Kinsella, J. E.; Damodaran, S.; German, B. Physiochemical and Functional Properties of Oilseed Proteins with Emphasis on Soy Proteins. *New Food Proteins* **1985**, *5*, 107–178.
Krebs, E. G. The Enzymology of Control by Phosphorylation. *The Enzymes*; Academic Press: New York, 1986; Vol. 17, pp 3–20.
Krebs, E. B.; Beavo, J. A. Phosphorylation-Dephosphorylation of Enzymes. *Annu. Rev. Biochem.* **1979**, *48*, 923–959.
Laemmli, U. K. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. *Nature (London)* **1970**, *227*, 680–685.
Matheis, G.; Whitaker, J. R. Chemical Phosphorylation of Food Proteins: An Overview and a Prospectus. *J. Agric. Food Chem.* **1984**, *32*, 699–705.
Matheis, G.; Penner, M. H.; Fenney, R.; Whitaker, J. R. Phosphorylation of Casein and Lysozyme by Phosphorus Oxychloride. *J. Agric. Food Chem.* **1983**, *31*, 379–387.
Moreira, M. A.; Hermodson, M. A.; Larkins, B. A.; Nielsen, N. C. Comparison of the Primary Structure of the Acidic Polypeptides of Glycinin. *Arch. Biochem. Biophys.* **1981**, *210*, 633–642.
Roskoski, R. Assay of Protein Kinase. *Methods Enzymol.* **1983**, *99*, 3–6.
Seguro, K.; Nio, S.; Motoki, M. The Manufacture Method of Modified Proteins. Japanese Patent No. 128,843, 1986.
Shoji, S.; Parmelee, D. C.; Wade, R. D.; Kumar, S.; Ericsson, L. H.; Walsh, K. A. Complete Amino Acid Sequence of the Catalytic Subunit of Bovine Muscle Cyclic AMP-dependent Protein Kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 848–851.
Shoji, S.; Ericsson, L. H.; Walsh, K. A.; Fischer, E. H.; Tetani, K. Amino Acid Sequence of the Catalytic Subunit of Bovine Type II Adenosine Cyclic 3′,5′-Phosphate-dependent Protein Kinase. *Biochemistry* **1983**, *22*, 3702–3709.
Smith, A. K.; Circle, S. J. *Soybeans: Chemistry and Technology*; Avi: Westport, CT, 1972.
Smith, S. B.; White, J. B.; Siegel, J. B.; Krebs, E. G. Cyclic-AMP-dependent Protein Kinase: Primary Steps of Allosteric Regulation. In *Protein Phosphorylation*; Rosen, O. R., Krebs, E. G., Eds.; Cold Springs Harbor Laboratory: Cold Springs Harbor, ME, 1981; Vol. 8, pp 55–65.
Staswick, P. E.; Hermodson, M. A.; Nielsen, N. C. The Amino Acid Sequence of the $A_2B_{1a}$ Subunit of Glycinin. *J. Biol. Chem.* **1984**, *259*, 13424–13430.
Sung, H. Y.; Chen, H. J.; Liu, T. Y.; Su, J. C. Improvement of the Functionalities of Soy Protein Isolate through Chemical Phosphorylation. *J. Food Sci.* **1983**, *48*, 716–721.
Sykes, G. E.; Gayler, K. E. Detection and Characterization of a New β-Conglycinin. *Arch. Biochem. Biophys.* **1981**, *210*, 525–530.
Uhler, M. D.; McKnight, G. S. Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent Protein Kinase. *J. Biol. Chem.* **1987**, *262*, 15202–15207.
Uhler, M. D.; Carmichael, D. F.; Lee, D. C.; Chrivia, J. C.; Krebs, E. G.; McKnight, G. Isolation of the cDNA Clones Coding for the Catalytic Subunit of Mouse cAMP-dependent Protein Kinase. *Proc. Natl. Acad. Sci. U.S.A.* **1986a**, *83*, 1300–1304.
Uhler, M. D.; Chrivia, J. C.; McKnight, G. S. Evidence for a second isoform of the catalytic subunit of cAMP-dependent Protein Kinase. *J. Biol. Chem.* **1986b**, *261*, 15360–15363.
Zoller, M. J.; Kerlavage, A. R.; Taylor, S. S. Structural Comparisons of cAMP-dependent Protein Kinases I and II from Porcine Skeletal Muscle. *J. Biol. Chem.* **1979**, *254*, 2408–2412.

Received for review August 30, 1988. Accepted January 26, 1989. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of this product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable.

# Oxygen Uptake by Gallic Acid as a Model for Similar Reactions in Wines

Vanna Tulyathan,[1] Roger B. Boulton, and Vernon L. Singleton*

Autoxidation in highly alkaline solution consumed 4.9 atoms of oxygen/molecule of gallic acid oxidized. Hydrogen peroxide was proven (for the first time at least in a wine context) to be produced during the oxidation and was quantitated at about 2 mol of hydrogen peroxide/mol of gallic acid. Production of carbon dioxide was not detected. After acidification, the oxidized products of gallic acid and ellagic acid (gallic acid dimer) were studied by paper chromatography and HPLC. Two major acidic products having polarity higher than gallic acid were produced, and they appeared to be the same for both gallic and ellagic acids. Analysis of the results indicates a gallic acid oxidation mechanism primarily via a dimer equivalent to ellagic acid resulting finally in opened-ring acidic products. The numerical integrations of the rate equations for the proposed scheme are in good agreement with the actual measurements of oxygen uptake. The results are discussed in relation to oxidations during wine aging and related food processing.

Oxidation of constituents other than lipids is an important cause of modification, sometimes spoilage and sometimes improvement, in foods and beverages. Sherries, raisins, black tea, and chocolate are examples in which such oxidation is part of desirable changes essential to the product. White table wine and many other fruit and vegetable products are examples in which oxidative browning and associated flavor modification are considered undesirable. Nutritional value can be decreased by reactions associated with or following oxidation of food phenols to quinones. In still other cases, notably red wines, optimum quality is associated with a shift to a somewhat tawny color and complexing flavor changes that result from limited oxidation and partial consumption of oxidizable substrates (Singleton and Esau, 1969). Important as it is, relatively little research beyond visible spectral studies has been devoted to such nonenzymic autoxidation in aqueous systems.

In a number of these foods and beverages, notably grape juices and wines and probably all such vegetable products without appreciable ascorbic acid content, naturally present phenolic substances are considered the major

Department of Viticulture and Enology, University of California, Davis, California 95616.
[1] Present address: Department of Food Technology, Chulalongkorn University, Bangkok, Thailand.

0021-8561/89/1437-0844$01.50/0 

substrates for direct oxidation whether enzymic or nonenzymic. In processing and aging of wines, the capacity of a wine to take up oxygen, the compositional and sensory effects of different levels of oxidation, and the kinetics of oxidation are vitally important, poorly known, and complex to study. Our laboratories have been studying these changes for some time (Singleton, 1982a,b, 1987). Under the acidic conditions normal for wines (pH 3–4) and fruit juices, the total oxygen taken up over a long period is often quite high and roughly proportional to the phenol content, but complete autoxidation has proven slow and too prolonged to monitor satisfactorily by techniques such as Warburg respirometry and oxygen electrode methods (Rossi and Singleton, 1966). Coupled oxidation of ethanol to acetaldehyde, as wine's vicinal diphenols are autoxidized nonenzymically, results from coproduction of a strong oxidant believed to be hydrogen peroxide along with the o-quinone (Wildenradt and Singleton, 1974). A reproducible measurement of a wine's total oxygen capacity can be made in about 30 min by Warburg respirometry after making the sample alkaline (Rossi and Singleton, 1966).

This alkaline procedure was shown to involve more oxygen uptake than expected, even allowing for hydrogen peroxide production. This large uptake is decreased but not proportionally so by previous reaction of the wine with oxygen under acidic conditions (Rossi and Singleton, 1966; Stern, 1968; Singleton et al., 1979); i.e., consumption of a sizable amount of oxygen under slow acidic conditions lowers the subsequent rapid alkaline uptake by a smaller amount. The alkaline-uptake procedure is considered the most useful analytical method available to estimate a wine's oxygen capacity (Stern, 1968). It was reasoned that a detailed study of one or more typical phenols under alkaline conditions of oxidation would serve as a base to guide further studies under conditions more typical for foods. To the extent that the reaction should be similar except for the relative phenolate ion content, the results may be directly applicable.

Gallic acid was chosen for this initial study because (1) it reached a constant maximum level of oxygen consumed rather quickly, (2) its dimer, ellagic acid, was available, and (3) it is present and readily detectable in wines (Singleton and Trousdale, 1983).

Previous studies by others of gallic acid oxidation have been relatively few, mostly by chemical agents such as chromic acid (Singh et al., 1985) or vanadium salts (Kustin et al., 1974) and under conditions not applicable to foods. Ionescu et al. (1976) studied oxygen uptake by gallic acid in aqueous alkaline solution but did not convert experimental values into generally useful terms and mainly were interested in the catalytic effect of cobalt on the reaction. Oszmianski et al. (1985) found gallic acid disappeared upon chemical oxidation, but much more slowly than in the presence of phenol oxidases and less rapidly than other phenols in a grape seed extract at pH 3.2.

Numerical integration of the applicable set of differential rate equations produces concentration–time relationships that can be compared to experimental values. Successive modification of the rate constants until the best agreement is obtained has been applied to a number of other reaction systems (Seinfeld and Lapidus, 1973) and was used here.

## MATERIALS AND METHODS

**Supplies and Equipment.** Gallic acid (99% grade) was purchased from J. T. Baker Chemical Co., Phillipsburg, NJ 08865. Before use, it was purified by recrystallization twice from glass-distilled water. Ellagic acid (97% grade) was purchased from Aldrich Chemical Co., Inc., Milwaukee, WI 53233. Catalase was purchased from Worthington Biochemical Corp., Freehold, NJ 07728.

Oxygen uptake of a phenolic substrate was determined with a differential respirometer (Gilson Medical Electronics, Middleton, WI 53562). Measurement of pH was with a Corning pH meter, Model 130.

HPLC was performed with a Model 6000A pump (Waters Associates, Milford, MA 01757), a 710B autoinjector (Waters), and a Perkin-Elmer (Norwalk, CT 06856) LC-55B spectrophotometric detector operated at 250 nm. The column was 250 mm × 4.6 mm from Brownlee Laboratories (Santa Clara, CA 95050) packed with 5-μm $C_{18}$ bonded reversed-phase spherical particles. The mobile phase was 0.01 M ammonium dihydrogen phosphate made to pH 2.6 with phosphoric acid and run at a flow rate of 1.5 mL/min.

**Oxidation Procedure.** *a. Oxygen Uptake.* Gallic acid (1.0 mL, 2 mM) was enclosed and brought to thermal equilibrium in the Warburg apparatus. Oxidation was initiated by mixing with 1.0 mL of 20% KOH solution from a one-side-arm reaction flank.

Ellagic acid, an exact weight, was oxidized in a two-side-arm 15-mL reaction flask containing 1.0 mL of 20% KOH and 1.0 mL of water because of the poor solubility of ellagic acid in water alone. Thermal equilibration was at 25 °C for 20 min, and air was used throughout the study. Five replicate samples were oxidized for each phenolic reaction series.

*b. Detection of Products from Alkaline Oxidation.* Gallic acid or ellagic acid (0.2 M) in 20.0 mL of 10% KOH was oxidized by stirring in air with a magnetic stirrer at room temperature. After 1 h, the reaction mixture was titrated with 10% $H_2SO_4$ and pH change was observed to pH 2. The acidified mixture was taken to dryness in a rotary vacuum evaporator (water bath at 30 °C).

The reaction mixtures after oxidation of gallic acid or ellagic acid were acidified and compared by means of paper chromatography, using the upper layer of a freshly made mixture of 1-butanol–acetic acid–water (4:1:5) as the solvent system. Bromcresol green (0.1%)–acetone mixture (1:4, v/v) spray was used to detect the acidic products on the chromatograms.

The acidified oxidation products of gallic acid were also analyzed by HPLC. The injecting volumes of the oxidation mixture and a nonoxidized gallic acid solution (2 mM) diluted with distilled water to one-fourth the original concentration were 5 and 1 μL, respectively.

Hydrogen peroxide from the oxidation was detected by using the enzyme catalase. After oxidation for 15 min, the mixture was neutralized and then catalase (in 0.05 M phosphate buffer, pH 7 at 25 °C) was added and hydrogen peroxide was analyzed by the oxygen released as measured by the differential respirometer. Five replicate experiments were used for detection. Controls without catalase and with heat-denatured catalase were used for comparison.

## RESULTS AND DISCUSSION

**Hydrogen Peroxide Production.** After gallic acid oxidation was complete, release of oxygen from this solution occurred as indicated by volume increase when the closed system was neutralized and treated with active catalase, but not when it was similarly treated with heat-inactivated catalase at the same concentration. Considering the specificity of catalase, this is considered to prove that hydrogen peroxide is the active oxidant produced in couple with phenol oxidation as hypothesized by Wildenradt and Singleton (1974). When the alkaline gallic acid oxidized solution was made strongly acid, release of $CO_2$ was not observed after correcting for that occurring as a contaminant in the KOH. The amount of apparent hydrogen peroxide produced averaged about 2 mol/mol of gallic acid completely oxidized. This amount is twice that expected from simple direct oxidation of gallic acid itself. It indicates that a great extent of polymerization has occurred, regenerating hydroquinones from quinones followed by reoxidation and additional hydrogen peroxide formation. Furthermore, the high yield shows that hydrogen peroxide accumulates in the alkaline solution as $HO_2^-$ without appreciable loss in this situation to other oxidations. That the yield appears to exceed theoretical for dimerization by about 25% is attributed to additional

**Table I. Experimental Oxygen Consumption of Gallic Acid and Ellagic Acid at pH 14 at 25 °C**

| phenol | mM/L | mL oxygen/mM phenol | M oxygen/M phenol | atoms oxygen/ ring |
|---|---|---|---|---|
| gallic acid | 2.0 | 55.2 | 2.45 | 4.9 |
| ellagic acid | 2.07 | 79.8 | 3.56 | 3.6 |

**Table II. Oxygen Consumption of Gallic Acid Oxidation via Polymerization According to the Mechanism of Figure 1**

| gallic acid rings involved ($n$) | oxygen atoms total[a] | oxygen atoms/ gallic acid |
|---|---|---|
| 1 | 2 | 2 |
| 2 | 6 | 3 |
| 3 | 10 | 3.3 |
| 4 | 14 | 3.5 |
| 100 | 398 | 3.98 |

[a] $2(2n - 1)$.

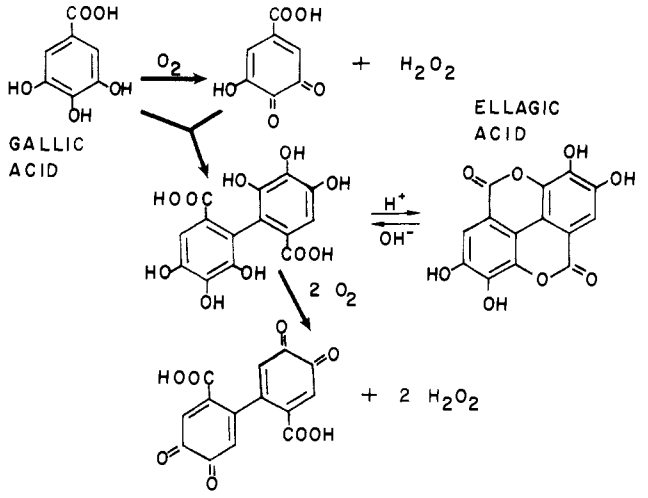


**Figure 1.** Autoxidation of gallic acid, dimerization (ellagic acid precursor), and reoxidation with coupled production of hydrogen peroxide consumes 3 mol equiv of oxygen total or 3 atoms per original gallic moiety or 2 atoms per aromatic ring of original ellagic acid.

polymerization or to difficulty in completely correcting in the small sized samples for experimental errors from carbonate in the KOH, large temperature effects from mixing concentrated solutions, etc.

**Oxygen Uptake.** Gallic acid consumed 4.9 atoms of oxygen/molecule of gallic acid (Table I). Note that gallic acid consumed only 1.3 atoms of oxygen more than did ellagic acid. If a gallic acid molecule is oxidized to its corresponding quinone and hydrogen peroxide, 2 atoms of oxygen are consumed (the first reaction, Figure 1).

The observed oxygen uptake was 2.9 atoms higher than the 2 atoms originally expected. About 2 mol of hydrogen peroxide/mol of gallic acid oxidized was found when only 1 would be expected from gallic acid going to its quinone. These facts demonstrated involvement of additional reactions such as polymerization occurring during oxidation and prompted further investigation.

**Regenerative Polymerization.** When gallic acid oxidizes and dimerizes, regenerating the hydroquinone form (regenerative polymerization; Singleton, 1982a,b, 1987), and then the dimer reoxidizes to give its quinone and hydrogen peroxide, 3 atoms of oxygen/gallic acid unit will be required to produce the oxidized dimer (Figure 1; Table II). Ellagic acid is the dilactone of the dimer of gallic acid. Its structure is shown in Figure 1. In strong alkali it would, of course, hydrolyze to the hexahydroxydiphenic acid.

**Table III. Oxygen Consumption of Gallic Acid Oxidation via Polymerization and Ring Opening According to the Mechanism of Figure 4**

| gallic acid rings involved ($n$) | oxygen atoms total[a] | oxygen atoms/ gallic acid |
|---|---|---|
| 1 | 4 | 4 |
| 2 | 10 | 5 |
| 3 | 16 | 5.33 |
| 4 | 22 | 5.55 |
| 100 | 598 | 5.98 |

[a] $2(3n - 1)$.

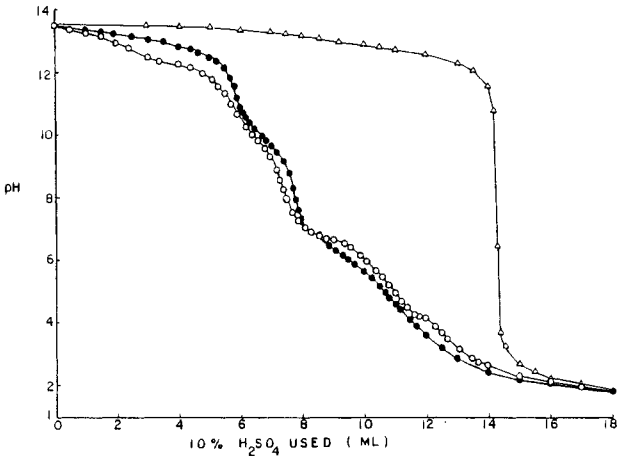


**Figure 2.** Neutralization curves of 10% potassium hydroxide solution alone (triangles), with oxidized gallic acid (solid circles), or with oxidized ellagic acid (open circles).

Note that the difference of 1.3 in atoms of oxygen uptake between gallic acid and ellagic acid is a bit more than the 1.0 expected if all the gallic acid were oxidized via dimerization. If more than dimerization occurs, more oxygen atoms will be consumed. The maximum number of atoms of oxygen required for infinite polymerization of gallic acid is 4/unit of gallic acid, which is still less than the observed total uptake. This assumes no participation of the coproduced hydrogen peroxide in oxygen-sparing reactions. This assumption appears valid in alkaline solution since the $H_2O_2$ accumulated as $HO_2^-$ apparently quantitatively. If such competing oxidation had occurred, the $O_2$ consumption would be lowered.

**Ring Opening.** The data show that additional drastic oxidations must have occurred beyond the polymeric quinone stage. The reaction that suggests itself is the degradation of oxidized phenolic rings to produce opened rings, nonbenzenoid unsaturated acids. That this further oxidation results in the conversion of the *o*-quinoids to *cis,cis*-muconic acid derivatives cannot be considered proven at this stage. This hypothesis is, however, consistent with other known phenolic ring degradations in biological systems, seems the most probable chemistry of further oxygen incorporation, and is supported by identification of products more acidic than phenols after complete oxidation, and numerical analysis of this reaction scheme fits the oxygen consumption data while other models do not. Pospisil (1984) has postulated oxidation of gallic acid to purpurogallic acid; however, this would not account for the oxygen consumption, should produce $CO_2$, and should give a red or purple color. None of these fit observed facts.

The *cis,cis*-muconic acid open-ring derivative of gallic acid would be expected to enolize to $\alpha$-keto acid and could also lactonize to $\gamma$- or $\delta$-lactones of the enol when the system was acidified. The estimation of atoms of oxygen

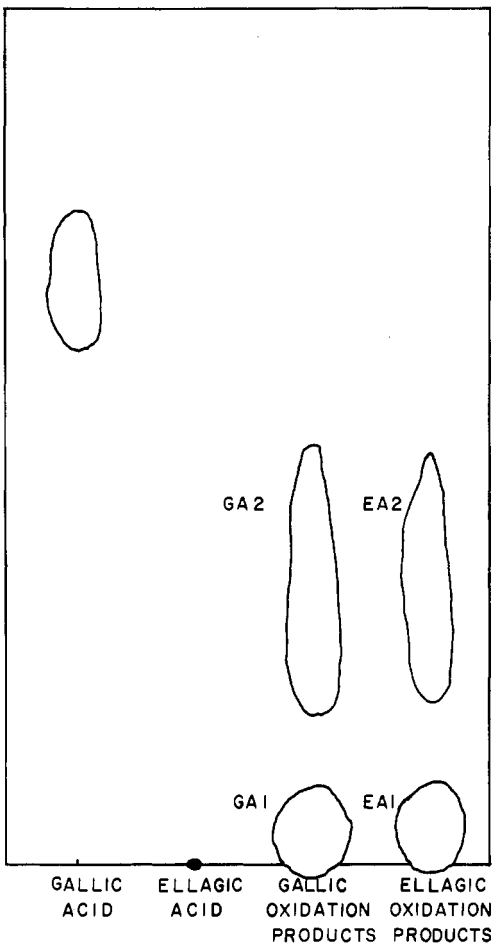


**Figure 3.** Paper chromatogram of gallic acid, ellagic acid, and their products at completion of alkaline oxidation followed by acidification (1-butanol–acetic acid–water (4:1:5), bromcresol green detection spray).

**Table IV. Apparent $R_f$ Values of the Acidic Products from the Gallic Acid and Ellagic Acid Oxidations**

| | $R_f$ |
|---|---|
| gallic acid | 0.69 |
| gallic acid oxidn products | |
| fraction GA 1 | 0.04 |
| fraction GA 2 | 0.33 |
| ellagic acid oxidn products | |
| fraction EA 1 | 0.04 |
| fraction EA 2 | 0.33 |

consumed by gallic acid if both polymerization followed by ring degradation occurred is shown in Table III.

When both gallic and ellagic acids were oxidized in air under the same experimental conditions and titrated with 10% $H_2SO_4$, neutralization curves of both acids coincided very well (Figure 2). There were at least three different acidic strength groups left after gallic acid oxidation. In strong alkali, the phenols would be ionized and conversion of two phenolic groups to carboxyls would give no net change in alkali consumption but the p$K$'s would be expected to be much lower. Data do not seem to be available in the literature regarding the actual p$K$'s of the opened ring products, but they should behave as carboxy acids with p$K_a$'s of the order of 5 compared to phenols near 10.

Paper chromatographic tests of the titrated acidic products of gallic acid and ellagic acid are shown in Figure 3. Bromcresol green is ordinarily sensitive to carboxy acids, but not phenols. Gallic acid gave two types of acidic products having essentially identical $R_f$ values as those from ellagic acid (Table IV). This reinforces the evidence

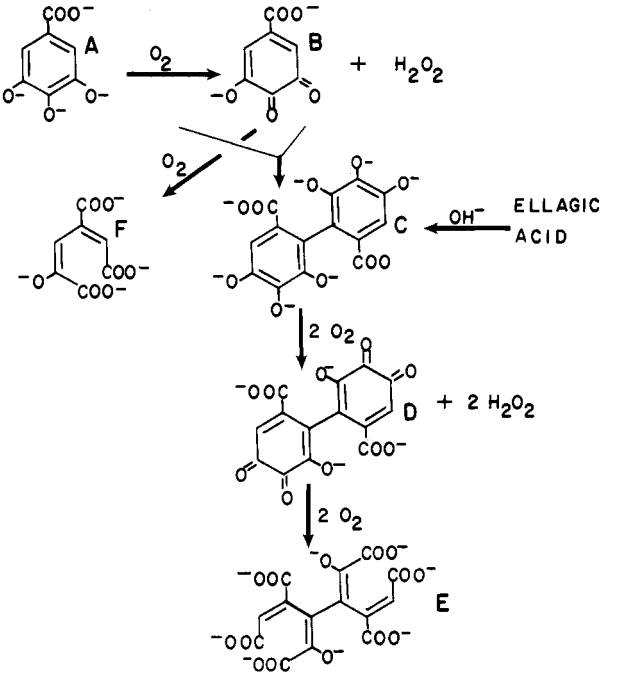


**Figure 4.** Proposed mechanistic sequence of autoxidation of gallic or ellagic acids in aqueous solution at pH 14: A = gallic acid, B = gallic acid quinone, C = gallic dimer, D = quinone of dimer, E = open-ring product of dimer, F = open-ring product of gallic acid.

that dimerization of gallic acid must have occurred during oxidation and that carboxy acids are produced.

**Proposed Reaction Scheme.** Thus, a reaction sequence is proposed for gallic acid oxidation in highly alkaline solution as shown in Figure 4. Since the alkaline oxygen uptake of gallic acid consumed 4.9 atoms/molecule of gallic acid (Table I), the reaction must lie between (1) oxidation of a gallic acid to opened ring acidic products and one hydrogen peroxide (this requires 4 atoms of oxygen/gallic acid) and (2) oxidation of a dimer of gallic acid through to acidic products (this requires 5 atoms of oxygen/gallic acid). To the extent that polymerization beyond the dimer occurred, a larger proportion of monomeric gallic acid oxidation would be required to compensate and return the uptake to 4.9.

Ansell and Palmer (1964) showed the formation of lactones from unsaturated acids by $H_2SO_4$, which is the most frequently used lactonizing agent. The dimer of gallic acid spontaneously forms ellagic acid when acidified. It was indicated by paper chromatography that some lactones must have formed (or remained in the case of ellagic acid) after the oxidation products were acidified.

The observed oxygen uptake, 4.9 atoms of oxygen/gallic acid, suggested that the reaction went almost exclusively to dimer formation. About 2% of gallic acid could have been oxidized as a monomer to the opened-ring acidic products, if no polymers larger than dimers were produced. Little product larger than dimer could have formed because 6 atoms of oxygen/gallic acid are required at infinite polymerization and ring openings (Table III). Additional carbon to carbon polymerization would appear difficult from a steric and a statistical viewpoint. Polymerizations via carbon to oxygen linkage are possible and may account for minor products shown in HPLC.

Separation of the acidified oxidized products by reversed-phase HPLC showed two major products and five other side products, all having higher polarity than gallic acid (Table V). Efforts to isolate and identify these products continue. Since acidification in the respirometer

**Table V. HPLC Resolution of Acidified Oxidized Products of Gallic Acid (250-nm Detection)**

| peak no. | ret time, min | % area |
|---|---|---|
| acidified oxidation products | | |
| 1 | 2.18 | 48.7 |
| 2 | 2.78 | 3.4 |
| 3 | 3.63 | 0.4 |
| 4 | 5.01 | 1.5 |
| 5 | 5.31 | 8.1 |
| 6 | 5.88 | 6.6 |
| 7 | 9.73 | 31.3 |
| gallic acid | 15.35 | 100 |

$$\frac{dA}{dt} = -k_1 \cdot O \cdot A - k_2 \cdot A \cdot B$$

$$\frac{dB}{dt} = k_1 \cdot O \cdot A - k_2 \cdot A \cdot B - k_6 \cdot O \cdot B$$

$$\frac{dC}{dt} = k_2 \cdot A \cdot B - k_3 \cdot O \cdot C$$

$$\frac{dD}{dt} = k_3 \cdot O \cdot C - k_4 \cdot O \cdot D$$

$$\frac{dE}{dt} = k_4 \cdot O \cdot D$$

$$\frac{dF}{dt} = k_5 \cdot O \cdot B$$

$$\frac{dO}{dt} = -(k_1 \cdot A + 2k_3 \cdot C + 2k_4 \cdot D + k_6 \cdot B) \cdot O$$

$$\frac{dP}{dt} = k_1 \cdot O \cdot A + 2k_3 \cdot O \cdot C$$

**Figure 5.** Set of rate equations for gallic acid oxidation by the sequence and conditions of Figure 4: A = gallic acid, B = gallic acid quinone, C = gallic dimer, D = quinone of dimer, E = open-ring product of dimer, F = open-ring product of gallic acid, O = $O_2$, P = $H_2O_2$.

failed to show any carbon dioxide generation as a result of alkaline oxidation of gallic acid, decarboxylation does not appear involved.

**Kinetic Modeling Studies.** From the proposed reaction sequences, the rate equations (Figure 5) were integrated to compare the calculated oxygen consumption over the reaction period with the experimentally determined values. All rate laws were assumed to be first order with respect to reacting species. A BASIC program was used for the numerical integration, using Euler's method with a time step of 5 s. The rate constants were systematically iterated until the predicted curve of oxygen uptake best fitted the experimental data. For most of these studies an AT&T PC6300 computer was employed.

It was assumed that (1) oxygen in the reaction cell was not limiting, (2) hydrogen peroxide did not participate in oxygen-sparing reactions, and (3) there was no hydrogen peroxide produced from the ring-opening reactions. That increasing the shaking rate of the Warburg apparatus did not increase the oxygen consumption rate agrees with assumption 1, and it was verified by obtaining twice the initial $O_2$ uptake rate when gallic acid concentration was doubled to 2 mM. Assumption 2 has already been discussed. Assumption 3 is less vigorously justified, but the amount of hydrogen peroxide did not appear to increase with further oxygen consumption over the level produced early in the reaction. The postulated ring splitting between the quinone carbonyls would use two oxygen atoms ($O_2$) simultaneously probably via a peroxide intermediate and preclude production of free hydrogen peroxide.

If oxygen is always in excess, the initial stage of the gallic acid oxidation reduces to a pseudo-first-order initial reaction rate. Pseudo-first-order rate plots were obtained from five replicate gallic acid oxidations in each of a series of solutions and buffers (20% KOH, NaOH/KCl, $Na_2HPO_4$/NaOH) covering initial pH's of 14, 12.48, 12.3, 11.98, 11.70, and 10.36. Final pH rose as much as 0.30 pH at the lower end of this range. The pseudo-first-order rate

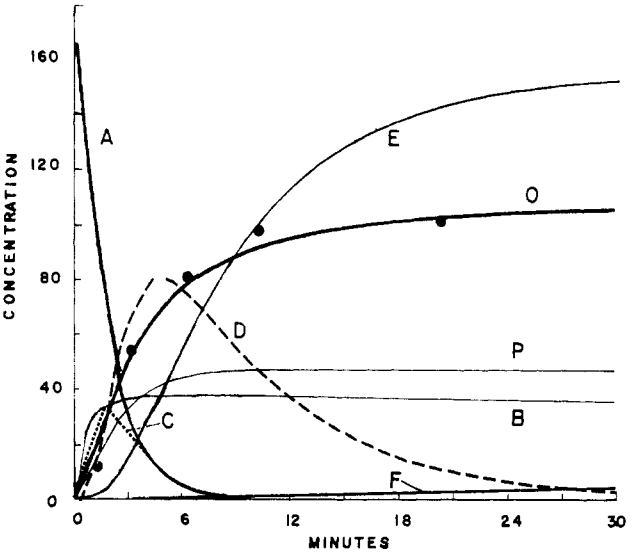


**Figure 6.** Oxygen uptake (calculated best fit = curve, experimental = solid circles) and oxidation reactions of gallic acid from kinetic analysis of the sequence of Figure 4 at pH 14.0 and 25 °C: A = gallic acid, B = gallic acid quinone, C = gallic dimer, D = quinone of dimer, E = open-ring product of dimer, F = open-ring product of gallic acid, O = $O_2$, P = $H_2O_2$. All in milligrams per liter except $O_2$ in milliliters × 2.

constants ($k_1' \times 10^2$, $min^{-1}$) were, respective to the above pH's, 12.9, 15.8, 18.8, 20.2, 12.7, and 7.8. That the rate was fastest at pH ~12 and slower above can be attributed to hydrogen bonding by the third phenol facilitating production of the quinone carbonyl. This effect would be lost in stronger alkali as all phenolic groups became phenolate ions. Below pH 12, decreased phenolate concentrations slowed the reaction with $O_2$. The $r^2$ was larger than 0.992 at every pH tested.

For the simple oxidation case of gallic acid to quinone plus hydrogen peroxide or for the additional ring-opening case without polymerization, agreement between kinetically calculated and experimental oxygen uptake was not possible and the predicted oxygen consumption was less than half the experimental value. Agreement was somewhat closer but still inadequate to explain the experimental curve for the dimerization model without ring opening. Close agreement between the proposed kinetic model of Figure 4 and the experimental data was found. Figure 6 shows the predicted concentration histories for the proposed reactions in Figure 4. Note good agreement between actual (dots) and predicted oxygen uptake (curve). The best fit terminal data are 4.3 atoms of oxygen and 1.4 mol of hydrogen peroxide/mol of gallic acid. The root sum of squares per point (or average deviation) for $O_2$ consumption is 0.8942 mL or approximately 2% of the final consumption value. These simulations gave $k$ values ($mol^{-1}$ $min^{-1}$) of $k_1$ = 1320, $k_2$ = 1320, $k_3$ = 4500, $k_4$ = 600, and $k_5$ = 12. The rate equations are for the change in concentration (M $L^{-1}$) with time of gallic acid (A), gallic quinone (B), gallic dimer (C), dimer quinone (D), open-ring dimer (E), and opened ring from gallic quinone (F) (see Figures 4–6).

These data show that the first reaction, oxidation of gallic acid to quinone, is initially the rate-limiting step for oxygen uptake. Oxidation of the dimer is the fastest step. Aromatic substitution on a hydroquinone is expected to make the compound easier to oxidize. The ring opening of both gallic acid and the dimer was even slower, but since the other reactions had to precede them were not rate limiting. In 30 min the open-ring gallic product was 2.5%

and the dimeric opened-ring product 97.5%.

Clearly, oxygen uptake continues after gallic acid depletion (agreeing with HPLC data), and the sequential accumulation of quinone, dimer, and open ring of the dimer is predicted. The oxidation–reduction potential of the dimer should be lower than that of gallic acid and helps explain the relatively high concentration of the dimer quinone and its open-ring derivatives.

Although the products have not been specifically isolated and chemically identified, the simulated model fits the data well, supports the postulated sequence and partial mechanism well, and explains the high oxygen uptake of gallic acid during alkaline oxidation.

**Concluding Comments.** Although the studies reported are at pH's much higher than would occur in foods or beverages, they are believed important for the further study of wines. They explain the puzzling observation of very high oxygen consumption values when wines are made alkaline. They have enabled postulation of a plausible mechanistic sequence of the oxidation. These studies will serve as the basis for studying other phenols of natural importance and devising tests for the more normal acidic conditions. The data have verified the production of hydrogen peroxide during autoxidation of gallic acid. Previously this was an hypothesis (Wildenradt and Singleton, 1974), but we believe that this is the first direct proof of this reaction under food-related conditions. Of course, the chemical synthesis of hydrogen peroxide from the cycled autoxidation of anthraquinones is a commercial process. The coproduction of hydrogen peroxide appears general in the autoxidation of ascorbic acid, melanoidins, phenols, and probably other substrates readily chemically attacked by oxygen.

Furthermore, the data offer an explanation of previous findings that exposure to oxygen of a wine in its normal acidic condition slowly but not proportionally reduced the alkaline uptake capacity of the same wine (Stern, 1968; Singleton et al., 1979). The hypothesis that this is because under slow (acidic) oxidation there is more polymerization of the quinone–phenol type that regenerates oxidizable hydroquinone forms appears reinforced (Singleton, 1982a,b, 1987). Under rapid oxidation (alkaline) the system would more rapidly reach a terminal stage with complete conversion of substrate to quinoids and more limited regenerative dimerization as phenols are quickly depleted.

Finally, the data are believed to offer the second direct demonstration of the important reaction of wines and plant-derived foods that we have termed "regenerative polymerization", which produces further oxidizable substrate as phenols are oxidized (Singleton, 1982a,b, 1987). The first was the regeneration of the hydroquinone as the quinone of caftaric acid dimerizes with glutathione (Cheynier et al., 1986).

ACKNOWLEDGMENT

The Wine Institute, Wine Growers of California, and the American Vineyard Foundation are thanked for financial support of this research.

**Registry No.** $O_2$, 7782-44-7; $H_2O_2$, 7722-84-1; gallic acid, 149-91-7; ellagic acid, 476-66-4.

LITERATURE CITED

Ansell, M. F.; Palmer, M. H. The cyclization of olefinic acids to ketones and lactones. *Q. Rev.* **1964**, *18*, 211–25.

Cheynier, V. F.; Trousdale, E. K.; Singleton, V. L.; Salgues, M. J.; Wylde, R. Characterization of 2-S-glutathionylcaftaric acid and its hydrolysis in relation to grape wines. *J. Agric. Food Chem.* **1986**, *34*, 217–21.

Ionescu, G.; Matei, F.; Duca, A. Kinetic aspects of the oxidation of gallic acid using an oxygen-selective electrode. *Rev. Roum. Chim.* **1978**, 1611–17.

Kustin, K.; Nicolini, C.; Topper, D. L. Interaction of catechol and catechol derivatives with dioxovanadium (V). II. Kinetics of ligand oxidation. *J. Am. Chem. Soc.* **1974**, *96*, 7416–20.

Oszmianski, J.; Sapis, J. C.; Macheix, J. J. Changes in grape seed phenols as affected by enzymic and chemical oxidation *in vitro*. *J. Food Sci.* **1985**, 1505–6.

Pospisil, F. Polarography of 3,4,5-trihydroxybenzoic acid. *Collect. Czech. Chem. Commun.* **1984**, 1459–63.

Rossi, J. A., Jr.; Singleton, V. L. Contributions of grape phenols to oxygen absorption and browning of wines. *Am. J. Enol. Vitic.* **1966**, *17*, 231–9.

Seinfeld, J.; Lapidus, L. *Mathematical methods in chemical engineering; process modeling, estimation, and identification*; Prentice-Hall: Englewood Cliffs, NJ, 1973; Vol. 3.

Singh, V. P.; Pandey, I. M.; Sharma, S. B. Kinetics of chromic acid oxidation of some hydroxy acids. *J. Indian Chem. Soc.* **1985**, *62*, 64–6.

Singleton, V. L. Grape and wine phenolics: background and prospects. Proceedings of a Symposium, University of California at Davis, Grape and Wine Centennial, 1980. **1982a**, 215–27.

Singleton, V. L. Oxidation of wine. In International Symposium, Viticulture, Vinification and the Treatment and Handling of Wine. *Inst. Masters Wine* **1982b**, *18*, 14 pp.

Singleton, V. L. Oxygen with phenols and related reactions in musts, wines, and model systems: Observations and practical implications. *Am. J. Enol. Vitic.* **1987**, *38*, 69–77.

Singleton, V. L.; Esau, P. Phenolic substances in grapes and wine, and their significance. In *Advances in Food Research*, Supplement 1; Academic Press: New York, 1969.

Singleton, V. L.; Trousdale, E. White wine phenolics: Varietal and processing differences as shown by HPLC. *Am. J. Enol. Vitic.* **1983**, *34*, 27–34.

Singleton, V. L.; Trousdale, E.; Zaya, J. Oxidation of wine. I. Young white wines periodically exposed to air. *Am. J. Enol. Vitic.* **1979**, *30*, 49–54.

Stern, P. M. Comparison of methods for determination of oxidizable substances in wine, particularly phenols. M.S. Thesis, University of California, Davis, 1968.

Wildenradt, H. L.; Singleton, V. L. The production of aldehydes as a result of oxidation of polyphenolic compounds and its relation to wine aging. *Am. J. Enol. Vitic.* **1974**, *25*, 119–26.

Received for review September 19, 1988. Accepted January 5, 1989.