Table 2. *Reaction of cystamine with sulphite at 25° in various buffers*

Values quoted from McPhee (1956). k_2 is the second-order rate constant with SO_3^{2-} ion.

Buffer	I	k_2 (l. mole^{-1} min.$^{-1}$)
Acetate	0·03	6700
Acetate + sulphate	0·10	1800
Phosphate	0·03	3900
Phosphate	0·05	2400

for k_2 taken from McPhee (1956) (Table 2). In view of these effects the value for k_2 of 8600 l. mole^{-1} min.$^{-1}$ obtained in formate buffer is consistent with McPhee's value of 6700 obtained in acetate buffer. It appears that multivalent ions such as phosphate and sulphate have a marked inhibitory effect on the reaction with SO_3^{2-} ion. Phthalate is no exception to this as the value of k_2, 2400 l. mole^{-1} min.$^{-1}$, obtained in this buffer shows. The interesting thing is that, under these conditions, there is for the first time a reaction with HSO_3^- ion since k_1 is 3 l. mole^{-1} min.$^{-1}$. It is possible that univalent ions such as formate inhibit the reaction with HSO_3^- ion in the same way that multivalent ions inhibit the reaction with SO_3^{2-} ion.

Although the reaction mechanisms in formate and phthalate buffers are different the apparent rate constants observed are of the same order. If therefore a disulphide reacts with sulphite in the acid range, as does one of the S·S bonds in insulin, it is reasonable to postulate that it has a positively charged environment. Without studying the reaction kinetics, however, it is not possible to say whether or not reaction is taking place with HSO_3^- ion.

SUMMARY

1. The reaction of cystamine with sulphite was studied in the range pH 3–5.

2. In the presence of formate buffer reaction took place with SO_3^{2-} ion but not with HSO_3^- ion.

3. In the presence of phthalate buffer a slow reaction took place with HSO_3^- ion. The reaction with SO_3^{2-} ion was slower than in the presence of formate.

REFERENCES

Cecil, R. (1955). *Biochim. biophys. Acta*, **18**, 154.
Cecil, R. & Leoning, U. E. (1960). *Biochem. J.* **76**, 146.
Cecil, R. & McPhee, J. R. (1955). *Biochem. J.* **60**, 496.
McPhee, J. R. (1956). *Biochem. J.* **64**, 22.

Biochem. J. (1960) **76**, 157

Synthesis of L-Ascorbic Acid in Rat-Liver Homogenates

CONVERSION OF L-GULONO- AND L-GALACTONO-γ-LACTONE AND THE RESPECTIVE ACIDS INTO L-ASCORBIC ACID

BY F. A. ISHERWOOD, L. W. MAPSON AND Y. T. CHEN
Low Temperature Research Station, Cambridge

(*Received* 21 *December* 1959)

Evidence has steadily accumulated, supporting the original contention of Isherwood, Chen & Mapson (1954), that L-ascorbic acid is synthesized in animals along the following route:

D-Glucose → D-glucurono-γ-lactone → L-gulono-γ-lactone → L-ascorbic acid.

This paper describes the results of an examination of cell-free extracts of rat liver prepared essentially by the method of Schneider (1948), either in iso-osmotic sucrose (0·25 M) or in potassium chloride (0·15 M). A detailed examination of the separate fractions of the rat-liver extract was carried out on the material prepared by homogenization in iso-osmotic sucrose. Differential centrifuging of these extracts into nuclear, mitochondrial and submicroscopic particles (microsomes) and soluble fractions showed that the mitochondrial–microsome fractions were alone responsible for the conversion of L-gulono- and L-galactono-γ-lactone into L-ascorbic acid. The nuclear and soluble fractions not only had no activity themselves but appeared to have no influence on the activity of the mitochondrial–microsome fractions. The microsomes were more active than the mitochondria. The free acids L-gulonic acid and L-galactonic acid were not converted by these washed particulate preparations into L-ascorbic acid. A preliminary account of this work was given at the Lind Bicentenary Symposium (Isherwood, 1953). The free acids, however, were slowly converted into L-ascorbic acid by whole homogenates of rat liver and later

experiments have been directed to elucidation of the mechanism by which the free acids can act as substrates (Hassan & Lehninger, 1956; Grollmann & Lehninger, 1957).

EXPERIMENTAL

Chemicals. The preparation of L-galactono- and L-gulonolactone has been described by Isherwood *et al.* (1954). The free acids (as their sodium salts) were prepared by dissolving an appropriate amount of the pure lactone (36 mg.) in a slight excess of warm NaOH (2·05 ml., 0·1 N), and the solution was allowed to stand overnight. The solution, which was still slightly alkaline (pH 8–9), was adjusted to pH 7·4 immediately before use. The methyl esters of the free acids were prepared as follows: the solution of the sodium salt was treated with several volumes of absolute ethanol, and the precipitated sodium salt was centrifuged and washed with absolute ethanol. It was then suspended in absolute methanol and treated with the calculated quantity of H_2SO_4 in absolute methanol. After shaking until free mineral acid disappeared the solution was centrifuged from precipitated Na_2SO_4. The methanolic solution of the free acid was treated with a slight excess of diazomethane and evaporated to dryness *in vacuo* at room temperature. The esters were used immediately. At room temperature they appear to be unstable and are slowly converted into the lactones.

The methyl esters of L-xylohexulonic acid and L-lyxohexulonic acid were prepared by refluxing the free acids in dry methanol containing 1% (w/v) of H_2SO_4 for 2 hr. The solutions were neutralized with Na_2CO_3, filtered and the filtrate was evaporated to dryness *in vacuo*.

D-Glucurono-, D-gulono- and D-galactono-γ-lactone, reduced glutathione (GSH), thiomalic acid and thioglycollic acid, L-cysteine, diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and adenosine triphosphate (ATP) were purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Chlorbutol, chloral hydrate and 2:4-dinitrophenol (DNP) were purchased from British Drug Houses Ltd., Poole, Dorset.

Preparation of enzyme material with iso-osmotic sucrose solution. Most of the experiments were carried out with rat-liver tissue and a typical procedure will be described for the preparation of enzyme material from rats. Enzymes were also prepared from sheep liver. The entire isolation was performed in a room at 0° as rapidly as possible. All equipment and fluids were precooled to this temperature. Young adult albino rats were used and each rat was killed by decapitation and the liver immediately exposed so that both the portal vein and the inferior vena cava could be clearly seen. In many experiments the blood was washed out of the liver before it was disintegrated. In these experiments the liver was perfused by way of the portal vein (the inferior vena cava was cut) with cold 0·9% NaCl until all the blood had been removed. At this stage the liver should be a uniform light-tan colour and free from reddish patches. In some cases the livers were finally perfused with 0·25 M-sucrose to remove the NaCl present. The liver was weighed and placed in a beaker immersed in an ice bath. It was cut into small portions and homogenized for 2 min. in 9 vol. of ice-cold 0·25 M-sucrose (Schneider, 1948) with a plastic homogenizing pestle (Schneider & Hogeboom, 1952) of the Potter–Elvehjem type (Potter & Elvehjem, 1936).

Mitochondria were isolated as follows: 200 ml. of the rat-liver homogenate (20 g. of liver) was centrifuged for 10 min. at 200 *g* to sediment nuclei, unbroken liver cells and any red blood cells which had not been removed during the perfusion. The sediment was rehomogenized in 50 ml. of 0·25 M-sucrose and again centrifuged for 10 min. at 200 *g*. The combined extracts were then centrifuged for 30 min. at 8000 *g* to sediment the mitochondria. These appeared as a pellet of firmly packed tan-coloured material covered with a fluffy lighter-coloured layer. The fluffy layer, according to Laird, Nygaard, Ris & Barton (1953), consists of particles distinct from mitochondria as normally sedimented but much larger than microsomes in size. However, Novikoff, Podber, Ryan & Noe (1953), from an examination of similar material with a phase-contrast microscope, suggest that it consists of a gross mixture of small mitochondria and microsomes (including most of the larger dense microsomes). The fluffy layer was removed by pipette and the sediment in the centrifuge tubes rehomogenized for 0·5 min. in 50 ml. of 0·25 M-sucrose and again centrifuged. For many experiments the mitochondria were washed once more with 0·25 M-sucrose, but in some experiments the last washing with sucrose was omitted and the mitochondria were washed instead with 0·25 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (pH 7·5). The mitochondria (and microsomes) appeared to be particularly stable in this buffer and retained their activity for several days if the suspension was stored at 0°.

The microsomes were isolated from the supernatant after removal of the mitochondria. The supernatant was centrifuged for 10 min. at 10 000 *g* to remove a small amount of particulate matter, which was mainly mitochondria (this was discarded) and then centrifuged for 1 hr. at 18 000 *g*. The sediment was translucent and pinkish in colour and consisted entirely of microsomes. It was rehomogenized in 0·25 M-sucrose or in 0·25 M-tris buffer (pH 7·5) and again centrifuged for 1 hr. at 18 000 *g*. It was noticeable that in tris buffer the sediment was less pink in colour whereas the supernatant was distinctly pink.

The supernatant after centrifuging at 18 000 *g* for 1 hr. to sediment the microsomes was not quite clear and still contained a small amount of particulate matter. If the supernatant was required for enzyme experiments this particulate matter was removed by centrifuging at 160 000 *g* for 30 min. in a refrigerated ultracentrifuge.

Nuclei were prepared separately from the mitochondria and microsomes by the method of Hogeboom, Schneider & Striebich (1952). A careful examination of the various preparations of mitochondria, nuclei and microsomes with a microscope with phase-contrast objective showed that each was substantially homogeneous and corresponded closely in appearance to what would be expected from the published photographs (Hogeboom, Schneider & Pallade, 1948). Microscopic examination was occasionally made of the appearance of the particulate preparations during the course of the enzyme reaction. Clumping in mitochondria was associated with loss of activity.

Preparation of enzyme material with 0·15 M-potassium chloride. Enzyme extracts were also prepared by homogenizing the rat liver in 2·5 vol. of 0·15 M-KCl at 0°. Under these conditions the nuclei and mitochondria clumped to-

gether and were removed when the extract was centrifuged at 200 *g* for 10 min. to remove unbroken liver cells. The extract was then centrifuged at 18 000 *g* for 90 min. to sediment the microsomes. The particulate fraction in these KCl extracts consisted almost entirely of microsomes. It was suspended in about 8 vol. of 0·15 M-KCl and centrifuged again. The particulate enzyme preparation is referred to as rat microsomes (KCl). The supernatant is described similarly as rat supernatant (KCl).

'Soluble' enzyme from rat microsomes (potassium chloride). The washed rat microsomes (from 24 g. of liver) were suspended in 0·1 M-phosphate buffer (Na_2HPO_4–KH_2PO_4, pH 7·4, 10 ml.) containing sodium deoxycholate (500 mg.) and the mixture was homogenized for 2 min. The suspension was left at −3° for 1 hr. and then centrifuged at 100 000 *g* for 1 hr. The clear light-brown solution was dialysed against 0·1 M-phosphate buffer (Na_2HPO_4–KH_2PO_4; pH 7·4; 500 ml., changed twice) overnight. This enzyme is described in the text as the 'soluble' microsomal enzyme. Attempts to extract a soluble enzyme from the microsomes by other methods have not been very successful; treatments with acetone, butanol, lipolytic enzymes and freezing and drying techniques have only yielded extracts of low activity (5–10% of original).

Isolation of purified 'aldonolactonase' enzyme from rat supernatant (sucrose). To rat supernatant (sucrose) (430 ml., equivalent to 64·5 g. of liver) was added 0·1 M-KH_2PO_4 (55–120 ml., depending on the liver) to bring the pH to 6·15. The solution was heated to 50±0·5° for 30 min. (in a water bath at 50°; this was conveniently done in a number of test tubes so that the time for the attainment of the temperature is not more than 5 min.), cooled to room temperature and then centrifuged. The clear supernatant which contained about 50% of the original protein retained all the lactonase activity of the original rat extract (fraction I). The solution was dialysed in Visking tubing (4 mm. diam.) against 0·15 M-KCl (2 × 15 l.) at 0° overnight to remove all the sucrose and soluble material. A small precipitate was centrifuged off and solid $(NH_4)_2SO_4$ was added until the solution reached 45% saturation (277 g. to 1 l. of soln.), the precipitated protein was centrifuged off and more $(NH_4)_2SO_4$ added (another 239 g. to 1 l. of soln.) to bring the solution to 75% saturation. The precipitated protein (fraction II), containing the bulk of the lactonase activity, was dissolved in 0·1 M-phosphate buffer (15 ml.; KH_2PO_4–Na_2HPO_4, pH 7·4; total vol. 25 ml.) and dialysed against 0·01 M-phosphate buffer (15 l.; Na_2HPO_4–KH_2PO_4, pH 6·8) at 0° overnight to remove $(NH_4)_2SO_4$. The solution (1875 mg. of protein in 25 ml.) was then treated with freshly prepared calcium phosphate gel (100 ml.; Keilin & Hartree, 1938) (1 mg. of gel/1 mg. of protein), left for 15 min. and centrifuged for 5 min. at 1500 *g*. The gel was washed with 0·01 M-phosphate buffer (pH 6·8). The combined supernatants, which contained all the enzymic activity (fraction III), were saturated with $(NH_4)_2SO_4$ and the precipitated protein was dissolved in 0·01 M-phosphate buffer (12 ml., pH 6·8) and dialysed overnight against 0·01 M-phosphate buffer (15 l., pH 6·8). The solution (115 mg. of protein in 12 ml.) was again treated with calcium phosphate gel (7 ml.; 1 mg. of gel/1 mg. of protein) as before and the supernatant and washings were combined (fraction IV). This solution (66·5 mg. of protein in 19 ml.) was given a further treatment with calcium phosphate gel (4 ml.; 1 mg. of gel/mg. of protein). The supernatant (38 mg. of protein in 22 ml.) contained practically all the lactonase activity of the original extract (fraction V). Further treatment with calcium phosphate gel removed appreciable amounts of enzyme from solution and the specific activity of that remaining in the supernatant was not increased. Fraction V represents the limit in purification by this method.

Attempts to fractionate either the crude or the partly purified enzyme on columns of diethylaminoethylcellulose by the method of Peterson & Sober (1956) were only partly successful. The specific activity of the most active fraction from the column was not higher than that obtained by the simpler calcium phosphate-gel method described above.

Lactic dehydrogenase. The pure crystalline enzyme was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. For enzyme experiments 5 mg. was suspended in 1 ml. of 0·6-saturated $(NH_4)_2SO_4$ and appropriate amounts of this were taken.

Assay methods

Protein. The protein content of the various enzyme preparations was estimated by the method of Lowry, Roseburgh, Farr & Randall (1951), or by the extinction at 280 mμ (Warburg & Christian, 1941). The results by either method were in good agreement.

Hydrolysis of lactones. The hydrolysis of γ-lactones in the presence of the enzymes was followed by estimating the excess of lactone present after different time intervals by the method of Hestrin (1949). This method depends upon the conversion of the lactone into the iron salt of the corresponding hydroxamic acid, which has a strong mauve–red colour and can be estimated from the extinction at 5400 Å.

Ascorbic acid. This was estimated by titration against 2:6-dichlorophenolindophenol (Harris & Olliver, 1942). When sulphydryl compounds such as cysteine were present the titration was carried out in the presence of formaldehyde according to the method described by Mapson (1943). 'Total' ascorbic acid including dehydroascorbic acid and 2:3-dioxogulonic acid was determined by the 2:4-dinitrophenylhydrazone method of Roe & Kuether (1943).

Estimation of '3-oxogulonic acid'. The method was based on observations of Ishikawa & Noguchi (1957) on the decarboxylation of L-gulonate to xylulose by a liver enzyme. The reaction is assumed to proceed as follows:

$$\text{L-gulonic acid} + \text{DPN} \xrightarrow{\text{dehydrogenase}} \text{'3-oxogulonic acid'} + \text{DPNH} \xrightarrow{-CO_2} \text{L-xylulose}$$

The '3-oxogulonic acid' is unstable and particularly in the presence of mineral acid immediately decarboxylates to give xylulose and CO_2; the CO_2 evolved on the acidification of the enzyme digest in the presence of L-gulonic acid was assumed to be a measure of the amount of '3-oxogulonic acid' present. To increase the yield, the DPNH formed in the reaction was oxidized with pyruvic acid in the presence of muscle lactic dehydrogenase. The reaction was carried out in a Warburg apparatus and at the appropriate time perchloric acid was tipped from a side compartment and the gas output measured within 5 min. Each Warburg flask contained the protein fraction which was precipitated between 30 and 50% saturation with $(NH_4)_2SO_4$ and which was derived from the centrifuged liver homogenate (KCl) of 2 g. of liver, muscle lactic dehydrogenase (0·2 ml.), sodium

L-gulonate (20 μM), sodium pyruvate (20 μM), 0·1 M-tris buffer (pH 7·4; 0·5 ml.), nicotinamide (1 mg.) and DPN (0·5 mg.). The side arm contained 60% (w/v) perchloric acid (0·3 ml.). Flasks were shaken at 37° and the gas phase was N_2. The reaction was usually allowed to proceed for 60–80 min., when the concentration of '3-oxogulonic acid' reached a maximum. A crude preparation of '3-oxogulonic acid' was made in a similar manner except that the reaction was carried out in a Thunberg tube and the protein precipitated by adding 4 vol. of ethanol. After centrifuging the supernatant was evaporated at room temperature *in vacuo* to a small bulk.

To examine the products of the reaction of L-gulonic acid dehydrogenase (Ishikawa & Noguchi, 1957) on its substrate, the reaction mixture was either deproteinized with perchloric acid, and then neutralized with KOH and treated with 4 vol. of ethanol, or merely treated with 4 vol. of ethanol. After centrifuging, the supernatants were evaporated at room temperature to about one-tenth of the original reaction volume and examined on a paper chromatogram. The paper had been previously soaked in 0·01 M-phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 7·4) and dried. The solvent was a mixture of propan-1-ol and 0·02 M-phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 7·4) (60:40, v/v) and the papers were developed overnight by downward displacement. After drying, the papers were sprayed with aniline phthalate or ammoniacal $AgNO_3$ (Isherwood & Jermyn, 1951).

Identification of indophenol-reducing substance. The indophenol-reducing substance produced during the mitochondrial–microsome enzyme reaction was identified by chromatographic examination (Chen, Isherwood & Mapson, 1953). The enzyme digest was acidified with solid oxalic acid, the precipitated protein was removed by centrifuging and the supernatant examined on a paper chromatogram with the phenol–water–oxalic acid system. This system will distinguish between related enediols.

Synthesis of L-ascorbic acid. To produce L-ascorbic acid from L-gulono-γ-lactone, a typical digest (total volume 8 ml.) contained the mitochondria (or microsomes) from 2·5 g. of liver (3 ml.), L-gulono-γ-lactone (2·5 mg.), GSH (50 mg.) and $NaHCO_3$ (16 mg.) in tris buffer (0·25 M, pH 7·4; 5 ml.). The reaction mixture was incubated at 37° with gentle shaking in the presence of air, the depth of liquid being 2–4 mm. In some experiments chlorbutol (2 mg.) was added and a phosphate buffer (0·1 M-KH_2PO_4–Na_2HPO_4) used in place of the tris buffer.

To produce L-ascorbic acid from L-gulonate, the digest contained the microsomes from 2·5 g. of liver (3 ml.), L-gulonate (sodium salt, 10 mg.), GSH (100 mg.), $NaHCO_3$ (to neutralize GSH, 33 mg.), supernatant from 2·5 g. of liver (or equivalent aldolactonase enzyme) and phosphate buffer (0·1 M-KH_2PO_4–Na_2HPO_4, pH 7·4; 5 ml.) to give a final volume of 10 ml.

Hydrolysis of L- or D-gulono-γ-lactone by liver aldolactonase. A typical digest for the assay of the aldolactonase contained: L- or D-gulono-γ-lactone (9 mg.) (D-gulono-γ-lactone is convenient to use because it can be purchased commercially and gives similar results to L-gulono-γ-lactone), phosphate buffer (0·1 M, pH 7·4; 3 ml.), manganese chloride (0·05 M, 0·1 ml.; the final solution is saturated and a small precipitate of manganese phosphate is present), cysteine hydrochloride (0·05 M, 0·1 ml.) and aldolactonase enzyme. The final volume was made to 5 ml. with water. The lactone was added when the enzyme digest had reached a temperature of 37°. The unit of enzyme activity was defined as that amount of enzyme hydrolysing 1 μmole of D-gulono-γ-lactone/min. at 37° under the conditions given above. In practice the amount of enzyme present was adjusted so that about 25% of the lactone was hydrolysed in 5 min. The observed rate of hydrolysis of the lactone was corrected for the hydrolysis which occurs even in the absence of the enzyme by running a control experiment without enzyme.

RESULTS

Formation of L-ascorbic acid by rat-liver homogenates

In preliminary experiments it was found that rat liver, homogenized in 0·25 M-sucrose–0·1 M-phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 7·4), would convert both L-gulono- and L-galactono-γ-lactone in the presence of air into a substance reducing 2:6-dichlorophenolindophenol; this was later identified as L-ascorbic acid. The amount of reducing material formed was very small (calculated as L-ascorbic acid it represented only 2–3% of the lactone present) and was difficult to measure because the homogenate already contained about 0·1 mg. of L-ascorbic acid/ml. from the liver itself. During the experiments L-ascorbic acid was progressively destroyed and its formation could only be detected by comparison with similar experiments in which no lactones were present. The results of a typical experiment are shown in Fig. 1 (*a*) and (*b*); the difference between the two curves indicates the amount of L-ascorbic acid synthesized.

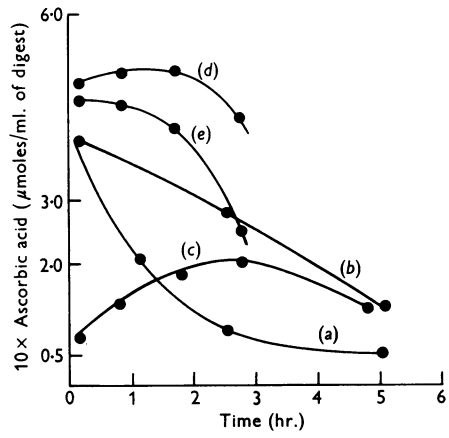


Fig. 1. Formation of L-ascorbic acid by liver homogenate and particulate fractions. All liver fractions were suspended in 0·25 M-sucrose, 0·1 M-phosphate buffer (pH 7·4). Temperature 37°. (*a*) Liver homogenate alone (1 ml. ≡ 0·3 g. of liver); (*b*) as (*a*) + L-gulono-γ-lactone (5 mM); (*c*) particulate fraction from liver homogenate (1 ml. ≡ 0·3 g. of liver) + L-gulono-γ-lactone (5 mM); (*d*) as (*c*) with L-ascorbic acid (0·5 mM); (*e*) as (*d*) without L-gulono-γ-lactone.

Table 1. *Enzymic hydrolysis of* L-*ascorbic acid precursors*

Each digest contained precursor (10 mg.), enzyme preparation (twice-washed mitochondria or microsomes in 0·25 M-sucrose, equivalent to 5 g. of rat liver) and buffer solution (0·25 M-sucrose in 0·1 M-phosphate buffer, KH_2PO_4–Na_2HPO_4, pH 7·4) to give a final volume of 10 ml. Incubation was at 37° in the presence of air. Chlorbutol (5 mg.) was added to the digest where indicated.

	Percentage hydrolysed after 1 hr.			
Enzyme preparation added	L-Gulono-γ-lactone	L-Galactono-γ-lactone	D-Galacturonic methyl ester	D-Glucuronic acid-γ-lactone
None	25	25	1·5	21
Mitochondria	50	55	1·5	55
Microsome	60	70	1·5	95
Microsome + chlorbutol	28	37	—	50

Fractionation of the liver homogenate by differential centrifuging into nuclear, particulate (mitochondria plus microsomes) and soluble fraction indicated that only the particulate fraction showed any activity. The course of the reaction was similar to that observed with homogenates. A typical experiment with the particulate fraction is shown in Fig. 1 (*c*). The conversion of the lactone into L-ascorbic acid reaches a maximum after 1–2 hr. and then declines. L-Ascorbic acid added to the particulate fraction is rapidly oxidized [Fig. 1 (*e*)] and it appears that the decline in Fig. 1 (*c*) is due to the destruction of the L-ascorbic acid synthesized. The addition of L-ascorbic acid in amounts equivalent to several times the maximum amount produced from the lactone did not noticeably affect the formation of L-ascorbic acid from the lactone; the difference between (*d*) and (*e*) (Fig. 1), which represents the amount of L-ascorbic acid synthesized in the presence of excess of L-ascorbic acid, was similar to that produced from L-gulonolactone in its absence [Fig. 1. (*c*)].

Hydrolysis of lactones. The low conversion of lactone into L-ascorbic acid is not due to the existence of an equilibrium between lactone and L-ascorbic acid unfavourable to the synthesis of L-ascorbic acid. It may, however, be due to hydrolysis of the lactone to the free acid, which is not converted. Hydrolysis occurs slowly at pH 7·4 and 37° even in the absence of liver extracts. The rate of hydrolysis in the enzyme digest was measured by the method of Hestrin (1949). Results are shown in Table 1 for the rate of hydrolysis of L-gulono- and L-galactono-γ-lactone, D-galacturonic methyl ester and D-glucurono-γ-lactone in aqueous solution and in the presence of washed mitochondrial and microsome preparations from rat liver respectively.

The results indicate that D-galacturonic acid methyl ester is only slowly hydrolysed and the presence of rat-liver extracts does not accelerate the hydrolysis. The γ-lactones are more rapidly hydrolysed in the presence of mitochondria and microsomes. Hydrolysis of the L-gulono-γ-lactone to the free acid probably accounts for the conversion into L-ascorbic acid reaching a maximum in about 2 hr. in the enzymic digest. The addition of chlorbutol partially inhibits enzymic hydrolysis. In many later experiments chlorbutol was added to reduce the enzymic hydrolysis of the lactones. Chloral hydrate has a similar effect and can be used in higher concentration because it is more soluble.

The hydrolytic activity observed with these particulate preparations may be due to contamination by traces of the very active lactonase present in the supernatant. In comparable experiments the supernatant hydrolyses the lactones at least 100 times as fast as the particulate preparations.

Stabilization of L-*ascorbic acid formed in digests.* In the preliminary experiments described above only the reduced form of L-ascorbic acid was measured and since L-ascorbic acid was readily oxidized in the enzymic digest attempts were therefore made to prevent the oxidation. Compounds containing the sulphydryl grouping were added to the enzyme digest; of these, GSH, cysteine, thiomalic acid and thioglycollic acid were tried. The effect on the synthesis of adding about equimolar quantities (0·03 M) of these compounds to enzyme digests is shown in Fig. 2.

Since the presence of some of the sulphydryl compounds interfered with the indophenol titration of the L-ascorbic acid formed, all the results described in Fig. 2 were obtained by the modified formaldehyde-titration method. Cysteine is particularly difficult because rat liver contains a very active cysteine desulphurase. The results show that the addition of sulphydryl compounds in relatively large amounts protects the L-ascorbic acid formed. GSH was chosen for subsequent work because it did not interfere with the simple indophenol titration and was the most effective of the compounds tried. The conversion of L-gulono-γ-lactone into L-ascorbic acid in a typical digest containing GSH, chlorbutol and the particulate fraction was 25–30 %. This is in marked contrast with the figure of 2–3 % observed in the early experiments.

The addition of GSH to rat-liver homogenates also made it possible to demonstrate that L-gulonic

acid was slowly converted into L-ascorbic acid in the homogenate. Results of an experiment in which L-gulonic acid (as sodium salt) was added to rat-liver homogenate (KCl) are shown in Table 3. This result is in marked contrast with the observations on pea mitochondria (Mapson, Isherwood & Chen, 1954), which indicated that the free acid of L-galactonic acid could not function as a substrate. Two separate aspects of the biosynthesis of L-ascorbic acid were therefore studied: the comparatively rapid conversion of the γ-lactone of L-gulonic acid into L-ascorbic acid by washed mitochondria and microsomes, and the comparatively slow conversion of L-gulonic acid by an homogenate.

Conversion of L-gulono-γ-lactone into L-ascorbic acid by particulate preparations. Although preliminary experiments had shown that the particulate fraction from rat-liver homogenates (sucrose) would convert L-gulono-γ-lactone into L-ascorbic acid, no attempt had been made to test whether other fractions were active or whether addition of these other fractions to the particulate fraction affected its activity. Experiments were therefore devised to find out which fractions of the cell homogenate were responsible. Four fractions were concerned: nuclei, mitochondria, microsomes and supernatant. These were separated by differential centrifuging and were refractionated until, with the particulate fractions, examination under a phase-contrast microscope indicated that they were homogeneous (Hogeboom *et al.* 1952; Hogeboom *et al.* 1948). The results obtained from the various fractions are shown in Fig. 3.

The results indicate that of the particulate fractions, only the mitochondria and microsomes were active. The nuclei were without activity and did not appear to affect the activity of the other fractions. The supernatant was also without activity but does contain a powerful aldolactonase enzyme which hydrolyses the lactone to the free acid within an hour. This means that the lactone is only converted to a slightly greater extent than the free acid in homogenates. The microsomes are much more active than the mitochondria (three to four times, calculated on an equal content of N, Kjeldahl) but neither will convert the free acid into L-ascorbic acid in the absence of the supernatant.

Conversion of related sugar-γ-lactones. In previous studies (Isherwood *et al.* 1954) it had been shown that whereas both L-gulono- and L-galactono-γ-lactone were converted into L-ascorbic acid in intact plants and animals, whole extracts or mitochondrial preparations from pea and bean seeds would only readily convert L-galactono-γ-lactone into L-ascorbic acid (Mapson *et al.* 1954). D-Altrono-γ-lactone, which was converted in the intact plant into D-araboascorbic acid, was also converted by enzyme preparations from pea and bean seeds. D-Mannono-γ-lactone, which was not converted in

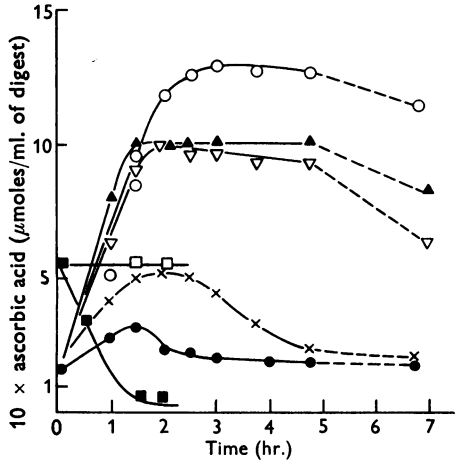


Fig. 2. Influence of sulphydryl compounds on the formation of L-ascorbic acid by particulate fractions of rat liver. The enzyme digest contained the particulate fraction in 0·25 M-sucrose and 0·1 M-phosphate buffer (pH 7·4, 8 ml.); 1 ml. of digest contained the equivalent of 0·5 g. of liver, chlorbutol (10 μM) and L-gulono-γ-lactone (5 mM). ●, No addition; ×, cysteine (30 mM); ○, GSH (30 mM); ▽, thiomalic acid (30 mM); ▲, thioglycollic acid (30 mM); □, no L-gulono-γ-lactone; L-ascorbic acid (0·5 mM) and GSH (30 mM) added; ■, no L-gulono-γ-lactone or GSH, only L-ascorbic acid (0·5 mM) added.

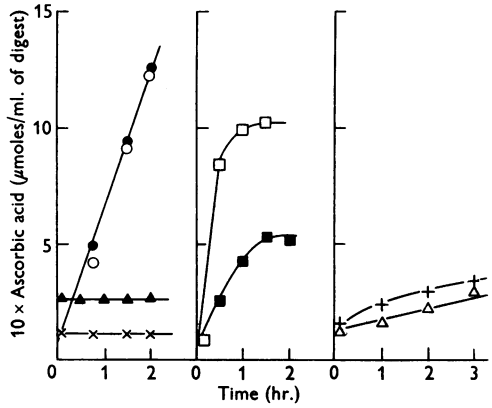


Fig. 3. Formation of L-ascorbic acid by different fractions of rat liver. The enzyme digests contained 5 ml. of tris buffer (0·25 M), pH 7·4, GSH (30 mM), chlorbutol (10 μM), liver fractions equivalent to 5 g. of liver. These fractions were all isolated by extracting perfused liver with 0·25 M-sucrose. Temperature 37°. All digests contained L-gulono-γ-lactone (5 mM) unless stated otherwise. ×, Nuclei; ○, nuclei + mitochondria + microsomes; ●, mitochondria + microsomes; ■, mitochondria; □, microsomes; ▲, supernatant; +, unfractionated homogenate; △, unfractionated homogenate with L-gulonate (5 mM) instead of L-gulono-γ-lactone.

the intact plant, was not converted by the enzyme extracts. It was of interest therefore to determine the specificity of animal particulate preparations towards the various lactones. In the intact animal, both L-gulono- and L-galactono-γ-lactone were converted into L-ascorbic acid, but of the related lactones of D-mannonic acid and D-altronic acid, only D-mannono-γ-lactone gave rise to D-araboascorbic acid. Experiments with rat-liver mitochondria showed that, as expected, L-galactono- and L-gulono-γ-lactone were converted into L-ascorbic acid and D-mannono-γ-lactone into D-araboascorbic acid. The rate of conversion with each lactone was about the same. D-Altrono-γ-lactone, which would not be expected to give D-araboascorbic acid, did in fact give a small yield, about 25% of that obtained with D-mannono-γ-lactone. This suggests that the animal-enzyme preparation does not show quite the same high substrate specificity as the plant preparations and that in the intact animal the difference between the response to the two lactones may be relative rather than absolute.

L-Xylohexulonic acid (2-oxo-L-gulonic acid) has been suggested (Smythe & King, 1942; Smith, 1952) as a possible precursor of L-ascorbic acid. We failed, however, to detect any formation of L-ascorbic acid when L-xylohexulonic acid was used as substrate for the rat-liver-enzyme preparations. Related compounds such as L-lyxohexulonic acid, D-xylohexulonic acid and D-lyxohexulonic acid also failed to give an ascorbic acid compound.

Rate of reaction as affected by concentration of L-gulono-γ-lactone and other related compounds. The maximum rate of conversion of L-gulono-γ-lactone into L-ascorbic acid occurs at low substrate concentrations. The apparent Michaelis constant is of the order of 0·1 mM, which is the same as that observed for pea mitochondria. At a concentration as low as 0·5 mM, 60–70% of the lactone was converted into L-ascorbic acid in a typical digest. The high affinity of the mitochondrial–microsomal fraction for the lactone is important when the mechanism of the conversion of the free acid into L-ascorbic acid by homogenates is considered below.

Effect of different extraction procedures and experimental conditions. Most of the early experiments were with rat liver that had been homogenized in 0·25 M-sucrose and, with this extracting fluid, it was possible to prepare both mitochondria and microsomes as comparatively homogeneous fractions. By a slightly modified technique the nuclei could be prepared free from mitochondria and microsomes.

The use of 0·15 M-KCl as extracting fluid caused the mitochondria and nuclei to clump, and this meant that the particulate fraction consisted almost entirely of microsomes. Since the microsomes were more active than the mitochondria and had similar enzymic properties the results obtained with enzymic preparations with 0·15 M-KCl as extraction fluid were similar to those made with 0·25 M-sucrose as extraction fluid. Variations in the strength of the sucrose from 0·1 to 0·8 M had little effect on the enzymic conversion of L-gulono-γ-lactone into L-ascorbic acid and the choice of a suitable buffer system was not critical. The reaction proceeded equally well either in 0·25 M-tris buffer (pH 7·5) or in 0·2 M-phosphate buffer (KH_2PO_4–Na_2HPO_4, pH 7·4). This suggests that phosphate is not essential for the reaction when particulate preparations are used. The apparent optimum pH for the reaction was about 8·5, but since the mitochondrial–microsomal preparations were rapidly inactivated at pH values above this and below 7·0, too much significance cannot be attached to this value. At pH 7·4 the particulate preparations were stable, and most experiments were made at this pH value and at a temperature of 37°. The comparative (initial) rate at which ascorbic acid was formed at temperatures of 15°, 20°, 25° and 37° in a typical digest was 0·14, 0·19, 0·36 and 0·86 μmole/ml. of digest/hr. At temperatures much above 37° the particulate preparations were rapidly inactivated.

As was to be expected, the oxidation of L-gulono-γ-lactone is dependent on O_2 and there is no reaction in its absence. The rate of the reaction varies with the partial pressure of O_2 (Fig. 4). Calculation of the affinity of the L-gulono-γ-lactone-oxidase system for O_2 gave K_m 0·2 mM, equivalent to an O_2 affinity ($1/K_m$) of 5×10^3. This affinity is considerably lower than the corresponding figure found with the system in pea mitochondria ($2{\cdot}5 \times 10^5$, Mapson *et al.* 1954).

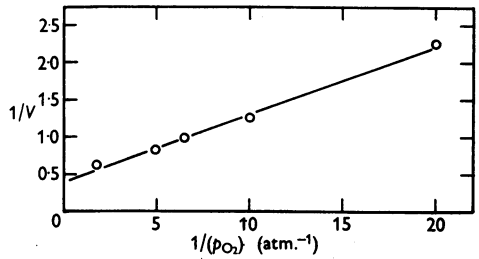


Fig. 4. Influence of partial pressure of oxygen on the rate of formation of L-ascorbic acid from L-gulono-γ-lactone by rat-liver microsomes. The enzyme digests contained, in a total vol. of 8 ml., 5 ml. of 0·1 M-phosphate buffer, pH 7·4, GSH (30 mM) and L-gulono-γ-lactone (5 mM) and the washed microsomes from 4 g. of rat liver. Each digest was incubated at 37° in atmospheres of different oxygen–nitrogen mixtures. V, μmoles of L-ascorbic acid formed. ml. of digest^{-1} hr.$^{-1}$.

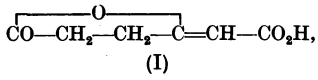
Action of enzymic inhibitors. The effect of the addition of various inhibitors to the enzyme digest was studied in order to determine whether the liver enzyme was similar to that in pea mitochondria investigated earlier. Mapson *et al.* (1954) found that with washed pea mitochondria, cyanide (1 mM) reduced the rate of formation of L-ascorbic acid from L-galactono-γ-lactone by 90% whereas 2:4-dinitrophenol (1 mM), azide (1 mM) or the use of $CO + O_2$ (95:5) was without effect unless a respirable substrate, e.g. succinate (10 mM), was present, when both azide and carbon monoxide inhibited the reaction. They also found that the addition of ATP (1 mM) decreased the formation of L-ascorbic acid by 40%. In the present investigation similar experiments have been made on particulate preparations from rat liver (either sucrose or KCl). These show that cyanide (10 mM), azide (4 mM) with or without succinate (10 mM), hydroxylamine (5 mM) or incubation in $CO + O_2$ (95:5) had no appreciable effect. The addition of ATP (5 mM) decreased by 25% the rate of formation of L-ascorbic acid, much as with pea mitochondria. The most strikingly different result was the effect of DNP (1 mM), which increased the rate of conversion of L-gulono-γ-lactone into L-ascorbic acid by 300% (Table 2). The results described in this and the preceding section indicate that the oxidation system in rat-liver-enzyme preparations is substantially different from that in pea mitochondria.

L-Ascorbic acid is necessary in the formation of connective tissue, and it was of interest to test whether inhibitors of the formation of connective tissue described by Heaton (1926) did so by inhibiting the formation of L-ascorbic acid from L-gulono-γ-lactone. Medawar, Robinson & Robinson (1943) suggested that one of these substances was possibly an unsaturated lactone similar to δ-hex-α-enolactone (parasorbic acid, III). Other similar lactones (I–VI) have been shown to have a marked effect on growth (Hauschka, Toennies & Swain, 1945) and experiments with a number of such lactones (concentrations 0·01 M) on the formation of L-ascorbic acid from L-gulono-γ-lactone in the presence of rat-liver mitochondria (sucrose) without the addition of GSH to the digest indicated that all the lactones inhibited the formation to some extent but that (I) almost entirely stopped it. Corresponding experiments with pea mitochondria and L-galactono-γ-lactone gave similar results

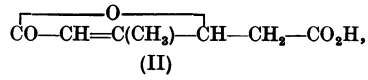
Table 2. *Effect of different phenols on the rate of oxidation of* L-*gulono-γ-lactone by microsomal-enzyme extracts*

Each digest contained 0·1 M-phosphate buffer (3·5 ml., pH 7·4), GSH (0·1 m-mole) L-gulono-γ-lactone (0·03 m-mole) enzyme (1 ml. equivalent to 1 g. of liver) and the phenol. Total vol., 5 ml.

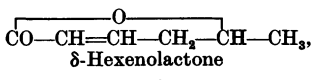
Enzyme extract	Phenol	Concn. (mM)	Rate of formation of L-ascorbic acid as percentage of control with no phenol
Microsomes in 0·15 M-KCl	2:4-Dinitrophenol	0·01	133
		0·1	235
		1·0	390
'Soluble' microsomal enzyme	Phenol	1·0	100
	o-Nitrophenol	1·0	126
	m-Nitrophenol	1·0	210
	p-Nitrophenol	1·0	155
	3:5-Dihydroxybenzene	0·1	100
	3:5-Dihydroxybenzene	1·0	100
	p-Chlorophenol	1·0	67
	2:4-Dinitrophenol	1·0	380
	2:4:6-Trinitrophenol	1·0	220



(I)

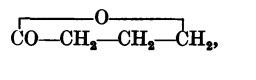


(II)

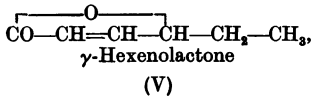


δ-Hexenolactone

(III)

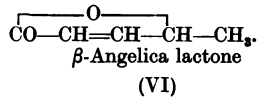


(IV)



γ-Hexenolactone

(V)



β-Angelica lactone

(VI)

except that the inhibition was most pronounced with (II). Many of these lactones inhibit the germination of seeds (Haynes, 1948).

Preparation of a 'soluble' enzyme from microsomes

Several attempts were made to solubilize the enzyme from the microsomes. These included treatment with acetone at $-20°$, freeze-drying followed by treatment with butanol, repeated freezing and thawing, treating with ethanol (10%) at 42° and treating with lipolytic enzymes before extraction. All these methods have been unsuccessful in that they have only yielded extracts of low activity (5%). The most promising method so far tested has been the 'solubilization' of the enzyme by treatment of the microsomes with deoxycholate solution. Extracts of high activity (90–100% of original activity) were obtained with sodium deoxycholate in a concentration of 5% (w/v) in phosphate buffer (0·1 M, pH 7·4), but subsequent experiments showed that the deoxycholate could be decreased to 0·5% (w/v) and yield extracts of equal activity. No loss of enzyme activity occurred when extracts were extensively dialysed against phosphate buffer (pH 7·4). This treatment with deoxycholate rendered the enzyme 'soluble' as judged by the fact that centrifuging at 100 000 *g* for 1·5 hr. failed to remove any activity; the enzyme could, however, still be precipitated by the addition of $(NH_4)_2SO_4$ to a concentration of 15% saturation. The enzyme activity of these preparations could also be precipitated by the addition of excess of acetone at $-20°$ and the precipitate, after removal of acetone, when dissolved in phosphate buffer (pH 7·4) still retained some of its activity.

The deoxycholate–enzyme preparations readily converted L-gulono-γ-lactone into L-ascorbic acid in the presence of O_2 and the latter could be substituted by phenazine methosulphate under anaerobic conditions, with 2:6-dichlorophenolindophenol as indicator according to the method described by Mapson & Breslow (1958). The formation of L-ascorbic acid from the lactone was not accelerated by the addition of either DPN or TPN, nor were these coenzymes reduced on the addition of the lactone; in fact no evidence was obtained that these compounds were necessary for the oxidation.

These enzyme preparations also oxidized D-altrono-, D-mannono- and L-galactono-γ-lactone, but not D-galactono-γ-lactone, the rate of oxidation of the first two lactones being, however, only about half that of the rate of oxidation of L-gulono-γ-lactone. As with the intact microsomes there was no oxidation of L-gulonic acid or L-galactonic acid. Similar results were obtained with phenazine methosulphate as electron acceptor, and there was the same stimulation of the oxidation of L-gulono-γ-lactone in the presence of dinitrophenol and related nitrophenols (Table 2).

Chatterjee, Kar, Ghosh & Guha (1960) have claimed that a lipid-soluble factor is involved in the oxidation of L-gulono-γ-lactone to L-ascorbic acid. Liver microsomes which had been only partially inactivated by treatment with fat solvents were reactivated by the addition of α-tocopherol; other substances including the lipids extracted from the microsomes were much less effective.

We have found that 2:4-dinitrophenol is much more effective than α-tocopherol and this effect can be simply explained as a stimulation of the residual enzyme activity by the nitrophenol. Thus if the microsomes are treated with acetone under the same conditions as these described by Chatterjee, Kar, Ghosh & Guha (1960), no residual activity remains and no reactivation occurs on addition of either 2:4-dinotrophenol or α-tocopherol. On the other hand, if the enzyme is only partially inactivated (treatment with light petroleum or diethyl ether), 2:4-dinitrophenol stimulates the activity to an extent which would be expected from the corresponding behaviour with the original enzyme (free microsomes, cf. Table 2, 380%; residual activity, 400%).

Microsomal cytochrome. Tests were carried out with the 'soluble' microsomal-enzyme extract to determine whether microsomal cytochrome participated in the oxidation of L-gulono-γ-lactone. The spectrum of this extract was similar to that reported for microsomal cytochrome by Strittmatter & Velick (1956). There was a peak at 413 mμ in the oxidized state, which on reduction with sodium dithionite shifted to 523 mμ, with the appearance of two additional peaks at 525 and 556 mμ. However, under anaerobic conditions there was no change in the oxidized absorption spectrum on the addition of L-gulono-γ-lactone, and, since the extract readily oxidizes this lactone in the absence of any added cofactors, it seems evident that microsomal cytochrome does not participate in the oxidative process.

Conversion of L-gulonate into L-ascorbic acid in rat-liver homogenates

Early experiments with L-gulonate as the substrate had suggested that no conversion of the acid into L-ascorbic acid occurred. The experimental conditions then were such that a small synthesis would have been obscured by the concomitant destruction of L-ascorbic acid present in the original rat-liver homogenate. The addition of GSH, however, prevents this destruction and it is then possible to detect a small synthesis of

Table 3. *Conversion of* L-*gulonate into* L-*ascorbic acid in rat-liver homogenates*

Each digest contained 0·02 M-phosphate buffer (7·2 ml., pH 7·4) and either rat-liver homogenate in 0·15 M-KCl (2·8 ml. ≡ 1 g. of liver) or washed liver microsomes suspended in 0·15 M-KCl (2·8 ml. ≡ 1 g. of liver); total vol., 8 ml. Incubation was at 37° in air.

Expt. no.	Addition to digest	L-Ascorbic acid (μ-moles synthesized in 10 ml. of digest) Time 1 hr.	Time 2 hr.	Time 3 hr.
1	Rat-liver homogenate, 0·2 m-mole of GSH, 0·2 m-mole of $NaHCO_3$, 0·04 m-mole of L-gulonate	0·8	1·3	2·2 (1·8)*
2	As for 1 but without GSH and $NaHCO_3$	0	0	0
3	As for 1 but 0·04 m-mole of L-gulono-γ-lactone in place of L-gulonate	0·8	1·3	2·2 (2·1)*
4	As for 3 without GSH and $NaHCO_3$	0	0	0
5	Washed rat-liver microsomes, 0·2 m-mole of GSH, 0·2 m-mole of $NaHCO_3$, 0·04 m-mole of L-gulono-γ-lactone	2·2	5·5	7·4
6	As for 5 but 0·04 m-mole of L-gulonate in place of lactone	0	0	0

* Estimated by the method of Roe & Kuether (1943).

Table 4. *Rate of conversion of* L-*gulonate into* L-*ascorbic acid at different concentrations in rat-liver homogenate*

Each digest contained 0·025 M-phosphate buffer (7·2 ml., pH 7·4) and liver homogenate in 0·15 M-KCl (2·8 ml. ≡ 1 g. of liver) together with 0·2 m-mole of GSH, 0·2 m-mole of $NaHCO_3$ and varying concentrations of L-gulonate. Incubation was at 37° in air.

Concn. of L-gulonate (mM)	Time of incubation (hr.) 0·75	1·75	3·0
	L-Ascorbic acid (μmoles synthesized in 10 ml. of digest)		
0·05	1·4	3·1	4·4
0·025	1·0	2·4	2·6
0·0125	0·7	1·6	2·0
0·005	0·5	0·9	1·3
0·0025	0·2	0·6	0·6

L-ascorbic acid. An experiment in which the increase in L-ascorbic acid was followed by titration of portions with 2:6-dichlorophenolindophenol and by the method of Roe & Kuether (1943) indicated that synthesis occurred only if GSH were present. The results are shown in Table 3.

It is noticeable that whereas the synthesis of L-ascorbic acid by homogenates is practically the same whether L-gulonate or L-gulono-γ-lactone is used as substrate, the washed particulate fraction from rat liver will convert the lactone at a very high rate into L-ascorbic acid but is entirely inactive towards the free acid. Similar results were obtained if a rat-liver homogenate in 0·25 M-sucrose was used in place of that in 0·15 M-KCl. The compound produced, which reacted as L-ascorbic acid towards 2:6-dichlorophenolindophenol and in the Roe & Kuether method, was positively identified as L-ascorbic acid by the chromatographic method of Chen *et al.* (1953). To preserve the small amount of L-ascorbic acid in the enzyme digest during deproteinization by the addition of ethanol and concentration, a few crystals of KCN were added to inactivate heavy metals. Tests on the supernatant after the rat-liver homogenate had been centrifuged at 100 000 *g* for 20 min. showed that it would not convert L-gulonic acid into L-ascorbic acid unless the particulate fraction was added. Dialysis against 0·15 M-KCl did not destroy its activity and in general it was found that the addition of TPN, DPN, ATP or ADP had no effect on the activity; GSH, however, appeared to be necessary for the conversion to occur. The supernatant contained a powerful lactone-hydrolysing enzyme which split added lactone within a few minutes so that the results obtained with rat-liver homogenate with either added lactone or free acid were substantially those recorded on the free acid. The effect of substrate concentration is shown in Table 4. It is clear that at the substrate concentration used in most experiments (0·04 m-mole in 10 ml. of digest) the enzyme system was not saturated; the apparent K_m for the system was 20 mM, which is about 100 times that for the lactone with washed particulate enzyme.

Since it was only possible to demonstrate the activity of the enzyme system when both supernatant and the particulate fraction were present, an attempt was made to investigate the enzyme in the supernatant by adding enzyme inhibitors which had no inhibitory effect on the activity of the particulate fraction towards L-gulono-γ-lactone.

Table 5. *Effect of inhibitors on the oxidation of* L-*gulonate to* L-*ascorbic acid by rat-liver homogenates*

Each digest contained 0·1 M-phosphate buffer (7·2 ml., pH 7·4) and liver homogenate in 0·15 M-KCl (2·8 ml. ≡ 1 g. of liver) together with 0·2 m-mole of GSH, 0·2 m-mole of $NaHCO_3$, 0·04 m-mole of L-gulonate and the appropriate compound; total vol., 8 ml. Incubation was at 37° in air.

Source of enzyme	Inhibitor	Concn. (mM)	Inhibition (%)
Sucrose homogenate	DNP	0·01	25
Sucrose homogenate	DNP	0·1	100
KCl homogenate dialysed	Hydroxylamine	20	60
KCl homogenate	Hydroxylamine + succinate (10mM)	20	60
KCl homogenate dialysed	Arsenate replaced phosphate as buffer	100	75
KCl homogenate	Adenosine monophosphate	10	0
KCl homogenate	EDTA	10	100
KCl homogenate	EDTA	1	50
KCl homogenate	Sodium benzoate	10	35
KCl homogenate	Sodium acetate	50	40
KCl homogenate	Sodium D-gulonate	10	0

The results obtained with a number of these inhibitors are given in Table 5 and Fig. 5.

Both DNP and arsenate inhibit the reaction in marked contrast with the fact that DNP definitely stimulates the activity of microsomes towards L-gulono-γ-lactone whereas arsenate is without effect. Ethylenediaminetetra-acetic acid (EDTA) chelates with heavy metals and the inhibition by this agent suggests that an enzyme inhibited by heavy metal may be involved in the conversion of L-gulonate into L-ascorbic acid. The inhibition caused by hydroxylamine may be due to reaction with acyl compounds or keto groups formed during the reaction. Estimation of 'hydroxamic acid' in solution by the method of Hestrin (1949) indicated that a maximum of 20% of the gulonate was present as a hydroxamic acid. Attempts to identify this compound chromatographically were not successful, mainly because its concentration was too small.

Chloral hydrate (Fig. 5), like chlorbutol, affects the lactone-splitting enzymes and the inhibition of the conversion of L-gulonate into L-ascorbic acid may be due to its action on a lactonase in the supernatant. Chloral hydrate stimulates the formation of L-ascorbic acid from the lactone.

Based on the action of these inhibitors and the facts described above, three possible mechanisms were considered for the conversion of L-gulonate into L-ascorbic acid in rat-liver homogenates. These are outlined in the following equations.

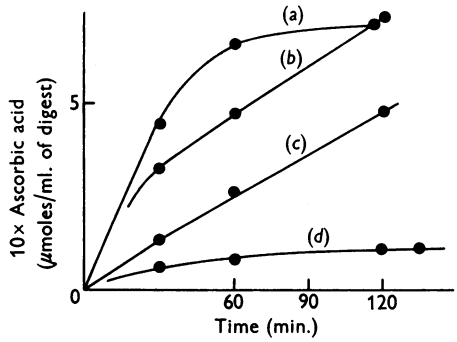


Fig. 5. Influence of chloral hydrate on the formation of L-ascorbic acid from L-gulono-γ-lactone or L-gulonate. All digests contained 2 ml. of phosphate buffer (0·1 M, pH 7·4), 3 ml. of rat-liver homogenate (KCl, equivalent to 1 g. of liver), with GSH (30 mM) and either L-gulono-γ-lactone (10 mM) or L-gulonate (10 mM). (*a*) L-Gulono-γ-lactone + chloral hydrate (0·1 M); (*b*) L-gulono-γ-lactone; (*c*) L-gulonate; (*d*) L-gulonate + chloral hydrate (0·1 M).

'*3-Oxogulonic acid*' *as intermediate.* This compound has not been synthesized chemically and therefore any experiments to test whether it was an intermediate in the formation of L-ascorbic acid had to depend upon its postulated formation in the enzyme conversion of L-gulonate into L-xylulose, which requires DPN as coenzyme (Grollman & Lehninger, 1957; Ishikawa & Noguchi, 1957). With an enzyme from guinea-pig liver (which does not

$$\text{L-Gulonic acid} \xrightarrow[\text{DPN}]{\text{Dehydrogenase}} \text{'3-oxogulonic acid'} \xrightarrow{\text{microsomes}} \text{L-ascorbic acid} \quad (1)$$

$$\text{L-Gulonic acid} + \text{acyl–S-compound} \xrightarrow[\text{transacylase}]{\text{Supernatant}} \text{L-gulonyl-S-compound} \xrightarrow{\text{microsomes}} \text{L-ascorbic acid} \quad (2)$$

$$\text{L-Gulonic acid} \xrightarrow[\text{lactonase}]{\text{Supernatant}} \text{L-gulonyl-enzyme complex} \xrightarrow{\text{microsomes}} \text{L-ascorbic acid} \quad (3)$$

form ascorbic acid) the progress of the DPN-dehydrogenase reaction was followed manometrically. At intervals samples were acidified with perchloric acid and the CO_2 evolved within 10 min. was measured. This evolution of CO_2 was assumed to be due to the decarboxylation of a '3-oxogulonic acid' and was used as a measure of the amount present. Samples of the enzyme digests, with and without L-gulonic acid, and before and after acidification, were deproteinized and examined on a paper chromatogram buffered at pH 7·4. The paper was sprayed with aniline phthalate. A substance was present on the paper (pink colour with aniline phthalate) which had R_F 0·22, somewhat greater than that of 2-oxogulonate and 2-oxogalactonate (0·12 and 0·13 respectively). This would be expected of a 3-oxogulonic acid since its ring structure is likely to be furanose (Isherwood & Jermyn, 1951). It was only present in the unacidified extracts when L-gulonate had been added to the enzyme digest and disappeared when the digests were acidified with the concomitant appearance of a similar spot in a position corresponding to xylulose (R_F 0·45).

Attempts to separate the '3-oxogulonic acid' by large-scale paper chromatography were unsuccessful as the compound was unstable. Experiments to test whether the '3-oxogulonic acid' was an intermediate were therefore made on the crude enzyme digest containing the '3-oxogulonic acid' by the addition of either rat-liver homogenate or a rat-liver particulate fraction together with GSH. The initial rate of the reaction was carefully studied because a true intermediate in the reaction would be expected to be more rapidly converted than the original substrate, L-gulonic acid. Some L-gulonate remained in the digest containing the '3-oxogulonic acid' and this was estimated by comparison with a standard concentration of L-gulonate on a paper chromatogram (Isherwood & Hanes, 1953). This residual L-gulonate gave rise to some L-ascorbic acid, the amount of which could be calculated from the known substrate–enzyme relation (cf. Table 4). The experiments, however, failed to reveal any evidence that '3-oxogulonate' was an intermediate or that the formation of L-ascorbic acid in the digests was not entirely due to the residual L-gulonate. In a typical experiment in which the amount of '3-oxogulonate' in the final digest was estimated to be 3·4 m-moles, the rate of production of L-ascorbic acid could be entirely accounted for on the basis of the L-gulonate estimated to be present. In particular the progress curve showed no greater rate at the beginning of the reaction than a control digest containing only L-gulonate in equivalent concentration. Experiments with rat-liver-enzyme preparations in place of the guinea-pig-liver enzyme gave similar results. The experiments did not support the mechanism outlined in Equation (1).

Acyl compound as intermediate. Since we have found that microsomes will convert not only L-gulono-γ-lactone but the methyl ester and amide of L-gulonic acid into L-ascorbic acid, it seemed possible that the true intermediate was a L-gulonyl–enzyme complex which could be formed directly from any of these compounds; it was this complex that was readily converted into L-ascorbic acid. With the free acid a possible mechanism for the formation of this L-gulonyl–enzyme complex was by transacylation with an acyl mercaptan according to Equation (2). Experiments in which precursors of these compounds, such as pyruvic acid and acetylglutathione, were added to rat-liver homogenate in the presence of L-gulonic acid, had no effect on the formation of L-ascorbic acid. The addition of acceptors for such acyl groups, aniline and nitroaniline (1 mM), also had no effect. Moreover a transacylation reaction did not seem likely since a very thorough dialysis of the homogenate for 48 hr. against several changes of 0·15 M-KCl did not decrease its activity towards L-gulonic acid; any low-molecular-weight soluble precursor of such acyl compounds would have been removed by the treatment and the reaction rate would have been affected.

Formation of L-gulonyl compound by the action of a lactonase enzyme. It has been shown (Winkelman & Lehninger, 1958) that two distinct lactonases occur in rat liver, one hydrolysing lactones (γ- or δ-) of a number of aldonic acids, but not D-glucurono-γ-lactone, and the other only hydrolysing D-glucurono-γ-lactone. The first is found in the soluble fraction of rat liver but not in the microsomes whereas the second is found only in the microsomes. The experimental work in this paper relates to the first enzyme, an aldonolactonase which is assumed to catalyse the reverse reaction to the hydrolysis of L-gulono-γ-lactone. In Equation (3) above the reverse reaction is pictured as far as the formation of a L-gulonyl–enzyme complex and it is this which is believed to react with the microsomal enzyme to give L-ascorbic acid.

To test whether the aldonolactonase enzyme in liver was involved in the conversion of L-gulonate into L-ascorbic acid, the liver extract was fractionated by heat-treatment at 50°, precipitation with $(NH_4)_2SO_4$ and calcium phosphate-gel adsorption, until the majority of the foreign protein had been removed. The activity of the aldonolactonase at each stage was tested (1) by its ability to hydrolyse D-gulono-γ-lactone, (2) by its ability, when added to a standard amount of washed microsomes or dialysed deoxycholate preparation of microsomes and L-gulonate, to synthesize L-ascorbic acid.

Table 6. *Isolation of aldonolactonase enzyme from rat and sheep liver and identification with soluble enzymic factor in conversion of* L-*gulonate into* L-*ascorbic acid*

To test each fraction for the soluble enzymic factor, each digest contained GSH (0·2 m-mole), $NaHCO_3$ (0·2 m-mole), L-gulonate (0·04 m-mole), washed microsomes (3 ml. ≡ 3 g. of liver) or a deoxycholate microsomal enzyme (≡ 3 g. of liver), the aldonolactonase enzyme (≡1 000 units), phosphate buffer (0·1 M, pH 7·4, containing mM-Mn^{2+} ions) to a total vol. of 8 ml.

Enzyme fraction	Total protein (mg.)	Specific activity (units/mg. of protein)	L-Ascorbic acid synthesized (μmole/hr.)
Rat supernatant	8 700	5·5	0·23
I	3 200	15	0·14
II	1 875	25	—
III	230	110	0·12*
IV	66	370	—
V	44	500	0·15, 0·20, 0·25
Sheep supernatant	13 000	4	—
II	5 000	8·5	—
III	500	90	0·1
IV	260	—	0·15*
V	158	300	0·105, 0·22

* Deoxycholate microsomal enzyme.

The results obtained are shown in Table 6. The specific activity of the enzyme as hydrolysing enzyme had risen from 5 units/mg. of protein in the original supernatant to 500 units/mg. in the purified protein preparation.

Comparison of the activities of the aldonolactonase as a hydrolysing enzyme and its ability to catalyse the conversion of L-gulonic acid into L-ascorbic acid shows that the two run closely parallel. There was no evidence that two separate enzymes were present even though the original enzyme was purified 100-fold. A combination of the purified aldonolactonase and the dialysed soluble microsomal enzyme was equally effective. An aldonolactonase prepared from sheep liver in a manner exactly similar to that described for rat liver appeared to be more stable but again there was no evidence that two separate enzymes were involved; combined with rat-liver microsomes it was equally effective. A study of the effect of inhibitors on the aldonolactonase and on the complete system for the conversion of L-gulonic acid into L-ascorbic acid also indicated that the enzyme present in the supernatant catalysing the conversion behaved like the aldonolactonase. Thus EDTA (1 mM) and *p*-chloromercuribenzoate (1 mM) inhibited both lactonase activity and synthesis from the free acid. The inhibition of *p*-chloromercuribenzoate was reversed by the addition of GSH (30 mM).

DISCUSSION

The isolation of homogeneous fractions from rat liver has shown that the ability to convert L-gulono-γ-lactone into L-ascorbic acid resides in both the mitochondria and microsomes but more especially in the latter. The comparative activity on the basis of the nitrogen content is about one to four respectively. We have no conclusive evidence that the enzymes present in these two fractions are the same but their general reactions appear to be identical. From the practical point of view the microsomes are much more stable in that the enzyme present remains active under conditions in which the mitochondria 'clump' and lose their activity. This may explain why Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960) did not find the enzyme to be present in the mitochondria.

For the demonstration of full activity the presence of sulphydryl compounds such as GSH is necessary with the intact particulate fractions. However, with 'solubilized' preparations from microsomes this requirement for sulphydryl compounds was not observed and no destruction of L-ascorbic acid occurred with these preparations. The failure to detect appreciable synthesis with the particulate fractions in the absence of GSH may be due to a concurrent oxidation of the synthesized L-ascorbic acid, since these preparations readily oxidize added ascorbic acid. This oxidation could be caused by the presence of heavy metals, haem compounds or enzymes. In all cases GSH may act either by chelating with the catalyst or by reducing the dehydroascorbic acid formed. Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960) found that the formation of L-ascorbic acid was increased in the presence of heavy-metal chelating agents.

The most striking difference between the rat-liver-enzyme system and the enzyme system as isolated from plant material is the stimulative effect of 2:4-dinitrophenol and related nitrophenols on both the 'soluble' microsomal enzyme and on the particulate fractions. The cause of this stimulation does not appear to be due to any uncoupling of

phosphorylation from oxidation since many other uncoupling agents (gramicidin, Ca^{2+} and Cd^{2+} ions) had no effect. It seems possible that the nitrophenol may be acting as an electron carrier. In the experiments recorded in this paper we have found no evidence that microsomal cytochrome is involved in the oxidation of the lactone. In the plant the oxidation pathway involves a cyanide- and carbon monoxide-sensitive cytochrome with a high oxygen affinity but this does not appear to be the case in the animal.

We have found that with the intact particulate or 'soluble' enzymes, L-gulonate, as opposed to L-gulono-γ-lactone, was not oxidized to L-ascorbic acid. However, in a homogenate which contained all elements of the tissue, L-gulonate was slowly converted into L-ascorbic acid. Hassan & Lehninger (1956), from similar experiments, suggested that this conversion proceeded through an intermediate '3-oxogulonic acid' [cf. formation of L-xylulose from L-gulonate; Touster, Hutcheson & Rice (1955)] and that the microsomes converted this hypothetical compound into L-ascorbic acid. In our experiments we have been unable to demonstrate the formation of L-ascorbic acid from such a compound. Winkelman & Lehninger (1958) have suggested that the lactone is formed by the reversal of the action of an aldonolactonase enzyme on the free acid. Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960) have advanced a similar hypothesis, the evidence for which was inconclusive in that they showed no correlation between the lactonase activity of the supernatant and its ability to catalyse, in conjunction with the microsomes, the synthesis of L-ascorbic acid from L-gulonate. The experiments recorded in our paper show that a highly purified aldonolactonase enzyme in conjunction with washed particulate enzyme, or 'soluble' microsomal enzyme, will convert L-gulonate into L-ascorbic acid. The most plausible explanation is that the free acid forms a small amount of the gulonyl–enzyme complex rather than the free lactone, as postulated by previous workers, which is then converted by the enzyme in the microsomes into L-ascorbic acid. The evidence for the formation of lactone described by Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960) involves the addition of hydroxylamine to the enzyme digest and isolation of the hydroxamic acid of L-gulonic acid. This could equally well be formed from the L-gulonyl–enzyme complex and there is no need to postulate the separate formation of a lactone.

The particulate preparations have been shown to contain a lactonase but the inability to demonstrate synthesis of L-ascorbic acid from L-gulonate with them is probably due to the fact that the enzyme is present only in small amount.

The influence of hypnotic drugs on the synthesis of L-ascorbic acid in crude homogenates is also in agreement with our hypothesis. The effect of these drugs on the synthesis of L-ascorbic acid from the lactone on the one hand and from the free acid on the other was different (cf. Fig. 5); the rate with the lactone was accelerated whereas with the free acid it was inhibited as compared with control experiments to which no drug was added. This can be explained simply on the basis of an inhibition of the aldonolactonase enzyme by the hypnotic drug. The significance of these results in relation to the formation of L-ascorbic acid in the intact animal is not clear. Hypnotic drugs stimulate synthesis and it is difficult to see how this can occur if the effect of these drugs is solely on a reaction pathway which proceeds via the free acid. As yet, we have found no evidence of the presence of aldonolactonase enzyme in plants (peas).

SUMMARY

1. Isolated mitochondria and microsomes from rat liver have been shown to convert L-gulono-γ-lactone into L-ascorbic acid. Microsomes were four times as active as mitochondria and nuclei were without effect. The presence of sulphydryl compounds was essential to prevent oxidation of the L-ascorbic acid.

2. The addition of cyanide, azide, hydroxylamine and carbon monoxide had no appreciable effect on the reaction. 2:4-Dinitrophenol and related nitrophenols caused a marked stimulation.

3. Treatment of the particulate fraction with deoxycholate gave a 'soluble' enzyme which behaved like the original particulate fraction except that the presence of sulphydryl compounds in the enzyme digest was not necessary.

4. Neither the mitochondria, nor the microsomes nor the soluble microsomal enzyme would convert L-gulonate into L-ascorbic acid. However, extracts of rats' liver containing all of the tissue elements could convert L-gulonate into L-ascorbic acid. The formation of L-ascorbic acid from the free acid has been shown to involve an aldonolactonase enzyme not present in the particulate fraction as well as an enzyme in the mitochondria and microsomes.

5. No evidence was obtained that '3-oxogulonic acid' was an intermediate in the conversion of L-gulonate into L-ascorbic acid.

6. A suggested reaction sequence for the conversion of L-gulonate into L-ascorbic acid is:

$$\text{L-gulonate} + \text{aldonolactonase enzyme} \rightleftharpoons \text{L-gulonyl–enzyme complex} \xrightarrow[\text{microsomal enzyme}]{} \text{L-ascorbic acid.}$$

The comments on the papers of Chatterjee, Chatterjee, Ghosh, Ghosh & Guha (1960) and Chatterjee, Kar, Ghosh & Guha (1960) were added after the present paper had been submitted for publication.

REFERENCES

Chatterjee, I. B., Chatterjee, G. C., Ghosh, N. C., Ghosh, J. J. & Guha, B. C. (1960). *Biochem. J.* **74**, 193.

Chatterjee, I. B., Kar, N. C., Ghosh, N. C. & Guha, B. C. (1960). *Arch. Biochem. Biophys.* **86**, 154.

Chen, Y. T., Isherwood, F. A. & Mapson, L. W. (1953). *Biochem. J.* **55**, 821.

Grollman, A. P. & Lehninger, A. L. (1957). *Arch. Biochem. Biophys.* **69**, 458.

Harris, L. J. & Olliver, M. (1942). *Biochem. J.* **36**, 155.

Hassan, M. & Lehninger, A. L. (1956). *J. biol. Chem.* **223**, 123.

Hauschka, T. S., Toennies, G. & Swain, A. P. (1945). *Science*, **101**, 383.

Haynes, L. J. (1948). *Quart. Rev. chem. Soc., Lond.*, **2**, 46.

Heaton, T. B. (1926). *J. Path. Bact.* **29**, 293.

Hestrin, S. (1949). *J. biol. Chem.* **180**, 249.

Hogeboom, G. H., Schneider, W. C. & Pallade, G. E. (1948). *J. biol. Chem.* **172**, 619.

Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1952). *J. biol. Chem.* **196**, 111.

Isherwood, F. A. (1953). *Proc. Nutr. Soc.* **12**, 335.

Isherwood, F. A., Chen, Y. T. & Mapson, L. W. (1954). *Biochem. J.* **56**, 1.

Isherwood, F. A. & Hanes, C. S. (1953). *Biochem. J.* **55**, 824.

Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.

Ishikawa, S. & Noguchi, K. (1957). *J. Biochem., Tokyo*, **44**, 465.

Keilin, D. & Hartree, E. F. (1938). *Proc. Roy. Soc.* B, **124**, 397.

Laird, A. K., Nygaard, O., Ris, H. & Barton, A. D. (1953). *Exp. Cell Res.* **5**, 147.

Lowry, O. H., Roseburgh, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Mapson, L. W. (1943). *J. Soc. chem. Ind., Lond.*, **62**, 223.

Mapson, L. W. & Breslow, E. (1958). *Biochem. J.* **68**, 395.

Mapson, L. W., Isherwood, F. A. & Chen, Y. T. (1954). *Biochem. J.* **56**, 21.

Medawar, P. B., Robinson, G. M. & Robinson, R. (1943). *Nature, Lond.*, **151**, 195.

Novikoff, A. B., Podber, E., Ryan, J. & Noe, E. (1953). *J. Histochem. Cytochem.* **1**, 27.

Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.

Potter, V. R. & Elvehjem, C. A. (1936). *J. biol. Chem.* **114**, 495.

Roe, J. H. & Kuether, C. A. (1943). *J. biol. Chem.* **147**, 399.

Schneider, W. C. (1948). *J. biol. Chem.* **176**, 259.

Schneider, W. C. & Hogeboom, G. H. (1952). *J. biol. Chem.* **195**, 161.

Smith, F. G. (1952). *Plant Physiol.* **27**, 736.

Smythe, C. V. & King, C. G. (1942). *J. biol. Chem.* **142**, 529.

Strittmatter, P. & Velick, S. F. (1956). *J. biol. Chem.* **221**, 253.

Touster, O., Hutcheson, R. M. & Rice, L. (1955). *J. biol. Chem.* **215**, 677.

Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.

Winkelman, J. & Lehninger, A. L. (1958). *J. biol. Chem.* **233**, 794.

Biochem. J. (1960) **76**, 171

A Comparison of the Nucleic Acids of Rat Liver and Novikoff Hepatoma

BY E. H. CREASER AND J. H. SPENCER

McGill-Montreal General Hospital Research Institute, 3619 University Street, Montreal, Canada

(*Received* 28 *September* 1959)

Several workers have shown that qualitative and quantitative differences occur between the ribonucleoproteins present in the various fractions obtained by differential centrifuging of homogenates of normal and neoplastic tissue. Fiala, Sproul, Blutinger & Fiala (1955) showed that several mouse tumours differed from normal mouse tissue in that the cancer cells contained less mitochondria and microsomes than did normal cells, but that the content of 'non-sedimentable' (after 18 000 *g* for 2 hr.) ribonucleic acid was much higher in the tumours. Petermann, Mizen & Hamilton (1956) compared the concentration of ribonucleoprotein particles in rat liver and in hepatoma by means of ultracentrifugal analysis. They observed that 'component B', which was associated with the endoplasmic reticulum and corresponded to the ribonucleoprotein particles described by Palade (1955), predominated in normal liver, spleen and pancreas. In tumours and rapidly growing cells such as regenerating liver, components C and E constituted the major part of the cytoplasmic ribonucleic acid. These ribonucleoprotein components were found to be free in the cytoplasm (Palade, 1955; Howatson & Ham, 1955) and correspond to the ribonucleoprotein found in the postmicrosomal fractions of Palade & Siekevitz (1956), and, in part, to the 'non-sedimentable' ribonucleic acid observed by Fiala *et al.* (1955). Comparison of the base ratios (molar proportions