of D-glyceric and L-ascorbic acids possibly exists in the rat, for treatment with the drug chloretone causes the rat to excrete in its urine increased amounts of both D-glyceric and L-ascorbic acids.

4. It is clear, however, that D-glyceric acid is not a direct precursor of L-ascorbic acid, since feeding the ethyl ester of D-glyceric acid (the ester was used as a convenient method of introducing D-glyceric acid into the cell) to cress seedlings or injecting it into the rat did not increase the formation of L-ascorbic acid. The connexion between the synthesis of D-glyceric acid and the synthesis of L-ascorbic acid remains obscure.

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Biological Synthesis of L-Ascorbic Acid: the Conversion of L-Galactono-γ-lactone into L-Ascorbic Acid by Plant Mitochondria

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Evidence presented by Isherwood, Chen & Mapson (1953) has indicated that the synthesis of L-ascorbic acid *in vivo* in both plants and animals can be represented in outline by one or both of the following sequences:

(1) D-glucose→D-glucurono-γ-lactone→L-gulono-γ-lactone→L-ascorbic acid;

(2) D-galactose→D-galacturonic acid methyl ester→L-galactono-γ-lactone→L-ascorbic acid.

In the present paper an account is given of the enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by extracts of plant tissues. A following paper will deal with the conversion both of L-gulono-γ-lactone and L-galactono-γ-lactone to L-ascorbic acid by extracts from animal tissues.

EXPERIMENTAL

Selection of material. Most plant tissues contain highly active enzymic systems capable of oxidizing ascorbic acid, and this fact had to be taken into account before selecting a tissue in which to demonstrate the synthesis of ascorbic acid. Clearly it is more difficult to study the subject in tissues which are actively oxidizing the vitamin, the use of inhibitors of oxidases, e.g. cyanide, being precluded by the knowledge, from our earlier studies *in vivo*, that this also inhibits the synthesis (Isherwood, Chen & Mapson, unpublished observations). It was desirable to choose a tissue in which the rate of synthesis *in vivo* was known to be rapid and, for this reason, seeds during the early stage of germination were selected. Most of the experiments reported in this paper have been carried out with pea seeds, but sufficient work has been done with mung bean seeds to show that the pea seed is not exceptional.

Pea seedlings contain an active ascorbic acid oxidase, but fortunately for our purpose the oxidative enzyme activity in the very early stage of germination is low, not reaching its full development until some 120 hr. after germination at 25°. The varieties of pea seeds used included Laxton Superb, Kelvedon Wonder and Onward, and differed only in their degree of activity.

Enzyme material—plant tissues. Dry pea seeds were soaked in water for periods of 12–48 hr. at 20°. The soaked seeds (30 g.) were ground up with sand and 40 ml. of medium of the desired composition in a mortar. The brei was centrifuged at approximately 500 *g* for 5 min. and the supernatant solution removed. This solution is referred to in the text as the whole extract and cytoplasmic particles (mitochondria) were separated from it by centrifuging at 10000 *g* for 20 min. Washed mitochondria were prepared by suspending the residue from the first centrifugation in 20 ml. of the medium and recentrifuging, this procedure

being repeated several times. All of these operations were carried out at a temperature of 1°, the extraction apparatus and extraction media being cooled to this temperature before use. Unless otherwise stated, the tests for enzymic activity were carried out at 37°.

Estimation of ascorbic acid. This was carried out by removing 1 ml. samples, acidifying with 2% HPO_3, removing protein by centrifugation, and titrating ascorbic acid against 2:6-dichlorophenol indophenol.

L-*Galactono-γ-lactone.* This was normally added in a concentration of 0·5 mg. to 1 mg./ml. of reaction mixture. The lactone was prepared as described previously (Isherwood, Chen & Mapson, 1954), as also were the L-gulono-γ-lactone and the other sugar γ-lactones used in this work.

Oxygen consumption. This was estimated by the standard Warburg technique.

RESULTS

Conversion of L-*galactono-γ-lactone into* L-*ascorbic acid*

After many unsuccessful attempts, it was found that preparational procedures in which the tissue was extracted in the cold with a solution containing 0·4 M sucrose, 0·1 M phosphate and 4×10^{-3} M-Mg^{2+} were successful. These are identical with those used by Millerd, Bonner, Axelrod & Bandurski (1951) for the extraction of plant mitochondria. The extracts so obtained catalysed the formation of L-ascorbic acid from L-galactono-γ-lactone and the ascorbic acid so formed was identified as L-ascorbic acid by the chromatographic technique which we have already described (Chen, Isherwood & Mapson, 1953). The enzymic activity of these extracts was found to be centred in the mitochondrial elements of the cell (Fig. 1), there being little or no activity in the soluble fraction. These mitochondrial preparations did not catalyse the oxidation of ascorbic acid, hence the synthesis could be studied without the complications due to oxidase activity. This, however, as will be shown later, depends critically on the stage of germination.

The reaction proceeded at an almost linear rate, ceasing when about 40% of the lactone had been converted to ascorbic acid. The addition of a further quantity of the lactone at this stage led to the synthesis of further amounts of ascorbic acid, which indicates that the reaction stopped owing to the disappearance of substrate rather than to destruction of enzyme activity (Fig. 1).

Three possible explanations of the failure to obtain a higher percentage of conversion suggested themselves: (1) that under the experimental conditions a 40% conversion represented the equilibrium point of the reaction L-galactono-γ-lactone ⇌ L-ascorbic acid, (2) that galactonic acid inhibited the reaction and (3) that the lactone was converted into free acid. The first explanation was shown to be incorrect when it was found that L-ascorbic acid, added at the start of the reaction in a concentration

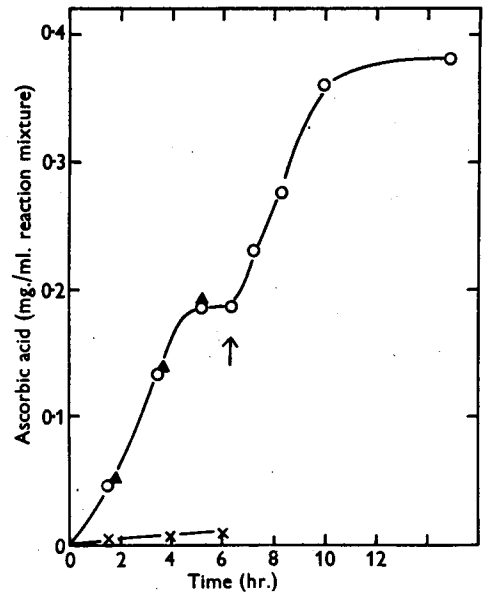

Fig. 1. The conversion of L-galactono-γ-lactone to L-ascorbic acid. Reaction mixture 0·4 M sucrose, 0·1 M-PO_4 (pH 7·4), 4×10^{-3} M-Mg^{2+}, containing initially 0·5 mg. L-galactono-γ-lactone/ml. ▲—▲, Whole extract (mitochondria + soluble fraction); O—O, mitochondria; ×—×, soluble fraction of whole extract; ↑ denotes addition of a further amount of L-galactono-γ-lactone (0·5 mg./ml. reaction mixture).

equal to that synthesized in control experiments, had no effect on the rate of synthesis; the second was disproved by the fact that galactonic acid, added in equivalent amount to L-galactono-γ-lactone, had no influence on the subsequent course of the reaction. At a neutral pH the γ-lactones of the sugar acids are known to be converted slowly to the free acids and we have found that galactonic acid is not oxidized to L-ascorbic acid by these enzyme preparations. It appears probable therefore that the third explanation is correct and, if so, this would explain both the continual synthesis which occurs on addition of more of the lactone (Fig. 1), and the higher percentage conversion of the lactone to ascorbic acid as the rate of synthesis is increased. When the rate of the enzymic oxidation is high, as with mitochondrial preparations of great activity, a higher proportion of the lactone is changed to ascorbic acid than with preparations of lower activity. This is undoubtedly due to the fact that the longer reaction times involved in the second case permit a greater proportion of the lactone to be transformed into the non-reactive galactonic acid.

The mitochondrial preparations could be washed two to three times with the sucrose : phosphate : magnesium mixture without any decrease in the enzyme activity, although each successive washing reduced

the oxygen consumption of the mitochondria themselves. We have not as yet ascertained whether more exhaustive washing would have any influence on the activity of these preparations. Preliminary experiments indicate that the addition of the lactone increases the oxygen consumption of both the washed and unwashed mitochondria more than can be accounted for by the conversion of L-galactono-γ-lactone to L-ascorbic acid.

Conversion of related sugar-γ-lactones

In view of the previous work (Isherwood *et al.* 1954) in which both L-gulono-γ-lactone and L-galactono-γ-lactone were converted to L-ascorbic acid in intact plants and animals, we anticipated that preparations active in respect of L-galactono-γ-lactone would also catalyse the synthesis of L-ascorbic acid from L-gulono-γ-lactone. This we have not been able to demonstrate either with the whole extract or with mitochondrial preparations from pea and mung bean seeds; the latter extracts resemble those from pea seeds in their ability to synthesize L-ascorbic acid from L-galactono-γ-lactone. The reason for this remains obscure, especially, as we have found that L-gulono-γ-lactone fed to excised pea embryos increases the formation of L-ascorbic acid, though the reponse is much less than with L-galactono-γ-lactone. The relative formation of L-ascorbic acid in excised embryos grown in water, 0·5 % L-gulono-γ-lactone, or 0·5 % L-galactono-γ-lactone over a period of 24 hr. at 25° was 100, 175 and 1000, respectively.

The formation of D-*arabo*ascorbic acid only from D-altrono-γ-lactone in the plant, and only from D-manno-γ-lactone in the animal (Isherwood *et al.* 1953) prompted us to inquire whether this substance could be produced enzymically from these lactones and it was found that with the plant mitocondria, D-mannono-γ-lactone was not converted to L-ascorbic acid; on the other hand, D-altrono-γ-lactone was converted to D-*arabo*ascorbic acid.

L-*xylo*Hexulonic acid (2-keto-L-gulonic acid) has been suggested by some workers (Smythe & King, 1942; Smith, 1952) as a possible precursor of L-ascorbic acid, and Galli (1946) has claimed that *Aspergillus niger* can convert it to ascorbic acid: we failed however to detect any formation of L-ascorbic acid when L-*xylo*hexulonic acid was added to our enzyme preparations.

Rate of reaction as affected by concentration of L-galactono-γ-lactone and other related compounds

The maximum rate of conversion of L-galactono-γ-lactone to L-ascorbic acid occurs at low substrate concentrations. A substrate/velocity curve is shown in Fig. 2. The apparent Michaelis constant is of the order of 10^{-4} M. The overall rate of the reaction would appear to be limited by the rate of hydrogen transfer from the lactone rather than by the rate of oxygen activation by the cytochrome system for, as shown later, inhibitors which are known to reduce the activity of the latter had little or no effect on the rate of formation of ascorbic acid from the lactone.

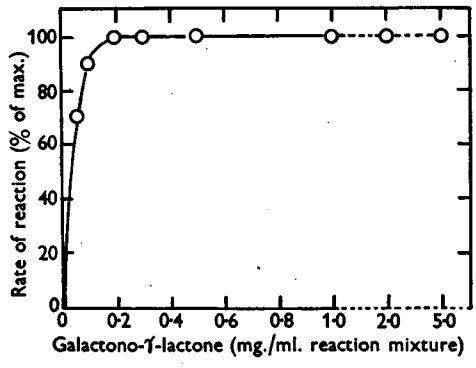

Fig. 2. The rate of formation of L-ascorbic acid as affected by substrate concentration.

Since we failed to find any evidence for the conversion of L-gulono-γ-lactone or of D-manno-γ-lactone to ascorbic acid by the plant mitochondria, it was of interest to determine whether these substances exerted any competitive inhibition on the formation of L-ascorbic acid from L-galactono-γ-lactone.

Neither L-gulono-γ-lactone, D-galactono-γ-lactone, nor D-manno-γ-lactone when used in an equivalent concentration of 2 to 5 times that of L-galactono-γ-lactone, had any appreciable effect on the rate of conversion of the latter substance to L-ascorbic acid; also neither L-*xylo*hexulonic acid nor, as mentioned earlier, galactonic acid had any influence on the course of the reaction.

Isherwood *et al.* (1954) noted that DL-glyceraldehyde but not D-glyceraldehyde when fed to intact cress seedlings depressed the synthesis of L-ascorbic acid. In conformity with these results we have observed that DL-glyceraldehyde in concentrations of 10^{-2} M and higher inhibits the enzymic formation of L-ascorbic acid from the γ-lactone. On the other hand, we have also observed inhibition by D-glyceraldehyde. The different result obtained *in vivo* as against that obtained *in vitro* may be due to the more rapid metabolism of the D-isomer in *vivo*.

Effect of different extraction procedures

As previously mentioned, extraction of the tissue with sucrose in a concentration of 0·4 M with 0·1 M phosphate yielded the most active preparations. Very similar results on the activity of mitochondria in oxidizing acids of the tricarboxylic acid cycle have been reported by Millerd (1953), who found that the mitochondrial preparations possessed the greatest

oxidative activity when prepared with a sucrose: phosphate medium similar to that used by us. If phosphate was eliminated, the particles failed to oxidize α-oxoglutarate but could still oxidize succinate, whereas if extracted with phosphate alone they could still oxidise α-oxoglutarate. With pyruvate both sucrose, or mannitol, and phosphate were necessary, at all stages during the preparation.

The ability of mitochondria to convert L-galactono-γ-lactone to L-ascorbic acid appears likewise to be dependent on their full activity. If either sucrose or phosphate is omitted during extraction from the tissue, inactive preparations result, even though these substances are added subsequently to the extract. The effect on the activity of these mitochondrial preparations of altering the sucrose content of the media is shown in Fig. 3*a*. Mitochondria were extracted from peas with media containing 0·1 M phosphate and 4×10^{-3} M-Mg^{2+}, with different concentrations of sucrose. The extracted mitochondria were washed and resuspended in the same media for the reaction. The results show that a concentration of 0·4 M of sucrose appears to be optimum, with a fairly sharp decrease in activity at higher or lower levels of sucrose. Millerd found 0·3 M sucrose to be optimum for the oxidation of pyruvate by mung bean mitochondria.

We have confirmed the observations of Laties (1951) that sucrose may be replaced by mannitol, and have also extended this to glucose. It seems probable that many other non-electrolytes would behave similarly.

Influence of phosphate

Millerd believed that the necessity for having high phosphate levels in order to retain maximum activity was due to the inhibition of a phosphorolytic breakdown of some essential component. In support of this she found that 0·1 M fluoride, a phosphatase inhibitor, could replace the phosphate to a large extent. The rate of oxidation of α-oxoglutarate by mitochondria prepared by extraction with 0·4 M sucrose, 0·01 M phosphate and 0·1 M fluoride, was as great as when they were prepared by the higher phosphate: sucrose medium, but it is worth noting that the oxidation of pyruvate was, however, lower under these conditions. Our experiments have shown likewise that fluoride can only partially replace phosphate.

To determine the optimum amount of phosphate necessary mitochondria, prepared by extraction with 0·4 M sucrose, 0·1 M phosphate and 4×10^{-3}-Mg^{2+}, were washed once with a sucrose: magnesium medium containing different levels of phosphate. The washed mitochondria were then re-suspended in the same medium. The results of such experiments are illustrated in Fig. 3*b*; they show that reduction of phosphate below a level of 0·1 M seriously affects the rate of the reaction. Such data give no indication as to whether phosphate is necessary for maintaining the integrity of the mitochondria, or as a participant in the reaction itself. Some evidence which indicates that the reaction is not dependent on the production of high-energy phosphate bonds is forthcoming from experiments in which it has been shown that mitochondrial preparations may be prepared by extraction with sucrose: arsenate mixtures, and washed 2 or 3 times with the same solutions, and yet possess as great an activity as when sucrose: phosphate mixtures are employed.

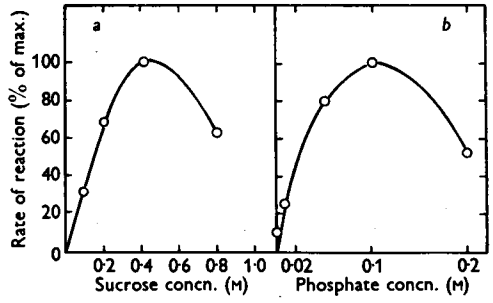

Fig. 3*a*. The influence of concentration of sucrose on the activity of mitochondria in converting L-galactono-γ-lactone to L-ascorbic acid.

Fig. 3*b*. The activity of mitochondria in converting L-galactono-γ-lactone to L-ascorbic acid, as influenced by the concentration of phosphate.

In view of such results, the influence of phosphate, as of arsenate, seems to us to be due to the effect these substances have in preserving and maintaining the integrity of the mitochondria. These results do not, of course, exclude the possibility that phosphate may be concerned in the oxidation of L-galactono-γ-lactone to L-ascorbic acid, though, so far, we have no direct evidence for this.

Influence of magnesium ions and adenosine triphosphate

Millerd (1953) showed in her experiments that both magnesium ions and adenosine triphosphate (ATP) when added to washed mitochondria caused a very considerable increase in the rate of oxidation of most of the acids of the tricarboxylic cycle. In contrast to this, the effect of magnesium ions on the oxidation of L-galactono-γ-lactone to L-ascorbic acid was small.

In these experiments, the mitochondria were prepared by extraction from the tissue with a magnesium-free sucrose: phosphate medium, washed once, and resuspended in the same medium. The omission of magnesium during extraction and washing reduced the rate by 10%, which was not increased if magnesium was subsequently added to the reaction mixture.

The addition of ATP in a concentration of 1×10^{-3}M to washed mitochondria cut down the rate of formation of ascorbic acid from galactono-γ-lactone by 40%. This inhibition was observed both in solutions of high (0·1M) and low (0·01M) phosphate concentrations and is not yet explained.

Effect of pH

The washed mitochondria were taken up in a sucrose:phosphate mixture containing 0·1M phosphate at different pH values and the results (Fig. 4*a*) show that the reaction is considerably affected by pH, the optimum value being in the region of 7·5 and falling sharply on either side. These results are similar to those reported by Slater & Cleland (1953) for the oxidation of α-oxoglutarate by cytoplasmic particles from heart muscle.

Hydrogen acceptors

As was to be expected, the oxidation of L-galactono-γ-lactone is dependent on oxygen and there is no reaction in its absence. Using washed mitochondria the oxygen tension may be reduced to 0·5% without any effect in the rate of formation of L-ascorbic acid (Fig. 4*b*).

Other substances have been tested to determine their ability to act as hydrogen acceptors. Under anaerobic conditions the rate of reduction of methylene blue by washed mitochondria was not increased by the addition of the lactone. Oxidized glutathione, dehydroascorbic acid and fumarate cannot function as hydrogen acceptors.

Action of cyanide, azide and carbon monoxide

The effects of cyanide, azide and carbon monoxide on the reaction were tested to determine whether the cytochrome system was involved. Cyanide (1×10^{-3}M) was found to reduce the rate of formation of ascorbic acid from the lactone very considerably, whether tested in the whole extract or on the mitochondria; with mitochondria alone an inhibition of 90% was obtained.

On the whole extract, azide ($1–4 \times 10^{-3}$M) inhibited the reaction almost completely during the first 2 hr. Subsequently, however, there was a considerable acceleration in the rate (Fig. 5*A*). When used in the same concentrations with mitochondria, azide inhibited the rate to only a small extent and in some experiments with azide at 1×10^{-3}M no inhibition was observed. This apparent anomaly was resolved when it was found that, with washed mitochondria, the inhibitory effect of azide was increased in the presence of respirable substrates, e.g. succinate or α-oxoglutarate (Fig. 5*B*, *C*). Evidently the residual cytochrome oxidase activity, left after treatment with azide, was sufficient to allow both the endogenous respiration of the mitochondria and any increase due to the addition of the galactono-γ-lactone to proceed at a maximum rate. In the presence of added substrates, competition for the available cytochrome was large enough in the case of succinate to suppress completely for a time, and in the case of α-oxoglutarate to reduce, the rate of formation of ascorbic acid from the lactone. As seen in Fig. 5*C*, with succinate the inhibition was only temporary but could be re-established by the addition of further succinate. In the absence of azide, in air, the addition of succinate at the levels tested had no adverse effect on the formation of ascorbic acid.

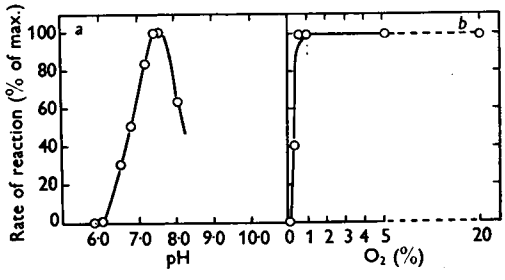

Fig. 4*a*. The influence of pH on the enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by mitochondria.

Fig. 4*b*. Effect of oxygen tension on the rate of formation of L-ascorbic acid from L-galactono-γ-lactone by mitochondria.

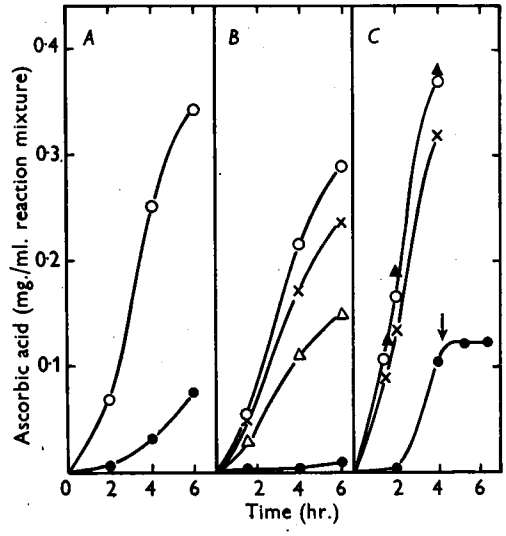

Fig. 5. The inhibitory effect of azide on the formation of L-ascorbic acid from L-galactono-γ-lactone on whole extracts (mitochondria + soluble fraction) and on washed mitochondria in the presence and absence of respirable substrates. (*A*) Whole extract: ○—○, control; ●—●, 4×10^{-3}M azide. (*B*) Mitochondria: ○—○, control; ×—×, 4×10^{-3}M azide; △—△, azide + 2×10^{-2}M α-oxoglutarate; ●—● azide + $2{\cdot}5 \times 10^{-2}$M succinate. (*C*) Mitochondria: ○—○, control; ▲—▲, 1×10^{-2}M succinate; ×—×, 4×10^{-3}M azide; ●—●, azide + 1×10^{-2}M succinate; ↓ addition of more 1×10^{-2}M succinate.

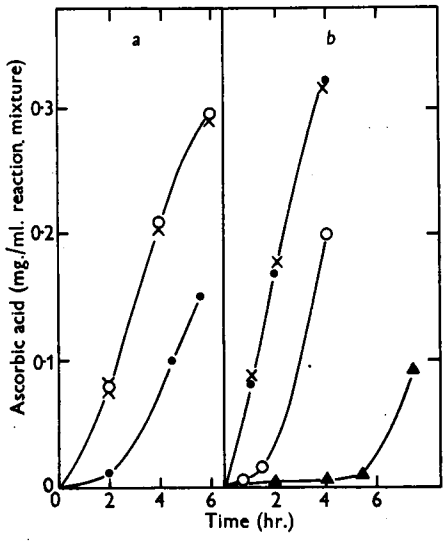

Fig. 6. The influence of CO on the formation of L-ascorbic acid from L-galactono-γ-lactone on whole extracts (mitochondria + soluble fraction) and on washed mitochondria in the presence and absence of succinate. All experiments carried out in the dark. (*a*) Whole extract: ○—○, in air; ×—×, in 95% N_2, 5% O_2; ●—●, in 95% CO, 5% O_2. (*b*) Mitochondria: ×—×, in 95% N_2, 5% O_2; ●—●, in 95% CO, 5% O_2; ○—○, in 95% N_2, 5% O_2 + succinate 2×10^{-2} M; ▲—▲, in 95% CO, 5% O_2 + succinate 2×10^{-2} M.

Very similar results were observed with carbon monoxide. With gas mixtures containing 95% CO and 5% O_2 in the dark, there was no inhibition of the reaction with washed mitochondria, but a temporary inhibition with whole extracts (Fig. 6*a*, *b*) when compared with controls containing 95% N_2, 5% O_2. As in the azide experiments, inhibition of the reaction due to the presence of carbon monoxide could be observed on the addition of succinate (Fig. 6*b*). A short temporary inhibition due to the addition of succinate was observed even with the controls in 95% N_2, 5% O_2, but a much longer one was observed with 95% CO, 5% O_2.

To demonstrate inhibition by carbon monoxide in mitochondria in the absence of added substrates, gas mixtures of 97·5% CO, 2·5% O_2 were made up. With these, an inhibition by carbon monoxide was observed, as compared with controls in gas mixtures containing 95% N_2, 2·5% O_2. This inhibition by carbon monoxide was reversed by light (Fig. 7).

The results described in this section indicate that the cytochrome system is involved as the terminal oxidase in the formation of L-ascorbic acid from L-galactono-γ-lactone. It seems clear also that, with mitochondria, the rate of formation of ascorbic acid is not limited by the activity of the cytochrome system.

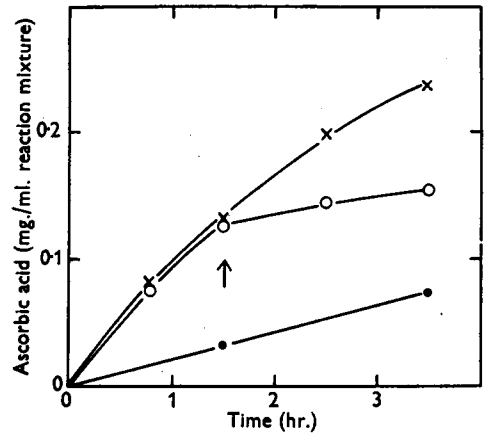

Fig. 7. Inhibition of the formation of L-ascorbic acid from L-galactono-γ-lactone by CO, and reversal by light. ×—×, Mitochondria in atmosphere of 97·5% N_2, 2·5% O_2; ○—○, mitochondria in atmosphere of 97·5% CO, 2·5% O_2 in light; ↑ denotes change from light to dark; ●—●, mitochondria in atmosphere of 97·5% CO, 2·5% O_2 in dark.

Effect of other enzyme inhibitors

We thought it of interest to examine the effect of 2:4-dinitrophenol (DNP) on the conversion of L-galactono-γ-lactone to L-ascorbic acid, because Loomis & Lippman (1948) have shown that this substance suppresses mitochondrial phosphorylative reactions without affecting those concerned with oxidation. Bonner & Millerd (1953) have further shown that 10^{-4} M DNP completely suppresses oxidative phosphorylation with plant mitochondria, without affecting oxygen consumption. The experiments we have carried out show that DNP (5×10^{-4} to 1×10^{-3} M) has absolutely no effect on the rate of conversion of L-galactono-γ-lactone to L-ascorbic acid. The same result is obtained whether the mitochondria have been washed or not. This evidence taken in conjunction with that given earlier in this paper suggests that phosphorylation reactions are not involved in the formation of L-ascorbic acid from L-galactono-γ-lactone.

Of the other inhibitors studied, fluoride (1×10^{-3} to 1×10^{-2} M) had no influence on the course of the reaction, whereas iodoacetate, arsenite and alloxan (each 1×10^{-3} M) inhibited the reaction by 30, 5 and 25%, respectively.

Activity of mitochondria prepared from peas at different stages of germination

Washed mitochondria were prepared from pea seeds after soaking for 17 hr. at 20°, and from seeds which had been germinated for periods varying from 1 to 5 days at 25°. Their activity in converting L-galactono-γ-lactone to L-ascorbic acid and in oxi-

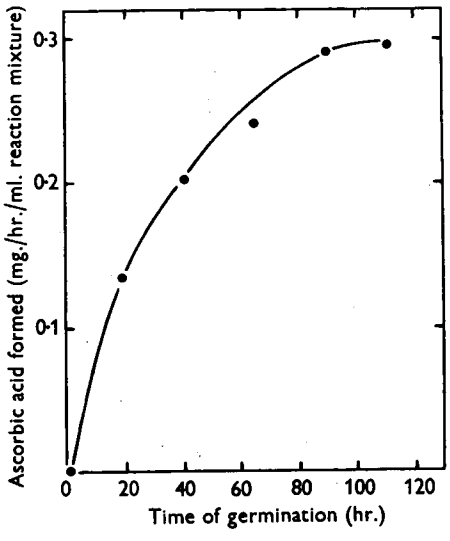

Fig. 8. Increase in enzymic activity of mitochondria prepared from peas at successive stages in germination. Washed mitochondria prepared from 5 g. of germinated peas suspended in 5 ml. of reaction mixture containing 0·4 M sucrose, 0·1 M phosphate (pH 7·4), and 4×10^{-3} M-Mg^{2+} with L-galactono-γ-lactone (1 mg./ml. reaction mixture).

dizing ascorbic acid was tested. The results (Fig. 8) show that extracts from ungerminated dry seeds did not synthesize ascorbic acid from the lactone but, on soaking, a very rapid increase of activity occurred and continued during the first few days of germination. It is worth noting here that this rate of increase of enzymic activity bears a striking resemblance to the rate of formation of ascorbic acid by intact pea seeds during germination, cf. Harris & Ray (1933). The ability of the mitochondria to oxidize ascorbic acid also increased during germination to such an extent that by the 4th to 5th day the initial rapid synthesis of ascorbic acid, which followed on the addition of galactono-γ-lactone to mitochondria prepared from such material, gave way to a very rapid oxidation of the ascorbic acid formed. The factors which govern the oxidation of ascorbic acid by mitochondria seem well worth further investigation. We have observed that unwashed mitochondria, prepared from pea seeds during the first 2 days of germination, do not catalyse the oxidation of added ascorbic acid but, on washing, the activity is noticeably increased. This problem requires further investigation.

DISCUSSION

The results presented in this paper show that cytoplasmic particles can, in the absence of added cofactors, oxidize L-galactono-γ-lactone to L-ascorbic acid. Our failure to observe a similar oxidation of L-gulono-γ-lactone suggests that the natural path of synthesis is via the galacturono-galactono-γ-lactone route rather than by that of glucurono-gulono-γ-lactone. Even more suggestive are the facts which will be presented in a later paper that D-galacturonic methyl ester is, and D-glucurono-γ-lactone is not, converted to L-ascorbic acid.

These observations do not however exclude the possibility that L-ascorbic acid may be synthesized by the gulono route, for the following reasons. The conversion of both glucurono-γ-lactone and L-gulono-γ-lactone to ascorbic acid by the intact seed during germination has been observed by us (Isherwood *et al.* 1954) for cress seedlings and we have found that this is also the case for pea seeds. It is true that the magnitude of the conversion of L-galactono-γ-lactone to ascorbic acid by the intact seedling is considerably greater than with L-gulono-γ-lactone, but this fact is susceptible to different interpretations.

Our failure to observe any synthesis of L-ascorbic acid from L-gulono-γ-lactone by mitochondrial preparations suggests that damage has been caused to some enzymic system necessary for the conversion. This presupposes either that there is a separate and more labile enzyme for the conversion of L-gulono-γ-lactone from that catalysing the corresponding conversion of L-galactono-γ-lactone, or, which seems more probable, that the route of synthesis is L-gulono-γ-lactone→L-galactono-γ-lactone→L-ascorbic acid, and that the ability of the mitochondria to effect the change L-gulono-γ-lactone to L-galactono-γ-lactone has been impaired.

An alternative explanation that L-gulono-γ-lactone may be metabolized along other lines besides being converted to L-ascorbic acid merits consideration, but seems unlikely in view of the fact that we have observed no trace of ascorbic acid being formed on addition of this lactone.

SUMMARY

1. The enzymic conversion of L-galactono-γ-lactone to L-ascorbic acid by mitochondria from pea and mung bean seeds has been demonstrated. Neither L-gulono-γ-lactone nor D-mannono-γ-lactone yield ascorbic acid in these circumstances.

2. Galactonic acid is not converted to L-ascorbic acid by these mitochondrial preparations, and the concurrent conversion of L-galactono-γ-lactone to galactonic acid during the reaction accounts for our failure to obtain quantitative yields of ascorbic acid from the lactone.

3. The conversion appears to be dependent on the integrity of the mitochondria. The most active preparations have been obtained by extraction with sucrose (0·4 M) and phosphate (0·1 M). Arsenate can

replace phosphate both in the extraction of the mitochondria and in the reaction medium.

4. The reaction is dependent on the presence of oxygen, but this may be reduced to a tension of 0·5% without any effect on the reaction velocity. Experiments with cyanide, azide and carbon monoxide suggest that the cytochrome system is involved.

5. 2:4-Dinitrophenol in high concentrations does not, but adenosine triphosphate (1×10^{-3} M) does inhibit the reaction. There is no evidence to suggest the necessity for the participation of compounds containing high-energy phosphate bonds in the formation of ascorbic acid from galactono-γ-lactone.

6. The enzymic activity of mitochondria prepared from peas increased rapidly in the early stages of germination.

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Oxidative Phosphorylation Coupled with the Oxidation of α-Ketoglutarate by Heart-muscle Sarcosomes

2. PHOSPHORUS:OXYGEN RATIO

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Ochoa (1944) was the first to show that the single step oxidation of α-ketoglutarate to succinate in dialysed heart extracts was accompanied by the esterification of inorganic phosphate. The number of atoms of phosphorus esterified per atom of oxygen consumed (the P:O ratio) in these experiments was 1·5, the same as obtained with pyruvate as substrate under otherwise identical conditions. Previously, Ochoa (1943) had shown that after correction for the heavy losses of esterified phosphorus due to the considerable adenosine triphosphatase and low hexokinase activities of his preparations, the P:O ratio with pyruvate was 3. Accordingly, Ochoa (1944) concluded that the corrected ratio with α-ketoglutarate as substrate was also 3.

In more recent work, the phosphorylation losses have been decreased by the addition of hexokinase and glucose. In this way Cross, Taggart, Covo & Green (1949) obtained a mean uncorrected value of $2{\cdot}43 \pm 0{\cdot}42$ (standard deviation) for 62 experiments with rabbit-kidney or rabbit-liver 'cyclophorase' preparations, while Hunter & Hixon (1949) obtained values, as amended by Hunter (1951), a little below 3 with a liver preparation. These determinations, as well as that given by Slater (1950) for a single determination with cat heart (2·79), agreed with Ochoa's conclusions.

In recent years, however, a number of workers have obtained ratios above 3. These, together with the earlier values, are summarized in Table 1. Values obtained with and without malonate have been listed separately. Unless malonate is used there will be some oxidation of the succinate formed from the α-ketoglutarate, resulting in somewhat lower ratios, since the P:O ratio for the oxidation of succinate to fumarate is lower than that obtained in the oxidation of other substrates. As a result of these recent findings and also on the basis of indirect arguments which will be considered in the Discussion, it is now widely believed that the true P:O ratio for the oxidation of α-ketoglutarate to succinate is 4 (e.g. see Hunter, 1951; Ochoa & Stern, 1952; Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). Ochoa's (1943) original data are probably not sufficiently precise to distinguish between a P:O ratio of 3 and 4.