Effect of Cyanide on the Biosynthesis of Ascorbic Acid by an Enzyme Preparation from Goat-Liver Tissue

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(*Received* 8 *April* 1958)

In exploring the mechanism of biosynthesis of ascorbic acid by the rat, King and his coworkers (Musulin, Tully, Longenecker & King, 1939; Longenecker, Musulin, Tully & King, 1939; Longenecker, Fricke & King, 1940) observed that rats treated with Chloretone (1:1:1-trichloro-2-methylpropan-2-ol) excreted more ascorbic acid than did the controls. By employing Chloretonized rats and isotopically labelled precursors they (Jackel, Mosbach, Burns & King, 1950; Horowitz, Doerschuk & King, 1952; Horowitz & King, 1953*a*, *b*) presented evidence for the following route of the synthesis:

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

Subsequently, Isherwood, Chen & Mapson (1954) and Mapson, Isherwood & Chen (1954) came to the conclusion from an extensive series of experiments that in plant and animal systems respectively the following sequences were involved:

(1) D-Galactose → D-galacturonic acid methyl ester → L-galactono-γ-lactone → L-ascorbic acid.

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

Hassan & Lehninger (1956) reported that rat-liver extracts containing the microsomes and soluble supernatant could convert D-glucurono-lactone, L-gulonolactone and also the corresponding free acids into L-ascorbic acid in the presence of cofactors.

In experiments previously carried out in this Laboratory it had been observed that rat-liver tissue incubated with sodium pyruvate showed a higher ascorbic acid value than did the controls incubated without the substrate (Roy, Roy & Guha, 1946). But in no experiment was the value so obtained higher than the original ascorbic acid content of the tissue before incubation, and it was possible therefore that pyruvic acid was not a direct precursor. In a search for a system in which a net synthesis over and above the original tissue ascorbic acid level takes place, Chatterjee, Ghosh, Ghosh & Guha (1957*a*) found, in later experiments, that a marked synthesis of ascorbic acid occurred in homogenates of rat-liver tissue, containing potassium cyanide, from D-glucurono-γ-lactone, the increase in ascorbic acid value being sometimes two to three times that originally present. No other substrate, including sodium D-glucuronate and sodium pyruvate, could be so converted into ascorbic acid. Subsequently, it was observed that L-gulonolactone, but not sodium L-gulonate, could also act as the substrate and this conversion took place in the absence of cyanide. Liver tissues of all the mammals investigated could bring about this cyanide-stimulated synthesis, but homogenates of the brain (rat), kidney (rat and goat) or adrenal gland (cow) were ineffective in this respect. Extracts from cow and goat livers were found to be the most active, and goat liver, being more freshly available, was chosen for further studies on the enzyme system concerned. The enzyme system was separated from goat-liver homogenates and obtained in the microsomes and the soluble fraction; it could be further concentrated from the extract by precipitation with ammonium sulphate (Chatterjee, Ghosh, Ghosh & Guha, 1958*a*). The present paper deals with detailed studies of this enzyme preparation.

EXPERIMENTAL

Enzyme preparation. The liver removed from the decapitated goat was immediately frozen and subsequently (within 3–4 hr.) homogenized in a Waring Blendor with 4 vol. of cold isotonic sucrose (with addition of 0·1 N-NaOH to keep the pH at 7·0) for 2 min., and centrifuged at 8500 *g* for 20 min. to sediment the nuclei and the mitochondrial fractions, following the method of Schneider & Hogeboom (1950). The supernatant, referred to in the text as the extract, was fractionally precipitated by addition of a saturated solution of ammonium sulphate at pH 6·0. The residue obtained at 30–50% saturation of the salt was suspended in 10 mM-sodium phosphate buffer (pH 7·0) and was used after dialysis. It was subsequently found that by spinning at 100 000 *g* the entire activity was concentrated in the sedimented material; it is therefore not a soluble preparation. The temperature throughout the whole procedure was not allowed to rise above 0°.

Materials. Solutions of D-glucuronolactone, L-gulonolactone, potassium cyanide and other relatively unstable compounds used in the various experiments were freshly made and neutralized where necessary before addition to the reaction media. Sodium gulonate was prepared by incubating 17·8 mg. of L-gulonolactone with 1 ml. of 0·1 N-NaOH at 40° for 24 hr.

D-Glucuronolactone, sodium glucuronate, sodium galacturonate and D-gulono-γ-lactone were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.

Diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN) and adenosine triphosphate (ATP) were purchased from Schwartz Laboratories Inc., N.Y., U.S.A.

Incubation media. The test system contained: 20 mM-phosphate buffer (pH 7·0), 10 mM-substrate, 0·25 ml. of enzyme preparation (equivalent to 50 mg. of wet liver/ml. of incubation mixture) and 50 mM-KCN. Total volume was 2·5 ml.; incubation was for 2 hr. at 37° in air.

Estimation of ascorbic acid. This was carried out by adding 0·5 ml. of 30% metaphosphoric acid to 2·5 ml. of the incubated mixture, and filtering and titrating against 2:6-dichlorophenol-indophenol. When cyanide was not present in the reaction media, 'total' ascorbic acid was estimated according to the method of Roe & Kuether (1943). In the presence of reducing substances such as sodium dithionite and sodium hypophosphite Roe & Kuether's method was used with the modification that charcoal was replaced by bromine water for converting reduced ascorbic acid into dehydroascorbic acid. Whenever glutathione was present in the incubation mixture, the ascorbic acid was estimated titrimetrically according to the modified method of Mapson (1943).

Identification of the biosynthesized ascorbic acid. The ascorbic acid biosynthesized in the presence of cyanide has been identified by paper chromatography, following the method of Chen, Isherwood & Mapson (1953) with formic acid as the irrigating solvent. The presence of ascorbic acid was also tested by feeding the synthesized material to a scorbutic guinea pig. Because of the scarcity of material only one animal could be tested, but this showed a positive growth response to two doses of 1 mg. each of the biosynthesized material. Deproteinization and removal of cyanide were effected by adding conc. HCl (0·15 ml.) to the incubated medium (2·5 ml.), and centrifuging, and evaporating to dryness under suction and taking up the residue with water and neutralizing with sodium bicarbonate.

Estimation of protein. The estimation of the protein content of the homogenate, the extract and the enzyme preparation was carried out according to the method of Gornall, Bardawill & David (1949).

RESULTS

Activity of the enzyme preparation. The relative activities of the homogenate, the extract (containing the microsomes and the soluble supernatant) and the enzyme preparation are shown in Table 1. Unless otherwise mentioned, all the results represent those obtained after incubation of the enzyme preparation with D-glucuronolactone in the presence of potassium cyanide.

Effect of concentration of potassium cyanide on the synthesis of ascorbic acid. Fig. 1 shows that an increase in the cyanide concentration above 5 mM steadily increases the synthesis until a concentration of 50 mM is reached, when no further increase in the ascorbic acid value is observed. When the concentration is below 5 mM, practically no synthesis of ascorbic acid takes place.

Mode of action of cyanide. That cyanide actually facilitates the biosynthesis and does not merely act as an agent preventing the oxidation of ascorbic acid is revealed from the fact that cyanide at mM-concentration can completely protect the ascorbic acid added to the incubation media (Fig. 1), whereas at this concentration no biosynthesis is observed. When cyanide is replaced by other protectors of ascorbic acid, such as 8-hydroxyquinoline or sodium diethyl dithiocarbamate, no increase in the ascorbic acid value has been observed.

In order to throw further light on the mode of action of cyanide, the enzyme concentrate was preincubated with potassium cyanide in the absence of substrate and then dialysed to free it from cyanide. The enzyme concentrate, thus treated, could not convert D-glucuronolactone into L-ascorbic acid; the activity could be restored only after addition of potassium cyanide. Sodium cyanide with sodium phosphate buffer is equally as effective as potassium cyanide with potassium phosphate buffer.

Specificity of cyanide. No other metabolic inhibitor examined, e.g. 2:4-dinitrophenol, phlor-

Table 1. *Synthesis of ascorbic acid by the homogenate, the extract and the enzyme preparation*

Enzyme material	Ascorbic acid synthesized (μg./mg. of protein) With cyanide	Without cyanide
Homogenate	7·0	0
Extract	10·5	0
Enzyme preparation	26·0	0

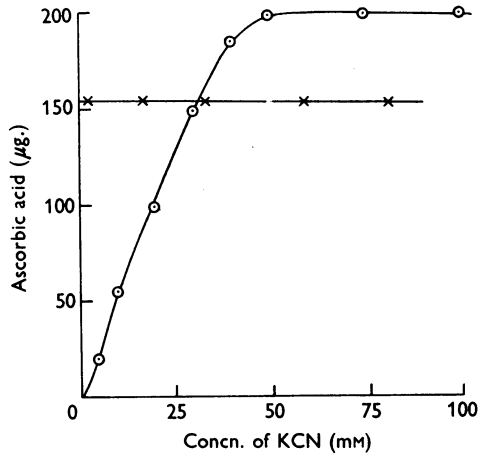

Fig. 1. Effect of cyanide concentration on the rate of conversion of D-glucuronolactone into L-ascorbic acid. ⊙, Influence of cyanide on the synthesis of ascorbic acid; ×, effect of cyanide on the protection of added ascorbic acid.

rhizin, Chloretone, each in mM-concentration, and sodium azide (mM, 50 mM), could replace cyanide for the conversion of D-glucuronolactone into L-ascorbic acid *in vitro*. In view of the possibility that cyanide might influence the biosynthesis by blocking an alternative metabolic pathway of D-glucuronolactone which might involve a cytochrome-linked enzyme system, incubation of D-glucuronolactone was carried out in a gas mixture of $CO + O_2$ (80:20) in the dark (and in the absence of cyanide) but without effect. When cyanide was replaced by reduced glutathione, sodium dithionite or sodium hypophosphite, each in a concentration of 50 mM, no increase in the ascorbic acid value was observed.

Specificity of substrate. With the exception of L-gulonolactone, no other substrate tested, e.g. sodium D-glucuronate, sodium L-gulonate, sodium D-galacturonate, D-gulono-γ-lactone, glucono-γ-lactone, sodium gluconate, sodium pyruvate, sodium pyruvate plus dihydroxyacetone or sodium citrate, was converted into L-ascorbic acid, all the substrates being used in 10 mM-concentration.

Table 2. *Synthesis of ascorbic acid from* D-*glucuronolactone,* L-*gulonolactone and the sodium salts of the corresponding free acids in the presence of cyanide*

Substrate	Ascorbic acid synthesized (μg.) Without cyanide	With cyanide
D-Glucuronolactone	0	210
Sodium glucuronate	0	0
L-Gulonolactone	450	188
L-Gulonate	0	0

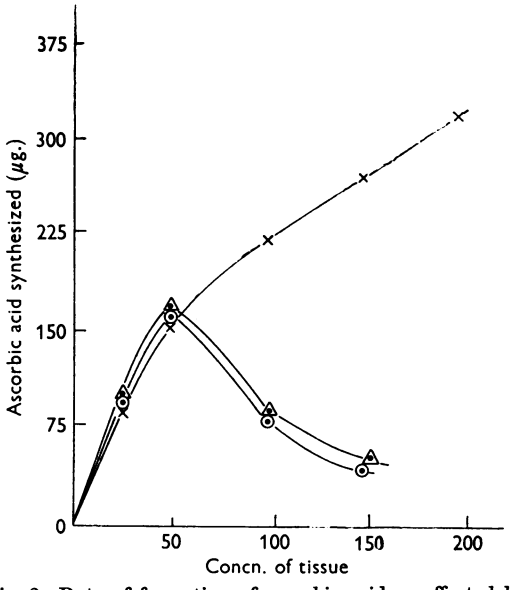

Fig. 2. Rate of formation of ascorbic acid as affected by tissue concentration (expressed as equivalent wet liver in mg./ml. of incubation mixture). ⊙, Homogenate; △, extract; ×, enzyme preparation.

The conversion of L-gulonolactone into L-ascorbic acid does not require the presence of cyanide and is, in fact, partially inhibited by it (Table 2).

Sodium glucuronate (10 mM) and sodium ascorbate (2–3 μmoles) did not exert any competitive inhibition on the conversion of D-glucuronolactone into ascorbic acid in the presence of cyanide.

Effect of variation of tissue concentration. It may be mentioned that under the influence of cyanide rat-liver slices could not convert D-glucuronolactone into L-ascorbic acid. It has also been noted that the homogenate as well as the extract contain some factors inhibitory to the synthesis of ascorbic acid under the conditions of the experiment, since it was found that the formation of ascorbic acid did not increase with increasing homogenate or extract concentration but rather decreased after a certain point (Fig. 2). But when the enzyme preparation was used instead, the synthesis became nearly proportional to the concentration of the former, the formation of ascorbic acid increasing with increasing enzyme concentration (Fig. 2). More recently it has been observed that the inhibitory factors are present in the supernatant fraction obtained after sedimentation of the liver extract at 88 700 *g* (Chatterjee, Ghosh, Ghosh & Guha, 1958*b*).

Other properties of the enzyme system. Treatment of the enzyme preparation with acetone or with alcohol (20%) or dilute acid (to pH 5·0), at 0°, resulted in inactivation of the enzyme system. When kept at −5°, it retained the full activity for about 2–3 weeks. When air was replaced by N_2 in the incubation media, no synthesis of ascorbic acid was observed either from D-glucuronolactone or L-gulonolactone. The enzyme concentrate, when dialysed against 10 mM-phosphate buffer at 0° even up to 60 hr., did not lose its activity for the conversion of D-glucuronolactone or L-gulonolactone. The optimum substrate concentration for the overall reaction was found to be 10 mM (Fig. 3), optimum pH was in the region of 6·5–7·0 (Fig. 4) and optimum temperature range was 37–42°. The maximum formation of ascorbic acid took place after an incubation period of 1·5 hr., after which no further increase was observed (Fig. 5).

Effect of metabolic inhibitors. The effects of some inhibitors on the cyanide-mediated biosynthesis were studied. Sodium azide, 2:4-dinitrophenol, phlorrhizin, arsenate, arsenite, pyrophosphate, each in mM-concentration, and sodium fluoride (up to 10 mM), were found to have no influence on

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

Effect of thiol reagents. In studying the effect of some thiol reagents it has been found that mercuric chloride or *p*-chloromercuribenzoate, when used at a concentration of mM, could inhibit the synthesis to about 50%. Iodoacetate up to a concentration of 5 mM was without effect. Reduced glutathione (GSH), which was found incapable of further stimulating the effect of cyanide, could reverse the inhibition by *p*-chloromercuribenzoate to about 94% (Table 3). This indicates a possible involvement of some thiol group in the enzyme system catalysing the conversion of D-glucuronolactone into L-ascorbic acid *in vitro*.

Hassan & Lehninger (1956), however, observed that concentrations of GSH above 8 mM were significantly inhibitory to the rate of formation of ascorbic acid in their system. It is likely that the inhibition observed by them might be due to the fact that the method of Roe & Kuether (1943), which was used by them for the determination of 'total' ascorbic acid, would give low values for ascorbic acid in the presence of 5 mM and higher

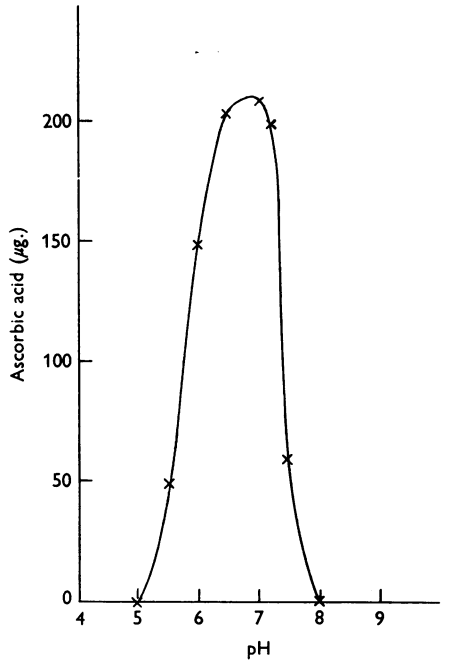

Fig. 4. Influence of pH on the enzymic conversion of D-glucuronolactone into L-ascorbic acid.

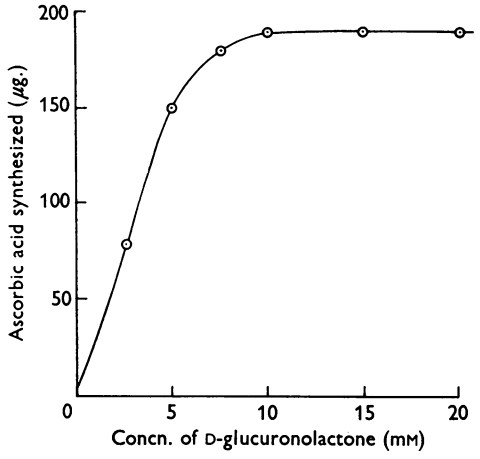

Fig. 3. Rate of formation of ascorbic acid as affected by substrate concentration.

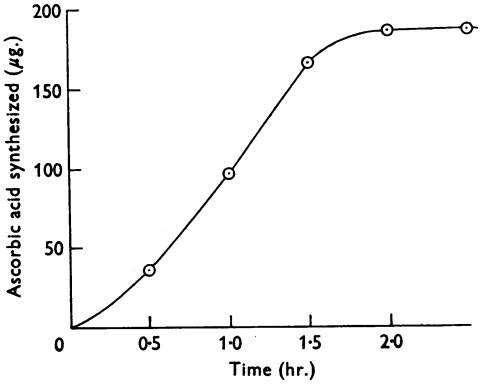

Fig. 5. Rate of formation of ascorbic acid.

Table 3. *Effect of thiol reagents on the synthesis of ascorbic acid from* D-*glucuronolactone* in vitro

Inhibitor	Concn. used (mM)	Ascorbic acid (μg.)	Inhibition (%)
None	—	200	—
Iodoacetate	5	200	0·0
Mercuric chloride	1	123	38·5
Mercuric chloride	5	0	100·0
p-Chloromercuribenzoate	1	88	56·0
p-Chloromercuribenzoate	5	0	100·0
GSH	10	200	0·0
p-Chloromercuribenzoate *plus* GSH	1 + 10	193	3·5

Table 4. *Inhibitory effects of adenosine triphosphate and uridine triphosphate on the biosynthesis of ascorbic acid* in vitro

ATP, adenosine triphosphate; UTP, uridine triphosphate.

Substrate	Inhibitor	Concn. used (mM)	Ascorbic acid (μg.)	Inhibition (%)
D-Glucuronolactone	None	—	228	—
	ATP	1	114	48
	UTP	1	89	61
L-Gulonolactone	None	—	180	—
	ATP	1	56	69
	UTP	1	36	74

concentrations of GSH. Thus in a recovery experiment starting with pure L-ascorbic acid it has been found that in the presence of 8 mM-GSH only 10–12 μg. out of 25 μg. of ascorbic acid could be estimated by that method.

Effect of other enzyme inhibitors. In studying the effects of other enzyme inhibitors it has been found that borate and Antabuse (disulfiram), each at mM-concentration, inhibited the cyanide-stimulated synthesis to about 50%. The inhibition by borate, however, was not reversed by sorbitol or ribose.

Effect of certain hydrogen acceptors. Dehydroascorbic acid (2–3 μmoles) or methylene blue (0·1 mM) had no influence on the rate of conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide.

Requirement of cofactors

The fact that cyanide in a concentration of 50 mM, which can bind the DPN and TPN of reaction media (Colowick, Kaplan & Ciotti, 1951), could effect the biosynthesis indirectly indicates that DPN and TPN are not involved in this synthesis under the experimental conditions used. Moreover the liver extract, after treatment with active charcoal to free it from nucleotides (Stadtman, Novelli & Lipmann, 1951), retained its original activity (without any added cofactor) for the conversion of D-glucuronolactone into L-ascorbic acid.

It has also been found that in the presence of cyanide none of the cofactors examined, e.g. DPN, TPN, coenzyme A, glucose 1-phosphate, glucose 6-phosphate, reduced DPN, reduced TPN (generated *in situ* by addition of TPN, glucose 6-phosphate and glucose 6-phosphate dehydrogenase), each at mM concentration, $MgCl_2$ (5 mM), nicotinamide (30 mM), flavinadenine dinucleotide (0·1 mM) and flavinadenine mononucleotide (0·1 mM), was needed for the conversion of D-glucuronolactone into L-ascorbic acid by the dialysed enzyme concentrate *in vitro*. With L-gulonolactone, its conversion into L-ascorbic acid, as mentioned above, did not require any cyanide, and was not further enhanced by addition of DPN.

ATP and uridine triphosphate, on the other hand, when used at mM-concentrations in the incubation media, were found to have pronounced inhibitory effects on the rate of synthesis (Chatterjee, Ghosh, Ghosh, Roy & Guha, 1957*b*). This inhibition was not reversed by glucose and Mg^{2+} ions. No inhibition was observed with adenosine diphosphate (ADP), adenosine monophosphate, adenosine, adenine chloride or uridine diphosphate. The fact that the inhibition obtained was not due to the destruction of synthesized ascorbic acid in the presence of added ATP was revealed from a recovery experiment in which all of the ascorbic acid (2–5 μmoles), added to the tissue in the presence of ATP, could be estimated after an incubation period of 2 hr. The inhibitory effects of ATP and uridine triphosphate were also observed when D-glucuronolactone was substituted by L-gulonolactone as the substrate (Table 4).

DISCUSSION

The fact that D-glucuronolactone is converted into L-ascorbic acid in the presence of cyanide and that the conversion of L-gulonolactone into L-ascorbic acid takes place in the absence of cyanide indicates that the mechanism of action of cyanide relates to the reduction of D-glucuronolactone to L-gulonolactone. The overall synthesis of L-ascorbic acid from D-glucuronolactone is inhibited by *p*-chloromercuribenzoate, which effect is reversed by reduced glutathione. These results indicate that some –SH group or groups are probably involved in this reduction and that at least one of the functions of cyanide is to protect the –SH group from its oxidation to the disulphide linkage. Since the conversion of D-glucuronolactone into L-ascorbic acid is not inhibited by 2:4-dinitrophenol or sodium azide it would appear that phosphorylative reactions are not involved in the biosynthesis stimulated by cyanide.

There are apparently some fundamental differences between the animal and the plant systems in both the intracellular localization of the enzymes concerned and the nature of the enzyme systems involved. Mapson *et al.* (1954) have found that in

the plant system it is the mitochondria which catalyse the conversion of L-galactono-γ-lactone into L-ascorbic acid. This conversion probably involves the cytochrome system, as indicated by its inhibition with cyanide. In the rat liver, microsomes are the site of the enzyme system converting L-gulono-γ-lactone into L-ascorbic acid (Burns, Peyser & Moltz, 1956; Chatterjee *et al.* 1958*b*) and, as shown in this paper, the overall synthesis from D-glucuronolactone is activated by cyanide. There seems to be one point of similarity between the plant and the animal systems, namely, the inhibitory effect of ATP on the biosynthesis of ascorbic acid in the plant system and on the cyanide-stimulated biosynthesis with rat-liver tissue. The inhibition by ATP and uridine triphosphate may possibly be due to their promoting the decarboxylation of the substrate (Rabinowitz & Sall, 1957), which may be competitive with ascorbic acid formation.

That no added cofactor is needed for the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide, and that the dialysed enzyme retains the full activity, point to the possibility that the coenzymes required for the overall conversion in the reaction studied are firmly bound to the enzyme system concerned. Preliminary experiments indicate that flavoproteins may have some role in the oxidation–reduction system involved in the biosynthesis. All efforts, however, to resolve the enzyme system into apoenzyme and coenzyme have so far failed.

In the presence of cyanide, only the lactone form and not the sodium salt of the free acid is converted into ascorbic acid. Although ascorbic acid synthesis increases with enzyme concentration (Fig. 2) a high conversion has not been obtained. This has been traced to the fact that the enzyme preparation has a strong lactonase activity, rapidly hydrolysing the lactone to the free acid. It should be mentioned that in plant systems also only the lactone has been found to be the active precursor (Mapson *et al.* 1954). Using rat-liver extract Hassan & Lehninger (1956) have found that both the lactones and the sodium salts of D-glucuronic and L-gulonic acids are equally effective; addition of ATP and other cofactors is essential for this system. On the contrary, as reported in this paper, ATP has been found to be distinctly inhibitory to the conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide. It is possible that there exist two separate pathways for the synthesis of ascorbic acid *in vitro*, one from the free acid requiring ATP and other cofactors, and catalysed by the enzyme system present in the supernatant as well as in the microsomes as suggested by Grollman & Lehninger (1957), and the other from the lactone only, which is stimulated by cyanide and requires no added cofactor. The latter enzyme system has recently been found to be localized entirely in the microsomes and is, in fact, inhibited by some factors present in the soluble supernatant (Chatterjee *et al.* 1958*b*). It has been observed that the addition of potassium cyanide to Hassan & Lehninger's system containing rat-liver microsomes, soluble supernatant and cofactors, further stimulates the synthesis of ascorbic acid from D-glucuronolactone (Chatterjee *et al.* 1957*a*). What exactly the relation is between the two systems will require further study.

SUMMARY

1. Potassium cyanide (50 mM), without any added cofactor, greatly stimulates the biosynthesis of L-ascorbic acid from D-glucuronolactone by an enzyme preparation from goat-liver extract containing the microsomes and the soluble supernatant.

2. The enzyme preparation can also convert L-gulonolactone into L-ascorbic acid with or without the presence of cyanide and in the absence of any added cofactor. But conversion is greater in the absence of cyanide.

3. The dialysed enzyme concentrate retains its full activity. Addition of cofactors such as di- or tri-phosphopyridine nucleotide, reduced di- or tri-phosphopyridine nucleotide, nicotinamide, magnesium chloride, coenzyme A, glucose 1-phosphate, glucose 6-phosphate and flavinadenine di- and mono-nucleotide does not further enhance the synthesis.

4. In the presence of cyanide only the lactone forms of the substrates are active. Neither the sodium salts of the free acids nor any other substrate tried are converted into ascorbic acid under the conditions studied.

5. The conversion of D-glucuronolactone is inhibited by *p*-chloromercuribenzoate and this inhibition is reversed by reduced glutathione, indicating the probability of the involvement of some thiol groups in the enzyme system concerned in the cyanide-mediated synthesis.

6. Adenosine triphosphate and uridine triphosphate have been found to be strikingly inhibitory to the synthesis of ascorbic acid from D-glucuronolactone and L-gulonolactone *in vitro* in the presence of cyanide.

7. The conversion of D-glucuronolactone into L-ascorbic acid in the presence of cyanide is not inhibited by sodium fluoride, sodium azide, 2:4-dinitrophenol, phlorrhizin, arsenite, arsenate or pyrophosphate.

8. Cyanide cannot be replaced by sodium azide, carbon monoxide and reducing substances such as reduced glutathione, sodium dithionite or sodium

hypophosphite for the conversion of D-glucurono-lactone into L-ascorbic acid.

9. The presence of borate or Antabuse, each at mM-concentration, inhibits the conversion of D-glucuronolactone into L-ascorbic acid to about 50%.

10. The optimum conditions for the action of the enzyme system have been studied.

11. The biosynthesized ascorbic acid has been identified by paper chromatography and by a biological test with one guinea pig.

The authors wish to express their thanks to Dr J. J. Burns, of the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md., U.S.A. and Dr M. L. Wolfrom, Ohio State University, Columbus, Ohio, U.S.A., for samples of L-gulono-γ-lactone, and to the Indian Council of Medical Research for financing this work.

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Identification of 6-*O*-Acetyl-D-Glucopyranose in *Bacillus megaterium* Cultures: Synthesis of 6-*O*-Acetyl-D-Glucopyranose and 6-*O*-Acetyl-D-Galactopyranose

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(*Received* 2 *April* 1958)

This paper deals with the characterization of the crystalline carbohydrate obtained from *Bacillus megaterium* (NCIB 8508) cultures (Duff & Webley, 1958; and Duff, Webley & Farmer, 1957).

Preliminary examination (including infrared analysis and paper chromatography) showed that this compound was an ester of equimolar proportions of glucose and acetic acid. One carbonyl group per molecule was found with assays made at pH values within a narrow range around neutrality to avoid removal of a possible labile acetyl group. Separate experiments (Table 1) showed that little or no hydrolysis occurred at these pH values. From the above-mentioned evidence and because an acetylated phenylhydrazone and osazone were formed, it seemed unlikely that the hydroxyl groups on $C_{(1)}$ or $C_{(2)}$ were esterified. The failure to form a triphenylmethyl (trityl) ether strongly suggested that there was no free primary hydroxyl group. Helferich (1948) states that no case has been reported of a primary hydroxyl group unreactive towards trityl chloride with the conditions