# CCXLVIII. THE NON-SPECIFICITY OF THE ASCORBIC ACID OXIDASE

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THE observation that the retention of *l*-ascorbic acid and its analogues in the body of the guinea-pig was controlled by their stereochemical structure as well as by their antiscorbutic potencies [Zilva, 1935, 1] suggested that the extension of this work to the investigation of the bearing of the spatial arrangement of the molecule on other biochemical reactions might be advisable. It was considered that the advance of the general problem of the mechanism of the biological action of *l*-ascorbic acid in the animal organism might be furthered by this means.

With this end in view attention was directed to the reversible oxidation of *l*-ascorbic acid and its related compounds by enzymes. In a series of investigations the following results emerged. The enzyme first described by Szent-Györgyi [1930; 1931], which oxidizes *in vitro* *l*-ascorbic acid but not phenols, also oxidized the analogues of ascorbic acid. In addition these compounds could be oxidized by the phenolases present in the apple or potato but only in the presence of mono- or polyhydric phenols or the juice of the plants [Zilva, 1934; 1935, 2; Johnson & Zilva, 1937, 1]. From this it was inferred that the dehydrogenation was brought about by quinones formed by the action of the phenolases on the above substrates. Keilin & Mann [1938] have since found that this also holds true of the polyphenol oxidase present in the cultivated mushroom. This enzyme system which is evidently distinct from the ascorbic acid oxidase apparently functions also *in vivo*, since it has been observed that the equilibrium between *l*-ascorbic acid and dehydroascorbic acid in the living tissue of the apple changes progressively with the development of the fruit [Zilva *et al.* 1935; 1938]. Although the ascorbic acid oxidase oxidized all the analogues of *l*-ascorbic acid investigated, the rate of oxidation was markedly influenced by the stereochemical structure of these compounds. In fact a certain parallelism existed between the influence of structure on their rate of oxidation on the one hand and their capacity of being retained in the animal organism on the other [Johnson & Zilva, 1937, 2]. Thus the compounds in which the oxygen ring engages the hydroxyl group to the right of the carbon chain and which invariably possess antiscorbutic potency were oxidized at a much higher rate than their antiscorbutically inactive enantiomorphs. In contradistinction to this the asymmetry of carbon 5 or the presence of a seventh carbon atom in the chain which conditions the intensity of the antiscorbutic activity of the compounds with the ring to the left of the carbon chain had no influence upon the kinetics of the enzymic oxidation.

It remained to ascertain whether the activity of the ascorbic acid oxidase was restricted to ascorbic acid and its analogues or whether it extended to other compounds capable of reducing indophenol. Preliminary experiments with reductone and dihydroxymaleic acid suggested that these substrates may also be oxidized by it [Johnson & Zilva, 1937, 1]. These results, however, were not

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sufficiently clear-cut to justify a final conclusion. Having overcome the technical difficulties first encountered it was possible to investigate the problem of the specificity of the enzyme in greater detail. The results obtained with substrates of different chemical constitution form the subject of this investigation.

### *Technique*

The oxidation of the compounds was assessed by means of a Barcroft-Warburg respirometer and the general technique was similar to that already described [Johnson & Zilva, 1937, 2]. The experiments were carried out at 26° and at *p*H 6·0. The *p*H at the end of the experiment never varied by more than 0·2. The volume of the oxygen was calculated for 760 mm. pressure. As a source of enzyme cucumber juice was employed, prepared by freezing the entire cucumber at −20° and pressing out the thawed pericarp. Some extraneous matter was then removed by adjusting the *p*H of the juice to 7·6–8·0, filtering and acidifying to its original *p*H (approximately 6·0). This enzyme preparation was dialysed in cellophane bags at 1° for 2 days against running distilled water. The quantity of enzyme which absorbed 10 $\mu$l. of $O_2$/min. in oxidizing 2·5 ml. 0·01 *N* *l*-ascorbic acid at 760 mm. pressure, 26° and *p*H 6·0 (*M*/15 phosphate buffer) was arbitrarily adopted to represent 10 units. The enzyme was always diluted to 1 ml. and the substrate in the buffer to 1·5 ml. Control experiments were performed in every case.

## Results

### *Reductic acid as substrate*

The reductic acid [Reichstein & Oppenauer, 1933; 1934] was a sublimed and recrystallized sample kindly placed at our disposal by Prof. T. Reichstein. It was found by us to be 99–100% pure by indophenol titration.

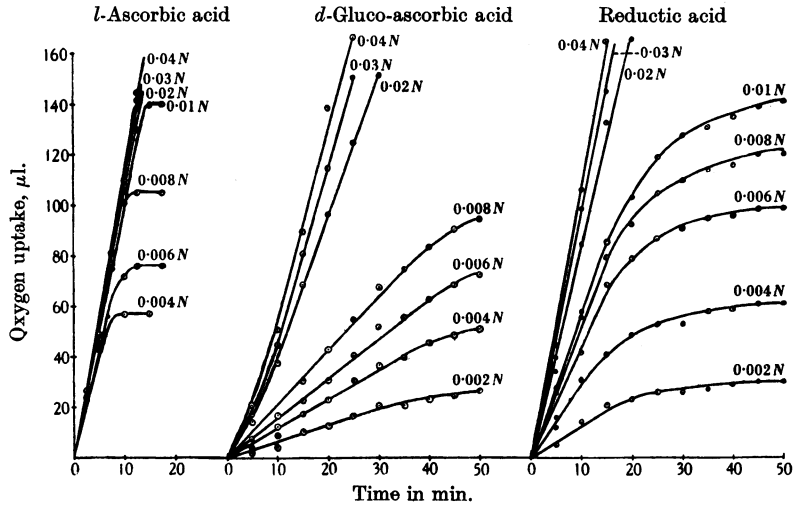



Fig. 1. Rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid at varying concentrations expressed in normality. Strength of enzyme (undialysed), 10 units.

The actions of dialysed and undialysed enzyme were studied on this substrate. Since, apart from a difference in the relative rates, the oxidations proceeded similarly, the results obtained with undialysed juice only will be given. Fig. 1

represents the course of oxidation of varying concentrations of *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid in the presence of 10 units of the enzyme. It will be seen that the rate of oxidation of reductic acid, like that of *d*-gluco-ascorbic acid, fell off after a time and was not linear as was found with *l*-ascorbic acid. This falling off was even more noticeable when dialysed juice was used, especially at high substrate concentrations.

In Fig. 2 the oxygen absorbed per min. during the initial stages, represented by linear portions of the graphs in Fig. 1, was plotted against concentrations of substrate. From this it may be observed that for low concentrations an increase of substrate raised the rate of oxidation of *l*-ascorbic acid much more than that of the other two compounds. With higher concentrations, however, such increase had hardly any perceptible effect on the oxidation velocity in the case of the former, but a considerable, although reduced, effect on that of the latter. This phenomenon may possibly be due to a lower combining affinity of the enzyme for *d*-gluco-ascorbic acid and reductic acid.

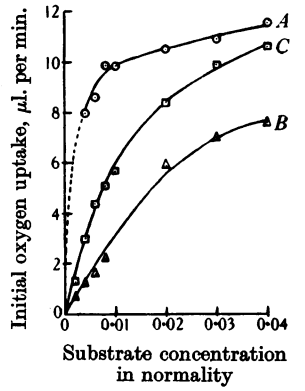



Fig. 2. Initial rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid plotted against substrate concentration. Derived from Fig. 1. ⊙—⊙ (*A*) *l*-ascorbic acid. △—△ (*B*) *d*-gluco-ascorbic acid. ⊡—⊡ (*C*) reductic acid.

The results obtained with varying quantities of enzyme and constant concentrations of substrate (0·01 *N*) are given in Figs. 3 and 4. As was to be expected the rate of oxidation was found to be almost directly proportional to the enzyme activity. From Fig. 4 the higher oxidation velocity for *l*-ascorbic acid emerges once more.

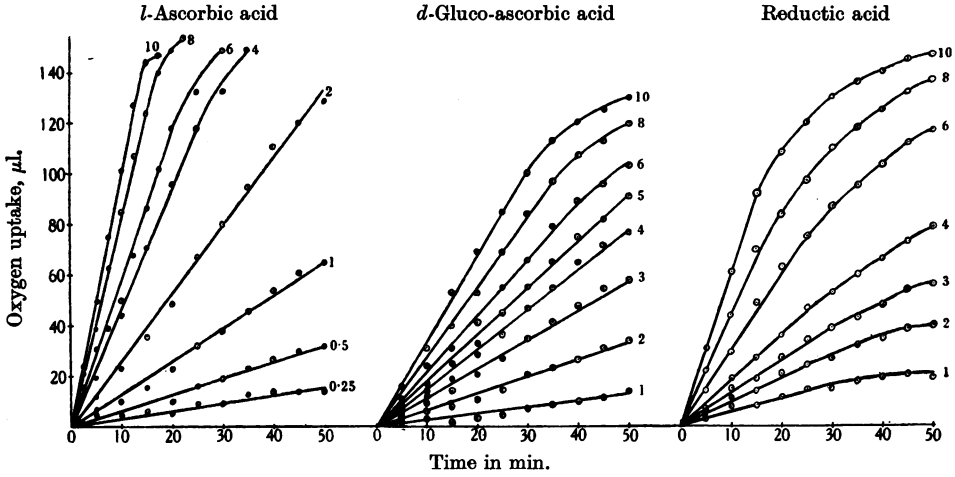



Fig. 3. Rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid at varying enzyme concentrations expressed in units. Substrate concentrations, 0·01 *N*. Undialysed juice used throughout.

In order to ascertain whether the amount of oxygen absorbed was equivalent to the amount of substrate destroyed, the following experiment was devised. Out of a battery of eight Warburg flasks treated in the usual manner, each containing the same amount of enzyme and substrate, one was reserved for

manometric readings; the remaining seven were removed singly at suitable intervals and immediately titrated with $N/1000$ indophenol. The results were calculated on the assumption that 1 mol. of each of the substrates required 1 atom of oxygen and the values obtained were corrected to 760 mm. pressure

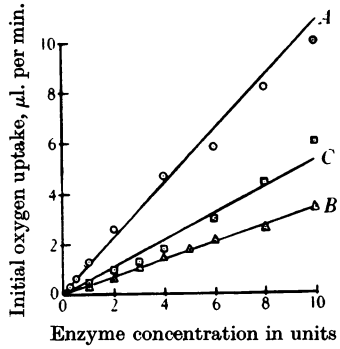



Fig. 4. Initial rates of oxygen uptake by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid plotted against enzyme concentration. Derived from Fig. 3. ⊙—⊙ (*A*) *l*-ascorbic acid. △—△ (*B*) *d*-gluco-ascorbic acid. ⊡—⊡ (*C*) reductic acid.

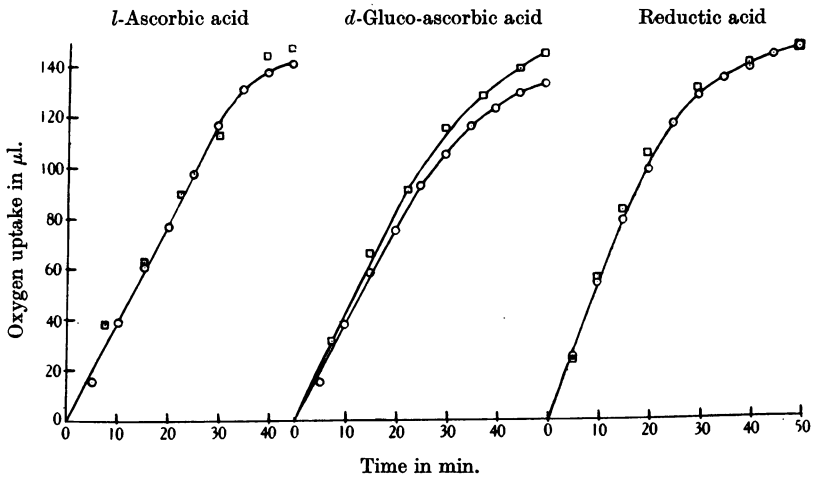



Fig. 5. Comparison of observed oxygen uptakes by *l*-ascorbic acid, *d*-gluco-ascorbic acid and reductic acid in the presence of undialysed cucumber juice, with that calculated from the disappearance of the substrates. ⊙—⊙ observed values. ⊡—⊡ calculated values.

and 26°. It will be seen from Fig. 5 that these figures agree well with those observed simultaneously by direct manometric measurements. We do not consider the slight deviation between the *d*-gluco-ascorbic acid graphs to be significant.

*Reductone as substrate*

Reductone was prepared by the method of Euler & Klussmann [1933] and Euler & Martius [1933, 1, 2, 3, 4]. The crude material was purified from hot ethyl acetate and then crystallized from butyl alcohol. The slightly brown product obtained in this way did not give a distinct M.P. (charring above 200°) thus resembling the product obtained by Euler & Martius. Further purification

revealed that this material contains a hitherto unsuspected impurity although by indophenol titration it was found to be 97% pure. All the experiments described below were therefore carried out with a preparation which was further purified by sublimation under low pressure at 125°. Two such treatments removed all the non-volatile impurities which remained as a feathery brown residue present to the extent of 8–10% of the crystallized product. The sublimed reductone, colourless needles, melted at 154–156° (decomp. uncorr.) according to the rate of heating. It must be pointed out that anomalous results were obtained when the non-sublimed substance was used as substrate.

Reductone reduces indophenol much more slowly than ascorbic acid and its analogues. It is, however, possible to obtain a definite end point if the titration is carried out at *p*H 4·5 and the indicator added slowly. Sublimed reductone was found to be 98–99% pure by this titration method.

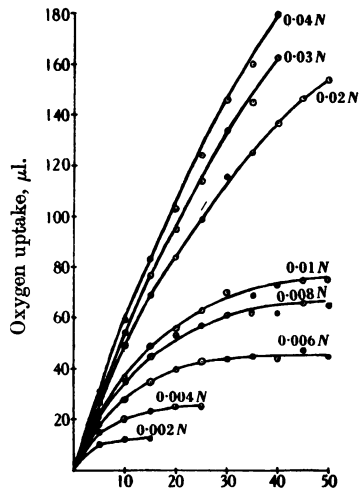



Fig. 6.

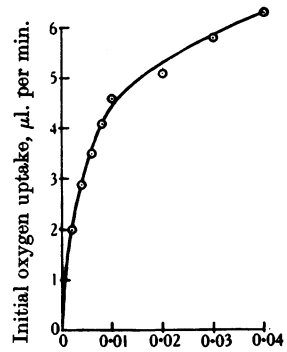



Fig. 7.

Fig. 6. Rate of oxygen uptake by reductone at varying concentrations expressed in normality. Strength of enzyme (undialysed juice), 10 units.

Fig. 7. Initial rate of oxygen uptake by reductone plotted against substrate concentration. Derived from Fig. 6.

As with *d*-gluco-ascorbic acid and reductic acid there was some inhibition as the oxidation of the reductone proceeded (Figs. 1 and 6). The increase in the initial velocity corresponding to the linear portions of the curves (Figs. 2 and 7) with the increase in substrate follows in this graph a form similar to that observed in the *d*-gluco-ascorbic acid and reductic acid graphs. These results which were obtained with undialysed juice were similar to those observed when the dialysed preparation was employed.

In the case of the undialysed juice, when the quantity of enzyme was varied and the concentration of the substrate (0·01 *N*) was kept constant the increase in enzyme at low concentrations brought about a very marked acceleration of the rate of oxidation. As the quantity of enzyme added was increased, however, a stage was reached when a further increase in the enzyme brought about

remarkably little response (Figs. 8 and 9). When dialysed juice was used the rate of $O_2$ uptake at low enzyme concentrations was greatly reduced (Fig. 10). The curve obtained by plotting initial velocities of $O_2$ uptake against enzyme

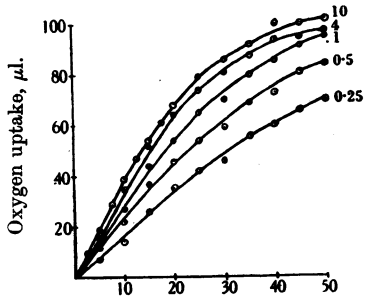



Fig. 8.

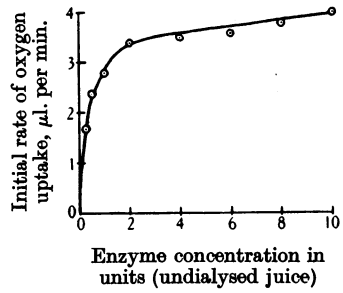



Fig. 9.

Fig. 8. Rate of oxygen uptake by reductone in the presence of varying amounts of *undialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration = 0·01 *N*.

Fig. 9. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 8 and containing some points corresponding to curves which could not conveniently be included in Fig. 8.

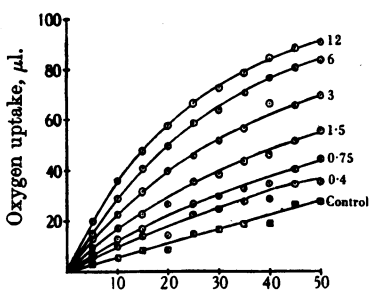



Fig. 10.

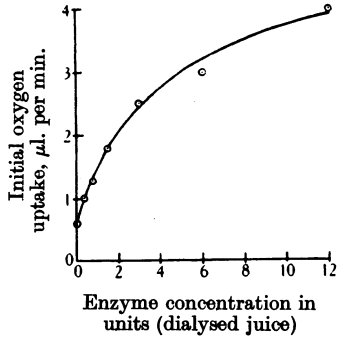



Fig. 11.

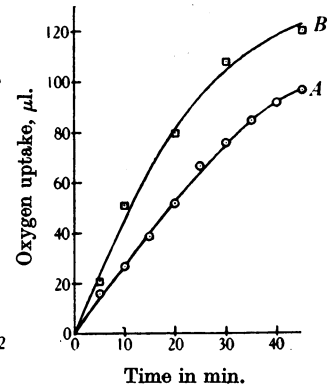



Fig. 12.

Fig. 10. Rate of oxygen uptake by reductone in the presence of varying amounts of *dialysed* cucumber juice. Enzyme concentrations expressed in units. Substrate concentration, 0·01 *N*.

Fig. 11. Initial rate of oxygen uptake by reductone plotted against enzyme concentration. Derived from Fig. 10.

Fig. 12. Comparison of observed oxygen uptake by reductone in the presence of undialysed cucumber juice with that calculated from the disappearance of the substrate. ⊙—⊙ (*A*) observed values. ⊡—⊡ (*B*) calculated values.

concentration (Fig. 11), however, shows that this relationship is again not linear. This behaviour of reductone therefore stands out in striking contrast to that of the other compounds (Figs. 3 and 4).

From Fig. 12 it is seen that the disappearance of the substrate as determined by the method described above was greater than would have been expected

from the amount of $O_2$ absorbed. The difference is significant since it was found to be reproducible. In this experiment undialysed juice was used but similar results were obtained also with dialysed juice.

*Dihydroxymaleic acid as substrate*

A commercial sample of dihydroxymaleic acid was purified by recrystallization at 60° from butyl alcohol. The scintillating white crystalline product became powdery on drying *in vacuo* over conc. $H_2SO_4$. As dihydroxymaleic acid is not very soluble in water it was found convenient to employ its Na salt which was prepared by treating the acid in ethyl alcohol with 2 mol. of sodium ethoxide. The precipitated salt was washed with ethyl alcohol and dried.

The oxidation of dihydroxymaleic acid is characterized by the formation of $CO_2$, and in order to obviate interference with the measurements of the $O_2$ uptake the former was absorbed by placing 0·3 ml. of 20 % KOH solution and a roll of filter paper in the central cup of each manometer flask.

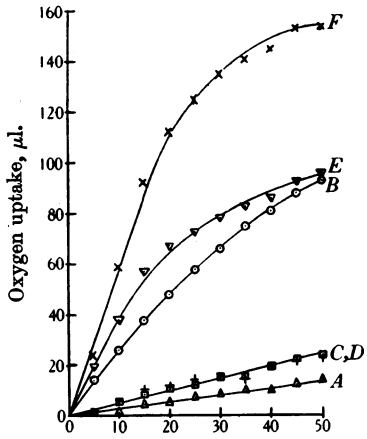



Fig. 13.

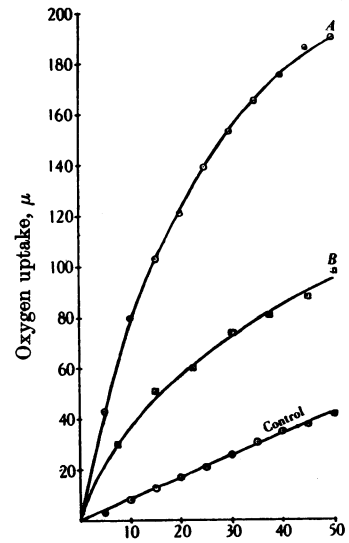



Fig. 14.

Fig. 13. Oxidation of 0·01 *N* dihydroxymaleic acid solutions. △—△ (*A*) with no addition (control). ⊙—⊙ (*B*) in the presence of 1 ml. undialysed cucumber juice. ⊡—⊡ (*C*) in the presence of 1 ml. dialysed cucumber juice. +—+ (*D*) in the presence of 1 ml. dialysed cucumber juice containing 10 mg. *l*-ascorbic acid per 100 ml. ▽—▽ (*E*) in the presence of 1 ml. dialysed cucumber juice and *M*/100 phenol. ×—× (*F*) in the presence of 1 ml. dialysed cucumber juice and *M*/100 catechol.

Fig. 14. Comparison of observed oxygen uptake by dihydroxymaleic acid in the presence of undialysed cucumber juice with that calculated from the disappearance of substrate. ⊙—⊙ (*A*) observed values. ⊡—⊡ (*B*) calculated values.

The action of cucumber juice upon dihydroxymaleic acid solutions differed from that on the preceding substrates in being greatly influenced by dialysis. Whilst the untreated juice caused a considerable $O_2$ uptake, after dialysis the uptake was hardly more than in the control. Further experiments showed that addition of quantities of ascorbic acid up to 10 mg. per 100 ml. of dialysed juice did not restore its catalytic activity towards dihydroxymaleic acid. On the

other hand, when phenol or catechol was added to the dialysed preparation, so that the final mixtures in the flasks were $M/100$ in respect to these compounds, considerable activity was imparted (Fig. 13).

It is of interest to note that there was a great discrepancy between the volume of $O_2$ calculated from the disappearance of the substrate in the presence of undialysed juice and the volume of $O_2$ actually taken up (Fig. 14). This fact, taken in conjunction with the observation made by us that $CO_2$ was evolved in the process, suggests that the mechanism involved in this oxidation was undoubtedly different from that associated with the enzymic oxidation of the other compounds.

*Sulphydryl compounds as substrates*

A crystalline sample of glutathione prepared by Pirie's method [1930; 1932] was employed and a commercial sample of cysteine hydrochloride was found to yield satisfactory results. Preliminary experiments have shown that at *p*H 6·0 oxidation was hardly, if at all, accelerated by the presence of the juice at the concentrations used (10 units of enzyme and 0·01 *N* substrate). Further trials have, however, shown that at *p*H 7·4 the oxidation was markedly catalysed and consequently the experiments were carried out at this *p*H. As no quantitative

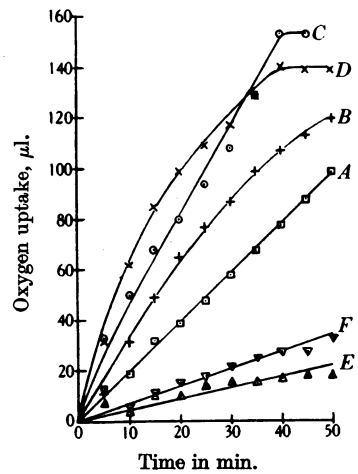



Fig. 15. Oxidation of 0·01 *N* solutions of glutathione and cysteine.

(*a*) In the presence of dialysed cucumber juice — ⊡—⊡ (*A*) GSH. +—+ (*B*) cysteine.

(*b*) In the presence of dialysed cucumber juice with the addition of 10 mg. ascorbic acid per 100 ml. — ⊙—⊙ (*C*) GSH. ×—× (*D*) cysteine.

(*c*) In the absence of enzyme (controls) — △—△ (*E*) GSH ▽—▽ (*F*) cysteine.

assessment of glutathione can be obtained by titration with indophenol, determinations of the substrate concentrations were carried out iodimetrically. The various concentrations of cysteine hydrochloride were made up on the assumption that this compound was pure.

Owing to the fact that undialysed cucumber juice usually contains traces of dehydroascorbic acid, the possibility could not be excluded that the oxidation of the sulphydryl compounds did not take place directly by the enzyme, but was due to the action of dehydroascorbic acid [cf. Hopkins & Morgan, 1936].

Additional experiments were therefore performed with dialysed juice. It will be seen from Fig. 15 that although the addition of 10 mg. of ascorbic acid to 100 ml. of the dialysed juice approximately doubled its oxidizing activity, both sulphydryl compounds were oxidized even in the absence of ascorbic acid at *p*H 7·4; the dialysed juice contained less than 0·1 mg. of dehydroascorbic acid per 100 ml.

A point of interest emerged from these experiments, namely that when different samples of dialysed juice were employed the relative rates of oxidation of the sulphydryl compounds and of *l*-ascorbic acid varied (Figs. 16 and 17).

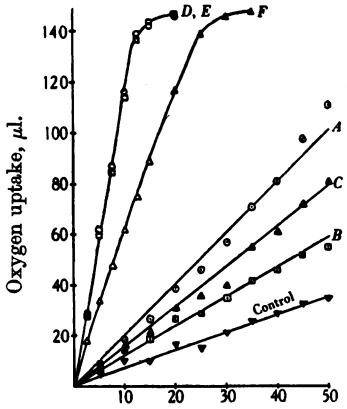



Fig. 16.

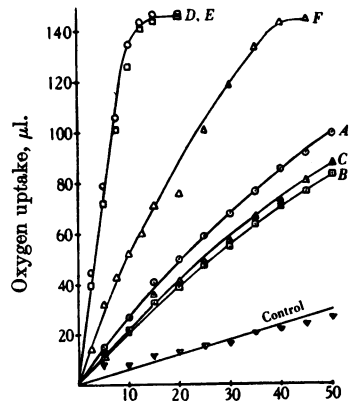



Fig. 17.

Fig. 16. Oxidation of 0·01 *N* solutions of glutathione and *l*-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

| | GSH | *l*-Ascorbic acid |
|---|---|---|
| Sample I | ⊙—⊙ (*A*) | ○—○ (*D*) |
| „ II | ⊡—⊡ (*B*) | □—□ (*E*) |
| „ III | △—△ (*C*) | △—△ (*F*) |

Fig. 17. Oxidation of 0·01 *N* solutions of cysteine and *l*-ascorbic acid in the presence of equal amounts of different samples of dialysed cucumber juice.

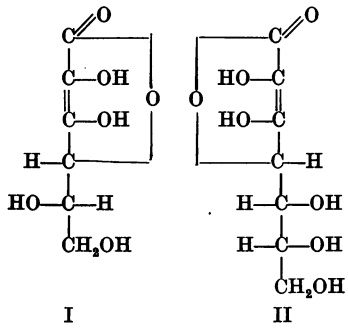
| | Cysteine | *l*-Ascorbic acid |
|---|---|---|
| Sample I | ⊙—⊙ (*A*) | ○—○ (*D*) |
| „ II | ⊡—⊡ (*B*) | □—□ (*E*) |
| „ III | △—△ (*C*) | △—△ (*F*) |

This suggests that in all probability different enzymes in the juice were involved in the oxidation of glutathione and cysteine on the one hand, and of *l*-ascorbic acid on the other.

## Discussion

The information obtained in this investigation suggests that the action of the ascorbic acid oxidase is not confined to *l*-ascorbic acid (I) and *d*-gluco-ascorbic acid (II), cyclic dienols with an oxygen bridge. It was shown that the oxidations of a cyclic dienol without an oxygen bridge, reductic acid (III) and an acyclic dienol, reductone (IV), both of which were capable of reducing indophenol, were catalysed by the enzyme. In most of the compounds the rate of oxygen absorption showed an inhibition as the oxidation proceeded. The oxidation of *l*-ascorbic acid and, as previously found [Johnson & Zilva, 1937, 2],

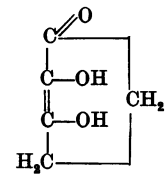
that of its analogues with the oxygen ring to the right of the carbon chain, were, however, directly proportional to the time.



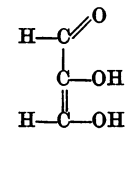
I



II



III



IV

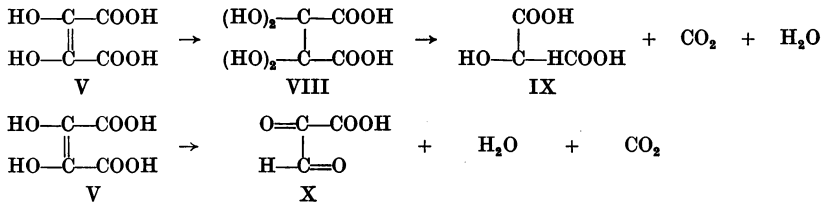
Amongst all the other substrates used in this investigation reductone stood out in its behaviour towards increasing enzyme concentrations in so far that beyond a certain stage the addition of more enzyme brought about very little response in the $O_2$ uptake (Figs. 9 and 11). The effect was specially noticeable when undialysed juice which showed greater activity towards reductone was employed. A possible explanation of this phenomenon may lie in the fact that reductone can exist in at least two tautomeric forms IV and VI. The high reducing properties of the compound in solution are presumably due to IV which would form the effective substrate and the concentration of which would therefore be controlled by the velocity of the tautomeric conversion. At high enzyme concentrations this may become the limiting factor in the rate of oxidation. The behaviour of reductone towards ascorbic acid oxidase is similar to that of fructose fermented by increasing quantities of yeast as observed by Hopkins & Roberts [1935, 1, 2]. In this case the formation of the fermentable component in the fructose solution could actually be observed by the rate of mutarotation.

In the compounds containing the 5-membered ring the $O_2$ uptake was consistent with the values calculated from the disappearance of the substrate on the assumption that two atoms of hydrogen were eliminated from each mol. In the case of reductone, however, the $O_2$ uptake was less than that calculated. This suggests that some of the substrate was utilized in a side reaction. By analogy with the easy conversion of methylglyoxal into lactic acid, the transformation of VI into glyceric acid (VII) provides a mechanism which would be consistent with such an assumption.

VI VII

The oxidation of dihydroxymaleic acid by the cucumber juice, unlike those of reductone and reductic acid, is not due to the presence of ascorbic acid oxidase but to another enzyme system, possibly a phenolase. The high $O_2$ uptake and the production of $CO_2$ in this case suggest a breakdown either of the acid or of a primary oxidation product, with the formation of a substance capable of absorbing $O_2$. It is well known that the products of oxidation of dihydroxymaleic acid depend on the nature of the oxidizing agent. Thus the halogens

oxidize it (V) to dihydroxytartaric acid (VIII) which easily decomposes into hydroxymalonic acid (IX) and $CO_2$, whilst ferric salts oxidize it to glyoxal-carboxylic acid (X), $CO_2$ and water. This behaviour of dihydroxymaleic acid towards oxidizing agents provides some suggestion for its more complete degradation in the presence of the juice.




It is of interest to note that the enzymic oxidation of *l*-ascorbic acid *in vivo* has so far been indicated to be due only to the indirect action of a phenolase [Zilva *et al.* 1938] and not to the direct oxidation by the ascorbic acid oxidase. This does not, however, exclude the possibility that the oxidase functions also *in vivo*. The marked capacity of the enzyme to act in dilute solutions of *l*-ascorbic acid as compared with other substrates in fact strongly favours this view, since the vitamin is present in plants in very low concentrations. Even in some of the exceptionally rich sources such as the juice of the mango (Alphonso variety) it is present only to the extent of 0·01 *N*. It would indeed be surprising if the ascorbic acid oxidase were not involved in any of the metabolic functions of the plant.

## Summary

Dialysed cucumber juice, apart from its ability to oxidise the ascorbic acid analogues directly, also oxidizes reductone and reductic acid.

Whilst in the compounds containing the five-membered ring the $O_2$ uptake agrees with the values calculated from the disappearance of the substrate, in the case of reductone the $O_2$ uptake is less than that calculated. It is suggested that some of the substrate is utilized in a side reaction.

Low concentrations of *l*-ascorbic acid are much more readily oxidized by dialysed juice than *d*-gluco-ascorbic acid, reductic acid and reductone, indicating a greater affinity of the enzyme for the naturally occurring vitamin.

Undialysed, but not dialysed, juice oxidizes dihydroxymaleic acid. Dialysed juice, however, regains its oxidizing activity towards this substrate on addition of catechol or phenol.

In the oxidation of dihydroxymaleic acid there is a formation of $CO_2$ and a higher $O_2$ uptake than that calculated from the disappearance of the substrate. The possible mechanism involved in this more complete degradation of the substrate is discussed.

Dialysed cucumber juice is capable of oxidizing glutathione and cysteine. The enzyme responsible for this oxidation appears not to be identical with the ascorbic acid oxidase present in the juice, since the relative rates of oxidation of the sulphydryl compounds and of ascorbic acid vary with different samples of dialysed juice.

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