about by the rat-liver or chick-kidney microsomes; but when any of the aforesaid-chelating agents or the boiled supernatant is added to the system, about 0·3–0·4 μmole of L-ascorbic acid is produced.

DPNH, TPNH or GSH does not further enhance the conversion of D-glucuronolactone into L-ascorbic acid already stimulated by 50 mM-potassium cyanide. This would probably indicate that when the activation of the thiol enzyme is maximum, the effect of the reduced pyridine nucleotide is not realised. Also the addition of any of the chelating agents or the boiled supernatant does not further promote the conversion of D-glucuronolactone into L-ascorbic acid stimulated by 50 mM-potassium cyanide. This is probably due to the fact that cyanide at such a high concentration inhibits the second stage of the reaction, namely conversion of L-gulonolactone into L-ascorbic acid (Chatterjee *et al.* 1960*a*), where the function of the chelating agents lies. The net result is that the stimulating effect of the chelating agent is not produced. This is supported by the observation that in the presence of 50 mM-potassium cyanide any of the aforesaid-chelating agents or the boiled supernatant does not enhance the conversion of L-gulonolactone into L-ascorbic acid by the rat-liver microsomes.

Synthesis by goat-liver microsomes. A low concentration of cyanide (5 mM), by itself, is ineffective with rat-liver and chick-kidney microsomes. But this concentration (5 mM) of cyanide can bring about the conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes and this conversion is not enhanced by the addition of any of the aforesaid agents. Perhaps the thiol groups in the microsomal enzyme from the goat liver are released more readily by a comparatively low concentration of cyanide.

It has recently been found (Chatterjee, Ghosh, Ghosh & Guha, 1959*d*; Chatterjee, Kar, Ghosh & Guha, 1959*e*, 1960*b*) that a microsome-bound lipid cofactor is involved in the oxidation of L-gulonolactone by the rat- and goat-liver microsomes. It has also been observed that goat-liver microsomes are extremely stable and can be stored at $-15°$ for months together without any loss of activity. The microsomes from rat liver and chick kidney, on the other hand, are labile and their activity falls by about 80% on storage at $-15°$ for 5–6 days. The activity, however, can be restored by addition of any of the aforesaid chelating agents. Lipid materials such as α-tocopherol and vitamin K_1 have been found to simulate the accelerating effect of the chelating agents for the conversion of L-gulonolactone into L-ascorbic acid by rat-liver and chick-kidney microsomes. These agents have, however, no influence on the synthesis by goat-liver microsomes apparently because their lipid cofactor is more stable. The action of the chelating agents on the microsomes from rat liver or chick kidney is perhaps to be attributed to the protection of the lipid cofactor against auto-oxidation by these agents. However, the mode of action of these agents and the reason for the difference in behaviour between the liver microsomes from the rat and the goat are not quite clear.

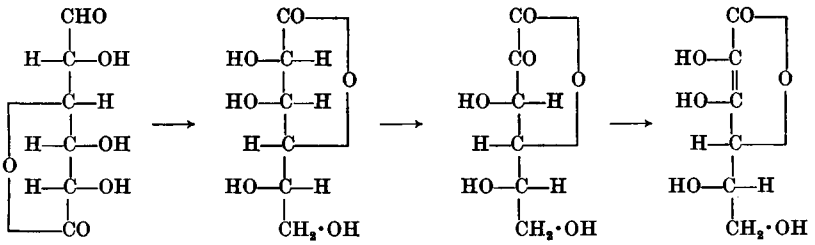
Effect of L-*gulonolactone on the conversion of* D-*glucuronolactone into* L-*ascorbic acid*

It has been reported in this paper that whereas the microsomes do not convert D-glucuronolactone into L-ascorbic acid unless cyanide is present in the incubation media, these do so when L-gulonolactone is added to the system. The amount of ascorbic acid produced is more than that obtained with L-gulonolactone alone and increases with increasing concentration of D-glucuronolactone up to a maximum. Similar results are obtained if increasing concentrations of L-gulonolactone are used with a given concentration of D-glucuronolactone. From Tables 4 and 5 it will be observed that L-gulonolactone in this system behaves similarly to cyanide towards the synthesis of ascorbic acid. It would appear that the cofactor reduced in the oxidation of L-gulonolactone to ascorbic acid functions like cyanide in the enzymic reduction of D-glucuronolactone to L-gulonolactone. The oxidation of L-gulonolactone and the reduction of D-glucuronolactone might appear to be a coupled reaction, but this does not seem strictly to be so, as a catalytic amount of L-gulonolactone in the presence of D-glucuronolactone produces only about double the amount of ascorbic acid that it produces in the absence of D-glucuronolactone and no more. On addition of more L-gulonolactone, however, more ascorbic acid is produced. Besides, this system does not produce ascorbic acid in the complete absence of oxygen. The mechanism of the reaction is not quite clear and is under further investigation.

Relative conversion of D-*glucuronolactone and sodium glucuronate into* L-*ascorbic acid*

The microsomal enzyme has a specificity for the lactone forms of the substrates: D-glucuronolactone and L-gulonolactone. The free acids are converted only when the soluble supernatant is present in the system. With sodium L-gulonate the soluble supernatant is needed only to supply the gulonolactonase, which lactonizes L-gulonate into L-gulonolactone, the latter being acted on by the microsomal enzyme. With D-glucuronate, however, a TPN-specific aldehyde dehydrogenase in the supernatant first reduces D-glucuronate to L-gulonate and L-gulonate is then lactonized to L-gulonolactone by the gulonolactonase of the supernatant (Hers, 1956; Hassan & Lehninger, 1956; Ishikawa & Noguchi, 1957; Chatterjee *et al.* 1960*a*). In this paper it has been indicated that an enzyme hydrolysing D-glucuronolactone to D-glucuronate is present in the soluble supernatant. This observation has also been made by Yamada (1959). In the absence of cyanide the amounts of ascorbic acid formed from D-glucuronolactone and from D-glucuronate, in the presence of microsomes, soluble supernatant and TPNH, are almost the same (Tables 7 and 8). This would indicate that D-glucuronolactone is probably first hydrolysed to D-glucuronate and the latter is then acted on by the aldehyde dehydrogenase of the supernatant. Recently a purified TPN-specific aldehyde dehydrogenase, free from the lactonase, has been obtained (Mano, Yamada, Suzuki & Shimazono, 1959) from the soluble supernatant, which reduces D-glucuronolactone to L-gulonolactone. This would indicate also the possibility of direct conversion of D-glucuronolactone into L-gulonolactone, thus eliminating the process of secondary lactonization of the L-gulonate formed from D-glucuronate. Since L-gulonolactone is the direct precursor of L-ascorbic acid, in this case the formation of ascorbic acid would be expected to be higher than that obtained from D-glucuronate. But owing to the presence of a strong gulonolactonase in the supernatant (Chatterjee *et al.* 1960*a*), the L-gulonolactone formed directly from D-glucuronolactone is probably competitively hydrolysed to L-gulonate. Hence, irrespective of the starting material, namely D-glucuronolactone or D-glucuronate, the net amount of ascorbic acid synthesized would represent that formed from the gulonate only. It has been found in this Laboratory that though with a high concentration of L-gulonolactone (5 mM) the synthesis of ascorbic acid in the presence of microsomes and the soluble supernatant is higher than that obtained from gulonate (5 mM) (Chatterjee *et al.* 1960*a*), with a low gulonolactone concentration (1–2 mM) the synthesis from gulonolactone is almost equal to that obtained from the corresponding amount of L-gulonate. This would explain why in the absence of cyanide the amount of ascorbic acid formed either from D-glucuronolactone or D-glucuronate in the presence of microsomes, soluble supernatant and TPNH is almost the same.

The mechanism of reduction of D-glucuronolactone to L-gulonolactone by the microsomal enzyme is apparently different from that of D-glucuronate to L-gulonate by the enzyme in the supernatant. The former is accelerated by the presence of a relatively high concentration of cyanide (50 mM), and no addition of cofactor is required, whereas the latter is completely inhibited at this cyanide concentration and addition of TPNH is necessary. With a low cyanide concentration (1–2 mM; Table 9) this inhibition is not, however, observed. The mechanism of the inhibition by a high concentration of cyanide in this system is not quite clear. It may be that D-glucuronate is converted into L-gulonate via an intermediary formation of D-fructuronate, and that a high concentration of cyanide arrests the reduction of D-fructuronate to L-gulonate by formation of a cyanohydrin derivative of the fructuronate. The



Scheme 1

intermediary formation of D-fructuronate in the conversion of D-glucuronate into L-gulonate in a bacterial system has been reported by Payne & McRorie (1958). Evidence of the formation of an unstable intermediate in the reduction of methyl D-galacturonate to L-galactono-γ-lactone has also been obtained by Mapson & Isherwood (1956). Table 6 shows that the rate of formation of ascorbic acid from D-glucuronate in the presence of soluble supernatant plus microsomes is about one-fourth of that obtained from D-glucuronolactone in the presence of the microsomes alone. The synthesis from the lactone increases with an increasing concentration of the microsomes (Chatterjee *et al.* 1958*c*), whereas that from the free acid does not increase with an increasing concentration of the soluble supernatant; the synthesis from the free acid, of course, appreciably increases with an increasing concentration of the microsomes (Table 7).

Yield of L-*ascorbic acid*

The yield of L-ascorbic acid from D-glucuronolactone is rather low. This is probably due to the fact that the lactone is readily hydrolysed into the free acid by the microsomal uronolactonase (Winkelman & Lehninger, 1958), the free acid being not acted on for ascorbic acid formation. The yield of L-ascorbic acid from L-gulonolactone, which is not hydrolysed by the microsomal lactonase, is considerably higher, up to 35% (Chatterjee *et al.* 1960*a*). With the soluble-enzyme preparation about 50% conversion is obtained (Fig. 7).

Mechanism of oxidation of L-*gulonolactone by the soluble-enzyme preparation*

The oxidation of L-gulonolactone by the soluble-enzyme preparation from the liver microsomes of the rat and goat and the kidney microsomes of the chick has been found to be an aerobic process, molecular oxygen being the ultimate electron acceptor. The products of oxidation have been identified as L-*xylo*hexulonolactone and hydrogen peroxide. Hence the enzymic-reaction pattern involved in the microsomal oxidation of L-gulonolactone can be represented as:

L-Gulonolactone + O_2 → L-*xylo*hexulonolactone + H_2O_2.

Since L-*xylo*hexulonolactone is spontaneously converted into L-ascorbic acid, the microsomal conversion of D-glucuronolactone into L-ascorbic acid in animal tissues may be represented as in Scheme 1. This rules out the possibility, postulated by Grollman & Lehninger (1957), of 3-keto-L-gulonate being the intermediate in the synthesis of ascorbic acid. 3-Keto-L-gulonate has recently been found to be the intermediate in the conversion of L-gulonate into L-xylulose (Ashwell, Kanfer & Burns, 1959).

SUMMARY

1. The enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid in the presence of potassium cyanide (50 mM) has been found to be located entirely in the microsomal fractions of the liver homogenates of the rat and goat and the kidney homogenate of the chick. This conversion requires no added cofactor.

2. A low concentration of cyanide (5 mM), by itself, is ineffective with rat-liver and chick-kidney microsomes for the synthesis of L-ascorbic acid from D-glucuronolactone. But the synthesis is stimulated by the addition of: (*a*) reduced di- or tri-phosphopyridine nucleotide, glutathione, adenosine tri- or di-phosphate, or sodium citrate; (*b*) a chelating agent such as sodium pyrophosphate, $\alpha\alpha'$-dipyridyl or 8-hydroxyquinoline; (*c*) the boiled supernatant obtained from the liver tissue of the rat, the goat or the guinea pig or the kidney tissue of the chick. In the absence of cyanide these agents are, however, ineffective. On the other hand, they do not further enhance the synthesis stimulated by 50 mM-potassium cyanide.

3. Potassium cyanide (5 mM) can bring about the conversion of D-glucuronolactone into L-ascorbic acid by the goat-liver microsomes. The conversion is not enhanced by the addition of any of the aforesaid agents.

4. The microsomes from the livers of the rat and goat and from the kidney of the chick can convert D-glucuronolactone into L-ascorbic acid even in the absence of cyanide if L-gulonolactone is added.

5. Potassium ferricyanide (mM), but not methylene blue or oxidized glutathione, completely

inhibits the conversion of D-glucuronolactone into L-ascorbic acid by rat-liver microsomes in the presence of 5 mM-potassium cyanide plus sodium pyrophosphate. The conversion of L-gulonolactone into L-ascorbic acid is not inhibited by potassium ferricyanide.

6. The microsomes can act only on D-glucuronolactone and not on the corresponding free acid. The soluble supernatant contains a strong lactonase hydrolysing D-glucuronolactone to D-glucuronic acid and thus inhibiting the cyanide-stimulated microsomal conversion of D-glucuronolactone into L-ascorbic acid.

7. Sodium glucuronate can also be converted into L-ascorbic acid but to effect this synthesis the presence of the soluble supernatant and reduced triphosphopyridine nucleotide along with the microsomes is necessary. This conversion is inhibited by 5 mM or a higher concentration of cyanide, in contrast with the microsomal conversion of D-glucuronolactone into L-ascorbic acid, which is stimulated by cyanide.

8. Soluble-enzyme preparations have been obtained from the microsomal fractions of the liver tissues of the rat and goat and the kidney tissue of the chick. Some properties of these enzyme preparations in the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid have been studied.

9. The oxidation of L-gulonolactone by the soluble-enzyme preparations from the liver microsomes of the rat and goat and the kidney microsomes of the chick is an aerobic process, oxygen being the ultimate electron acceptor. Hydrogen peroxide is formed in the process.

10. The oxidation of L-gulonolactone by microsomes from rat liver and chick kidney and by the soluble-enzyme preparation from goat-liver microsomes is not inhibited even by 50 mM-potassium cyanide.

11. The immediate product of oxidation of L-gulonolactone by the soluble-enzyme preparations obtained from the rat, goat and chick has been identified as L-*xylo*hexulonolactone.

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