REFERENCES

Bicknell, F. & Prescott, F. (1948). *The Vitamins in Medicine*, 2nd ed. London: Heinemann.
Cailleau, R. (1939). *C.R. Soc. Biol., Paris*, **130**, 1089.
Dalmer, O. & Moll, T. (1933). *Hoppe-Seyl. Z.* **222**, 116.
Demole, V. (1934). *Biochem. J.* **28**, 770.
Dyke, S. C., Della Vida, B. L. & Delikat, E. (1942). *Lancet*, **2**, 278.
Kligler, I. J., Guggenheim, K. & Warburg, F. M. (1938). *J. Path. Bact.* **46**, 619.
Knox, W. E. & LeMay-Knox, M. (1951). *Biochem. J.* **49**, 686.
Krayer, O., Linstead, R. P. & Todd, D. (1943). *J. Pharmacol.* **77**, 113.
Leibowitz, J. & Guggenheim, K. (1938*a*). *Z. Vitaminforsch.* **8**, 1.
Leibowitz, J. & Guggenheim, K. (1938*b*). *Z. Vitaminforsch.* **8**, 8.
Lwoff, M. (1951). The Nutrition of Parasitic Flagellates. In *Biochemistry and Physiology of Protozoa*, ed. by A. Lwoff. New York: Acad. Press.
McChesney, E. W., Barlow, O. W. & Klinck, G. H. (1944). *J. Pharmacol.* **80**, 81.
May, C. D., Nelson, E. N., Lowe, C. U. & Salmon, R. J. (1950). *Amer. J. Dis. Child.* **80**, 191.
Nichol, C. A. & Welch, A. D. (1950). *Proc. Soc. exp. Biol., N.Y.*, **74**, 52.
Sauberlich, H. E. & Baumann, C. A. (1948). *J. biol. Chem.* **176**, 165.
Sealock, R. A., Goodland, R. L., Summerwell, W. N. & Brierly, J. M. (1952). *J. biol. Chem.* **196**, 761.
Smith, F. (1946). *Advanc. Carbohyd. Chem.* **2**, 79.
Vilter, R. W. (1947). *Symposium on Nutrition of the Robert Gould Research Foundation*, Cincinnati, **1**, 179. Ed. by Lejwa, A.
Welch, A. D., Nichol, C. A., Anker, R. M. & Boehne, J. W. (1951). *J. Pharmacol.* **103**, 403.

The Effect of L-Ascorbic Acid on the *in vitro* Activity of Polyphenoloxidase from Potato

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In recent years many studies of the action of polyphenoloxidase on various substrates, *in vitro*, have been carried out in the presence of L-ascorbic acid (Asimov & Dawson, 1950; Miller, Mallette, Roth & Dawson, 1944; Robinson & Nelson, 1944; Sreerangachar, 1943). In these studies the L-ascorbic acid has been assigned the role of a reducing agent for any *o*-quinones produced during the oxidation of the substrates. It has been generally assumed (Asimov & Dawson, 1950; Miller & Dawson, 1941; Kendal, 1949; Sreerangachar, 1943) that the ascorbic acid has no other effect on the system, although many observers have reported that the rate of oxidation was sometimes significantly different in the presence of ascorbic acid than in its absence (Kendal, 1949; Roberts & Wood, 1950; Krueger, 1950).

Krueger (1950) made a study of the effect of ascorbic acid on the rate of oxidation of tyrosine by mushroom polyphenoloxidase and concluded that initially the ascorbic acid had a 'pro-oxidant' effect on the system since the initial oxygen uptake was substantially increased and the induction period was decreased (cf. Kendal, 1949), but later in the reaction it had an antioxidant effect since the rate of production of L-β-3:4-dihydroxyphenylalanine (dopa) was decreased below that for the ascorbic-acid-free system.

The investigations of Kertesz (1951, 1952) have shown that the observed oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid are markedly dependent on the concentration of cupric ion, and since Krueger apparently did not use copper-free water for his experiments, it seemed worth while to re-investigate this system.

Our experiments, using a colorimetric procedure to determine enzyme activity, show that ascorbic acid has an inhibitory action on potato polyphenoloxidase which is not elicited in the presence of ionic copper. However, when the enzyme was treated with ascorbic acid in the absence of oxygen and the ascorbic acid subsequently removed by dialysis, it was irreversibly changed so that reactivation with cupric salts was not possible. Oxidation products of ascorbic acid, including dehydroascorbic acid, were found to have little effect on the enzyme.

It is suggested that the ascorbic acid affects specific structures of the enzyme (cf. Kertesz, 1951, 1952) in which copper is involved.

EXPERIMENTAL

Materials

Reagents. All salts used were of A.R. quality and distilled water freed from copper (lowered from 95 μg./l. to undetectable amounts) by passage through a mixed-bed ion-exchange resin column (Amberlite MB 1) was used throughout.

Polyphenoloxidase. Potato peelings (50 g.) were macerated in a Waring blendor with ice-cold water (50 ml.), the mixture centrifuged, the supernatant liquid treated with one-third of its volume of acetone (at $-20°$) and the precipitate discarded. The enzyme was precipitated by the addition of more acetone (final concentration 50%, v/v), taken up in 10 ml. of ice-cold water, centrifuged, and the supernatant stored at 0°. It was diluted before use.

Ascorbic acid solution. This was freshly prepared for each experiment.

Dehydroascorbic acid. This was prepared according to Mapson (1946), and its concentration determined by the microbiological method of Mapson & Ingram (1950).

Measurement of enzyme activity

Colorimetric method. The enzyme solution, ascorbic acid or other constituents and 0·1 M-phosphate-citrate buffer (McIlvaine) (determined pH, 5·98) to give a volume of

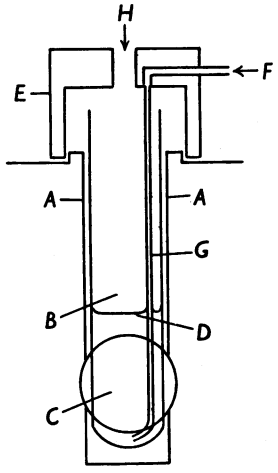

Fig. 1. Diagram of apparatus. *A*, spectrometer case; *B*, 0·5 in. test tube; *C*, light source and photocell perpendicular to plane; *D*, liquid level; *E*, air-lead cap; *F*, air inlet; *G*, hypodermic tubing; *H*, hole for addition of substrate, etc.

3·5 ml. were placed in a 0·5 in. diameter tube of a Unicam D.G. spectrophotometer, and the air-lead cap (Fig. 1) placed in position. Air was bubbled through the mixture at a constant rate (700 ml./hr.) and the galvanometer adjusted to read 100% transmission at 480 mμ. ($E = \log I_0/I = 0$). Catechol (0·5 ml.; 4 mg./ml.) was quickly introduced from a hypodermic syringe (final volume, 4·0 ml.) and readings taken every 15 or 30 sec. All results for each series of experiments were obtained in the shortest possible time since no temperature control was possible. Experimental results were reproducible with a maximum error of ± 2%.

Although the passage of air bubbles caused slight oscillation of the galvanometer, the effect was constant and readings could be taken with ease. Interruption of the air flow was not nearly so satisfactory because of galvanometer drift. The rate of air flow specified was found to be the most satisfactory, lower rates being unsteady while much higher rates caused excessive foaming, although this could be avoided by using methylheptanol. To ensure uniformity of air flow, stainless steel hypodermic tubing (int. diam. 0·3 mm.) was used as a capillary lead throughout these experiments, and had no apparent effect on the system.

Sreerangachar's (1943) *method.* Enzyme, ascorbic acid and buffer solution were equilibrated in tubes (200 × 25 mm.) under nitrogen for 10 min. at $35° \pm 0·1°$, catechol introduced (total vol. 10 ml.) and air drawn through at 8 l./hr. (Moores, Greninger & Rusoff, 1951). After 5 min. an aliquot of the solution was added to excess 1% oxalic acid and the ascorbic acid determined colorimetrically (Ponting & Joslyn, 1948).

RESULTS

Investigation of the colorimetric method for the measurement of enzyme activity

The shape of the curve. Using the colorimetric method, Ponting & Joslyn (1948) and Warner (1951) stated that the plot of extinction against

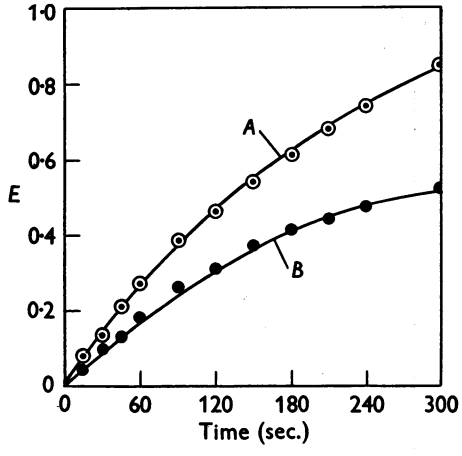

Fig. 2. The increase of extinction with time in the catechol-polyphenoloxidase system, showing the distinct curvature of the plot. Vol. of enzyme used: *A*, 1·0 ml.; *B*, 0·5 ml. Catechol (2 mg.) and total vol. (4·0 ml.) in each case.

time in a catechol-polyphenoloxidase system gave a straight line, although the latter author found that this relationship held only for the first 2·5 min. period. In our hands these results held true when using low enzyme concentrations giving extinction differences of 0·01–0·05/min. (cf. Ponting & Joslyn, 1948). With the concentrations we have used for our work, however, the plot usually had a distinct curvature, as can be seen from Fig. 2, in which two different enzyme concentrations are used. This

corresponds to the findings of Wallerstein, Alba & Hale (1947), who used macerated potato tissue and catechol.

It was shown by Miller *et al.* (1944), using the chronometric technique, in which the time taken for small amounts of ascorbic acid added to the enzyme-substrate system to be oxidized is measured, that during the first 2 or 3 min. of oxidation of catechol, catalysed by polyphenoloxidase, *o*-benzoquinone is produced according to the equation

$$Q = at/(b+t), \qquad (1)$$

where Q is the concentration of quinone, t is the time in sec., and a and b are constants determined by the extent of enzyme inactivation during the initial stage of the reaction. The values of a and b which determine the curvature of the reaction course may be evaluated from the slope and the intercept of the linear curve obtained from the reciprocal equation

$$\frac{1}{Q} = \frac{b}{a}\left(\frac{1}{t}\right) + \frac{1}{a}, \qquad (2)$$

and since the initial rate of reaction $dQ/dt (t \geqq 0)$ is given by

$$dQ/dt = a/b, \qquad (3)$$
$$(t \geqq 0)$$

it is thus equal to the reciprocal of the slope of the linear curves obtained from equation 2. Miller *et al.* (1944) evaluated the quinone concentration to the

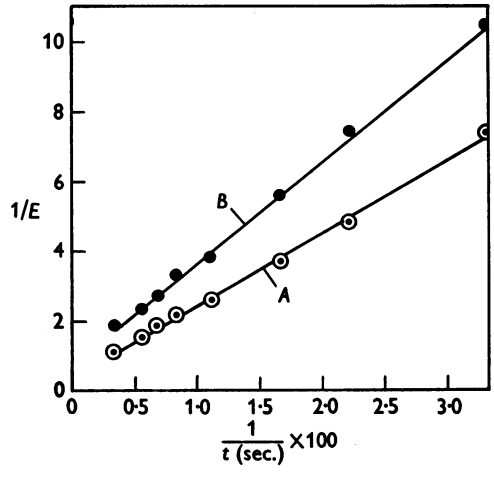

Fig. 3. The results of Fig. 2 shown as a reciprocal plot.

ascorbic acid oxidized by the system. A consideration of the proposed mechanism of the oxidation of catechol by polyphenoloxidase (Nelson & Dawson, 1944) suggests that, since the transformation of the initial oxidation product is independent of enzyme action and virtually instantaneous, the quinone produced can be related directly to absorbance. Consequently, a plot of $1/E$ (E = extinction) against $1/t$ should produce a straight line. This is confirmed by Fig. 3 where the results obtained from Fig. 2 have been plotted in this way. Hence the initial rates of reaction can readily be determined.

Miller *et al.* (1944) showed that the initial rate of reaction could also be deduced from the equation

$$\frac{a}{b} = \left(\frac{1}{t_1} - \frac{1}{t_2}\right) \Big/ \left(\frac{1}{Q_1} - \frac{1}{Q_2}\right). \qquad (4)$$

A comparison of the rates derived from the slopes of the reciprocal curves (Fig. 3), calculated from equation 4 ($Q = E$) and estimated from the original curves (Fig. 2) is shown in Table 1.

Table 1. *Initial rate of oxidation of catechol by potato polyphenoloxidase*

(In each experiment 2 mg. of catechol were used, in total vol. 4 ml. Initial rate given as increase in E/min.)

Enzyme solution (ml.)	Initial rate		
	From Fig. 2	From Fig. 3	From equation 4
0·5	0·18	0·187	0·190
1·0	0·27	0·275	0·284

Since, however, the initial rates obtained from the plots of absorbance against time give satisfactory agreement with those derived from both the reciprocal plots and from equation 4 they have been used throughout and are designated R (graph).

In using the extinction as a measure of *o*-benzoquinone produced we have assumed that the same coloured condensation product is produced regardless of conditions. Warner (1951) has pointed out that in his system the colour produced changed progressively from red-brown to bright yellow with increasing substrate concentration. He was working with relatively crude enzyme preparations, and indicated that the colour was probably due in part to secondary reactions between the oxidation products of catechol and other compounds such as amino acids present in his system (cf. Jackson & Kendal, 1949; Trautner & Roberts, 1950). We have found that crude enzyme preparations from different sources give easily observed differences in colour with catechol (unpublished results). However, with the enzyme used here the absorption maximum of the colour product was always at 480 mμ., and it may be concluded that our assumption was justified.

The effect of the rate of air flow. The effect of the rate of air flow through the system is shown in Fig. 4. At the two lower rates of air flow (440 and 700 ml./hr.; curve A) the result was found to be virtually the same (cf. Asimov & Dawson, 1950). At the highest rate of flow (Fig. 4, curve B) the initial rate of enzyme activity is slightly higher, but as can be seen the rate of increase of absorbance falls off more rapidly than at the lower rates of air flow. This may

be due to surface denaturation of the enzyme by the air stream (Tenenbaum, 1940), a possibility which is rendered more likely by the observed diminution of the activity of a polyphenoloxidase solution through which air has been passing (1500 ml./hr.) for 2 hr. (Fig. 4, curve *C*). The initial rate of reaction determined without air flow is virtually the same (Fig. 4, curve *D*), but the rate falls off considerably after 1·5–2 min., and an examination of the reaction tube after 10 min. showed that there was a dark-brown top layer. Careful mixing of the two layers gave no

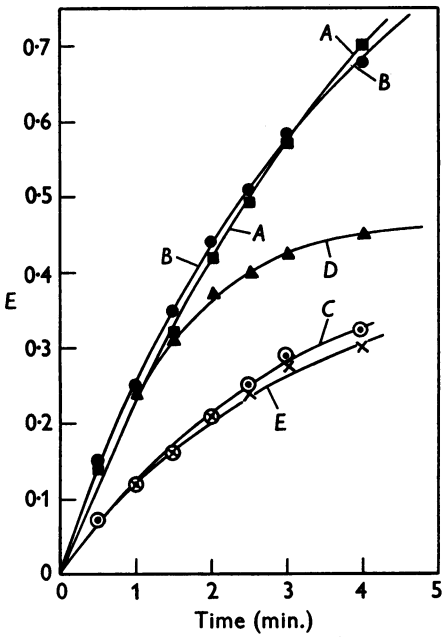

Fig. 4. Effect of rate of air flow on the measurement of enzyme activity. *A*, air passed at either 440 or 700 ml./hr.; *R* (graph) = 0·32 *E*/min.; *B*, air passed at 1440 ml./hr.; *R* (graph) = 0·36 *E*/min.; *C*, air passed at 700 ml./hr. using enzyme solution which previously had air at 1500 ml./hr. passing through for 2 hr.; *R* (graph) = 0·125 *E*/min. *D*, no aeration, but enzyme and substrate solutions completely mixed initially; *R* (graph) = 0·32 *E*/min. *E*, no aeration, but enzyme and substrate solutions incompletely mixed initially; *R* (graph) = 0·12 *E*/min. In each case 0·5 ml. enzyme and 2 mg. catechol in a total volume of 4·0 ml. were used.

significant increase in absorbance even after some minutes, indicating that the oxygen tension of the solution was too low for enzyme action to take place (cf. oxidation of ascorbic acid in boiling water, Mapson, 1941). This was confirmed by subsequent passage of air through the solution when the total absorbance was raised to almost the same level as curve *A*. Curve *E* (Fig. 4) shows the result of incomplete mixing. In this case, the lower half of the reaction tube was virtually colourless.

From these experiments it can be seen that although there is no need to aerate the solution in order to measure initial rates in the catechol-polyphenoloxidase system, the oxygen tension drops considerably during the first 2 min. Thus in systems containing ascorbic acid where the quinone condensation products are not formed until all the ascorbic acid has been oxidized, it is essential to bubble air through to ensure a constant oxygen tension in solution during the whole course of the reaction.

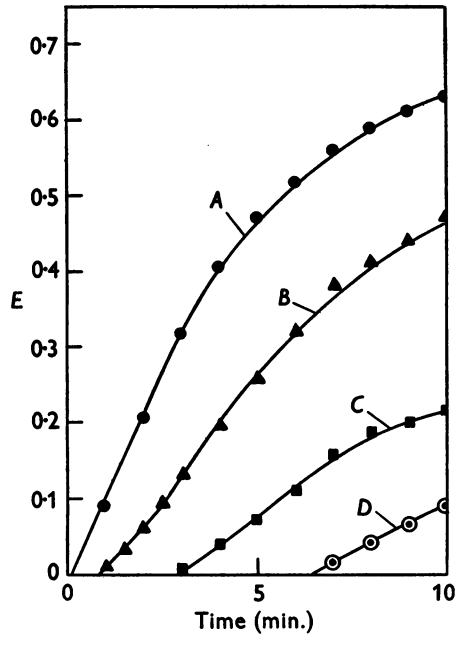

Fig. 5. Effect of increasing ascorbic acid concentration on the rate of enzyme action. *A*, no ascorbic acid added; *R* (graph) = 0·10 *E*/min. *B*, 0·4 mg. ascorbic acid added; *R* (graph) = 0·06 *E*/min. *C*, 0·8 mg. ascorbic acid added; *R* (graph) = 0·04 *E*/min. *D*, 1·2 mg. ascorbic acid added; *R* (graph) = 0·025 *E*/min. In each experiment 0·5 ml. enzyme, 2 mg. of catechol and ascorbic acid as shown in a total volume 4·0 ml. was used.

The effect of ascorbic acid on the catechol-polyphenoloxidase system

Colorimetric measurements. When ascorbic acid is present in the catechol-polyphenoloxidase system there is a delay in the onset of colour development until all the ascorbic acid has been oxidized. If the activity of the enzyme is not affected by the vitamin the rate of colour development should then be the same as if no ascorbic acid had been added. The results presented in Fig. 5 show that this is not the case, and that ascorbic acid in increasing concentration has a marked inhibitory effect on the initial rate of reaction. Amounts of ascorbic acid above 3 mg. prevented colour development completely.

Measurement by Sreerangachar's method. In order to demonstrate that the results obtained in the experiment described above were due to a loss in the oxidizing activity of the enzyme, a series of determinations were carried out using Sreerangachar's method, with increasing amounts of ascorbic acid and the same concentration of enzyme and catechol. It was argued that if ascorbic acid has no inhibitory effect, the same concentration of enzyme should be capable of oxidizing the same quantity of ascorbic acid irrespective of the concentration of the latter. The results in Table 2 show that this is not the case, and hence it must be concluded that ascorbic acid does inhibit the enzyme.

Table 2. *The effect of ascorbic acid on polyphenol-oxidase activity measured by Sreerangachar's method*

(Reaction volume 10 ml.; enzyme 0·5 ml., catechol 2 mg., with ascorbic acid as shown. These represent the average of duplicate determinations.)

Ascorbic acid (mg.)		Loss of ascorbic acid (mg.)	Per cent of fastest rate
At start	After 5 min.		
0·87	0·43	0·44	100
1·69	1·32	0·37	84
4·00	3·77	0·23	52
7·94	7·80	0·14	32

The effect of ascorbic acid oxidation products on the reaction

From the results obtained above it was not clear whether ascorbic acid itself or the oxidation products formed during the course of the reaction were the true inhibitors. In order to determine between these alternatives, the effect of dehydroascorbic acid and solutions of ascorbic acid oxidation products obtained by aerating a solution of ascorbic acid both in the presence and absence of added copper were tested. For the preparation of such solutions, air was drawn through a mixture of ascorbic acid and buffer for 2 hr. The results are shown in Tables 3 and 4.

It is apparent that in similar concentrations to those in which ascorbic acid shows a marked effect (about 0·4 mg.) neither dehydroascorbic acid nor the complicated oxidation products of ascorbic acid have much inhibitory action. In much higher concentrations, however, the oxidation products of ascorbic acid do affect the enzyme (Table 4). The smaller inhibition shown by the solution containing copper is explicable by the results shown later, where the effect of copper on the system is demonstrated.

It can be seen that the tube with no added copper (*A*) must have contained traces of a metal catalyst, since little ascorbic acid remained after 2 hr. (cf. Mapson, 1945), and this emphasizes the need for thorough precautions in determining the oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid.

Table 3. *The effect of dehydroascorbic acid on the catechol-enzyme system*

(0·5 ml. enzyme + 2 mg. catechol in a total volume of 4·0 ml. in each experiment.)

Compound added	Amount (mg.)	Initial rate (*R* (graph))	Per cent maximum
None	—	0·16	100
Dehydroascorbic acid	0·34	0·16	100
Ascorbic acid	0·40	0·05	31

Table 4. *The effect of ascorbic acid oxidation products on the catechol-enzyme system*

(0·5 ml. enzyme, 2 mg. catechol plus additions as shown in a total vol. of 4·0 ml. in each experiment. Soln. *A* contained originally 625 μg./ml. of ascorbic acid in buffer solution. Soln. *B* contained originally 625 μg./ml. of ascorbic acid and 5 μg./ml. of copper in buffer solution. Both solutions aerated in buffer for 2 hr. before testing.)

Solution added	Volume (ml.)	Total ascorbic acid present (μg.)	Initial rate (*R* (graph))	Per cent maximum
Nil	—	0	0·21	100
A	0·5	1·3	0·21	100
B	0·5	0	0·21	100
A	3·0	7·8	0·09	43
B	3·0	0	0·14	67
Ascorbic acid	0·5	400	0·08	38

Correlation of the inhibitory activity with a direct effect of ascorbic acid on the enzyme

The effect of ascorbic acid on the substrate. Although it was clear from the foregoing experiments that, unless very high concentrations were used, ascorbic acid and not its oxidation products is involved in the inhibition of the enzyme-catechol system, there was a possibility that it reacted with the substrate and prevented the formation of brown condensation

Table 5. *Effect of added enzyme or substrate on the catechol-oxidized ascorbic-acid-enzyme system*

(Originally, 0·5 ml. enzyme, 2 mg. catechol + ascorbic acid as shown in total volume 4·0 ml. in each experiment. Additions made after 10 min.)

Addition after all ascorbic acid had disappeared	Ascorbic acid originally present (mg.)	Initial rate (*R* (graph))	Per cent maximum
None	0	0·125	100
None	1	0·05	40
Catechol (2 mg.; 0·5 ml.)	1	0·055*	44
Enzyme (0·5 ml.)	1	0·105*	84

* Corrected for volume increase.

products. However, when all the ascorbic acid had just disappeared (10 min.) from an ascorbic-acid-enzyme-catechol system only the addition of extra enzyme, and not extra catechol, increased the activity. The results are shown in Table 5.

It is evident that if the ascorbic acid reacted with the substrate or its oxidation products to prevent the formation of coloured compounds, addition of extra catechol after all the ascorbic acid had disappeared would increase the rate to that of the control, whereas addition of extra enzyme to such a system would have little or no effect. Since the reverse is the case, ascorbic acid must act on the enzyme itself.

The effect of ascorbic acid on the enzyme. That the conclusions reached in the previous experiment were correct was demonstrated in the following way.

Polyphenoloxidase solution (2 ml.) and varying amounts of ascorbic acid were diluted to constant volume (10 ml.) with buffer and oxygen-free nitrogen passed through the solutions at a slow rate (about 100 ml./hr.) for 2 hr. at room temperature. The solutions were then dialysed overnight against running (copper-free) water until completely free of ascorbic acid and made up to volume. One ml. of each solution was diluted with buffer (2·5 ml.) and its activity against catechol (2 mg.; 0·5 ml.) tested in the usual way. The results are shown in Table 6.

Table 6. *Effect of ascorbic acid on polyphenoloxidase under anaerobic conditions*

(Enzyme solution (2·0 ml.) in total volume of 10 ml. treated with varying amounts of ascorbic acid as shown under N_2 for 2 hr. After removal of excess ascorbic acid by dialysis, 1 ml. of the enzyme solution was taken with 2 mg. catechol in total volume 4·0 ml.)

Ascorbic acid added initially (mg.)	pH of initial solution	Ascorbic acid destroyed after 2 hr. under N_2 (% initial)	Initial rate (R (graph))	Per cent maximum
0	5·98	0	0·075	100
4	5·94	5	0·055	73
8	5·94	3	0·045	60
12	5·93	0	0·03	40

More reactive enzymes were naturally less affected, but it is immediately apparent from the results that ascorbic acid, since little or none is lost during the period of contact under nitrogen, itself inhibits the enzyme. It must be stressed that in the activity measurements shown in Table 6 no ascorbic acid is present and there is no delay in the onset of colour development.

The effect of copper on the system

In the absence of ascorbic acid. We have found with our enzyme system that the addition of extra cupric ion greatly increases the measurable activity, although catechol itself is not affected by copper in the absence of the enzyme (Table 7).

Kertesz (1952) has recently shown that when polyphenoloxidases are prepared by extraction with potassium cyanide solution, the resulting copper-free protein (apoenzyme) may be reactivated by the addition of cupric ion. He found that the activity of his preparation against dopa was unaffected by the presence of excess copper, but with tyrosine, increasing amounts of copper gave increased rates of oxygen uptake and suggested that cupric ion catalysed the non-enzymic oxidation of tyrosine by traces of *o*-quinone present.

Table 7. *Influence of copper on polyphenoloxidase activity*

(Enzyme 0·5 ml., catechol 2 mg. and Cu^{2+} as shown. Total vol. 4·0 ml.)

Cu^{2+} added (μg.)	Initial rate (R (graph))	Per cent control
0	0·11	100
25·5	0·20	182
51	0·32	290

Polonovski & Gonnard (1951) found that addition of cupric ion to polyphenoloxidase-dopa systems increased the production of secondary coloured quinone compounds but inhibited the formation of melanin. It has been shown that cupric ion alone increases the rate of oxidation of several phenolic compounds as shown by the colour produced and the oxygen uptake (Bhagvat & Richter, 1938).

It is likely, therefore, that the observed acceleration with added copper in our system is mainly due to the effect on the formation of coloured condensation products from the *o*-benzoquinone and not to a direct increase in the enzyme activity as such.

In the presence of ascorbic acid. As was expected from the results presented above, the presence of copper had a marked effect on enzyme systems containing ascorbic acid. Kertesz (1951) has shown that for his system of apoenzyme (copper-free), cupric ion and catechol, the oxidation of ascorbic acid is dependent on two different mechanisms: (i) oxidation by free cupric ion (Barron, de Meio & Klemperer, 1936), and (ii) oxidation by the protein-bound copper in the presence of catechol (Kubowitz, 1938).

The addition of excess cupric ion to our system involves mainly mechanism (i) and, as can be seen from the Table 8, there is a large decrease in the lag period for the onset of browning, indicating that ascorbic acid is oxidized rapidly. It was then expected that the rates obtained when all the ascorbic acid had been oxidized would be comparable with those obtained in its absence (Table 7). The initial rates are, however, much lower, although it is apparent from Fig. 6 that there is an autocatalytic effect after 1–1·5 min. This suggests that the unbound Cu^{+} produced during the oxidation of

ascorbic acid by mechanism (i) does not activate the system to the same extent as the cupric ion but, when re-oxidized by the air stream, exerts its effect producing the 'autocatalytic' appearance of the curves in Fig. 6. There is also the possibility that some copper may be lost by chelation with the oxidation products (cf. Mapson, 1941).

Table 8. *Influence of copper on the ascorbic-acid-enzyme systems*

(0·5 ml. enzyme, 2 mg. catechol and additions of ascorbic acid and of Cu^{2+} as shown. Total volume 4·0 ml.)

Cu^{2+} added (μg.)	Ascorbic acid added (μg.)	Delay period before onset of colour (min.)	Initial rate (R (graph))	Per cent control
0	0	0	0·11	100
0	400	0·9	0·095	86
51	400	0·2	0·145	127
0	800	5·7	0·055	50
51	800	1·3	0·10	91

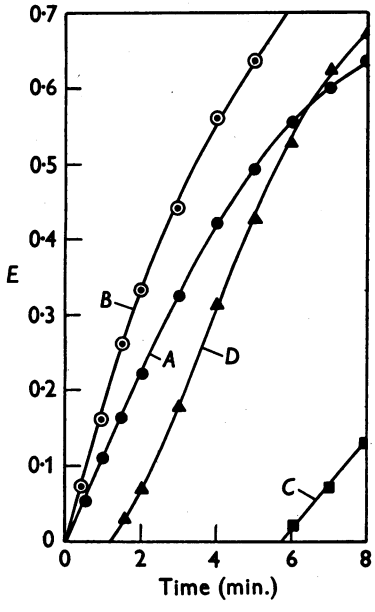

Fig. 6. Effect of Cu^{2+} on the polyphenoloxidase-catechol system, with and without ascorbic acid. *A*, no addition; *B*, 51 μg. Cu^{2+} added; *C*, 0·8 mg. ascorbic acid added; *D*, 51 μg. Cu^{2+} and 0·8 mg. ascorbic acid added. 0·5 ml. enzyme, 2 mg. catechol and additions as shown. Total volume 4·0 ml. in each case.

Influence of copper on the ascorbic-acid-'inactivated' enzyme. It has already been shown that the polyphenoloxidase is inactivated by ascorbic acid. It was thought that this might well have been due to the effect of the ascorbic acid on the enzymically-bound copper, and that addition of extra copper to the system would reverse the inhibition. Consequently, excess copper (51 μg. as $CuSO_4$) was added to enzyme solutions which had been treated as in Table 6 and their activity measured in the usual way. The results are shown in Table 9.

Table 9. *Influence of copper on the inactivated enzymes*

(Enzyme solution (2·0 ml.) in a total volume of 10 ml. treated with varying amounts of ascorbic acid as shown under N_2 for 2 hr. After removal of excess ascorbic acid by dialysis 1 ml. of the enzyme solution was taken with 2 mg. of catechol and Cu^{2+} as shown. Total volume 4·0 ml.)

Ascorbic acid added initially (mg.)	Cu^{2+} present (μg.)	Initial rate (R (graph))	Per cent control	Per cent initial rate with added Cu^{2+}
0	0	0·035	100	100
0	35	0·06	172	172
8	0	0·02	57	100
8	35	0·035	100	175
16	0	0·013	37	100
16	35	0·025	71	192

It can be seen that although the addition of excess copper does indeed bring the rate back to that of the untreated control, the increase in rate is roughly the same for each enzyme used (about 75%). Kertesz (1952) has shown that extremely small amounts of copper (<5 μg.) are required to reactivate his apoenzyme completely. Hence if the enzymically-bound copper had merely been removed during the treatment with ascorbic acid, the addition of the great excess of copper ion would have been expected to increase the observed activity of the inactivated enzyme to a much greater extent than the untreated control, since the excess copper, over the amount required for enzyme reactivation, would then increase the observed activity as shown in Table 7. Thus it appears that the active centres of the protein moiety of the enzyme have been irreversibly affected by the treatment with ascorbic acid.

DISCUSSION

From the results obtained in our experiments there can be no doubt that ascorbic acid exerts a considerable inhibitory effect on potato polyphenoloxidase which is not completely reversed when copper is added to the ascorbic-acid-free system.

It is apparent, therefore, that if the enzyme activity is measured in the presence of ascorbic acid by the chronometric method, by Sreerangachar's method (Asimov & Dawson, 1950) or manometrically (Krueger, 1950), erroneous results may be obtained even when precautions are taken to exclude ionic copper from the solution.

Krueger's results may be partially explained on the assumption that he used ordinary distilled water for his experiments (Kertesz (1951) reported that his 'ordinary' distilled water contained 0·4 μg. copper/ml.). The 'pro-oxidant' effect of ascorbic acid on the system which he observed, could then be due to oxidation of ascorbic acid by ionic copper without the intervention of the enzyme at all. His observations on the decreased rate of formation of dopa from tyrosine in the presence of ascorbic acid are in accord with our results.

Asimov & Dawson (1950), working on the inactivation of mushroom polyphenoloxidase during the oxidation of catechol, stated that 'the possibility of a "slow down" effect due to ascorbic acid itself, or to its degradation products, would also appear to be non-existent, since the course of oxygen uptake (as measured manometrically) has been shown, in these laboratories, to be unaffected by the presence or absence of ascorbic acid.' They measured enzyme activity both by the chronometric method and by Sreerangachar's method and, in view of our results, it is not surprising that they could find no simple equation to fit their inactivation rates.

It appears likely therefore that in much recent work on the nature of polyphenoloxidase systems, in which measurements have been made in the presence of ascorbic acid, the role of ionic copper has been insufficiently considered. The effect of ascorbic acid on polyphenoloxidase may be different *in vivo* and we hope to investigate this point later.

SUMMARY

1. The effect of ascorbic acid and its oxidation products on the activity of potato polyphenoloxidase has been studied using a modified colorimetric procedure.

2. It is shown that the enzyme undergoes a marked denaturation in the presence of ascorbic acid which is not reversed by ionic copper.

3. It is deduced that copper ions catalyse the rate of formation of coloured condensation products from *o*-benzoquinone.

4. Previous work on polyphenoloxidase systems is discussed in the light of our findings.

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REFERENCES

Asimov, I. & Dawson, C. R. (1950). *J. Amer. chem. Soc.* **72**, 821.

Barron, E. S. G., de Meio, R. H. & Klemperer, F. (1936). *J. biol. Chem.* **112**, 625.

Bhagvat, K. & Richter, D. (1938). *Biochem. J.* **32**, 1397.

Jackson, H. & Kendal, L. P. (1949). *Biochem. J.* **44**, 477.

Kendal, L. P. (1949). *Biochem. J.* **44**, 442.

Kertesz, D. (1951). *Bull. Soc. Chim. biol., Paris*, **33**, 1400.

Kertesz, D. (1952). *Biochim. biophys. Acta*, **9**, 170.

Krueger, R. C. (1950). *J. Amer. chem. Soc.* **72**, 5582.

Kubowitz, F. (1938). *Biochem. Z.* **298**, 32.

Mapson, L. W. (1941). *Biochem. J.* **35**, 1332.

Mapson, L. W. (1945). *Biochem. J.* **39**, 228.

Mapson, L. W. (1946). *Biochem. J.* **40**, 240.

Mapson, L. W. & Ingram, M. (1950). *Biochem. J.* **48**, 551.

Miller, W. H. & Dawson, C. R. (1941). *J. Amer. chem. Soc.* **63**, 3375.

Miller, W. H., Mallette, M. F., Roth, L. J. & Dawson, C. R. (1944). *J. Amer. chem. Soc.* **66**, 514.

Moores, R. G., Greninger, D. M. & Rusoff, I. I. (1951). *J. Amer. chem. Soc.* **73**, 928.

Nelson, J. M. & Dawson, C. R. (1944). *Advanc. Enzymol.* **4**, 99.

Polonovski, M. & Gonnard, P. (1951). *C.R. Soc. Biol., Paris*, **145**, 1612.

Ponting, J. D. & Joslyn, M. A. (1948). *Arch. Biochem.* **19**, 47.

Roberts, E. A. H. & Wood, D. J. (1950). *Nature, Lond.*, **165**, 32.

Robinson, E. S. & Nelson, J. M. (1944). *Arch. Biochem.* **4**, 111.

Sreerangachar, H. B. (1943). *Biochem. J.* **37**, 653.

Tenenbaum, L. E. (1940). Dissertation, Columbia University. Quoted in Nelson & Dawson (1944) p. 127.

Trautner, E. M. & Roberts, E. A. H. (1950). *Aust. J. sci. Res.* B, **3**, 356.

Wallerstein, J. S., Alba, R. T. & Hale, M. G. (1947). *Biochim. biophys. Acta*, **1**, 197.

Warner, C. (1951). *Aust. J. sci. Res.* B, **4**, 554.