SUMMARY

1. A partially purified preparation of tryptophan synthase from *Neurospora crassa* has been shown to catalyse the conversion of all four *Bz*-methylindoles into the corresponding methyltryptophans.

2. The four *Bz*-methylindoles are much less effective substrates than indole itself, the 7- and 4-methyl compounds being the most effective, followed by the 6- and 5-methyl compounds, in descending order of effectiveness.

3. The equilibrium constants, K_m , for the dissociation of the enzyme-substrate complexes with indole and 4- and 7-methylindoles have been evaluated.

4. The low affinity of the enzyme for 4-methylindole is ascribed to shielding of the 3-position by the 4-methyl group.

5. The 5- and 6-methylindoles inhibit the conversion of indole into tryptophan by the enzyme and the equilibrium constants, K_i , for the dissociation of the enzyme-inhibitor complexes have been evaluated.

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Further Observations on the Intracellular Location and Mechanism of Action of Liver Enzymes Catalysing the Synthesis of L-Ascorbic Acid

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Evidence has been presented that the enzyme systems catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid in animal tissues reside in the microsomal fractions of liver homogenates of rat and goat, and kidney homogenate of the chick (Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*, 1960*a*; Suzuki, Mano & Shimazono, 1960). It was also reported that the enzymes can convert

only the lactone forms of the precursors, namely D-glucuronolactone and L-gulonolactone, but not the corresponding free acids: the sodium salts of the corresponding free acids can be converted into L-ascorbic acid only after lactonization by incubating them with an aldonolactonase preparation from the soluble supernatant (Chatterjee *et al.* 1958*a*, 1960*a*).

However, Isherwood, Mapson & Chen (1960) reported that, not only the microsomes, but also the mitochondrial fraction of rat-liver homogenate can convert L-gulonolactone into L-ascorbic acid,

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and they have postulated that, with gulonic acid, a gulonoyl-enzyme complex, rather than the lactone, is the intermediate in the formation of L-ascorbic acid.

Chatterjee *et al.* (1960a) indicated that the observed activity of the mitochondria is probably due to contamination by microsomes. In this paper further experimental evidence is presented in support of this view. It has also been confirmed that, in the formation of L-ascorbic acid from L-gulonic acid, the latter is first converted into the lactone form and then acted on by the L-gulonolactone-oxidase system, and there is no need to postulate the intermediate formation of a gulonoyl-enzyme complex. This paper also gives evidence of the formation of L-gulonolactone and L-xylohexulonolactone as intermediates in the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide. That L-xylohexulonolactone is formed as an intermediate in the conversion of L-gulonolactone into L-ascorbic acid has already been shown by Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1959b, 1960b).

MATERIALS

L-Gulonolactone and sodium L-gulonate were prepared as described by Chatterjee *et al.* (1960a). Methyl L-gulonate was prepared according to Isherwood *et al.* (1960). L-Gulonic amide was prepared by the method of Hudson & Komatsu (1919) by adding 10 ml. of ethanol to a solution of 500 mg. of L-gulonolactone in 2 ml. of ammonia (sp.gr. 0.90).

Ethyl D-glucuronate and methyl D-glucuronate were prepared by the method of Jansen & Jang (1945). Samples of ethyl D-glucuronate and of D-glucuronic amide were supplied by Professor M. Ishidate, Faculty of Pharmaceutical Sciences, University of Tokyo, Japan.

Solutions of substrates, sodium pyrophosphate, potassium cyanide and the other relatively unstable compounds used in the various experiments were freshly made and neutralized where necessary before addition to the incubation media.

METHODS

Preparation of tissue homogenate. Young albino rats were used. The rats were killed by decapitation, and the livers immediately removed and chilled. The livers were then weighed and homogenized for 2 min. in 9 vol. of ice-cold 0.25 M-sucrose (Schneider, 1948) in an all-glass Potter-Elvehjem homogenizer with the addition of 0.01 N-NaOH to keep the pH at 7.0. The temperature throughout the procedure was kept at 0–2°. All equipment was precooled to this temperature.

Isolation of subcellular fractions.

(a) *Mitochondria.* Rat-liver homogenate (200 ml., equivalent to 20 g. of liver) was centrifuged for 10 min. at 800g to sediment the nuclei, unbroken liver cells and red blood cells, and the process was repeated to sediment the residual nuclei. The supernatant was centrifuged for 20 min. at

8500g to sediment the mitochondria. These appeared as a pellet of firmly packed buff-coloured material covered with a fluffy layer. The fluffy layer was removed by decantation and the sediment washed six times with approx. 10 vol. of iso-osmotic sucrose each time. Each washing was collected separately and then centrifuged at 88 700g for 60 min. in a Spinco Model L Ultracentrifuge with a Type 30 Rotor. The sediment from each washing was collected and dispersed in iso-osmotic sucrose. In one experiment the sixth washing was done with 0.25 M-tris buffer, pH 7.5, instead of 0.25 M-sucrose. The mitochondria thus prepared were used immediately or stored at 0° for determining the stability of the mitochondrial enzyme.

(b) *Microsomes.* The supernatant together with the fluffy layer obtained after the removal of mitochondria was centrifuged for 10 min. at 11 730g to sediment the residual mitochondria. The supernatant was then fractionated by centrifuging at 26 390, 59 380, 88 700, and 109 500g for 60 min. each time. The sedimented fractions were collected separately, and were washed twice with 10 vol. of iso-osmotic sucrose and dispersed in iso-osmotic sucrose so that 1 ml. of the dispersion contained microsomes from 1 g. of liver. The microsomal dispersions were then stored at –18°. In some experiments the microsomes were washed six times.

(c) *Soluble supernatant.* The supernatant obtained after the removal of the microsomes was centrifuged at 100 000g for 90 min. and the clear supernatant from the middle portion of the tube was collected carefully with a teated pipette, by rejecting the slight sediment at the bottom and the fatty layer at the top. This was stored at –18°.

Preparation of soluble enzyme. The term 'soluble enzyme preparation' refers throughout to the enzyme prepared from microsomes as described by Chatterjee *et al.* (1960b).

Preparation of supernatant lactonase. The preparation of the supernatant lactonase was carried out by the method described by Chatterjee *et al.* (1960a). The preparation contained both uronolactonase and aldono-lactonase, reversibly hydrolysing D-glucuronolactone and L-gulonolactone respectively, as observed by Yamada (1959) and also in this Laboratory.

Incubation media. Unless otherwise mentioned, the test system contained 20 mM-sodium phosphate buffer, pH 7.4, 10 mM-D-glucuronolactone or its derivatives, 0.25 ml. of mitochondrial or microsomal dispersion or the soluble enzyme preparation (equivalent to 250 mg. of fresh liver) and 50 mM-KCN. The total volume was 2.5 ml. and the mixture was incubated in air at 37° for 1 hr.

When L-gulonic acid, L-galactonic acid or any of their derivatives (5 mM) was used as substrate, cyanide (mM) was added to prevent oxidation of the L-ascorbic acid formed.

Estimation of ascorbic acid. Ascorbic acid was identified and estimated as described by Chatterjee *et al.* (1958a).

Identification and estimation of L-gulonolactone formed from sodium L-gulonate. L-Gulonolactone formed by the action of the supernatant lactonase on sodium L-gulonate was identified and estimated as described by Chatterjee *et al.* (1960a), with the modification that hydroxylamine was added, not during incubation of L-gulonate with the supernatant lactonase, but after incubation and deprotonization of the incubation mixture. The amount of L-gulonate used was 50 μ moles and that of the lactonase was 10 mg. protein equivalent. The amount of L-gulonolactone formed was 0.5 μ mole.

Identification of L-gulonolactone formed from methyl L-gulonate. L-Gulonolactone formed during incubation of methyl L-gulonate with rat-liver microsomes in sodium phosphate buffer (0.02M), pH 7.4, was identified by paper chromatography with butan-1-ol-acetic acid-water (4:1:1, by vol.) as the solvent; R_F values for the methyl ester and the lactone were 0.63 and 0.21 respectively. This conversion of methyl L-gulonate into L-gulonolactone occurs non-enzymically to approximately the same extent under the conditions of incubation.

Identification of L-gulonolactone formed during incubation of D-glucuronolactone with the soluble enzyme preparation in the presence of 0.05M-potassium cyanide. D-Glucuronolactone (10 mm) was incubated at 37° in an atmosphere of nitrogen for 2 hr. with the soluble enzyme preparation from goat liver (20 mg. protein equivalent) in 0.02M-sodium phosphate buffer, pH 7.0, and in the presence of 0.05M-KCN, the total volume being 5 ml. After incubation the mixture was deproteinized with 1 ml. of 30% (v/v) HClO_4 and filtered. The filtrate was neutralized with 40% (w/v) KOH and the precipitated KClO_4 was filtered off. The filtrate was then evaporated to a small volume at 40° under reduced pressure and refluxed with hot ethanol (20 ml.) for 15 min. The ethanolic extract was evaporated to dryness under reduced pressure and the residue was taken up in water (0.20 ml.). A portion (60 μl .) was chromatographed on Whatman no. 1 paper with ethyl acetate-acetic acid-water (3:1:3, by vol.; upper phase, descending), butan-1-ol-acetic acid-water (4:1:1, by vol.; ascending) and acetone-water (4:1, v/v; ascending) as solvents. A parallel run was made with authentic samples of D-glucuronolactone and L-gulonolactone. After the run was over (5-6 hr.), the papers were air-dried, sprayed with neutralized 2M-hydroxylamine and again air-dried. Finally the papers were sprayed with 0.5% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution containing HCl (N). Two reddish-brown spots corresponding to those of authentic samples of D-glucuronolactone and L-gulonolactone were identified. No L-gulonolactone formation could be detected after incubating D-glucuronolactone with a boiled-enzyme preparation under identical conditions.

In some incubation experiments carried out under identical conditions, the filtrate obtained after the removal of KClO_4 was heated with acetic acid (0.1 ml.) for 30 min. on a water bath at 60° and then evaporated to a small volume at 40° under reduced pressure. After extracting with hot ethanol, as described above, the ethanolic extract was concentrated and chromatographed as above. More-intensely coloured spots with R_F values corresponding to those of both D-glucuronolactone and L-gulonolactone were obtained under these conditions. It seemed that the lactones were largely hydrolysed during deproteinization and neutralization after the incubation, but the free acids formed were again lactonized by acetic acid, thus producing strongly coloured spots for both the lactones.

Identification and estimation of D-glucuronolactone formed from methyl D-glucuronate and ethyl D-glucuronate. D-Glucuronolactone, formed during incubation of methyl D-glucuronate and ethyl D-glucuronate with rat-liver microsomes, was identified by paper-chromatographic analysis with butan-1-ol-acetic acid-water (4:1:1, by vol.) as solvent, R_F values for the methyl ester, ethyl ester and the lactone being 0.57, 0.50 and 0.40 respectively.

D-Glucuronolactone was estimated by the method of Eisenberg & Field (1956). In the presence of ethyl D-

glucuronate, D-glucuronolactone was still estimated by this procedure, since the intensity of the colour obtained with the lactone was significantly greater than that obtained with the ethyl ester: the reading obtained in a Klett-Summerson Photoelectric Colorimeter from the hydroxamic acid derivative of 1 mg. of ethyl D-glucuronate was 22, whereas that obtained from 1 mg. of the lactone was 212. After incubation of ethyl D-glucuronate (4 mg.) with rat-liver microsomes for 1 hr., the Klett reading increased from 87 to 125. This increase agreed approximately with the value obtained with 1 μmole of D-glucuronolactone. This amount of the lactone is also formed by keeping the solution of the ester (4 mg.) in sodium phosphate buffer, pH 7.4, for 1 hr. At pH 6.6 the lactonization is not, however, appreciable.

Assay of glucose 6-phosphatase activity in mitochondria and microsomes. Glucose 6-phosphatase activity was measured by the method of Swanson (1950).

Estimation of inorganic phosphorus. Inorganic phosphorus was estimated by the method of Fiske & Subbarow (1925).

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

RESULTS

Site and substrates involved in the synthesis of L-ascorbic acid

Subcellular site of synthesis of L-ascorbic acid. Chatterjee *et al.* (1960a, b) reported that, of the nuclei, mitochondria, microsomes and soluble supernatant, only the microsomes can convert D-glucuronolactone and L-gulonolactone into L-ascorbic acid. The activity of the microsomes sedimenting under different centrifugal forces, in each case for 60 min., was studied. It was found that the rat-liver fraction sedimenting at 26 390g, which was composed of the heavier microsomes, possessed the highest specific activity (Table 1). With goat liver, however, the fraction sedimenting at 59 380g was more active than the fractions sedimented at 26 390, 88 700 and 109 500g.

Effect of washing on the activity of the microsomes. The activity of the microsomes for the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid increased during the first two washings (Fig. 1), but on further washing it declined to approximately the original value. This activity of the enzymes responsible for the synthesis of L-ascorbic acid has been found in the present work to go hand-in-hand with the glucose 6-phosphatase activity (Fig. 2), which is known to be specifically associated with the microsomes (de Duve, Pressman Gianetto, Wattiaux & Appelmans, 1955). The increased activity after the first two washings is apparently due to the removal of the soluble supernatant which contains some soluble proteins and also the enzyme lactonase, which is inhibitory to the synthesis from lactone. The first two wash-

Table 1. Conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid by rat- and goat-liver microsomes sedimented under different centrifugal forces for 60 min. in each case

The conditions were as described in the Methods section.

Centrifugal force (g)	Ascorbic acid synthesized (μ mole/mg. of protein)			
	By rat-liver microsomes		By goat-liver microsomes	
	From D-glucuronolactone	From L-gulonolactone	From D-glucuronolactone	From L-gulonolactone
26 390	0.06	0.12	0.10	0.16
59 380	0.03	0.09	0.15	0.30
88 700	0.02	0.05	0.05	0.08
109 500	0	0	Trace	Trace

Fig. 1. Effect of washing on the conversion of L-gulonolactone into L-ascorbic acid by microsomes from the livers of the rat and goat. Δ , Rat-liver microsomes; $\circ$, goat-liver microsomes.

ings were found to contain lactonase but not any inhibitor of glucose-6-phosphatase activity. The rise in the specific activity of glucose 6-phosphatase (Fig. 2) is probably due to the removal of soluble proteins from the microsomal fraction. The decline in activity of both enzymes after two washings is probably due to gradual denaturation.

Six sections of the twice-washed microsomal fraction were examined under the electron microscope: no mitochondria could be detected. Even if a small number of mitochondria were present in the entire microsomal fraction, they could not account for its high biosynthetic activity, since twice-washed mitochondrial fractions possess only 10–20 % of the activity of the microsomes on an equal basis of protein content.

Observed activity of the mitochondria and the effect of washing on this activity. Isherwood *et al.* (1960) reported that not only microsomes from rat liver but also the mitochondria convert L-gulonolactone into L-ascorbic acid, the activity of the

Fig. 2. Effect of washing on the glucose 6-phosphatase activity of microsomes from the livers of rat and goat. Δ , Rat-liver microsomes; $\circ$, goat-liver microsomes.

mitochondria being about one-third of that of the microsomes. We have also found that the mitochondrial fractions from the liver of rat and goat convert D-glucuronolactone and L-gulonolactone into L-ascorbic acid; the activity varied from one-fifth to one-tenth of that of the microsomes on an equal basis of protein content. In contrast with the microsomes, however, this mitochondrial activity decreased with the very first washing (Table 2). The decrease was sharp up to the second washing, and most of the lost activity could be recovered from the washings. However, the fall in the activity was less sharp after the second washing, and no activity could then be recovered from the washings (Table 3). The pellets obtained after centrifuging the first two washings at 88 700g for 60 min. were identified as microsomes by their high specific activity for the conversion of

D-glucuronolactone and L-gulonolactone into L-ascorbic acid, high glucose 6-phosphatase activity and by electron-microscopic examination.

The possibility of contamination of the mitochondria by microsomes was tested by measuring the glucose 6-phosphatase activity of the mitochondria. The mitochondria were found to possess significant glucose 6-phosphatase activity and, in contrast with the microsomes, this activity of the mitochondria decreased with washing; however, the mitochondria still had some glucose 6-phosphatase activity left (Table 3) after six washes.

Careful examination of the mitochondria under the electron microscope revealed that not only the twice-washed but also the six-times-washed mitochondrial fraction was not entirely homogeneous but contained a small amount of microsomes. These were not easily removed under the conditions of centrifuging used and most probably accounted for the observed residual activity of the mitochondria.

Stability of the mitochondrial enzyme. Isherwood *et al.* (1960) stated that the mitochondria and microsomes appeared to be particularly stable

when dispersed in 0.25M-tris buffer, pH 7.5, and that they retained their activity for the conversion of L-gulonolactone into L-ascorbic acid for several days when the suspension was stored at 0°. Isherwood *et al.* (1960) also observed that the microsomal enzyme was much more stable than that of the mitochondria. However, we have observed that both the mitochondrial and microsomal fractions retained 90–95% of their original activity and their enzymes were approximately equally stable whether the dispersion medium was sucrose or tris buffer or whether the particulate fractions were stored at 0° or at –18°. The storage stability of the enzyme in both the mitochondrial fraction and in the microsomes was unaffected by washing up to six times.

Conversion of L-gulonate into L-gulonolactone as an intermediate in the synthesis of L-ascorbic acid. The conversion of L-gulonate into L-gulonolactone by the lactonase of the soluble supernatant before L-gulonate could be converted into L-ascorbic acid was demonstrated by Chatterjee *et al.* (1960a) by trapping the lactone formed with hydroxylamine added to the incubating medium. Isherwood *et al.* (1960), however, argued that the hydroxylamine would not specifically estimate the lactone formed in the reaction, but would also estimate any L-gulonoyl-enzyme complex formed during incubation. We have now found that, even when sodium L-gulonate was incubated with the lactonase preparation in the absence of any trapping agent, L-gulonolactone was formed. The lactone was identified by paper chromatography, as described in the Methods section.

Conversion of ethyl D-glucuronate and methyl D-glucuronate into L-ascorbic acid. Suzuki *et al.* (1960) reported that incubation of ethyl D-glucuronate with rat-liver microsomes in the presence of 0.05M-potassium cyanide led to the formation of L-ascorbic acid. We have also observed this with

Table 2. *Effect of washing on the rat-liver mitochondria for the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid*

The conditions were as described in the Methods section.

No. of washings	Ascorbic acid synthesized (μmole/mg. of protein)	
	From D-glucuronolactone	From L-gulonolactone
0	0.015	0.027
1	0.010	0.018
2	0.006	0.010
3	0.006	0.009
4	0.005	0.008
5	0.004	0.007
6	0.004	0.007

Table 3. *Relative activity of the rat-liver mitochondria and of the microsomes sedimented from mitochondrial washings for the synthesis of L-ascorbic acid in relation to the glucose 6-phosphatase activity*

The conditions were as described in the Methods section.

No. of washings	Mitochondria				Microsomes sedimented from the washing			
	Protein content (mg.)	Ascorbic acid synthesized (μmoles)		Inorganic phosphate liberated (μmoles)	Protein content (mg.)	Ascorbic acid synthesized (μmoles)		Inorganic phosphate liberated (μmoles)
		From D-glucuronolactone	From L-gulonolactone			From D-glucuronolactone	From L-gulonolactone	
0	480	7.20	12.96	120.00	—	—	—	—
1	440	4.40	7.20	72.00	40	2.40	4.16	26.00
2	410	2.46	4.10	41.00	28	1.12	2.80	19.32
3	408	2.40	3.68	28.56	Trace	Trace	Trace	Trace
4	402	2.20	3.22	24.12	—	Trace	Trace	0
5	402	1.92	3.16	20.10	—	Trace	Trace	0
6	400	1.92	3.14	20.00	—	Trace	Trace	0

microsomes and with soluble enzyme preparations from rat and goat liver; the synthesis from the ester was about one-third of that obtained from D-glucuronolactone (Table 4). We have further observed that incubation of ethyl D-glucuronate with sodium phosphate buffer, pH 7.4, in the presence or absence of enzyme (cf. Methods section) leads to the formation of D-glucuronolactone, and the amount of lactone formed (approx. 1 μ mole) can account for the formation of L-ascorbic acid (0.26 μ mole) from the ester (Table 4). This is further supported by the fact that the supernatant lactonase strongly inhibits synthesis of L-ascorbic acid from the ester. The percentage inhibition of synthesis also approximately parallels that observed with D-glucuronolactone as the substrate (Table 5). Methyl D-glucuronate behaved similarly to ethyl D-glucuronate, and here also the L-ascorbic acid formed (0.28 μ mole) could be accounted for by the D-glucuronolactone formed (approx. 1 μ mole) from the methyl ester (4 mg.). The lactone formed from methyl D-glucuronate and ethyl D-

glucuronate was identified by paper chromatography as described in the Methods section.

Conversion of methyl L-gulonate into L-ascorbic acid. Isherwood *et al.* (1960) reported that methyl L-gulonate was converted into L-ascorbic acid by rat-liver microsomes. We have also observed this with microsomes and soluble enzyme preparation from rat and goat liver, the synthesis from the methyl ester being about one-third of that obtained from the lactone (Table 4). Methyl L-gulonate was slowly converted into L-gulonolactone during incubation with sodium phosphate buffer, pH 7.4, in the presence or absence of enzyme (comparable with methyl D-glucuronate above), the lactone being identified by paper chromatography as described in the Methods section. The synthesis of L-ascorbic acid from the methyl ester was strongly inhibited by the addition of supernatant lactonase (Table 5), which further supports the view that the lactone is the intermediate in the conversion of the ester into L-ascorbic acid.

Conversion of L-gulonic amide and D-glucuronic amide into L-ascorbic acid. Contrary to the report of Isherwood *et al.* (1960) that L-ascorbic acid could be formed from L-gulonic amide by rat-liver microsomes, we have found that L-gulonic amide is ineffective as a precursor for the synthesis of L-ascorbic acid by the soluble enzyme preparations from rat and goat liver (Table 4). We have also observed that D-glucuronic amide is not converted into L-ascorbic acid in the presence of 0.05M-potassium cyanide (Table 4). The samples of D-glucuronic amide and L-gulonic amide used were free of lactone, as shown by the hydroxamic acid reaction. The amides could not be converted into their respective lactone forms by the action of supernatant lactonase.

Effect of reduced glutathione, chelating agents and some other compounds on the synthesis of L-ascorbic acid. Isherwood *et al.* (1960) stated that L-gulonate was not converted into L-ascorbic acid by rat-liver homogenate in the absence of reduced glutathione (GSH), and considered that GSH functioned as a protector of synthesized ascorbic acid from oxidative breakdown. However, Chatterjee *et al.* (1960a) observed that rat-liver homogenate converts L-gulonate into L-ascorbic acid in the absence of GSH or any other added agent. Addition of GSH to the system, however, increases the synthesis by 15–20%. As indicated by Chatterjee *et al.* (1960a), this increased synthesis is very probably due to the observed stimulative effect of GSH on the microsomal conversion of L-gulonolactone, the intermediate in the conversion of L-gulonate into L-ascorbic acid. The stimulative effect of GSH, which is also produced with some chelating agents, such as sodium pyrophosphate and ethylenediaminetetra-acetate (EDTA), and a variety of

Table 4. *Synthesis of L-ascorbic acid from different substrates*

The conditions were as described in the Methods section.

Substrate	Ascorbic acid formed by soluble enzyme preparation (μ moles)	
	From rat liver	From goat liver
D-Glucuronolactone	0.63	1.20
Sodium D-glucuronate	0	0
Ethyl D-glucuronate	0.26	0.32
Methyl D-glucuronate	0.28	0.34
D-Glucuronic amide	0	0
L-Gulonolactone	1.14	2.72
Sodium L-gulonate	0	0
Methyl L-gulonate	0.30	0.83
L-Gulonic amide	0	0
L-Galactonolactone	1.00	2.10
Sodium L-galactonate	0	0

Table 5. *Effect of supernatant lactonase on the synthesis of L-ascorbic acid*

The amount of ester used was 4 mg. in each case. The amounts of D-glucuronolactone and L-gulonolactone were 1 μ mole each. The source of the lactonase and microsomes was rat liver. Other conditions were as described in the Methods section.

Substrate	Ascorbic acid formed (μ mole)	
	Without lactonase	With lactonase
Ethyl D-glucuronate	0.25	0.03
D-Glucuronolactone	0.26	0.02
Methyl L-gulonate	0.32	0.02
L-Gulonolactone	0.35	0.02

other compounds, such as vitamin K₁, sodium chloride and ribose 5-phosphate (Chatterjee, Kar, Ghosh & Guha, 1961*a*), is not merely due to the protection of the synthesized ascorbic acid, since these agents could not be replaced by 2 mM-potassium cyanide which fully protected ascorbic acid (2 μ moles) added to the system. Rat-liver microsomes, washed twice with iso-osmotic sucrose followed by 0.5 M-sodium chloride, did not oxidize added ascorbic acid (2 μ moles), as estimated by the method of Roe & Kuether (1943), under the conditions of incubation (Methods section), but still the synthesis by these washed microsomes was increased by addition of any of the above agents as revealed by the increase in 'total' ascorbic acid (Table 6). Thus the function of GSH, chelating agents and the other substances mentioned is not to protect the synthesized ascorbic acid. These agents actually stimulate the synthesis but the mechanism of stimulation is not yet clear.

Mechanism of conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide

Experiments *in vivo* with radioactive compounds (Burns & Evans, 1956; Burns, 1957*a*) have shown that the intermediate in the conversion of D-glucuronolactone into L-ascorbic acid is L-gulonolactone. This has been confirmed by the following experiments *in vitro*.

When D-glucuronolactone was incubated with the soluble enzyme preparation from goat liver in the absence of cyanide, no oxygen consumption leading to the formation of ascorbic acid was detected, and practically all the glucuronolactone (25 μ moles, identified by paper chromatography and estimated by the hydroxamic acid procedure after non-enzymic lactonization with dilute hydrochloric acid) could be recovered at the end of incubation. However, when incubation was carried out in the presence of cyanide, the oxygen con-

sumed (2.04 μ moles) approached that consumed (2.20 μ moles) with L-gulonolactone as the substrate, indicating that the product of reduction of D-glucuronolactone is probably L-gulonolactone (Chatterjee *et al.* 1960*b*). L-Gulonolactone could not, however, be identified on a paper chromatogram when the incubation was carried out in air. When air was replaced by nitrogen during incubation, L-gulonolactone could be identified as an intermediate. Failure to make L-gulonolactone accumulate in the system under aerobic conditions is apparently due to its rapid conversion into L-ascorbic acid by the L-gulonolactone-oxidase system; in the anaerobic incubation L-gulonolactone is not further metabolized, and it accumulates.

Identification of L-gulonolactone as an intermediate. L-Gulonolactone was chromatographically identified (cf. Methods section) as the product of incubation of D-glucuronolactone with soluble enzyme preparations from liver of rat and goat in the presence of 50 mM-potassium cyanide and in a nitrogen atmosphere (Table 7). L-Gulonolactone was also identified by Suzuki *et al.* (1960) as a product of anaerobic incubation of D-glucuronolactone with rat-liver microsomes in the presence of cyanide. Table 7 shows that incubation of D-glucuronolactone with liver or kidney microsomes from man, monkey, guinea pig, Indian fruit bat and red-vented bulbul in the presence of cyanide did not result in the formation of L-gulonolactone, indicating that D-glucuronolactone reductase, the enzyme converting D-glucuronolactone into L-gulonolactone, is specifically absent in these species which cannot synthesize L-ascorbic acid (Roy & Guha, 1958*a, b*).

The formation of L-gulonolactone as an intermediate in the enzymic conversion of D-glucuronolactone into L-ascorbic acid by rat and goat liver in the presence of cyanide has been further shown as follows. D-Glucuronolactone (20 μ moles) was incubated with soluble enzyme preparations from rat and goat liver in the presence of sodium phosphate buffer (0.02 M, pH 7.0) and potassium cyanide (50 mM) in a nitrogen atmosphere. After incubation, the mixture was deproteinized with perchloric acid, neutralized with potassium hydroxide and the precipitated potassium perchlorate was filtered off. The cyanide was then removed by acidification with N-hydrochloric acid and evaporating to dryness at 40° under reduced pressure. The residue thus obtained, when incubated with fresh enzyme preparation in the absence of cyanide, led to the formation of a small amount of ascorbic acid (0.2 μ mole), indicating that L-gulonolactone was formed as a product of incubation of D-glucuronolactone in the presence of cyanide. The residue obtained from a control experiment with

Table 6. *Effect of certain factors on the conversion of L-gulonolactone into L-ascorbic acid by washed rat-liver microsomes*

The microsomes were washed twice with iso-osmotic sucrose and then with 0.5 M-sodium chloride. Other conditions were as described in the Methods section.

Factors	Concn. (M)	'Total' ascorbic acid formed (μ moles)
None	—	0.90
Reduced glutathione	0.01	1.15
Sodium pyrophosphate	0.005	1.30
Sodium chloride	0.50	1.15
Vitamin K ₁	0.5 mg./2.5 ml.	1.20
Ribose 5-phosphate		1.20
2,4-Dinitrophenol		1.45

Table 7. *Paper-chromatographic identification of L-gulonolactone formed from D-glucuronolactone*

The conditions were as described in the Methods section. The solvent systems used were: (a) ethyl acetate-acetic acid-water (3:1:3, by vol.; upper phase); (b) ethanol-acetic acid-water (8:1:1, by vol.); (c) acetone-water (4:1, v/v).

Solvent system	R_F		
	(a)	(b)	(c)
Authentic samples			
D-Glucuronolactone	0.51	0.62	0.70
L-Gulonolactone	0.43	0.54	0.55
Products obtained with soluble enzyme preparations from:			
Rat liver	{ 0.51 0.44	{ 0.61 0.56	{ 0.72 0.56
Goat liver	{ 0.50 0.45	{ 0.62 0.55	{ 0.70 0.55
Products obtained with microsomes from:			
Human liver	0.51	0.61	0.71
Monkey liver	0.50	0.62	0.72
Guinea-pig liver	0.51	0.61	0.70
Indian-fruit-bat liver	0.51	0.61	0.70
Red-vented-bulbul liver and kidney	0.50	0.60	0.71

boiled-enzyme preparation, D-glucuronolactone and cyanide did not give L-ascorbic acid when incubated with fresh enzyme preparation.

Identification of L-xylohexulonolactone as an intermediate. The aerobic incubation of D-glucuronolactone with soluble enzyme preparations from the liver of rat and goat in the presence of cyanide led to the formation of L-ascorbic acid, and L-xylohexulonolactone could be identified in this system. L-xylohexulonolactone was identified as the *o*-phenylenediamine derivative by the paper-chromatographic and spectrophotometric analysis which was used previously for the identification of the same compound as the intermediate in the conversion of L-gulonolactone into L-ascorbic acid (Chatterjee *et al.* 1959*b*, 1960*b*). D-Glucuronolactone by itself gives a condensation product with *o*-phenylenediamine having a bluish fluorescence like that of the similar derivative of L-xylohexulonolactone but without the characteristic absorption spectrum of the latter. As regards dehydroascorbic acid, contrary to our previous report (Chatterjee *et al.* 1960*b*), its condensation product with *o*-phenylenediamine has an absorption spectrum in the region 325–360 $m\mu$ similar to that of the quinoxaline derivative of L-xylohexulonolactone, but, unlike the latter, the quinoxaline derivative of dehydroascorbic acid has a typical yellow fluorescence. Also, its position on a paper chromatogram, though very near to that of L-xylohexulonolactone, was distinct from it (Chatterjee *et al.* 1960*b*). Moreover, the ascorbic acid synthesized from D-glucuronolactone or L-gulonolactone in the presence of cyanide remained in the reduced form, and none in the dehydro form was found. Nearly all the ascorbic acid (0.5–2.0 μ moles) added to the system containing potassium cyanide

(0.001–0.05 M) could be recovered as reduced ascorbic acid at the end of incubation. There would, therefore, be no interference from dehydroascorbic acid in the identification of L-xylohexulonolactone through its quinoxaline derivative in this system. That L-xylohexulonolactone is spontaneously converted into L-ascorbic acid has been shown by Reichstein & Grüssner (1934).

DISCUSSION

Isherwood *et al.* (1960) reported that not only the microsomes but also the mitochondria of rat liver contained the enzyme system converting L-gulonolactone into L-ascorbic acid, since even twice-washed mitochondria could bring about this conversion, although to a less extent than microsomes. They showed the homogeneity of the twice-washed mitochondrial fraction by examining it by a phase-contrast microscope. Examination with a light microscope would not, however, reveal microsomes. In the present work, even the six-times washed mitochondrial fraction was found to contain some microsomal particles as judged by electron microscopy and by the glucose 6-phosphatase activity, since this activity is associated specifically with microsomes (Table 3). Further, two washings considerably decreased simultaneously the activity of the mitochondrial fraction in synthesizing L-ascorbic acid and the glucose 6-phosphatase activity. Most of the lost activity of both the enzyme systems could be recovered in the washings, which contained mostly microsomes (Table 3). The two washings, on the other hand, increased the activity of both enzymes in the main microsomal fraction, apparently by removing the soluble proteins and lactonase of the adhering supernatant (Figs. 1

and 2). These results support the conclusion (Chatterjee *et al.* 1960*a, b*) that the activity of the mitochondrial fraction in the synthesis of L-ascorbic acid is probably due to the presence of microsomes.

Of the rat-liver microsomes, the heaviest fraction sedimenting at 26 390*g* for 60 min. contained the highest activity; in goat-liver microsomes, however, the fraction sedimenting at 59 380*g* for 60 min. was the most active (Table 1). Decken & Campbell (1961) have reported that the heavier microsomal fraction of rat liver was particularly effective in the synthesis of microsomal albumin, whereas the lighter fraction was responsible for the synthesis of the soluble protein.

Chatterjee *et al.* (1960*a*) showed that L-gulonolactone was an intermediate in the conversion of L-gulonate into L-ascorbic acid by adding hydroxylamine to the incubation mixture containing L-gulonate and the supernatant lactonase to trap the

which in turn would be converted into L-ascorbic acid. However, Chatterjee *et al.* (1960*a*) reported that the supernatant lactonase strongly inhibits the formation of L-ascorbic acid in the above system. It does so by hydrolysing the lactone, which appears to be the obligatory intermediate in the synthesis of L-ascorbic acid.

It was tentatively assumed that, *in vitro*, the intermediate in the conversion of D-glucuronolactone into L-ascorbic acid was L-gulonolactone, but no experimental evidence was given previously. In the present work, L-gulonolactone and L-xylohexulonolactone have been identified as the products of incubation of D-glucuronolactone with the soluble enzyme preparations from microsomes of rat and goat liver in the presence of cyanide. This provides direct evidence for the following mechanism of conversion of D-glucuronolactone into L-ascorbic acid, previously indicated by Chatterjee *et al.* (1960*a, b*):

lactone formed. Isherwood *et al.* (1960), however, postulated the formation of a L-gulonoyl-enzyme complex as the intermediate by incubating L-gulonate with the supernatant lactonase, and they argued that the use of hydroxylamine in the incubation media would not specifically estimate the lactone formed in the reaction but would also estimate any L-gulonoyl-enzyme complex formed. In the present work, L-gulonolactone has been identified as an intermediate in the conversion of L-gulonate into L-ascorbic acid even without using hydroxylamine. The formation of the L-gulonoyl-enzyme complex was postulated since microsomes converted not only L-gulonolactone but also methyl L-gulonate into L-ascorbic acid. In this paper the methyl and ethyl esters of D-glucuronic acid and also methyl L-gulonate have been shown to be converted into L-ascorbic acid by the microsomes, but only via the intermediate formation of the respective lactones. D-Glucuronic amide and L-gulonic amide have not been found to be converted into L-ascorbic acid under the same experimental conditions.

Further, if the L-gulonoyl-enzyme complex were the true intermediate and were directly acted on by the enzyme system in the microsomes for the synthesis of L-ascorbic acid, the addition of the supernatant lactonase to a system containing L-gulonolactone and microsomes would not lead to any inhibition in the synthesis, since the L-gulonate formed by the hydrolysis of the lactone would be converted into the L-gulonoyl-enzyme complex,

Previously it was reported (Chatterjee *et al.* 1960*a*; Burns, 1957*b*) that the failure of the guinea pig to synthesize L-ascorbic acid was due to the absence of the L-gulonolactone-oxidase system which oxidizes L-gulonolactone to L-xylohexulonolactone. Chatterjee, Kar, Ghosh & Guha (1961*b*) have since found that not only the L-gulonolactone-oxidase system but also the microsomal enzyme, D-glucuronolactone reductase, which converts D-glucuronolactone into L-gulonolactone, is absent in man, monkey, guinea pig, Indian fruit bat and red-vented bulbul, all the species known to be unable to synthesize L-ascorbic acid (Roy & Guha, 1958*a, b*).

SUMMARY

1. The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid has been found to be located particularly in the heavier microsomal fractions of the liver homogenates of the rat and goat.

2. Some enzymic activity is also associated with the mitochondrial fraction and evidence has been given to show that this is very likely due to contamination of the mitochondrial fraction by microsomal particles.

3. The microsomes can convert only the lactone forms of D-glucuronic acid and L-gulonic acid into L-ascorbic acid. The methyl and ethyl esters of D-glucuronic acid and L-gulonic acid are converted into L-ascorbic acid only through the intermediate formation of their respective lactones. D-Gluc-

uronic amide and L-gulonic amide are not converted into L-ascorbic acid by the microsomes.

4. Reduced glutathione and some metal-binding agents enhance the microsomal synthesis of L-ascorbic acid by stimulating the synthesis and not indirectly by protecting the synthesized ascorbic acid.

5. L-Gulonolactone and L-xylohexulonolactone have been identified as the intermediates in the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide by the soluble enzyme preparations from the liver of rat and goat.

6. Both D-glucuronolactone reductase and L-gulonolactone oxidase are absent from the tissues of the primates, guinea pig, Indian fruit bat and red-vented bulbul—species which cannot synthesize ascorbic acid.

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Biochem. J. (1962) **84**, 25

Liver Damage in Acute Heliotrine Poisoning

1. THE INTRACELLULAR DISTRIBUTION OF PYRIDINE NUCLEOTIDES

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Heliotrine belongs to the group of pyrrolizidine alkaloids of which many members are selectively hepatotoxic when ingested in the diet or administered by injection (Bull, 1955; Bull, Dick, Keast & Edgar, 1956; Schoental & Magee, 1957, 1959; Bull & Dick, 1959). The purified alkaloid can produce extensive liver-cell necrosis in rats within 24 hr. (Bull, Dick & McKenzie, 1958; Christie, 1958a), is rapidly metabolized in the rat, and the liver appears to be the main and possibly the only

site of its metabolism (A. T. Dann, personal communication).

Administration of a dose of heliotrine which might be expected to kill 70% of rats (LD₇₀) within 72 hr. (Bull *et al.* 1958) was found to result, after an interval of 16–20 hr., in a loss of the capacity of homogenate and isolated mitochondrial preparations of liver to oxidize certain substrates (Christie, 1958b). The enzyme systems affected were all pyridine nucleotide-dependent, and could