

Biological Synthesis of L-Ascorbic Acid in Animal Tissues: Conversion of L-Gulonolactone into L-Ascorbic Acid

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The enzyme system catalysing the conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid in animal tissues has been previously reported to be entirely located in the microsomal fractions of rat- and goat-liver homogenates (Burns, Peyser & Moltz, 1956; Chatterjee, Ghosh, Ghosh & Guha, 1957*a, b*; 1958*a, b*). The kidney tissue has been found to be the site of this conversion in amphibian, reptilian and some of the avian species examined (Roy & Guha, 1958), and in chick kidney the enzyme system concerned has also been found to be located in the microsomes. Isherwood (1953) stated that mitochondria from rat liver catalyse the transformation of L-gulonolactone into L-ascorbic acid. However, our experiments indicate that the observed activity of mitochondria is probably due to their contamination by microsomes. Potassium cyanide greatly accelerates this synthesis from D-glucuronolactone but the synthesis from L-gulonolactone does not require cyanide or any other added factor (Chatterjee *et al.* 1958*b*). Subsequently the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes has been found to be greatly accelerated by boiled rat-liver supernatant or a metal-binding agent such as sodium pyrophosphate, $\alpha\alpha'$ -dipyridal and 8-hydroxyquinoline (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1958*c*). The microsomal enzyme concerned in the oxidation of L-gulonolactone to L-ascorbic acid is inhibited by heavy-metal ions (Hg^{2+} , Cu^{2+} and Zn^{2+}) and reversibly by *p*-chloromercuribenzoate, indicating the involvement of some essential thiol groups in the enzyme (Chatterjee *et al.* 1958*c*).

It has already been reported that the microsomes can convert only the lactone forms of the precursors, namely D-glucuronolactone and L-gulonolactone. The sodium salts of the corresponding free acids are not acted on (Chatterjee *et al.* 1957*b*; 1958*a, b*). The soluble supernatant has been found to inhibit the rate of conversion of L-gulonolactone into L-ascorbic acid to a great extent and, though L-gulonate is not converted by the microsomes alone, it leads to the formation of L-ascorbic acid when soluble supernatant is added to the system. The factor in the supernatant which inhibits the rate of microsomal synthesis of ascorbic acid from L-gulonolactone but, curiously, helps in

the microsomal synthesis from L-gulonate as the substrate has been traced to a strong lactonase, which has been termed gulonolactonase in this paper (Chatterjee *et al.* 1958*b*; Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*a*). This enzyme has been partially purified and characterized as an enzyme which acts reversibly by hydrolysing gulonolactone to the free acid and also lactonizing the free acid to gulonolactone, the equilibrium being more favourable for the formation of the free acid. When the lactone is used as the substrate, its conversion into ascorbic acid is diminished in the presence of the supernatant owing to this enzyme hydrolysing the lactone. On the other hand, when gulonate is used as the substrate, a small part of it is converted into lactone and this lactone is then acted on by the microsomal enzyme producing ascorbic acid (Chatterjee *et al.* 1959*a*). Therefore it is the gulonolactone and not the gulonate which is specifically acted on by the enzymes concerned, for the formation of ascorbic acid. In guinea-pig liver the gulonolactonase has been found to be present but L-gulono-oxidase, the enzyme catalysing the oxidation of L-gulonolactone to L-ascorbic acid, has been found missing (Chatterjee *et al.* 1958*c*). Recently a 30-fold concentrated soluble preparation of goat-liver microsomal enzyme (L-gulono-oxidase), converting L-gulonolactone into L-ascorbic acid, has been obtained (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959*b*). The soluble enzyme acts only on the lactone and not on the free acid, again confirming the earlier observation from this Laboratory (Chatterjee *et al.* 1957*b*; 1958*a, b*) that the lactone and not the free acid is the precursor of ascorbic acid.

With this and other information in hand a detailed investigation was carried out on the mechanism of synthesis of ascorbic acid in animal tissues. This paper deals with the conversion of L-gulonolactone into L-ascorbic acid by liver microsomes of the rat and the goat and kidney microsomes of the chick as well as with some other features of the reaction mechanism involved. Since the publication of the above-mentioned papers from this Laboratory, a paper by Kanfer, Burns & Ashwell (1959) has appeared which confirms our previous observations on the specificity of the

lactone form of the precursor for the biosynthesis of ascorbic acid (Chatterjee *et al.* 1957a, b; 1958a, b; 1959a).

EXPERIMENTAL

Materials

Preparation of microsomes. The tissue extract (liver from rat and goat and kidney from chick), freed from the nuclear and mitochondrial fractions and containing the microsomes and the soluble supernatant, was prepared as described before (Chatterjee *et al.* 1958b). The extract was centrifuged at 88 700 *g* for 1 hr. in a Spinco model L preparative ultracentrifuge with rotor type no. 30. The supernatant was drained off, separated from the fatty layer and again centrifuged for 1 hr. to sediment any microsomal matter present. The clear supernatant layer which stood at the top of a fluffy layer was drawn off and kept aside and is referred to in the text as the soluble supernatant. The sedimented microsomes, after the supernatant had been completely drained out and the walls of the centrifuge tubes wiped with filter paper, were pooled, washed once with iso-osmotic sucrose solution by recentrifuging at 88 700 *g* for 2 hr. and dispersed in cold iso-osmotic sucrose in a Waring Blender for 15 sec. The concentration of the dispersion was so adjusted that 1 ml. of the dispersion contained an amount of microsomes equivalent to 1 g. of wet tissue. The temperature throughout the procedure was kept at 0–2°. When potassium chloride solution was used in place of the sucrose solution as the homogenizing medium which was done especially for xylulose determination, the concentration of potassium chloride was 150 mM and the nuclear and mitochondrial fractions were sedimented at 4000 *g*, other procedures being the same as those carried out with the sucrose solution.

The dispersed rat-liver microsomes were generally stored at 0° in the lyophilized condition in sealed tubes of 5 ml. or 10 ml. capacity under vacuum. In this condition they were stable at least for a year without any loss of activity. Goat-liver microsomes, however, could be stored in the frozen state (–5°) without lyophilization for months together, but with rat liver the activity of the frozen microsomes fell gradually after 4–5 days.

Preparation of gulonolactonase. The soluble supernatant was heated at 50° for 30 min. with constant stirring, centrifuged and the clear centrifugate was fractionally precipitated with saturated ammonium sulphate at pH 6.0. The protein fraction at 50–80% saturation of the salt was collected and dissolved in water. This enzyme preparation acted reversibly on gulonolactone, hydrolysing it to the free acid as well as converting the free acid into the lactone and is referred to in the text as gulonolactonase. This enzyme is probably identical with the aldolactonase described by Winkelman & Lehninger (1958).

Boiled supernatant. The soluble supernatant was heated at 100° on a water bath for 5 min., with constant stirring, and centrifuged. The clear pale-yellow centrifugate is referred to in the text as the boiled supernatant.

L-Gulonic dehydrogenase. The protein fraction of rat-liver soluble supernatant obtained at 30–50% saturation with ammonium sulphate at pH 7.4, followed by dialysis, is referred to in the text as L-gulonic dehydrogenase.

Chemicals. L-Gulonolactone was prepared by a modified method of Wolfrom & Anno (1952), Dowex 50 being used

as the cation-exchange resin. A sample was also kindly supplied by Dr S. Ishikawa, Tokyo University, Japan. Sodium gulonate was prepared as by Chatterjee *et al.* (1958b). Sodium L-xylulohexulonate (sodium 2-keto-L-gulonate) was prepared from the 2:3:4:6 diacetone derivative of L-xylulohexulonic acid (2-keto-L-gulonic acid) following the method of Reichstein & Grüssner (1934). The diacetone derivative was a gift from Takeda Pharmaceutical Industries Ltd., Osaka, Japan, who also kindly supplied samples of flavinadenine dinucleotide (FAD), 93% pure and flavin mononucleotide (FMN). Riboflavin monosulphate was kindly supplied by Dr K. Yagi, Nagoya University, Japan. A sample of D-xylulose was obtained through the courtesy of Dr G. Ashwell, Bethesda, Md., U.S.A. Diphosphopyridine nucleotides (DPN and DPNH, over 90% purity) were from Sigma Chemical Co., U.S.A.; pancreatic lipase (steapsin) was from Nutritional Biochemical Corp., U.S.A. and ribonuclease from the Worthington Biochemical Corp., U.S.A.

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

Methods

Lyophilization. Lyophilization was carried out in an Edwards lyophilizer (Speedivac model 30P Centrifugal freeze-drier).

Incubation media. Unless otherwise mentioned, the test system contained 20 mM-sodium phosphate buffer, pH 7.4, 5 mM-L-gulonolactone or sodium L-gulonate and 0.25 ml. of microsomal dispersion (equivalent to 250 mg. of wet tissue). The total volume was 2.5 ml. and the digest was incubated in air at 37° for 1.5 hr.

Estimation of ascorbic acid. Ascorbic acid was identified and estimated by the procedure described before (Chatterjee *et al.* 1958b).

Identification of L-gulonolactone. L-Gulonolactone was identified as the hydroxamic acid derivative. The identification of L-gulonolactone formed from L-gulonate by the action of gulonolactonase was carried out by the following procedure.

L-Gulonate (10 mM) was incubated for 2 hr. with gulonolactonase (1 mg. equivalent of protein) in 20 mM-sodium phosphate buffer, pH 7.0, containing 0.5 ml. of neutral 2M-hydroxylamine. The total volume was 2 ml. At the end of incubation 1 ml. of the mixture was mixed with 1 ml. of neutral 2M-hydroxylamine and allowed to stand for 30 min. The hydroxamic acid derivative of L-gulonolactone formed was extracted with 20 ml. of hot absolute ethanol, centrifuged and the protein-free clear centrifugate was evaporated under suction at 40° to about 3 ml. The solution was evaporated to dryness at 0° in a vacuum desiccator and the residue taken up with 0.25 ml. of water. A portion of it (50 μ l.) was applied on paper for chromatographic separation with formic acid–water (4:1, v/v) as the irrigating solvent. A parallel run was given with an authentic sample of L-gulonolactone. After development for 4 hr. the paper was air-dried and sprayed with a 0.5% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in 95% ethanol containing mM-HCl. A single spot corresponding to the hydroxamic acid derivative of authentic L-gulonolactone, R_f 0.8, was obtained. No lactone formation could be detected by incubating L-gulonate with boiled gulonolactonase under identical conditions.

Estimation of lactone. This was carried out by an adaptation of the hydroxamic acid procedure of Lipmann & Tuttle (1945). In the presence of tissue the modified method of Eisenberg & Field (1956) was used. Since the rate of hydrolysis of the lactone to the free acid is several times greater than the formation of lactone from free acid, estimation of the lactone formed from sodium L-gulonate by the action of gulonolactonase was carried out by using hydroxylamine as a trapping agent in the system, as mentioned above under 'Identification of L-gulonolactone'.

Estimation of DPNH. DPNH was estimated by measuring the extinction at 340 m μ in a Beckman model DU Spectrophotometer.

Identification and estimation of L-xylulose. L-Xylulose was identified by paper chromatography by the method of Touster, Hutcheson & Rice (1955), D-xylulose being used as the standard. The estimation of L-xylulose was carried out according to the method of Kulka (1956), a sample of authentic D-xylulose being taken as the standard.

Estimation of protein. Protein was estimated by the Biuret method described before (Chatterjee *et al.* 1958b), checked frequently by the micro-Kjeldahl method.

RESULTS

Microsomal conversion of L-gulonolactone into L-ascorbic acid by different species. It has already been reported that the enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid resides en-

Table 1. Microsomal conversion of L-gulonolactone into L-ascorbic acid by different species

Conditions are given in the Methods section.

Species	Tissue	Ascorbic acid synthesized (μ mole)
Rat	Liver	0.80
Goat	Liver	2.75
Chick	Kidney	0.85

tirely in the microsomes (Chatterjee *et al.* 1958a). Table 1 shows the conversion of L-gulonolactone into L-ascorbic acid by liver or kidney microsomes from the different species studied.

Kinetics of the enzymic conversion of L-gulonolactone into L-ascorbic acid. The Michaelis constants for L-gulonolactone with microsomal enzymes from goat, chick and rat, as calculated from Fig. 1, are 16, 10 and 10.4 mM, respectively. With a given concentration of the substrate the rate of synthesis increases proportionally with increasing concentration of the microsomes up to a certain point, beyond which no such proportionality is noticed (Fig. 2). The maximum formation of ascorbic acid takes place after an incubation period of 1.5 hr. (Fig. 3).

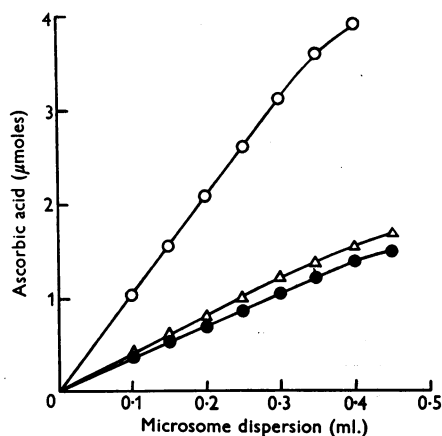


Fig. 2. Rate of formation of L-ascorbic acid as affected by enzyme concentration. Conditions are given in the Methods section. O, Goat-liver microsomes; Δ , chick-kidney microsomes; \bullet , rat-liver microsomes.

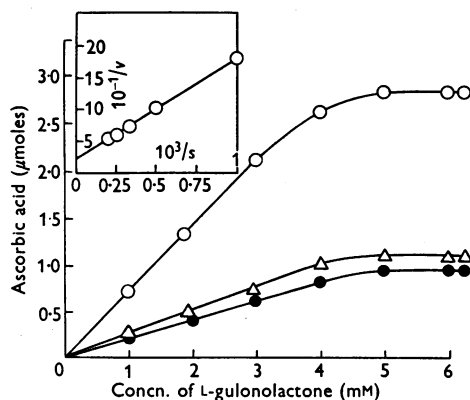


Fig. 1. Rate of formation of L-ascorbic acid as affected by substrate concentration. O, Goat-liver microsomes; Δ , chick-kidney microsomes; \bullet , rat-liver microsomes. Inset figure represents $1/v \times 1/s$ for L-gulonolactone with goat-liver microsomes, where s is L-gulonolactone in moles/l. and v is μ moles of ascorbic acid formed/hr.

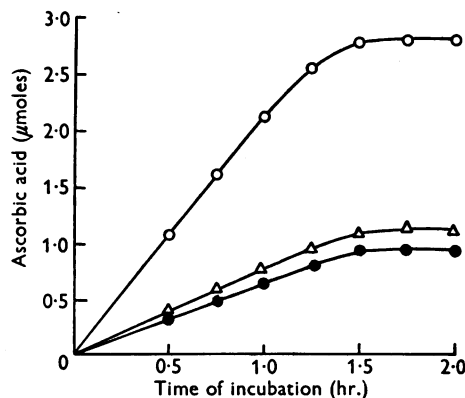


Fig. 3. Rate of formation of L-ascorbic acid from L-gulonolactone by microsomes. Conditions are given in the Methods section. O, Goat-liver microsomes; Δ , chick-kidney microsomes; \bullet , rat-liver microsomes.

Accelerating effect of certain metal-binding agents on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Certain metal-binding agents, i.e. sodium pyrophosphate, $\alpha\alpha'$ -dipyridyl

Table 2. *Effect of metal-binding agents on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes*

Conditions are given in the Methods section.

Addition	Concn. (mM)	Ascorbic acid synthesized (μ moles)
None	—	0.82
Sodium pyrophosphate	5	1.40
$\alpha\alpha'$ -Dipyridyl	2	1.35
8-Hydroxyquinoline	0.5	1.30

Table 3. *Effect of boiled supernatant on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes*

Concentration of boiled supernatant used was 100 mg. equivalent of wet tissue. Other conditions are given in the Methods section.

Source of boiled supernatant	Ascorbic acid synthesized (μ moles)
None	0.80
Rat liver	1.45
Goat liver	1.20
Chick kidney	1.35
Guinea-pig liver	1.40

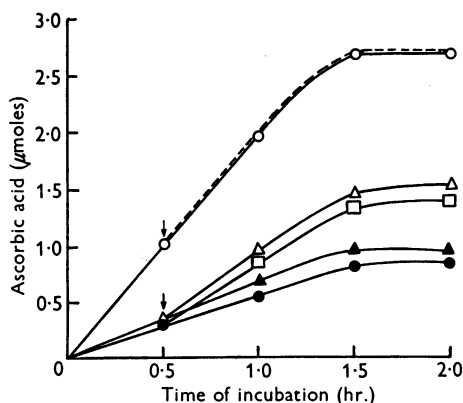


Fig. 4. Species difference in the requirement for chelating agent and boiled supernatant in the synthesis of L-ascorbic acid from L-gulonolactone by microsomes. Other conditions are given in the Methods section. \circ — \circ , Goat-liver microsomes; \circ — \circ — \circ , after addition of either sodium pyrophosphate or boiled supernatant. Δ , Chick-kidney microsomes; Δ , after addition of sodium pyrophosphate or boiled supernatant. \bullet , Rat-liver microsomes; \square , after addition of sodium pyrophosphate or boiled supernatant. Arrows indicate addition of 5 mM-sodium pyrophosphate or boiled rat-liver supernatant (100 mg. equivalent of liver).

and 8-hydroxyquinoline, have been found to accelerate the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes (Table 2).

Accelerating effect of boiled supernatant on the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Boiled rat-liver supernatant has been found to accelerate to a great extent the conversion of L-gulonolactone into L-ascorbic acid by rat-liver microsomes. Boiled supernatant from rat liver, however, is not specifically active; boiled supernatants obtained from goat liver and chick kidney, as well as from guinea-pig liver, are also similarly active (Table 3).

Species difference in the requirement for chelating agent and boiled supernatant. It has been observed that, unlike rat-liver microsomes, the synthesis of ascorbic acid by goat-liver microsomes is not accelerated in the presence of any of the above-mentioned chelating agents or boiled supernatant (Fig. 4). The microsomes from chick kidney behave in a similar way to rat-liver microsomes.

Effect of heavy-metal ions on the conversion of L-gulonolactone into L-ascorbic acid. Results showing the effect of some added heavy-metal ions on the synthesis of ascorbic acid from L-gulonolactone are given in Table 4. The heavy-metal ions inhibit the synthesis probably by blocking the essential thiol groups involved in the synthesis (Chatterjee *et al.* 1958c).

Effect of thiol reagents. The rate of conversion of L-gulonolactone into L-ascorbic acid is strongly inhibited by *p*-chloromercuribenzoate, the inhibition

Table 4. *Effect of some heavy-metal ions on the conversion of L-gulonolactone into L-ascorbic acid by microsomes from different species*

Conditions are given in the Methods section.

Microsomes	Inhibition (%)		
	Hg ²⁺ (0.5 mM)	Cu ²⁺ (0.5 mM)	Zn ²⁺ (1.0 mM)
Rat liver	85	98	75
Goat liver	80	95	70
Chick kidney	82	96	73

Table 5. *Effect of thiol reagents on the conversion of L-gulonolactone into L-ascorbic acid by microsomes from different species*

Reduced glutathione (GSH) was added 10 min. after *p*-chloromercuribenzoate (PCMB). Conditions are described in the Methods section.

Microsomes	Inhibition (%)	
	PCMB (0.5 mM)	PCMB (0.5 mM) with GSH (5 mM)
Rat liver	90	15
Goat liver	80	10
Chick kidney	85	10

being reversible with reduced glutathione. The results are given in Table 5.

It will be seen from Tables 4 and 5 that microsomes from goat liver and chick kidney behave in a similar manner to rat-liver microsomes in the inhibition by heavy-metal ions and by *p*-chloromercuribenzoate.

Solubilization and purification of the microsomal enzyme. Treatment of the microsomes with sonic or ultrasonic oscillations, irradiation with ultraviolet light, precipitation with protamine sulphate or treatment with digitonin or ribonuclease did not give fruitful results. Treatment with 20% ethanol in the presence of 0.5M-sodium chloride or incubation with pancreatic lipase resulted in significant loss in activity. The microsomes could, however, be effectively solubilized by treatment with either snake venom (cobra, *Naja naja*) or sodium deoxycholate. The use of deoxycholate for the solubilization of the microsomal enzyme has also been mentioned by Mapson (1958). The solubilized microsomes could be further purified by precipitation with ammonium sulphate, the details of which are described elsewhere (Chatterjee *et al.* 1959b). The enzyme, thus purified, appears to be a soluble one. After being centrifuged at 100 000 *g* for 1 hr. almost the entire activity of the original microsomes was found to reside in the supernatant.

Nature of the cofactor involved in the oxidation of L-gulonolactone into L-ascorbic acid. When rat-liver microsomes were treated with acid ammonium sulphate (Warburg & Christian, 1938), flavin was split off in the supernatant and the activity of the precipitated fraction in the conversion of L-gulonolactone into L-ascorbic acid was completely destroyed, but the activity was not restored by the addition of FAD or FMN. The soluble-enzyme preparation (30-fold concentrated), obtained by treatment with sodium deoxycholate followed by fractionation with ammonium sulphate, has been found to contain flavin (Chatterjee *et al.* 1959b). It was inhibited by Antabuse (disulfiram), riboflavin and a specific flavin inhibitor such as flavin monosulphate (Egami & Yagi, 1956) (Table 6). Even after treatment with active charcoal or dialysis

against phosphate buffer for 3 hr. with constant stirring, the purified enzyme preparation retained its full activity and converted L-gulonolactone into L-ascorbic acid in the absence of any added cofactor. The results indicate that some flavin, rather strongly bound with the protein moiety of the enzyme, is probably involved in the oxidation of L-gulonolactone into L-ascorbic acid.

Specificity of the lactone structure of the precursor in the synthesis of ascorbic acid. Fig. 5 shows that, irrespective of the species, the supernatant inhibits the microsomal conversion of L-gulonolactone into L-ascorbic acid. This is apparently due to the presence of gulonolactonase in the supernatant, an enzyme fairly stable at 50°, which hydrolyses the lactone into L-gulonic acid (Chatterjee *et al.* 1959a), as will be evident from Fig. 7. The amount of gulonolactone (9.6 μ moles), which had disappeared at the end of incubation for 2 hr. by the action of gulonolactonase (Fig. 7), presumably by conversion into gulonate, was recovered by non-enzymic lactonization in the presence of dilute hydrochloric acid and estimated as described above. Fig. 6 shows that L-gulonate is not converted into L-ascorbic acid by the microsomal enzyme but is so converted after the addition of a preparation of gulonolactonase, obtained from the soluble supernatant from rat liver, to the system. Fig. 7 shows the corresponding amount of L-gulonolactone formed from gulonate under these conditions, the action of gulonolactonase being reversible. It will be observed from Figs. 6 and 7 that the rate of formation of gulonolactone can account for the rate of conversion of

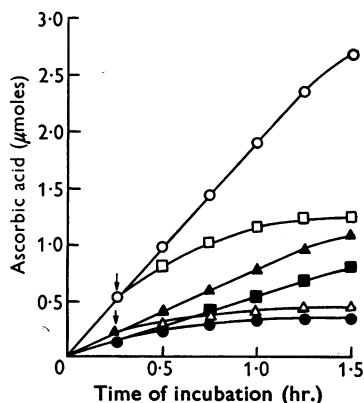


Fig. 5. Effect of soluble supernatant from rat liver on the rate of microsomal conversion of L-gulonolactone into L-ascorbic acid. Conditions are given in the Methods section. ○, Goat-liver microsomes; □, after addition of soluble supernatant. ▲, Chick-kidney microsomes; △, after addition of soluble supernatant. ■, Rat-liver microsomes; ●, after addition of soluble supernatant. Arrows indicate addition of soluble supernatant from rat liver (100 mg. equivalent of liver).

Table 6. Effect of flavin inhibitors on the conversion of L-gulonolactone into L-ascorbic acid by a soluble enzyme system from goat-liver microsomes

The system contains 3 mg. protein equivalent of a soluble enzyme preparation (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1959b). Other conditions are given in the Methods section.

Inhibitor	Concn. (mm)	Inhibition (%)
Disulfiram	1	50
Riboflavin	0.5	40
Flavin monosulphate	0.01	25

L-gulonate into L-ascorbic acid in the presence of the gulonolactonase. The conversion of L-gulonate into L-gulonolactone to a small extent in the presence of gulonolactonase, with the consequent formation of a small amount of ascorbic acid, is due to the reversible action of the enzyme, the equilibrium being more favourable towards the hydrolysis of the lactone.

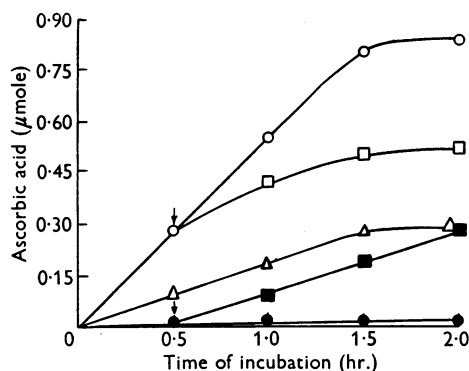


Fig. 6. Rate of formation of L-ascorbic acid from L-gulonolactone by rat-liver microsomes in the presence of gulonolactonase. Conditions are given in the Methods section. ○, With 5 mM-L-gulonolactone; □, after addition of gulonolactonase; △, with 5 mM-L-gulonolactone; ●, with 5 mM-sodium L-gulonate; ■, after addition of gulonolactonase. Arrows indicate addition of gulonolactonase (1 mg. equivalent of protein).

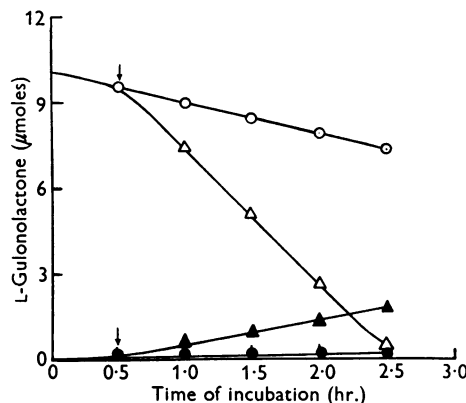


Fig. 7. Effect of gulonolactonase from rat liver on the formation and disappearance of lactone from sodium L-gulonate and L-gulonolactone respectively. With gulonate, neutral 0.4M-hydroxylamine was used as a trapping agent. Other conditions are given in the Methods section. ○, With 10 μmoles of L-gulonolactone; △, after addition of gulonolactonase; ●, with 10 μmoles of sodium L-gulonate; ▲, after addition of gulonolactonase. Arrows indicate addition of gulonolactonase (1 mg. equivalent of protein).

Gulonolactonase from different species. The gulonolactonase does not occur specifically only in the soluble supernatant from rat liver. It is also present in goat-liver and chick-kidney supernatants as well as in guinea-pig-liver supernatants (Table 7).

Effect of heat on gulonolactonase activity. Table 8 shows the effect of heat on the activity of gulonolactonase in the rat-liver supernatant. The activity is expressed in terms of μmoles of L-gulonolactone formed from 10 mM-sodium L-gulonate after an incubation period of 2 hr.

Relationship between syntheses of L-ascorbic acid and L-xylulose. The L-gulonic dehydrogenase preparation catalysed the reduction of added DPN when L-gulonate was used as the substrate, leading to the formation of L-xylulose, as will be observed in Figs. 8 and 9. The figures also show that, unlike L-gulonic dehydrogenase, neither the soluble microsomal-enzyme preparation nor the gulonolactonase, separately, or conjointly, could catalyse this reduction of added DPN leading to the formation of xylulose from L-gulonate.

The microsomes could convert L-gulonolactone into L-ascorbic acid without any added factor. L-Gulonate, however, was converted only when it was lactonized by prior incubation with gulonolactonase. In the latter case also no added cofactor was needed and, except L-ascorbic acid, no other

Table 7. Effect of gulonolactonase obtained from different species on the synthesis of L-ascorbic acid from sodium L-gulonate by goat-liver microsomes

Each flask contains 1 mg. protein equivalent of gulonolactonase, 10 μmoles of sodium gulonate and 0.25 ml. of goat-liver microsomes. Other conditions are given in the Methods section.

Source of gulonolactonase	Ascorbic acid synthesized (μmole)
Rat-liver supernatant	0.66
Goat-liver supernatant	0.55
Chick-kidney supernatant	0.60
Guinea-pig-liver supernatant	0.27

Table 8. Effect of heat on the activity of gulonolactonase of rat-liver supernatant

Figures in parentheses indicate the times for which the enzyme was kept at the corresponding temperature. Each flask contained 10 μmoles of sodium gulonate and 0.4M-hydroxylamine (neutral). Other conditions are given in the Methods section.

Temperature	Gulonolactone formed (μmoles)
37° (30 min.)	2.50
40 (30 min.)	2.45
50 (30 min.)	2.40
60 (15 min.)	1.20
70 (10 min.)	0.01

product of reaction was noticed, even in the presence of added DPN. The preparation of gulonolactonase was thus free from contamination with L-gulonic dehydrogenase. Whereas L-gulonate was converted into L-ascorbic acid by the microsomes in the presence of gulonolactonase and, in the absence of any added cofactor, it was not converted into L-xylulose by the L-gulonic dehydrogenase unless DPN was added to the system (Table 9). It is clear from the results given in Table 9 that the conversion of L-gulonate into L-ascorbic acid is a quite different and independent pathway from that of L-gulonate into L-xylulose. The former takes

place via the formation of L-gulonolactone by the gulonolactonase and the latter via the hypothetical 3-keto-L-gulonate by the DPN-specific L-gulonic dehydrogenase.

Microsomes could not convert L-gulonolactone into L-xylulose (Table 9). This would indicate that the intermediate product in the microsomal oxidation of L-gulonolactone to L-ascorbic acid is most probably L-xylohexulonolactone (2-keto-L-gulonolactone); 2-keto-L-gulonate could not be decarboxylated into L-xylulose (Table 9). That the product of oxidation might be the 3-keto compound, which, owing to the absence of a necessary decarboxylating enzyme in the microsomes is not decarboxylated, is unlikely in view of the fact that even after addition of a partially purified soluble supernatant containing the decarboxylase no formation of L-xylulose was detected (Table 9).

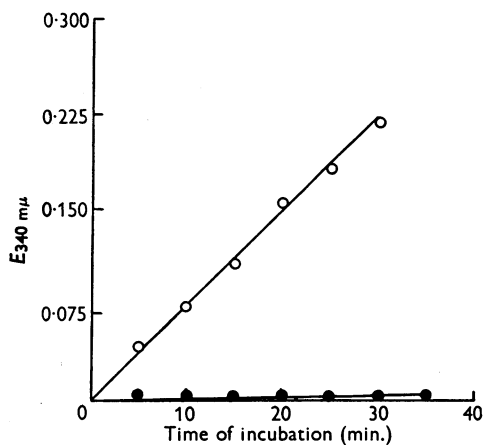


Fig. 8. Rate of reduction of DPN by L-gulonic dehydrogenase. ○, L-Gulonic dehydrogenase; ●, gulonolactone or soluble microsomal enzyme preparation added separately or conjointly. Each cuvette (1 cm.) contained 40 mM-sodium phosphate buffer, pH 7.8, 0.5 μmole of DPN, 10 μmoles of sodium L-gulonate, 4 mg. protein equivalent of L-gulonic dehydrogenase or 1 mg. protein equivalent of gulonolactonase obtained from rat liver or 5 mg. protein equivalent of the soluble microsomal enzyme, incubated at 30°. Total volume was 3 ml.

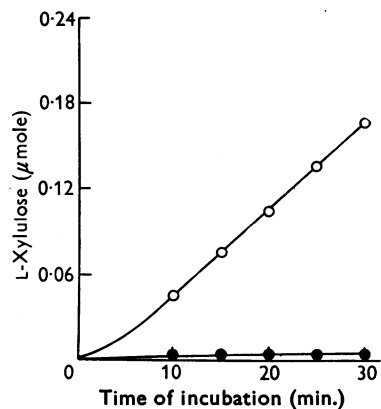


Fig. 9. Rate of synthesis of L-xylulose by L-gulonic dehydrogenase. ○, L-Gulonic dehydrogenase; ●, gulonolactonase or soluble microsomal enzyme preparation added separately or conjointly. Other conditions are the same as described for Fig. 8.

Table 9. Relative conversion of L-gulonolactone and sodium L-gulonate into L-ascorbic acid

The system contained 40 mM-sodium phosphate buffer, pH 7.4, 10 μmoles of substrate, 1.5 μmoles of DPN, 1 mg. protein equivalent of gulonolactonase (rat liver) or 4 mg. protein equivalent of soluble supernatant (obtained after fractionation with ammonium sulphate at 30–80% saturation of the salt at pH 7.8), 5 mg. protein equivalent of soluble microsomal enzyme (goat liver); incubated at 37° in air for 2 hr.; total volume 2.5 ml.

Source of enzyme	Substrate	Addition	L-Ascorbic acid formed (μmoles)	DPNH formed (μmole)	L-Xylulose formed (μmole)
Microsomes	L-Gulonolactone	—	2.50	—	Nil
		DPN	2.50	Nil	Nil
		—	Nil	—	Nil
Microsomes and gulonolactonase	L-Gulonate	—	Nil	Nil	Nil
		DPN	0.65	—	Nil
		—	0.65	Nil	Nil
Microsomes and soluble supernatant	L-Gulonolactone	DPN	0.50	0.70	0.40
		—	1.0	—	Nil
		—	—	—	Nil

Missing link in the guinea pig. Guinea-pig liver is known to contain the enzyme system converting D-glucuronic acid into L-gulononic acid (Hill & Chaikoff, 1956; Burns, 1957) as well as L-gulononic acid into L-xylulose (Touster *et al.* 1955; Ishikawa & Noguchi, 1957). As stated before, it has also been observed to contain the necessary gulonolactonase converting L-gulonate into the corresponding lactone. But it could not convert L-gulonolactone into L-ascorbic acid even in the presence of boiled supernatant or any of the above-mentioned chelating agents. This indicates that the biochemical step missing in the guinea-pig liver is the oxidation of L-gulonolactone to L-ascorbic acid. The presence of an inhibitor in the guinea-pig liver microsomes is excluded by the fact that when these microsomes were added to rat-liver microsomes no inhibition of the rate of conversion of L-gulonolactone into L-ascorbic acid was observed. Guinea-pig liver has, however, been found to contain the heat-stable factor which accelerates the conversion of gulonolactone into ascorbic acid (Table 3).

DISCUSSION

The enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid has been found to be located entirely in the microsomal fractions of the liver homogenates of rat and goat (Chatterjee *et al.* 1958*a*). It has also now been found to be located in the microsomal fraction of the kidney homogenate of chick (Table 1). The synthesis from rat-liver microsomes is accelerated in the presence of boiled supernatant or chelating agents such as sodium pyrophosphate, $\alpha\alpha'$ -dipyridyl and 8-hydroxyquinoline. Boiled supernatant or chelating agent, however, has no influence on the synthesis by goat-liver microsomes. The possibility that boiled supernatant and chelating agents act by protecting the synthesized ascorbic acid from oxidative breakdown has been eliminated by the fact that all of the ascorbic acid (2 μ moles) added to the system could be recovered in the absence of boiled supernatant or chelating agent. The metal-binding agents might act by chelating some metal [e.g. Zn^{2+} , present in the rat-liver microsomes (Thiers & Vallee, 1957)], which has been found to be inhibitory to the conversion of L-gulonolactone into L-ascorbic acid (Table 4). The action of the boiled supernatant might also be due to some chelating agent present in it. The mode of action of these factors and the reason for the difference in the behaviour between rat- and goat-liver microsomes in the requirement for these factors are under further investigation.

The synthesis of ascorbic acid from L-gulonolactone has been found to be inhibited by heavy-metal ions and by *p*-chloromercuribenzoate, the inhibition by the latter being reversible by reduced

glutathione, indicating that some essential thiol groups are involved in this reaction.

The conversion of L-gulonolactone into L-ascorbic acid by the microsomal enzyme requires the presence of oxygen (Chatterjee *et al.* 1957*b*; 1958*a, b*) but is not inhibited by 2 mM-potassium cyanide, indicating that a cytochrome system is not involved in this oxidation.

The oxidation of L-gulonolactone to L-ascorbic acid is significantly inhibited by flavin inhibitors such as disulfiram, riboflavin and flavin monosulphate. The partially purified soluble enzyme contains flavin (Chatterjee *et al.* 1959*b*); it retains its full activity, without any added factor, even after prolonged dialysis. The oxidation of L-gulonolactone to L-ascorbic acid is thus probably mediated by a thiol-containing flavoprotein, the flavin part of which is rather firmly bound with the protein moiety of the enzyme. This confirms the earlier report from this Laboratory (Chatterjee *et al.* 1957*b*, 1958*b*) that a flavoprotein might be involved in the oxidation of L-gulonolactone to L-ascorbic acid. The involvement of a flavoprotein in the conversion of L-galactonolactone into L-ascorbic acid by plant systems has been indicated by Mapson & Breslow (1958).

The microsomes have been found to utilize L-gulonolactone and not the sodium salt of the corresponding free acid. This failure to metabolize the free acid may be due to difficulties of penetration of the ionized compound through the microsomal lipid barrier, since it is known that polar compounds generally do not penetrate the microsomes whereas non-polar compounds do (Mitoma, Posner, Reitz & Udenfriend, 1956). But this explanation appears improbable in view of the fact that a preparation of soluble enzyme from the microsomes still catalyses the oxidation of L-gulonolactone and not of sodium L-gulonate. The free acid can, however, be converted into ascorbic acid by the microsomal enzyme if it is lactonized beforehand by incubation with gulonolactonase, an enzyme present in the soluble supernatant, which reversibly converts the lactone into free acid. The synthesis of L-ascorbic acid then runs parallel with the formation of L-gulonolactone, the latter having been identified by paper chromatography. The rate of synthesis of ascorbic acid from L-gulonolactone in the presence of microsomes alone is about four to five times that obtained from gulonate in the presence of microsomes plus gulonolactonase. The action of gulonolactonase is reversible, the equilibrium being more favourable towards the hydrolysis of the lactone. The conversion of L-gulonate into L-gulonolactone is therefore the rate-limiting step in the synthesis. With L-gulonolactone as the substrate, addition of gulonolactonase to the microsomes inhibits the synthesis to a great extent but

the rate is still significantly higher than that obtained with L-gulonate. Contrary to the postulation of Grollman & Lehninger (1957) and of Bubltz, Grollman & Lehninger (1958) that the free gulonic acid is the precursor of ascorbic acid and that the lactone is acted on only after hydrolysis, it is clear that the lactone is the precursor of ascorbic acid and that the free acid is acted on only after lactonization. This confirms earlier observations from this Laboratory (Chatterjee *et al.* 1957*a, b*; 1958*a, b*; 1959*a*). Moreover, it has been observed, with L-gulonate as the substrate, that, unlike the soluble supernatant, microsomes alone or microsomes in conjunction with gulonolactonase do not catalyse the reduction of added DPN and also no xylulose is formed. This indicates that in the presence of soluble supernatant containing L-gulonic dehydrogenase and gulonolactonase the microsomal formation of ascorbic acid from L-gulonate depends upon the amount of L-gulonolactone formed by the action of gulonolactonase and not on the hypothetical 3-ketogulonate formed by the action of L-gulonic dehydrogenase. Hence the synthesis of L-ascorbic acid is a process competitive with the formation of L-xylulose at the stage of oxidation of L-gulonate by the DPN-specific L-gulonic dehydrogenase and not with the subsequent decarboxylation of the hypothetical '3-keto-L-gulonate'. Since one-step oxidation of gulonolactone would lead to the formation of ketogulonolactone and since ketogulonolactone is non-enzymically converted into ascorbic acid, there is no necessity to postulate, as has been done by Grollman & Lehninger (1957), the presence of a specific lactonizing enzyme in the microsomes converting 3-keto-L-gulonic acid into the corresponding lactone.

When, instead of L-gulonate, D-glucuronate is used as the substrate, addition of gulonolactonase to the microsomal system does not lead to the formation of ascorbic acid. In this case addition of soluble supernatant and TPNH is necessary for the synthesis. Soluble supernatant is known to contain a TPN-dependent aldehyde dehydrogenase (Hers,

1956; Hassan & Lehninger, 1956) which reduces D-glucuronic acid probably to L-gulonate, though it has not so far been identified. L-Gulonate is then lactonized to L-gulonolactone by the gulonolactonase of the supernatant, and the latter by the action of the microsomal enzyme is converted into L-ascorbic acid. The formation of ascorbic acid from D-glucuronate is thus limited by two rate-determining steps, namely D-glucuronate to L-gulonate and L-gulonate to L-gulonolactone. Actually it has been found in this Laboratory that the rate of formation of ascorbic acid from D-glucuronate in the presence of soluble supernatant plus microsomes is about one-sixth of that obtained from the lactone in the presence of microsomes alone.

Taking into consideration the above described facts, the sequences of reactions involved in the biosynthesis of L-ascorbic acid as well as of L-xylulose may be represented by the scheme shown in Fig. 10.

Thus there is a remarkable difference between the enzymic oxidation of L-gulonate and that of L-gulonolactone. With the former, the free acid is the substrate and the oxidation is catalysed by the DPN-specific L-gulonic dehydrogenase of the soluble supernatant, and addition of DPN is necessary; whereas, with the latter, the lactone is the substrate and the enzyme, very probably a flavoprotein, is located entirely in the microsomes and no addition of cofactor is needed. With gulonate the product of oxidation should be sufficiently unstable to be decarboxylated into L-xylulose and has usually been considered to be 3-keto-L-gulonate. But in ascorbic acid formation the intermediate product should be rather stable in order to maintain its C₆ chain, and there is every possibility of 2-keto-L-gulonolactone being this intermediate. 2-Keto-L-gulonolactone has not been found to lead to the formation of L-xylulose (Table 9), showing that the former is not decarboxylated by the enzyme of the soluble supernatant, an observation which has also been made by Burns, Kanfer & Dayton (1958) using [¹⁴C]2-keto-L-gulonolactone. Further, even after addition to the system containing microsomes and L-gulonolactone, of a partially purified soluble supernatant

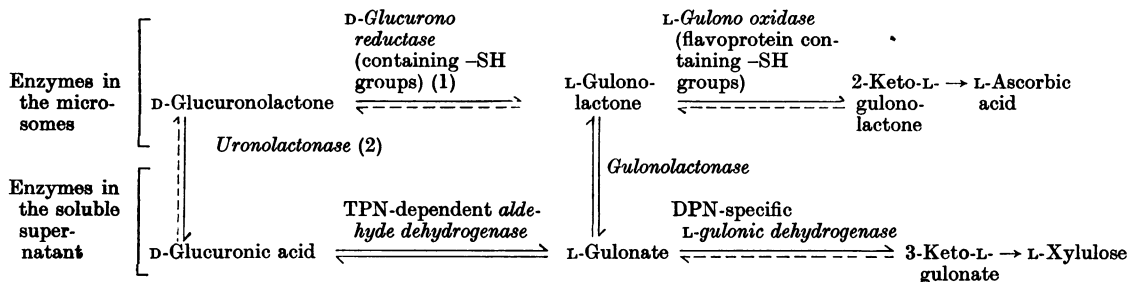


Fig. 10. Schematic representation of the pathways of biosynthesis of L-ascorbic acid and of L-xylulose. (1) Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1958*c*). (2) Winkelman & Lehninger (1958). - - - -, Reversibility not known.

containing the decarboxylating enzyme, no detectable formation of L-xylulose was noticed (Table 9). Had the 3-keto compound been the intermediary in the microsomal oxidation of L-gulonolactone to L-ascorbic acid, the formation of L-xylulose in the above system could have been expected.

In species requiring dietary ascorbic acid, e.g. the guinea pig, the liver enzyme has been found to convert D-glucuronic acid into L-gulonic acid (Hill & Chaikoff, 1956; Burns, 1957). It has also been observed to contain the necessary lactone-forming enzyme converting L-gulonate into L-gulonolactone. But L-gulono-oxidase, the microsomal enzyme catalysing the oxidation of L-gulonolactone into L-ascorbic acid, is absent. This missing biochemical step is perhaps a general characteristic of all the species which are unable to synthesize L-ascorbic acid.

SUMMARY

1. The enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid has been found in the liver microsomes of the rat and the goat and kidney microsomes of the chick.

2. The synthesis of ascorbic acid from gulonolactone by rat-liver and chick-kidney microsomes is accelerated by the metal-binding agents sodium pyrophosphate, $\alpha\alpha'$ -dipyridyl and 8-hydroxyquinoline and by the boiled supernatant from livers of rat, goat and guinea pig and kidney of the chick. These factors do not accelerate the synthesis of ascorbic acid by goat-liver microsomes.

3. The enzyme converting L-gulonolactone into L-ascorbic acid is inhibited by heavy-metal ions (Hg^{2+} , Cu^{2+} , Zn^{2+}) and by *p*-chloromercuribenzoate, the inhibition by the last-named being reversible with reduced glutathione, indicating the involvement of thiol groups in the enzyme.

4. The conversion of L-gulonolactone into L-ascorbic acid by a soluble and purified preparation of the microsomal enzyme is inhibited by the flavin inhibitors riboflavin, Antabuse (disulfiram) and riboflavin monosulphate, indicating the involvement of a flavoprotein.

5. With sodium L-gulonate as substrate the soluble enzyme preparation does not reduce diphosphopyridine nucleotide and no xylulose formation was detected.

6. L-Gulonolactone and not L-gulonate is the precursor of ascorbic acid. Gulonate is converted into ascorbic acid only after lactonization by the action of gulonolactonase, an enzyme present in the soluble supernatant. The action is reversible and the equilibrium favours hydrolysis of the lactone.

7. A scheme showing the pathways of the biosyntheses of L-ascorbic acid and of L-xylulose is given. The conversion of L-gulonolactone into L-ascorbic acid is shown to follow a different and in-

dependent pathway from that of L-gulonolactone into L-xylulose. The former is catalysed by an enzyme present entirely in the microsomes and the latter by an enzyme system present only in the soluble supernatant.

8. The possibility of L-xylulohexulonolactone (2-keto-L-gulonolactone) being an intermediate in the synthesis of L-ascorbic acid is discussed.

9. In guinea-pig liver, the microsomal enzyme catalysing the conversion of L-gulonolactone into L-ascorbic acid is missing.

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The Determination of Blood Glutathione

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The nitroprusside method for estimating glutathione is non-specific and suffers from the defect of an unstable colour, although recent improvements (Thompson & Watson, 1952; Grunert & Phillips, 1951) appear to make the method more satisfactory. Binkley, Fufii & Kimmell (1950) used a method based on the Sullivan & Hess (1936) reaction to estimate glutathione, γ -glutamylcysteine, cysteinylglycine and cysteine, but in our hands it failed to give reproducible results. We therefore had to seek another method and investigated the observation by Patterson, Lazarow & Levey (1949) that alloxan and glutathione interact to form a compound characterized by an absorption band at 305 $m\mu$. Other sulphhydryl compounds, e.g. cysteine, react with alloxan but produce dialuric acid by reduction. This last-named substance has an absorption maximum at 275 $m\mu$ and does not interfere with measurements at 305 $m\mu$. Some proteins react with alloxan but these can be removed by precipitation, as in the proposed method.

It appeared possible therefore that the reaction with alloxan could be used to estimate glutathione in blood, thus avoiding the interference of the peptides cysteinylglycine and γ -glutamylcysteine, which, present in blood, react in other methods as glutathione. A very sensitive method, using this reaction, has been developed which readily detects 15 μg . of glutathione and can be used to estimate it in as little as 0.2 ml. of blood. We have not attempted to extend the method to the estimation of glutathione in tissues. The method was used to investigate the blood-glutathione concentration in some cases of therapeutic deep insulin coma.

METHOD

Principle. Whole lysed blood is deproteinized with trichloroacetic acid, neutralized with buffered NaOH and

alloxan is added to develop the compound with glutathione (GSH), which has a characteristic absorption at 305 $m\mu$.

Reagents. 10% (w/v) Trichloroacetic acid.

0.24 M-Phosphate buffer, pH 7.6, prepared by mixing 0.24 M- Na_2HPO_4 with 0.24 M- KH_2PO_4 (88:12, v/v).

Neutralizing NaOH: this contains in 1.5 ml. of solution both 0.24 M-phosphate buffer and enough N-NaOH to neutralize 0.5 ml. of supernatant from protein precipitation (219:21, v/v).

Alloxan reagent: 1 mg. of alloxan monohydrate/ml. in dilute HCl (1 drop of conc. HCl/100 ml. of water). Prepared freshly and used within 5 min.

Standard GSH: 10 mg./100 ml. of water, freshly prepared.

0.1 M-Glycine solution.

Procedure. A specimen (0.2 ml.) of whole blood from a finger prick is added to 0.6 ml. of water. After it has been allowed to stand for 10 min. to haemolyse, 0.8 ml. of trichloroacetic acid is added, the liquids are mixed immediately with a glass rod and centrifuged for 5 min.

For each sample of blood two tubes are prepared: test (tube 1) and control (tube 2); each contains 0.5 ml. of glycine solution, and 2.9 and 3.9 ml. of phosphate buffer are added to (1) and (2) respectively. To each tube 0.4 ml. of supernatant from the deproteinized blood and then 1.2 ml. of neutralizing NaOH are added, with mixing.

An alloxan blank (tube 3: 0.5 ml. of glycine solution and 4.5 ml. of phosphate buffer), a phosphate blank (tube 4: 6 ml. of phosphate buffer) and a standard tube (tube 5: 0.5 ml. of glycine solution, 4.3 ml. of phosphate buffer and 0.2 ml. of standard glutathione) are also prepared.

Fresh alloxan solution (1 ml.) is added to tubes 1 (test), 3 (alloxan blank) and 5 (standard) and the contents of each tube are mixed. The final volume in each tube is 6 ml. This procedure is summarized in Table 1.

Readings. All extinctions are read in 2 cm. silica cells in a spectrophotometer set at 305 $m\mu$. (A Uvispek spectrophotometer was used with hydrogen-arc lamp and slit width 19 = 0.38 mm., waveband width 5 $m\mu$.) The control tube 2 is read against the phosphate blank immediately, as its absorption at 305 $m\mu$ increases slightly on standing. For completion of the reaction 20 min. is allowed and the test tube 1 and standard tube 5 are then read against the alloxan blank (tube 3).

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