Interference by Ascorbic Acid in Test Systems Involving Peroxidase. I. Reversible Indicators and the Effects of Copper, Iron, and Mercury

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I describe the mechanism whereby ascorbic acid can hamper test systems involving a peroxide-generating system, peroxidase, and a benzidine-type indicator. In test media such as urines, abnormally high concentrations of ascorbic acid may give rise to false negatives in the determination of analytes such as glucose. Absorbance measurements in solution or reflectance measurements on commercially available paper reagent strips demonstrate either inhibition of visible activity by ascorbic acid or a lag time in the development of oxidized indicator color.

The duration of the lag time is proportional to the ascorbic acid concentration, is inversely proportional to the enzyme concentration, and is also affected by concentrations of hydrogen peroxide and o-tolidine indicator. The same results were seen in both citrate buffer pH 5 and phosphate buffer pH 7. Because the complete system (o-tolidine indicator, hydrogen peroxide, and peroxidase) must be present if the ascorbate is to be oxidized rapidly, this indicates that ascorbic acid inhibits color development by re-reducing oxidized indicator as fast as it is formed; the o-tolidine then acts catalytically in oxidizing ascorbic acid. Added Cu²⁺ and Fe³⁺, both known to react with ascorbic acid, had measurable but small effects on the system. In contrast, Hg²⁺ abolished the ascorbic acid-elicted lag time, even when present in near-stoichiometric concentration. Hg²⁺ showed little inhibitory effect on the activity of either glucose oxidase or peroxidase. Presumably it rapidly oxidizes ascorbic acid to dehydroascorbate. The reaction of mercuric ion with ascorbate was measured by reflectance measurements of paper reagent strips in addition to absorbance measurements of solution assays; equivalent results were obtained. Incorporation of Hg²⁺ into reagent strips can thus render both strips and solution diagnostic tests insensitive to interfering substances such as ascorbic acid.

Additional Keyphrases: enzymic methods · "kit" methods · glucose oxidase · glucose · urine

The use of ascorbic acid in large doses as a dietary supplement has been advocated as a preventative for maladies ranging from the common cold (1) to atherosclerosis (2) and even cancer (3). Within 3 to 6 h after its ingestion, ascorbic acid appears in the urine (4)—after doses of 2 to 5 g/day, as much as 1.5 g per liter may be found in urine (5). With no deliberate supplemental ascorbic acid in the diet, concentrations in urine are expected to be 50 to 100 mg/L. Concentrations of ascorbic acid between these extremes can cause decreased color development with enzymic glucose tests that are based upon the glucose oxidase/peroxidase/indicator test system. Several papers report such interference by ascorbic acid (6-8) as well by other "reducing metabolites" such as homogentisic acid, gentisic acid, dopamine, and 5-hydroxy-indole acetic acid (9). Moreover, glucose assay is not the only analysis that is interfered with by ascorbic acid. Other tests involving the peroxidase redox indicator test principle may likewise be inhibited, e.g., tests for uric acid, occult blood, and galactose.

Here, I focus on the glucose oxidase/peroxidase (EC 1.1.3.4/1.11.1.7) coupled-enzyme test for glucose in general and a commercially available version of it (Clinistix® Reagent Strips) in particular. The commercial product consists of the coupled-enzyme system glucose oxidase/peroxidase, with o-tolidine as indicator, all of which is impregnated into filter paper and mounted on a plastic support handle (10). When this reagent strip is dipped into a urine specimen that contains glucose, a change in color, which can either be read visually by comparison with an appropriate color chart or measured with a reflectometer, indicates the presence of glucose.

In this coupled-enzyme test, glucose is aerobically oxidized to δ-glucuronolactone by the flavoprotein glucose oxidase, with concomitant evolution of H₂O₂. The H₂O₂ in turn reacts with horseradish peroxidase and reduced o-tolidine to generate the blue color of oxidized o-tolidine. In some assay systems, the indicator is oxidized further to produce a yellow color. Other indicators such as o-dianisidine can serve in place of o-tolidine.

Ascorbic acid could interfere with such assay systems in one or more of five ways: interference with the oxidase reaction, competition as a substrate for peroxidase, reversal of dye color, auto-oxidation of ascorbate to generate free radicals, and (or) peroxidase-catalyzed production of radicals with ascorbate.

Interference with the oxidase reaction seemed to me unlikely, because the standard electrode potential for most flavoproteins lies below that for the dehydroascorbate/ascorbic acid couple (0.08 V at pH 6.4) (11). Glucose oxidase containing flavin adenine dinucleotide is not known to be so affected. The remaining four possible routes of inhibition all deal with H₂O₂ and peroxidase and would, therefore, be common to all systems involving these two species.

A likely possibility seemed to be the competition of ascorbic acid with the indicator. It is well known that horseradish peroxidase reacts first, and extremely rapidly, with H₂O₂ to form "Compound I," a spectrophotometrically identifiable heme complex (12, 13). The peroxidase subsequently reacts with a wide variety of substrates, including ascorbate and o-tolidine. Rate constants for the reaction of peroxidase/H₂O₂ (written to include all intermediates) and some substrates have been determined, and they vary over many orders of magnitude (13). Thus ascorbate could compete with o-tolidine for peroxidase. Data to be presented indicate that o-tolidine reacts with peroxidase >100-fold faster than ascorbate, so that for this indicator system the competition mechanism of interference is not important; for slower-reacting indicators, it might be.

The reversal of dye color represents a simple nonenzymic reaction:

\[
\text{Ascorbate/H}_2 + \text{In (colored)} \rightarrow \text{dehydroascorbate} + \text{InH}_2 \text{ (colorless)}
\]

Such a reaction can be predicted for any indicator (In) that
has a higher oxidation-reduction potential than that for ascorbic acid. Many such examples have been reported (14).

The fourth and fifth of the above-listed possible mechanisms of ascorbate inhibition are more complicated and involve evolution of free-radical species in solution. Ascorbate oxidizes spontaneously in aerobic solution to produce radicals (15):

\[ \text{AsH}_2 + \text{O}_2 \rightarrow \text{AsH}^+ + \text{O}_2^- \]

The radical species, especially superoxide \( \text{O}_2^- \), can break down \( \text{H}_2\text{O}_2 \) via the Haber-Weiss cycle (16), which has the net effect of dissipating hydrogen peroxide to oxygen and water. In the presence of metals such as iron, ascorbate and \( \text{H}_2\text{O}_2 \) generate free-radical mixtures that are capable even of hydroxylating aromatic ring compounds (17). Possibly the enzyme preparations used in clinical tests may contain metal-ion impurities. Both peroxidase and glucose oxidase contain iron; the utility of the 2 mol of iron per mole of enzyme found even in highly purified glucose oxidase is not known (18).

The peroxidase-catalyzed production of radicals with ascorbate as a substrate is more clearly understood (19). Peroxidase/\( \text{H}_2\text{O}_2 \) (Compound I) is reduced by ascorbate to give peroxidase "Compound II" and ascorbate free radicals. The interaction of ascorbate radical with oxidized \( \text{o-tolidine} \) is a distinct possibility in view of the suggestion that \( \text{o-tolidine} \) is oxidized one electron per molecule at a time. Johnson and Overby (20) suggest that the typical one-electron \( \text{o-tolidine} \) blue oxidation product is that of a quinhydrone imine dimer. Yamazaki and Piette (21) suggest that some peroxidase substrates, including ascorbate, readily form long-lived free radical intermediates, while others do not.

In understanding the relative importance of these various mechanisms, one must consider the element of turnover, namely, which mechanism(s) predominate(s). To see how much \( \text{H}_2\text{O}_2 \) is lost or degraded by each route discussed was the object of this research.

An understanding of the mechanism in turn suggested suitable approaches for eliminating the interference of ascorbic acid.

Materials and Methods

Reagents

Horseradish peroxidase was obtained as a lyophilized powder from the Research Products Division, Miles Laboratories, Inc. The enzyme, assayed at 2460 kU/g, was used without further purification, and was made up as a 0.75 g/L solution in potassium phosphate buffer (107 mmol/L, pH 7.0). This solution was diluted 100-fold in de-ionized water immediately before use. When stored in concentrated form, no loss in activity was detectable during several months. Glucose oxidase, also from Miles Research Products, was the highly purified grade, and assayed at 1063 kU/g. It was also used without further purification.

\( \text{o-tolidine dihydrochloride} \) (Fisher Scientific Co., Pittsburgh, PA 15219) was dissolved in de-ionized water. Hydrogen peroxide was analytical-reagent grade (Mallinckrodt, Inc., St. Louis, MO 63147) and was assayed spectrophotometrically each day before use; the concentration was determined with use of the value for molar absorptivity, 43.6, at 240 nm (22). Glucose was Mallinckrodt reagent grade and was prepared as a 20 g/L aqueous solution. Mercuric acetate and ferric chloride, from Fisher, and cupric sulfate, from Mallinckrodt, were prepared as 0.1 mol/L aqueous solutions. The mercuric acetate, being light sensitive, was stored in the dark.

Ascorbic acid (Eastman Kodak Co., Rochester, NY 14650; reagent grade) was made up as a 100 g/L solution in water, and diluted either 10- or 100-fold in water immediately before use. Ascorbic acid auto-oxidizes rapidly in phosphate buffer. Consequently, assays in this medium involved addition of ascorbate just before addition of enzyme. Ascorbic acid deteriorates slowly in dilute (100 \( \mu \text{mol/L} \)) solution in water, which created difficulties in obtaining an accurate absorption spectrum. Accordingly, I picked a reference point, 283 nm, and measured the absorbance at this point every four or five readings. The rate of decline of absorbance at 283 nm was sufficiently linear that I could apply a correction factor to each point and thus obtain a more nearly accurate spectrum.

The phosphate buffer mentioned above was prepared from Mallinckrodt analytical reagents in the proportions of 61 mol of dibasic to 39 mol of monobasic potassium phosphate. Citrate buffer (pH 5.0, final concentration in the cuvette, 211 mmol/L) was prepared from reagent-grade citric acid and trisodium citrate. Reagent filter paper from which reagent strips were made was from Eaton-Dikeman, Mt. Holly Springs, PA 17065.

Solution Assays

Assays were run in either 3-mL glass cuvettes or 1-mL quartz cuvettes, depending on wavelength. Changes in absorbance were monitored with a Gilford 2000 recording spectrophotometer. Reactions were initiated by rapidly adding rate-limiting enzyme to the cuvette from a Hamilton syringe while the spectrophotometer chart was moving. This method permitted an accurate measurement of the lag time in color development elicited by ascorbic acid. Kinetic assays were run either in the citrate buffer, where the blue oxidation product of \( \text{o-tolidine} \) predominated, or in phosphate buffer, where a brown oxidation-product predominated. The blue color was measured at 630 nm, the brown color at 411 nm. Initial concentrations of components in the cuvette were: 56.8 \( \mu \text{mol/L} \) ascorbate, 333 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \), 127.5 \( \mu \text{mol/L} \) \( \text{o-tolidine} \), and 55 or 27.5 ng (0.073 U) of peroxidase per milliliter of reaction mixture. In glucose oxidase assays, I used 333 \( \mu \text{mol/L} \) glucose in place of \( \text{H}_2\text{O}_2 \), plus 5500 ng of peroxidase (14.5 U) and 4200 ng of glucose oxidase (0.81 U) per milliliter of reaction mixture. Other components were the same.

In some studies, \( \text{o-tolidine} \) in solution was oxidized nonenzymically, by means of potassium perchromate, \( \text{K}_3\text{CrO}_4 \), a compound that decomposes in aqueous solution with the release of molecular singlet (\( \Delta^g \)) oxygen (23). This reagent was prepared from potassium chromate and 30% \( \text{H}_2\text{O}_2 \) as partially described (23). For electrolytic oxidation of \( \text{o-tolidine} \), I used two platinum electrodes at a potential difference of 22 V.

In studies with assays involving ascorbic acid and possible ascorbate-sensitive metallic agents, a certain order of addition had to be observed. Assays with just peroxidase involved the addition of mercuric ion or other metal, as a 0.1 mol/L solution, 6 s before enzyme was added. With the glucose oxidase assays, peroxidase concentrations were so much higher (200-fold), that ascorbic acid was added 6 s before addition of mercury and 12 s before addition of glucose oxidase.

Assays on Strips

Clinistix\textsuperscript{R} reagent strips (Ames Division, Miles Laboratories) were used in visual experiments. Strips were dipped only in freshly prepared solutions of glucose and ascorbic acid and the stopwatch readings were recorded at that time when the first darkening in color or points of color change were observed.

1 The potassium perchromate was a gift of Dr. M. T. Skarstedt of our laboratory. Personal communication with the authors of this paper (23) revealed an error in their publication on page 3306. Preparation of \( \text{K}_3\text{CrO}_4 \) involved 16.8 g of potassium chromate, instead of potassium hydroxide as described, plus 3.5 g of potassium hydroxide. Other procedures were the same.

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Glucose oxidase/peroxidase reagent strips in which mercuric acetate was added involved first the aqueous impregnation of plain filter paper in a 60 mmol/L solution of mercuric acetate. This paper was then dried in a forced-draft oven at 52–56 °C for 20 to 30 min. The paper containing the mercuric acetate, if overheated, would turn yellow orange, indicating decomposition of mercuric acetate to mercuric oxide, so I took care to avoid this. The paper impregnated with mercuric acetate was next impregnated with the glucose oxidase/peroxidase formula. For some formulations containing mercuric acetate I also used higher concentrations of glucose oxidase and peroxidase enzymes.

I studied development of color on the strip as a function of time by using a reflectance spectrophotometer that can scan a reagent strip over the visible spectrum twice a second. Reagents were automatically dispensed with an automatic pipette pump (Micromedic Systems, Inc., Horsham, PA 19044), and reflectance data were stored on tape by a PDP-12 computer (Digital Equipment Corp., Maynard, MA 01754).

The rapid scanner referred to here was a laboratory-constructed device, consisting of a tungsten iodide light source, appropriate collimating lenses and diaphragms, a monochromator wheel containing 16 narrow-band interference filters (every 20 nm, 380 to 680 nm inclusive) that was motor driven at 120 rpm, and an aluminum integrating sphere, coated on the inside with high-reflectance white paint. The construction and use of this scanner has been described (24). Paper strips mounted upon a holder were inserted in the bottom of the sphere. Light reflected from the sample was amplified by photomultiplier tubes, and data were converted by computer to percent reflectance by comparison with a BaSO₄ white standard calibrator. Percent reflectance data were stored on tape and could be plotted either as spectra or as percent reflectance vs time.

**Results and Discussion**

**The Ascorbate-Elicited Lag Time: Solution Studies**

The object of this research was to define the point at which, and the mechanism by which, ascorbic acid interferes with coupled assay systems involving H₂O₂ and peroxidase. As mentioned earlier, it was postulated that ascorbic acid would not interfere with the flavoprotein glucose oxidase. Consequently, solution assays were devised in which the glucose oxidase was omitted and as few components as possible were included: buffer, peroxidase enzyme, hydrogen peroxide, and o-tolidine, in the concentrations specified in Materials and Methods. An assay mixture containing buffer, hydrogen peroxide, and o-tolidine developed color rapidly after peroxidase was added. In pH 5 citrate buffer, the color was a deep blue; in pH 7 phosphate buffer the color initially was light blue, but quickly turned brown. The results were identical if glucose plus glucose oxidase were substituted for H₂O₂.

Ascorbic acid rapidly injected into a pH 5 assay in progress caused the blue color formed to instantly disappear. The same procedure at pH 7 resulted in a brown color, which would not disappear (these studies will be described in detail shortly). If the ascorbic acid was present in the assay cuvette initially, before addition of peroxidase, no rapid color development occurred, regardless of pH, and no absorbance increase could be seen at either 411 nm or 630 nm. After a certain lag time, color would appear and a rate of color development could be measured. The duration of the lag time was highly reproducible and highly dependent upon ascorbic acid concentration. Figure 1 shows curves illustrating the lag time at pH 5.0 at four concentrations of ascorbic acid, and subsequent formation of the blue color read at 630 nm. Lag time was measured from the point on the chart when enzyme was added to that point where the recorder-pen deflected upward. When the ascorbic acid concentration was decreased by half from 56.8 μmol/L (10 mg/L) to 28.4 μmol/L (5 mg/L), the lag time also decreased by half, from 3.07 to 1.54 min. The relationship between lag time and ascorbic acid concentration is highly linear. Regression analysis of such lag time data in Figure 1 gave a standard error of the estimate of 0.007 min and a correlation coefficient of r = 0.999997. One possible interpretation of these data would be that all ascorbate must be removed before any oxidized o-tolidine color can be seen. The rates at which color formed after the lag time were not very different for low concentrations of ascorbic acid.

Varying the concentration of peroxidase used in the assay had a similar effect on the lag time (Figure 2). In these experiments, ascorbate was held constant at 56.8 μmol/L, other reagents were as described previously, and the concentration of enzyme was varied. Doubling the amount of enzyme present had the effect of halving the lag time: 3.06 min to 1.55 min, whereas halving the enzyme essentially doubled the lag time. One might conclude from this that the lag time is inversely proportional to the enzyme concentration. In addition, the rate of reaction, once it started, was directly enzyme dependent: twice the enzyme gave twice the reaction rate. Table 1 summarizes the effects of variations in reaction components at pH 5.0 on the ascorbate-produced lag time.

**Ascorbate Effects at pH 7.0**

Peroxidase assays at pH 7.0 with all assay components present except ascorbic acid rapidly developed a brown color absorbing at 411 nm. Although a fleeting light-blue color was seen initially, attempts to follow this spectrophotometrically at 630 nm were not useful. Unlike the pH 5.0 assays, when ascorbic acid was added to a pH 7.0 assay already in progress, the brown color did not disappear as did the blue color at pH 5. Instead, further color development was halted until an appropriate lag time had passed, after which color development again proceeded normally. Addition of ascorbate to a
finished reaction did reduce the absorbance at 411 nm somewhat, but this was due to precipitation of the brown-colored material in the cuvette.

If ascorbic acid was present initially in the assay cuvette at pH 7.0 at the time peroxidase enzyme was added, a characteristic lag time in color development was seen. Like similar assays run at pH 5.0, no color formation at all was evident until the lag time had passed. Again, like the pH 5.0 assay, the length of the lag time was directly proportional to the concentration of ascorbic acid added. If ascorbic acid was held constant and peroxidase enzyme varied, an inverse dependence of lag time upon peroxidase concentrations was seen. These data are summarized in Table 2.

Reaction Rate Dependence on Peroxide Concentration

Two other variables, H₂O₂ and o-tolidine concentrations, were also tested, and the lag times for these are summarized in Tables 1 and 2 for each pH. Evidently, increases in either peroxide or o-tolidine concentrations shorten the lag times, but the relation between lag time and concentration is not as straightforward as was the case for variations in ascorbate or peroxidase concentration. The relationship between lag time and peroxide concentration can be better understood when one considers the reaction rate dependence upon H₂O₂ concentration.

Both the length of the ascorbate-elicited lag time and the reaction rate after the lag changed as the H₂O₂ concentration was varied. Decreasing the peroxide concentration had the effect of both prolonging the lag time and slowing the reaction rate. At double the peroxide concentration, 666 μmol/L, the rate was 0.75 A/min at 630 nm in pH 5.0 citrate buffer. Conversely, at one-half as much peroxide, 167 μmol/L, the rate was 0.253 A/min in this buffer. From Table 1, one may note that the lag time changed from 2.52 to 4.99 min, respectively. Both the reduction of H₂O₂ and the oxidation of ascorbate involve two electrons. If one assumes a 1:1 stoichiometry between ascorbate and H₂O₂, and a decrease in the H₂O₂ concentration by that ascorbate concentration added initially, the effective H₂O₂ concentration presented to the o-tolidine is decreased by that concentration of ascorbate (56.8 μmol/L), to 609 μmol/L H₂O₂ at double, or to 110 μmol/L H₂O₂ at half, the concentration of H₂O₂.

The relationships between lag time and variation in either H₂O₂ or o-tolidine concentration as seen in Table 2 at pH 7.0 are also similar to those depicted at pH 5.0 in Table 1. The

**Table 1. Ascorbate Lag Time of Peroxidase Reaction at pH 5.0**

<table>
<thead>
<tr>
<th>Component and &quot;normal&quot; concn</th>
<th>Lag time, min, at component concns varied by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Double</td>
</tr>
<tr>
<td>Ascorbate, 56.8 μmol/L</td>
<td>6.17</td>
</tr>
<tr>
<td>Peroxidase, 27.5 μg/L</td>
<td>1.55</td>
</tr>
<tr>
<td>Hydrogen peroxide, 333 μmol/L</td>
<td>2.52</td>
</tr>
<tr>
<td>o-Tolidine, 127.5 μmol/L</td>
<td>2.85</td>
</tr>
</tbody>
</table>

In citrate buffer, 0.311 mol/L; reversible blue color appearing at 630 nm.

**Table 2. Ascorbate Lag Time of Peroxidase Reaction at pH 7.0**

<table>
<thead>
<tr>
<th>Component and &quot;normal&quot; concn</th>
<th>Lag time, min, at component concns varied by</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Double</td>
</tr>
<tr>
<td>Ascorbate, 10 mg/L (56.8 μmol/L)</td>
<td>7.12</td>
</tr>
<tr>
<td>Peroxidase, 27.5 μg/L</td>
<td>1.95</td>
</tr>
<tr>
<td>Hydrogen peroxide, 333 μmol/L</td>
<td>3.6</td>
</tr>
<tr>
<td>o-Tolidine, 127.5 μmol/L</td>
<td>3.48</td>
</tr>
</tbody>
</table>

In phosphate buffer, 0.107 mol/L; irreversible brown color, appearing at 411 nm.

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rates of enzyme activity after the lag are also comparable at pH 7.0 and pH 5.0.

This effect of H₂O₂ concentration on the reaction rate was tested directly, as seen from the data presented in Figure 3. If one varies H₂O₂ concentration, keeping all other species constant, a plot of H₂O₂ concentration vs reaction rate gives a saturation curve. The corresponding double-reciprocal plot of 1/v in ΔA/min (reaction rate) vs 1/[H₂O₂] is a straight line, from which an apparent Kₘ for H₂O₂ of 92.6 μmol/L can be calculated. This is about 100-fold greater than previously reported values (12, 25) with different indicators. From this, one would predict a dropoff in reaction rate as a relatively high substrate concentration approached the apparent Kₘ. These data also explain why the curves in Figure 1 are parallel. At 14.2, 28.4, and 56.8 μmol/L ascorbate concentrations, H₂O₂ concentrations would be 319, 306, and 276 μmol/L, respectively. These are all too close together compared with a Kₘ of 93 μmol/L for H₂O₂; hence, the similarity in reaction rates in Figure 1. The data in Figure 3, though, were determined with use of 107 mmol/L phosphate buffer, pH 7.0. One would not necessarily expect the same apparent Kₘ when determined with use of 0.3 mol/L citrate buffer, pH 5.0, although the overall saturation pattern should be the same. And this peroxide saturation pattern should explain the effects of variations in H₂O₂ concentration seen in Tables 1 and 2.

Variations in o-tolidine, like those in H₂O₂, had a similar inverse effect upon the ascorbate-induced lag time. Higher o-tolidine concentrations resulted in shorter lag times, both at pH 5 and pH 7. A saturation curve for o-tolidine was not prepared; presumably, the same sort of phenomenon is occurring with o-tolidine as was seen for H₂O₂.

Two oxidation products of o-tolidine are described: a blue product, thought to be quinhydrone imine dimer (20), and the brown product, which is regarded as a monomeric quinone imine and is also the predominant oxidation product in the pH range near 3.0 (20, 26). Interconversion of the two forms is reported (20, 26) to be dependent upon pH, although the data herein suggest that the brown form is less soluble in water than the blue form. The absorptivity of the blue form of o-tolidine appears to be at least 3.5-fold that of the brown form, but accurate measurements of this are difficult because of the instability of the blue color. Even at pH 5.0, the blue color formed initially after several minutes became green and, finally, brown. The data in Table 1 refer only to initial activity and would therefore reflect formation only of blue color.

The Role of Ascorbate in the Reaction

All of the above data can be explained with the assumption that in an assay system containing peroxidase, o-tolidine, hydrogen peroxide, and ascorbic acid, all ascorbate present in solution must be oxidized before any o-tolidine present oxidizes. Neither of the two oxidation products of o-tolidine appear until a lag time absolutely proportional to ascorbate concentration has elapsed. This suggests that ascorbate is possibly a highly active substrate for peroxidase, very much more active than o-tolidine. I tested this hypothesis spectrophotometrically.

Figure 4 illustrates absorption spectra for ascorbic acid, hydrogen peroxide at the assay concentration (333 μmol/L), and dehydroascorbate. The dehydroascorbate spectrum was obtained by allowing that reaction, involving ascorbic acid, H₂O₂, peroxidase, and phosphate buffer, to go to completion. The absorption spectrum for ascorbic acid in water diminished with time; the half-time for decay measured at 261 nm was 111 ± 2 min. Although H₂O₂ and dehydroascorbate both absorb in the far ultraviolet, at the concentrations and wavelengths of interest these species are essentially transparent. Contributions of buffer and peroxidase enzyme to the ultraviolet absorption at the concentrations indicated were also negligible.

Table 3 gives the observed rates of decrease in the absorbance of ascorbic acid at 261 nm as each component of the assay system was added. The Table includes both buffer systems: phosphate buffer at pH 7.0 and citrate buffer at pH 5.0. The phosphate buffer appeared to accelerate the rate of ascorbate auto-oxidation (8.1-fold), whereas the citrate buffer depressed that rate (3.4-fold), both as compared with distilled water. Under these conditions, ascorbate was not active as a substrate for the peroxidase. By comparison, the relative number for o-tolidine oxidation (ΔA/min measured at 630 nm rather than 261 nm, in citrate buffer) is 895. The above might indicate that
Table 3. Rate of Oxidation of Ascorbate, 100 
μmol/L, under Different Conditions

<table>
<thead>
<tr>
<th>Condition</th>
<th>ΔA_215nm/min</th>
<th>Relative ΔA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ascorbic acid in distilled water</td>
<td>0.0041</td>
<td>1</td>
</tr>
<tr>
<td>2. Add phosphate buffer (107 mmol/L, pH 7) to 1</td>
<td>0.0332</td>
<td>8.1</td>
</tr>
<tr>
<td>3. Add H₂O₂ (333 μmol/L) to 2</td>
<td>0.0866</td>
<td>21.1</td>
</tr>
<tr>
<td>4. Add peroxidase (55 μg/L) to 3</td>
<td>0.0657</td>
<td>16.0</td>
</tr>
<tr>
<td>5. Ascorbic acid in citrate buffer (0.3 mol/L, pH 5)</td>
<td>0.00120</td>
<td>1</td>
</tr>
<tr>
<td>6. Add H₂O₂ (333 μmol/L) to 5</td>
<td>0.00147</td>
<td>1.23</td>
</tr>
<tr>
<td>7. Add peroxidase (55 μg/L) to 6</td>
<td>0.00233</td>
<td>1.94</td>
</tr>
<tr>
<td>8. Add o-tolidine (127.5 μmol/L) to 7</td>
<td>0.371</td>
<td>309</td>
</tr>
</tbody>
</table>

Ascorbate is not a substrate for peroxidase. Such is not true. One should note that Yamazaki et al. (19), for example, used 200-fold these concentrations of ascorbate and peroxidase in their assays. Relative to o-tolidine, ascorbate is simply very slowly acted on by peroxidase.

When o-tolidine (100 μmol/L) was now added to the assay system consisting of citrate, H₂O₂, ascorbate, and peroxidase, the absorbance at 261 nm very rapidly decreased. At the point where the decrease became less rapid, the cuvette contents had turned blue. This point corresponded with the lag time predicted from previous experiments. It seemed as if the addition of o-tolidine catalyzed the rapid oxidation of ascorbate. The data in Table 3 indicate a 159-fold increase in activity at 261 nm when o-tolidine was added to the system. I did not attempt to follow the o-tolidine-enhanced oxidation of ascorbate in phosphate buffer because, in this buffer, o-tolidine oxidizes to a brown product, which rapidly precipitates. The light scattering from any precipitate formation would only interfere at the 261-nm wavelength for ascorbate observation.

During the lag time, then, ascorbate is clearly being oxidized, as observed at 261 nm, the absorbance peak of ascorbate. It is of interest to know if any of the absorbance changes occurring at 261 nm can be attributed to o-tolidine. Reduced o-tolidine shows considerable absorption at this wavelength. Because no color is developed during the lag time, it may be concluded that neither of the oxidation products of o-tolidine is produced. This does not answer the question of what, if anything, is happening to reduce o-tolidine during the lag. The answer to this was tested spectrophotometrically. An absorption spectrum of reduced o-tolidine is shown in Figure 5. This spectrum shows a peak at 281 nm (ε = 20 340 L·mol⁻¹·cm⁻¹), as well as considerable absorption at 261 nm (absorptivity equivalent to that for ascorbic acid). There is considerable overlap between absorptions for ascorbic acid and reduced o-tolidine in the ultraviolet, except above 310 nm, where ascorbic acid is transparent. At 315 nm, o-tolidine still has appreciable absorption (ε = 5460 L·mol⁻¹·cm⁻¹).

Accordingly, an assay was performed in citrate buffer and observed at 315 nm for possible changes in the absorption spectrum of reduced o-tolidine; none was found during the lag time. At the same time that the absorbance at 630 nm increased, the absorbance at 315 nm began to increase. Before this point, the cuvette contents were colorless; afterward, they had turned blue. Clearly, nothing that is spectrophotometrically detectable happens to the reduced o-tolidine during ascorbate oxidation, yet o-tolidine must be present for this oxidation to occur.

There are two possible explanations for the activity of o-tolidine in the peroxidase-mediated oxidation of ascorbate. Possibly o-tolidine binds to the peroxidase enzyme as an effector, thus promoting the catalysis of ascorbate oxidation. The other, more viable explanation consistent with the above observations is a nonenzymic reduction by ascorbate of a small amount of oxidized o-tolidine produced enzymically. Experimentally, o-tolidine has been shown to be a much faster substrate for peroxidase than is ascorbate. If one considers the low redox potential for ascorbate (0.06 V at pH 7.0 vs. 0.55 V at pH 7.0 for o-tolidine) (14, 27), it is entirely conceivable that o-tolidine is first oxidized by peroxidase, but reduced so readily by ascorbate that no spectral changes in o-tolidine (at 315, 411, or 630 nm) can be detected. Thus o-tolidine effectively catalytically cycles all the oxidation power from H₂O₂ to ascorbate without appearing to be affected itself. When all the ascorbic acid is oxidized, the normal o-tolidine blue color begins to appear.

I devised a further experiment to test the ability of ascorbate to react rapidly in a nonenzymic fashion with oxidized o-tolidine. As already mentioned, addition of ascorbate to the peroxidase-produced blue o-tolidine color caused immediate loss of color. Whether this could occur in the absence of enzyme was tested experimentally. Chemical oxidation was effected by adding a small amount of K₂CrO₅ to a buffered solution of o-tolidine, producing a deep-blue color. A few milligrams of added ascorbic acid immediately reversed the color.

Oxidized o-tolidine was also prepared electrolytically as described in Methods. About 3-5 min of electrolysis of o-tolidine in citrate buffer sufficed to produce a blue color; addition of ascorbate quickly reversed this color.

Both of these nonenzymic experiments demonstrate that peroxidase enzyme is not at all essential for the o-tolidine-mediated oxidation of ascorbate. Perhaps o-tolidine can function as an enzyme-bound effector that facilitates ascor-
bating oxidation. However, the rates at which ascorbate bleached oxidized o-tolidine in the two cases above were much too fast to measure by conventional techniques. This suggests that the simpler explanation involving nonenzymatic reduction of oxidized indicator is probably correct.

Because the o-tolidine is envisaged as the species interfered with by ascorbic acid, the same relative effects of ascorbic acid should be seen in the glucose oxidase/peroxidase coupled assay system, and this was, in fact, found to be the case. Glucose was then substituted for H$_2$O$_2$ at the same concentrations, the peroxidase concentration was increased, and glucose oxidase was added at a rate-limiting concentration. The same linear lag time dependence upon ascorbate concentration was obtained: 10 mg/L ascorbate gave a lag of 2.38 min, 5 mg/L ascorbate gave a 1.14-min lag, and 2.5 mg/L a 0.60-min lag. These data suggest that the effect of ascorbate on the glucose oxidase/peroxidase/tolidine system is indistinguishable from the effect on the peroxidase/tolidine system and can be explained entirely on the basis of ascorbate reduction of oxidized indicator, with no effect on the glucose oxidase-catalyzed reaction.

Effects of Ascorbic Acid on Clinistix Reagent Strips

The studies described previously in solution experiments were now extended to reagent strips containing the coupled enzyme assay system for determination of glucose. Several commercial products are available for the determination of glucose in biological fluids such as urine. Most of these contain glucose oxidase, peroxidase, and an indicator such as o-tolidine, o-dianisine, 2,7-diaminofluorone, iodide, or 3-amino-6-chloro-9(γ-dimethylaminopropyl)carbazole.

The present observations are restricted to Clinistix reagent strips manufactured by Ames Co., Division of Miles Laboratories. When dipped in a urine specimen containing glucose, this reagent strip turns purple or dark blue, due to oxidation of the o-tolidine indicator. These Clinistix reagent strips were tested by dipping first into a glucose solution and then into ascorbate. The blue color that initially formed with glucose would fade if dipped into ascorbate, but this depended upon the concentrations of ascorbate and glucose relative to each other. When ascorbate concentration approached 50% of the glucose concentration, fading of the strip’s blue color was readily apparent. Such fading was time dependent as well; the blue color faded more slowly if the reagent strip was allowed to stand longer before being dipped in ascorbate.

When Clinistix reagent strips were wet with an aqueous solution containing both glucose and ascorbic acid, a lag time was observed between wetting and the onset of color. This lag time was dependent on ascorbate concentration. Data obtained at several glucose and ascorbate concentrations are given in Table 4. The lag time is proportional to ascorbate concentration, though not as strictly so as in the solution experiments. Increasing the glucose concentration in strip experiments elicited essentially the same response as did increasing the H$_2$O$_2$ concentration in solution experiments. When ascorbate concentration reached about 50 to 60% of the glucose concentration, the lag times became very long. Strip measurements at long times (greater than 2 to 3 min) were less meaningful because drying of the strip slowed the reaction rate.

The time course of development of Clinistix blue color in the presence of glucose with and without ascorbic acid may be followed with a reflectance spectrophotometer. Figure 6 is typical of data gathered in this laboratory. Two strips were wet automatically and the strip reflectance recorded every half-second, over the course of 100 s, with the rapid scanning reflectance spectrophotometer described in Methods. The graph shows the development with time of blue color by showing the drop in reflectance of red light at 640 nm. Both strips were wet with 2.5 g/L (13.88 mmol/L) glucose in the presence and absence of 1 g/L (5.68 mmol/L) ascorbic acid. The curve with ascorbic acid shows a "plateau" of no color development lasting some 22 s, after which color development begins slowly. This time lag is in agreement with that seen in Table 4 (22 s).

The data in Table 4 and Figure 6 agree with the solution assay results reported above, namely, that ascorbic acid elicits a lag time in color development in such peroxidase-dependent assays. The length of this lag time is strictly proportional to ascorbic acid concentration. The results obtained with the solution assays involving the coupled-enzyme system are also in good agreement with the simplified system containing just peroxidase, H$_2$O$_2$, and oxidizable indicator. Although ascorbic acid is a substrate for peroxidase, its rate of oxidation in the absence of o-tolidine is several hundred times slower than the comparable rate for o-tolidine itself. Oxidation of ascorbic acid in the presence of o-tolidine is 159-fold faster than in the absence of o-tolidine. Nothing spectrophotometrically detectable happens to the o-tolidine during such ascorbate oxidation.

All of these results suggest that o-tolidine acts catalytically to oxidize ascorbic acid. The fact that ascorbate will very quickly bleach oxidized o-tolidine blue color in the absence of enzyme suggests that ascorbic acid serves to re-reduce a catalytic and very small quantity of oxidized o-tolidine as fast as it is formed enzymically. This is the most likely mechanism of action of the ascorbic acid inhibition of peroxidase systems involving reversible indicators such as o-tolidine.

This mechanism appears to operate at both pH 5 and at pH 7 where an irreversible brown color is formed. The brown color could not be bleached by ascorbic acid after it had formed. If

![Fig. 6. Reflectance changes of Clinistix wet with glucose (2.5 g/L), with and without ascorbic acid](image-url)
ascorbic acid were present initially, no color formed at pH 7.0. These results, consistent with the literature, support the belief that o-tolidine oxidation at pH 7 proceeds first to a reversible blue color and then to the irreversible brown color.

**Effect of Copper and Iron on Ascorbic Acid Lag Time**

The ascorbic acid-dependent lag time assay with peroxidase/o-tolidine permitted evaluation of various metallic cations as possible oxidants of ascorbic acid, by measuring the length of the lag time for oxidizing o-tolidine color development in the presence of both ascorbic acid and metal cation.

The chemical oxidation of ascorbic acid by copper has long been known (28, 29). The products of such aerobic chemical oxidation are dehydroascorbate and hydrogen peroxide (28). By itself, ascorbic acid will auto-oxidize only at a pH >7.6 (29). Under alkaline conditions, such products are salts of oxalic and L-threonic acids (29). Copper is also involved biochemically in the oxidation of ascorbic acid. It is a necessary component in the active site of at least two enzymes that interact with ascorbic acid, namely, ascorbic acid oxidase (30) and dopamine β-hydroxylase (31).

For these and other reasons, I considered copper a possible candidate for removing ascorbic acid from test solutions. Accordingly, I added increasing quantities of 0.1 mol/L CuSO₄ to separate solution assays of peroxidase, all of which contained, per liter, 56.8 μmol (10 mg) of ascorbate. The results are depicted in Figure 7 as the development of color at 630 nm vs time.

In the absence of copper, no oxidized o-tolidine blue color formed until after a certain interval, in this case 2.95 min. After this time, color development was that expected for a peroxidase/o-tolidine assay without added ascorbic acid. The addition of 0.3 mmol of cupric sulfate per liter to this assay 6 s before addition of peroxidase shortened the lag time from 2.95 to 2.84 min. A 10-fold increase in copper, to 3 mmol/L, shortened the lag to 2.59 min, only a 12% decrease. Much higher concentrations of copper had little further effect. The higher absorbance at 620 nm at which the 10 and 30 mmol/L CuCl₂ assays began merely reflects the blue color of the copper. The data in Figure 7 suggest that copper is not as effective in oxidizing ascorbic acid as is oxidized o-tolidine. The reduction of oxidized o-tolidine by ascorbic acid is too fast to be measured by conventional spectrophotometric techniques.

Iron has been studied in systems with ascorbic acid, hydrogen peroxide, and ethylenediaminetetraacetic acid (EDTA) in attempts to simulate the peroxidase reaction (17, 32). Either ferrous or ferric iron in combination with ascorbic acid was effective in hydroxylating aromatic rings; copper and cobalt were only 5–10% as effective as iron (17).

Ferric chloride then was tried in the same assay system illustrated in Figure 7. Again, FeCl₃ was added 6 s before addition of peroxidase. The results were only slightly better than those achieved with copper. A concentration of 3 mmol/L FeCl₃ reduced the ascorbate-elicited lag time from 3.07 to 2.00 min and 30 mmol/L FeCl₃ reduced it to 1.10 min. Although the literature suggests that iron in the presence of H₂O₂ is effective in oxidizing ascorbic acid, kinetically the reaction does not seem to be rapid. Iron concentrations of 30 mmol/L were 528-fold the concentration of ascorbic acid.

**Effects of Mercuric Ion on Ascorbic Acid Lag Time**

In the case of mercuric ion, however, a dramatic decrease in lag time was seen. A concentration of mercuric ion of only 300 μmol/L, added as the acetate, reduced the lag time in the above assay system to only 0.1 min. A concentration 10-fold this abolished the lag time entirely (Figure 8) under assay conditions.

[Fig. 7. Effects of cupric ion on the ascorbate-induced lag time. Absorbance at 630 nm vs time in minutes ('). Conditions were as specified in Methods. Cupric sulfate at the concentrations indicated was added 6 s before 82.5 ng of peroxidase (POD). Fig. 8. Elimination of the ascorbate-induced lag time by mercuric ion. See text for H₂O₂, o-tolidine, and pH 5.0 citrate buffer content in reactions A, B, and C; ascorbic acid (AsCH₃) at the concentrations indicated. Mercuric acetate at concentrations specified was added 6 s before 82.5 ng of peroxidase (POD). Initial rates of color development at 630 nm are indicated. Reaction D included 333 μmol/L glucose in place of H₂O₂, peroxidase at 5500 μg/L, and the same o-tolidine and buffer as reactions A–C. Ascorbate, mercuric ion, and glucose oxidase (25.1 μg) were added 6 s apart as indicated. For reaction C, v is 51% of that for reaction A.]
conditions the same as those in Figure 1. Considering the 2:1 stoichiometry needed between mercuric ion and ascorbic acid, the first assay (A) represents a ratio of 2.64 equivalents of mercuric ion to 1 of ascorbic acid. Curves B and C in Figure 8 represent the same ratio of mercury to ascorbic acid as Curve A, except that the concentrations of mercury and ascorbic acid are each increased 10-fold (Curve B) and 100-fold (Curve C). In both cases, the lag time is minimal to none. The only apparent difference among the three curves, A, B, and C, is a 49% loss in enzyme activity at 100-fold increases of ascorbate and mercury concentrations.

The last curve, D, represents the complete assay with glucose substituted for H₂O₂ at the same concentration and with glucose oxidase now rate-limiting instead of peroxidase. No essential difference was seen between the glucose/glucose oxidase assay and the H₂O₂/peroxidase assay.

The rapid oxidation of ascorbic acid by mercuric ion, as compared with copper and iron, was certainly not expected from consideration of the standard potentials of these metals, as given in Table 5. One would have expected ferric iron to be more reactive with ascorbic acid than it was. The lag times are all with metal concentrations at 3 mmol/L and ascorbic acid at 56.8 mmol/L. The short lag time of 0.02 min for mercuric ion was obtained by extrapolation of the curve for solution absorbance vs time. Assay conditions otherwise were identical to those in Figure 7.

Little is known of the reaction of ascorbic acid with mercuric ion, although it has been studied as part of a program of ascorbinometry of various heavy metals (34). The possible clinical utility of mercuric ion was advanced by Ku in a patent covering the use of metals as trapping agents for interfering reducing substances, such as ascorbic acid, that are found in body fluids (35). The examples suggested by Ku pertain to test reactions run on strips. The data presented herein constitute further documentation of this phenomenon with solution studies.

Apparently ascorbic acid reduced mercuric ion only to mercurous ion, there being no evidence of the production of elemental mercury at any of the concentrations studied here. The standard potentials are certainly favorable for this reduction to occur; the Hg²⁺/Hg and ½ Hg₂⁺²/Hg reactions have standard potentials of 0.851 and 0.799 V, respectively (33).

Effects of Mercuric Ion on the Enzyme Reactions

The loss in enzymatic activity at the higher mercuric acetate concentration in Figure 8 suggested possible inhibition of enzyme activity. The possible inhibitory effects of mercuric acetate upon both the peroxidase and glucose oxidase/peroxidase coupled reactions were studied in the absence of ascorbic acid; the results are given in Table 6. For the glucose oxidase/peroxidase reaction, the peroxidase was increased 400-fold, such that glucose oxidase was rate-limiting.

The degree of inhibition was roughly the same for peroxidase as for glucose oxidase. In fact this "inhibition," if that term can be used, should be regarded as minimal to nonexis-

**Table 6. Effects of Mercuric Acetate on Peroxidase and Glucose Oxidase/Peroxidase Reactions**

<table>
<thead>
<tr>
<th>Mercuric acetate, mmol/L</th>
<th>Peroxidase reaction</th>
<th>Glucose oxidase/peroxidase reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔA/min</td>
<td>Relative velocity</td>
</tr>
<tr>
<td>0</td>
<td>0.2983</td>
<td>100</td>
</tr>
<tr>
<td>0.3</td>
<td>0.2968</td>
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</tr>
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<td>3</td>
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<td>80.5</td>
</tr>
<tr>
<td>30</td>
<td>0.1213</td>
<td>40.7</td>
</tr>
</tbody>
</table>

Peroxidase reaction conditions: 333 μmol/L H₂O₂, 127.5 μmol/L o-tolidine, and 0.311 mol/L citrate buffer, pH 5.0, with 13.76 μg/L peroxidase added to start the reaction. Glucose oxidase reaction conditions: the same o-tolidine and buffer, 333 μmol/L o-glucose, with 5500 μg/L peroxidase and 4185 μg/L glucose oxidase added to start the reaction.

The enzymes in which sulphydryl groups participate in catalysis are inactivated by mercury concentrations several orders of magnitude lower than those reported in Table 6. Both peroxidase (36) and glucose oxidase (37) have been reported to be insensitive to Hg²⁺ ion, although the concentrations used were not as high as those in the present study.

The same level of inhibition seen at 30 mmol/L Hg²⁺ in Table 6 and Figure 8 would suggest that mercuric ion rather than mercurous ion or dehydroascorbate is responsible for the inhibition seen. Obviously, very little mercurous ion could be present in the assays without added ascorbic acid.

If, at high concentrations mercuric ion, the amount of rate-limiting enzyme (peroxidase or glucose oxidase) was increased, the rate of color development also increased in the same proportion as the enzyme. This suggests that the inhibitory effect of mercuric ion upon the rate of color development acts on the enzyme system and not on the indicator used.

Effect of Mercury on Ascorbic Acid with Reagent Strips

The above studies by solution experiments were now extended to Clinistix reagent strips. The data obtained with Clinistix are shown in Figure 9 as percent reflectance at 640 nm vs time. Only the first 5 s are shown; curves 1 and 2 are the same unaltered Clinistix shown in Figure 6, and glucose and ascorbic acid concentrations are the same as Figure 6. The initial decrease in reflectance during the first second represents wetting of the reagent strip. Curve 3 illustrates the change in reflectance with time for a Clinistix strip containing 60 mmol/L mercuric acetate wet with 2.5 g/L glucose and 1 g/L ascorbic acid. Unlike curve 2, this strip shows considerable reactivity in the presence of ascorbic acid. The rate of color development with this strip, though, is slower than that of a Clinistix without mercury dipped just in glucose (curve 1). A possible reason for the reduced reactivity of such strips might be the inhibition of enzyme activity by the presence of mercuric ion; if so, this inhibition could be overcome by increasing the amounts of glucose oxidase and peroxidase in the strip.

In visual experiments, Clinistix strips containing 60 mmol/L mercuric acetate were dipped in five tubes, all containing 2.5 g/L glucose and with ascorbic acid at 0, 0.5, 1.0, 1.5 and 2.0 g/L. All of the strips changed color, but the rate of color development was much slower than that of a normal Clinistix strip wet just with 2.5 g/L glucose. The mercury strip with 2 g/L ascorbate added to the glucose lagged behind the others in color development.

The studies with the glucose oxidase/peroxidase system on
paper strips, with mercuric acetate added, demonstrate the ability of mercuric ion to offset the otherwise inhibitory effects of ascorbic acid on such a strip test. The studies with reagent strips are in agreement with the solution studies reported above, namely, that mercuric acetate will rapidly remove the inhibitory effects of reducing species such as ascorbic acid upon peroxidase-mediated test systems. The slight inhibition of enzymic activity caused by mercuric ion was easily offset by increasing the amount of enzyme, both in solution and on strips.

The peroxidase/indicator system is a convenient though indirect means for testing the ability of oxidizing agents to oxidize reducing species such as ascorbic acid. Low concentrations of ascorbic acid and other similar reductants can be detected by their ability to produce a lag time in color development, the duration of which is proportional to the reductant concentration. The ability of an oxidant to oxidize the reducing species is then easily assessed by its effect upon the reductant-produced lag time. The assay system is most effective if neither the oxidant nor the reductant, nor their reaction products, are absorbant in the visible spectrum. Such is the case with mercuric acetate and ascorbic acid.

References


