analyzed, the size of the sample depending on the initial perester concentration in the kinetic run. In order to avoid crystallization of the phenyl ether on the inside of the pipets, the perester samples were warmed in water at a temperature of about 32° before pipetting. The fourth flask was used as a blank.

The flasks were placed in a black desiccator and after being flushed with carbon dioxide for about 25 minutes, 2 to 3 ml. of 57% hydriodic acid was quickly added to each flask. The desiccator was then closed and allowed to stand under continued flushing of carbon dioxide for a period of 30 minutes to one hour, the shorter reaction time for the methoxy, methyl and unsubstituted peresters and the longer one for the less reactive nitro and chloro compounds. At the end of the standing time, about 50 ml. of distilled water was added to each flask, and the liberated iodine was titrated with standard thiosulfate of 0.01 *N* while the flask was vigorously agitated to prevent retention of any iodine by the immiscible chloroform layer. The presence of chloroform was necessary to dissolve all colored organic substances, thus permitting the iodine to be the only colored substance in the aqueous layer and improving the ease of detection of the starch-iodide end-point considerably. From the titration value minus the blank titration, the concentration of the perester was calculated. The blank values were generally zero and never greater than 0.20 ml. The precision of the method is probably no better than ±3%.

To avoid difficulties in pipetting samples from kinetic experiments run on the 0.2 *M* solutions, in which considerable amounts of insoluble decomposition products resulted, a rapid, convenient method of filtration by centrifugation was developed. The bottom of a drying tube, cut off just above the bulb, was fitted into a 15-ml. centrifuge tube with a cork. After placing a small piece of filter paper and glass wool in the drying tube bulb, the sample of liquid and solid was poured into the bulb. Separation of solids by their retention on the glass wool and filter paper was complete after centrifugation for about one minute.

When the peresters were analyzed for their purity, *i.e.*, the active oxygen content, the only variations in the method were the following: if liquids, the samples were weighed from a Lunge pipet and, if the peresters were solids, in a weighing bottle. The greater amount of iodine liberated in the purity determinations was titrated against 0.1 *N* thiosulfate solution.

Apparatus and Procedure for the Kinetic Studies.—The apparatus and procedure for the decompositions of the peresters were essentially the same as previously described by Blomquist and Buselli.[5]

The decomposition flasks were immersed in an oil-bath whose temperature was controlled by a bimetallic thermostat operating through an electronic time-delay relay. With this control apparatus, the temperature of the bath could be held to within ±0.1° of the desired temperature. The thermometer used in all the experiments was calibrated against a platinum resistance thermometer previously calibrated by the National Bureau of Standards and made available by Dr. E. R. VanArtsdalen. Decompositions were allowed to proceed to 50–70% of the initial concentration. After the perester samples for a given run had been taken, aliquot portions of the samples were analyzed for peroxide content according to the analytical method previously described. If the samples were not to be analyzed immediately or rechecks were to be made they were stored in a refrigerator, and on being stored for as long as several weeks, they evidenced no perceptible change in perester content.

ITHACA, N. Y. RECEIVED MARCH 5, 1951

[CONTRIBUTION FROM THE WESTERN REGIONAL RESEARCH LABORATORY[1]]

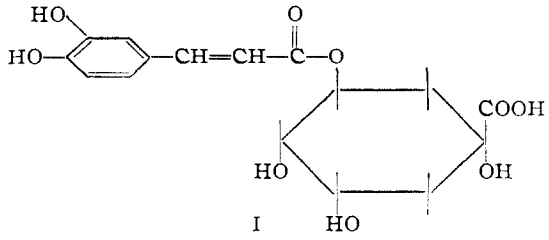
Enzymatic Browning of Fruits. I. Autoxidation of Chlorogenic Acid

BY LLOYD L. INGRAHAM AND JOSEPH CORSE

The rate of absorption of oxygen by chlorogenic acid has been studied under various conditions. It has been found that the initial rates obey the expression: $-\frac{dO_2}{dt} = \frac{k\,(\text{chlorogenic acid})\,P_{O_2}}{(H^+)}$ over the *p*H range of 7.49 to 8.74 and are little affected by light or cupric ions. The over-all activation energy for autoxidation was found to be 13.6 ± 1 kcal./mole.

Introduction

It has long been recognized that rapid browing on the cut surfaces of fresh fruits is an enzymatic oxidation process and can be prevented by heat inactivation of the enzymes involved, but the kinetics and mechanism are not well understood and require further study. The part played by the non-enzymatic reaction (autoxidation) must also be known in such studies. There are indications that chlorogenic acid (I) which is a widely distributed compound in the plant kingdom,[2] occurs in fruits,[3] and may be a substrate in the enzymatic darkening. The present paper describes autoxidation studies of chlorogenic acid.



I

Experimental

The chlorogenic acid hemihydrate was isolated from coffee by the method of Moores, McDermott and Wood.[2d] It melted at 208–209° and appeared homogeneous when examined in a 25-plate Craig countercurrent extraction apparatus[4] with ethyl acetate and water as solvents. The solutions were made in water redistilled in an all-glass still. The rates were measured by the oxygen absorption in constant-volume manometers of the Summerson type[5] employing flasks of about 50-ml. capacity. One flask was kept as a control to compensate for temperature and pressure fluctuations. This flask contained buffer and oxygen but not the substrate. The center well of each flask was filled with paraffin; on this was placed a platinum cup containing a weighed sample of solid chlorogenic acid. Five milliliters of 1 *M* potassium phosphate buffer was placed in the bottom of the flask. The manometer was filled with Brodie's solution. The flask and manometer were evacuated with a water-pump and filled with oxygen. This was repeated

(1) Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

(2) (a) K. Freudenberg, *Ber.*, **53**, 232 (1920); (b) J. Politis, *Compt. rend.*, **225**, 954 (1947); (c) G. O. Rudkin and J. M. Nelson, THIS JOURNAL, **69**, 1470 (1947); (d) R. G. Moores, D. L. McDermott and T. R. Wood, *Anal. Chem.*, **20**, 620 (1948).

(3) K. Spitzer, *Biochem. Z.*, **231**, 309 (1931); W. V. Cruess and J. Sugihara, *Arch. Biochem.*, **16**, 39 (1948); G. Johnson, E. M. Foreman and M. M. Mayer, *Food Tech.*, **4**, 237 (1950); J. Corse and L. L. Ingraham, unpublished results.

(4) L. C. Craig and O. Post, *Anal. Chem.*, **21**, 500 (1949).

(5) W. W. Umbreit, R. H. Burris and J. F. Stauffer, "Manometric Techniques," Burgess Pub. Co., Minneapolis, Minn., 1945, pp. 119–120.

five times. After an equilibration period of from 15 to 20 minutes, shaking was commenced with 3-cm. strokes at a rate of 110 cycles per minute. This knocked the sample of chlorogenic acid into the buffer solution. The sample dissolved in about half a minute and the manometer readings were commenced. The measurements lasted from two to eight hours.

The oxygen uptake was measured at constant volume. The *p*H was determined in duplicate solutions of chlorogenic acid in the given buffer with a Beckman glass electrode calibrated against 0.05 *M* potassium acid phthalate (*p*H assumed to be 4.00 at 25°).

The accuracy of the rate constants is limited primarily by uncertainties in the measurement of *p*H (*e.g.*, an error of 0.02 in *p*H will affect the rate constant about 5%). The errors listed in Tables I and IV are standard errors.[6]

The rates were measured to determine effects of the concentration of chlorogenic acid, *p*H, oxygen pressure, copper ion concentration, temperature and light. The temperature variation during an experiment was ±0.03°.

Experimental Results and Discussion.—The forms of the curves for oxygen uptake *vs.* time are shown in Fig. 1. In the *p*H range of 7.5 to 7.9 (curve A) an autocatalytic period was evident. No quantitative study of this autocatalysis was made in the present research. It is very probable that the observed autocatalysis is quite similar to that found in some hydroquinone autoxidations.[7] The rates were measured from the linear portions of the curves after steady-state conditions had been reached (usually within the initial 5 to 10% of reaction). In the *p*H range of 7.9 to 8.6 no autocatalytic period was noted and the curves were linear throughout the runs (curve B, Fig. 1).

In the *p*H range 8.6 to 8.9 the curves were no longer linear (curve C, Fig. 1). The curvature is probably due to the decrease in chlorogenic acid concentration from oxidation (in effect, because of lower initial concentration, the reactions were carried proportionally further) or hydrolysis. Since there was no induction period, the rates in this case were calculated by finding the instantaneous rates at various times graphically and extrapolating to zero time. There was no observable change in *p*H during experiments 10 and 20, taken as typical runs.

TABLE I

EFFECT OF CHLOROGENIC ACID CONCENTRATION AND *p*H 24.43°; 1 *M* PHOSPHATE BUFFER; 0.968 ATM. OF OXYGEN

Expt.	Chlorogenic acid concn., mole/liter	*p*H	$k \times 10^7$, ml. O_2/liter/min./atm.
	Effect of chlorogenic acid concentration		
1	0.00334	7.82	4.76
2	.00568	7.80	4.72
3	.0118	7.74	4.64
4	.0149	7.72	4.97
5	.0223	7.68	5.61
6	.0335	7.61	5.59
7	.0367	7.60	5.87
8	.0399	7.58	5.26
9	.0458	7.54	5.72
10	.0499	7.52	5.40
11	.0516	7.51	4.62
12	.0524	7.51	5.65
13	.0533	7.51	5.38
14	.0568	7.50	5.16
15	.0568	7.50	5.70
16	.0621	7.49	5.16
17	.0682	7.49	4.95
		Av. 5.24 ± 0.39	
	Effect of *p*H		
16	0.0621	7.49	5.16
17	.0682	7.49	4.95
15	.0568	7.50	5.70
18	.0136	8.63	5.59
19	.0124	8.67	5.96
20	.0101	8.74	5.38
21	.00922	8.76	6.13
22	.00893	8.79	6.39
23	.00866	8.79	6.59

(6) F. C. Mills, "Statistical Methods," Henry Holt and Company, New York, N. Y., 1938, p. 330.

(7) See J. E. Lu Valle and A. Weissberger, THIS JOURNAL, **69**, 1576 (1947), for leading references.

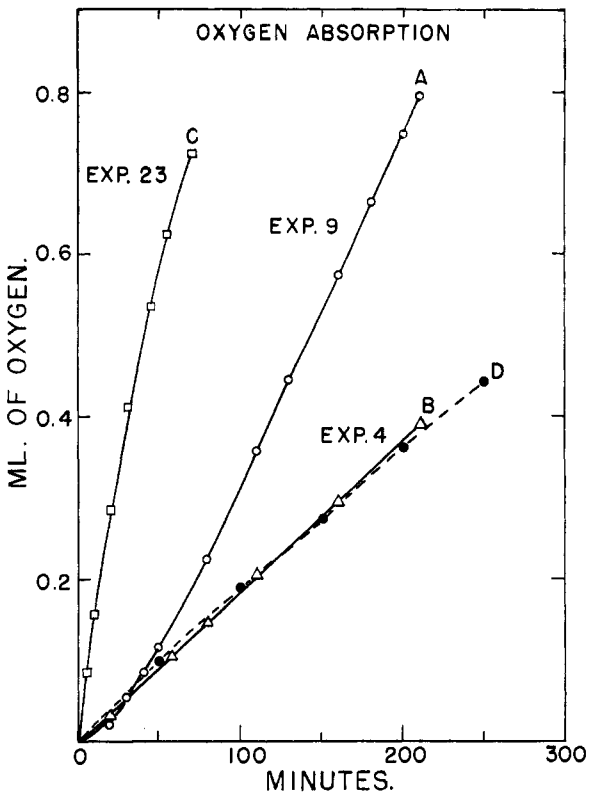


Fig. 1.—Course of oxygen uptake by various buffered solutions of chlorogenic acid: curve A, 5 ml. of 0.0458 *M* chlorogenic acid at *p*H 7.54; curve B, 5 ml. of 0.0149 *M* chlorogenic acid at *p*H 7.72; curve C, 5 ml. of 0.00866 *M* chlorogenic acid at *p*H 8.79; curve D, calculated oxygen uptake assuming first order disappearance of chlorogenic acid under the conditions of curve B.

The rate constant k for the initial rates was calculated from the expression

$$\frac{d(O_2)}{dt} = \frac{k\,(\text{chlorogenic acid})P_{O_2}}{H^+} \qquad (1)$$

where $d(O_2)/dt$ represents the milliliters of oxygen, measured at standard conditions, absorbed by one liter of solution per minute. The concentrations of chlorogenic acid and hydrogen ion are in moles per liter and the oxygen pressure is in atmospheres.

Effect of Chlorogenic Acid and Hydrogen Ion Concentration.—Chlorogenic acid is a relatively strong organic acid ($pK \sim 3.5$), and the *p*H of the reaction mixtures even in 1 *M* phosphate buffers was somewhat affected by varying its concentration. Thus, in successively adding larger amounts of chlorogenic acid to the same stock buffer to effect a 20-fold increase in substrate concentration (experiments 1–17, Table I), a decrease in *p*H of 0.3 *p*H unit occurred (about a two-fold change in hydrogen ion concentration). If sufficiently low concentrations of chlorogenic acid were used to minimize the effect on *p*H, the rates would have been almost immeasurably slow. It was not possible, therefore, to completely dissociate the study of the two variables, *p*H and chlorogenic acid concentration, and get measurable rates. In experiments 16 to 20 the hydrogen ion concentrations varied about 16-fold and the chlorogenic acid concentration variation was six-fold.

There is no trend in the rate constants obtained in these experiments (1–20, Table I) and therefore the hydrogen ion and chlorogenic acid concentration terms in Equation (1) are verified.

Above *p*H 8.74 there is an increase in rate constant which is discussed later.

Effect of Oxygen Pressure.—Experiments at three different oxygen pressures were performed by filling the manometers with pure oxygen, air and 10% oxygen–90% nitrogen, respectively. The rate constants calculated from equation 1 and listed in Table II show no significant trend when the partial pressure of the oxygen is varied by a factor of ten. This indicates that the rate is proportional to the partial pressure of oxygen.

TABLE II

EFFECT OF OXYGEN PRESSURE 24.43°; 1 *M* PHOSPHATE BUFFER

Expt.	Chlorogenic acid, mole/liter	*p*H	P_{O_2} atm.	$k \times 10^7$, ml. O_2/liter/min./atm.
1–17			0.968	5.24 ± 0.39[a]
24	0.0137	7.73	.203	5.85
25	.0176	7.71	.203	5.76
26	.0218	7.68	.203	5.87
27	.0127	8.41	.0968	4.50
28	.0137	8.40	.0968	5.75
29	.0120	8.42	.0968	5.66

[a] Average of Experiments 1–17.

Effect of Light.—Experiments 10, 12, 13 and 14 were performed in the dark by wrapping the flasks in aluminum foil, and experiments 4, 8, 9, 11 and 16 were performed with a 500-watt Photoflood lamp 3 to 5 inches above the thermostat. The average of the rate constants in the dark (5.40) is sufficiently close to that in the light (5.15) to make it justifiable to measure the other rates in diffuse laboratory light.

Effect of Copper.—Since polyphenol oxidase, which is the enzyme responsible for the rapid browning of injured fruit tissue,[8] is known to contain copper, it was of interest to determine the effect of copper ions in solution on the rate of autoxidation (*cf.* Table III). There were no effects due to the addition of small amounts of copper, as cupric sulfate, or of saturating the buffer with a copper complexing agent, thenoyltrifluoroacetone (TTA).[9] Nikolaev[10] reported that the autoxidation of pyrogallol was unaffected by cupric ion.

TABLE III

EFFECT OF CUPRIC ION

24.43°; 1 *M* PHOSPHATE BUFFER, 0.968 ATM. OXYGEN

Expt.	Chlorogenic acid concn., mole/liter	*p*H	Added copper (as Cu^{++}), mg./liter	$k \times 10$, ml. O_2/liter/min./atm.
1–17			None	5.24[a]
30	0.0154	7.72	0.30	4.81
31	.0107	7.74	.30	4.85
32	.00823	7.76	.30	5.46
33	.00994	7.75	.30	4.28
34	.0199	7.68	.30	4.89
35	.0178	7.70	7.6	4.83
36	.0159	7.71	7.6	4.91
37	.0217	7.68	7.6	4.95
38	.0138	7.73	7.6	4.46
39	.0153	7.72	7.6	4.68
Buffer saturated with thenoyltrifluoroacetone				
40	0.0135	7.72	None	4.92
41	.0125	7.73	None	4.49
42	.00961	7.75	None	5.09
43	.0112	7.74	None	4.85

[a] Average of experiments 1–17.

Effect of Temperature.—An activation energy of 13.6 ± 1 kcal./mole was calculated for the over-all reaction from the ratio of rate constants measured at 39.89° and 24.43°.

TABLE IV

EFFECT OF TEMPERATURE

39.89°; 1 *M* PHOSPHATE BUFFER; 0.928 ATM. OXYGEN

Expt.	Chlorogenic acid concn., mole/liter	*p*H	$k \times 10^7$, ml. O_2/liter/min./atm.
44	0.0132	7.46	17.2
45	.0129	7.47	17.4
46	.00723	7.49	16.8
47	.00888	7.48	15.7
48	.00814	7.48	16.8
49	.0138	7.46	16.7
50	.00936	7.49	15.8
51	.0136	7.46	15.7
52	.0108	7.47	15.7
		Av.	16.4 ± 0.7

Discussion

Since chlorogenic acid is a derivative of catechol, it seems of interest to determine whether any similarity exists in the oxidation mechanisms of these two substances.

Joslyn and Branch[11] have carefully studied the autoxidation of catechol, and found that the rate of oxygen absorption V, over the *p*H range of 6 to 10, was given by the expression

$$V \alpha \frac{(\text{cat.}^-)(O_2)(C)}{(C) + A} \qquad (2)$$

where A is a constant, (O_2) is the pressure of oxygen, (C) the catechol concentration and (cat.$^-$) the concentration of the monoanion of catechol.

LuValle and Weissberger[12] in their excellent classification of oxidation reactions on the basis of semiquinone formation, express the rate of oxygen uptake after the steady-state conditions have been obtained

$$\frac{-d(O_2)}{dt} = \frac{-k_1k_4(R^-)(R)(O_2)}{(R) + A} = \frac{k_1k_4K(R)^2(O_2)}{(H^+)((R) + (A))} \qquad (3)$$

where k_1 and k_4 are the rate constants for the reactions[13]

$$R + O_2 \xrightarrow{k_1} S + O_2^-$$

$$R + T \xrightarrow{k_4} D$$

and K is the dissociation constant for the acid R

$$R \underset{}{\overset{K}{\rightleftharpoons}} R^- + H^+$$

We have shown that the rate of oxygen uptake by solutions of chlorogenic acid fits equation 1, which differs from that found by Joslyn and Branch[4] (*cf.* equation 2) for catechol by the constant A. The constant A has been postulated by LuValle and Weissberger[12] to be equal to k_6 (X), where X

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

(9) J. C. Reid and M. Calvin, Atomic Energy Commission Report MDDC-1405 (1947).

(10) A. Nikolaev, Vestnik Moskov. Univ., 1947, No. 1, 71–7; *C.A.*, **42**, 1617 (1948).

(11) M. A. Joslyn and G. E. K. Branch, THIS JOURNAL, **57**, 1779 (1935); G. E. K. Branch and M. A. Joslyn, *ibid.*, **57**, 2388 (1935).

(12) J. E. LuValle and A. Weissberger, *ibid.*, **69**, 1567 (1947).

(13) The terms R, S and T are the standard Michaelis abbreviations for reduced compound, semiquinone and totally oxidized compound, respectively; D is some sort of dimerization product.

is solvent or hydrogen peroxide and k_6 is the rate constant for the reaction between quinone (T) and X. In the case of chlorogenic acid k_6 is probably small, since the quinone formed is more stabilized by resonance than *o*-benzoquinone. In addition, one of the two reactive positions (the 4,5-carbons) is substituted and is unavailable. This substitution must offer some steric hindrance to further reaction at carbon atom 5.

The autoxidation of chlorogenic acid also differs from that found for catechol by the occurrence of a measurable autocatalytic period up to *p*H 7.9. Joslyn and Branch[11] found no autocatalytic period for catechol.

The inverse hydrogen ion relationship shows that the catechol ion with one hydroxyl group ionized is very probably the reactive species. At *p*H values greater than 8.74 the rate constants increase as Joslyn and Branch[11] also found for catechol at high *p*H values. Their explanation that this is due to the oxidation of the ion with both hydroxyl groups ionized may also be true here. However, it is also possible that the high rate constants at the higher *p*H values may be due to an inaccuracy in the extrapolation of rate to zero time.

In some runs, which were carried up to 30% reaction, the oxygen uptake curve (*e.g.*, B of Fig. 1) appeared to be linear throughout. This linearity would seem to indicate a zero-order relationship for chlorogenic acid during the reaction, which might be expected if the products absorbed oxygen at a comparable rate to chlorogenic acid.

In order to determine what the course of the oxygen uptake would be during the reaction if the products did not absorb oxygen, equation 1 was integrated. Integration of equation 1 gives the equation

$$\text{Vol. } O_2 = 22400C_0[1 - \exp(-kP_{O_2}/(H^+)22400)] \quad (4)$$

where C_0 is the initial concentration of chlorogenic acid and C is the concentration of chlorogenic acid at any time during the reaction. The assumptions were made that (a) there is a 1:1 stoichiometry between oxygen and chlorogenic acid, (b) *p*H and P_{O_2} remain constant during the reaction, and (c) chlorogenic acid disappears by the first order rate law

$$C = C_0 \exp.(-k\,P_{O_2}t/(H^+)22400)$$

Using an average value of k for experiments 1–17 and the initial conditions of curve B (Fig. 1), curve D (Fig. 1) was calculated from equation 2.

As curve B does not differ significantly from curve D, it is not possible to draw any conclusions concerning the order of chlorogenic acid during the reaction or whether or not the products of the chlorogenic acid oxidation themselves absorb oxygen. Further research is being undertaken on this point.

Since the k we found is a product of k_1, k_4 and K, the energy of activation contains contributions from three sources and is therefore difficult to compare with the corresponding value found by Joslyn and Branch for catechol, which also includes contributions from similar terms.

Acknowledgment.—We wish to thank Dr. Benjamin Makower for many stimulating discussions during this work.

ALBANY 6, CALIFORNIA RECEIVED MAY 8, 1951

[CONTRIBUTION FROM THE CHEMOTHERAPY DIVISION, STAMFORD RESEARCH LABORATORIES, AMERICAN CYANAMID COMPANY]

Preparation of Peptides Using Mixed Carboxylic Acid Anhydrides

BY JAMES R. VAUGHAN, JR., AND RUTH L. OSATO

Mixed anhydrides of N-substituted aminoacids with α- or β-branched chain, low molecular weight aliphatic acids are formed under anhydrous conditions at low temperature and react with aminoacid esters to give good yields of the corresponding peptide esters. Anhydrides with isovaleric acid have been found particularly advantageous and have been used to prepare a series of peptides. The theoretical background for the work is discussed.

The use of mixed carboxylic acid anhydrides for the synthesis of peptides was reported recently by Wieland and Sehring,[1] who found that anhydrides of benzoic or acetic acid with N-substituted aminoacids react readily with aqueous solutions of a salt or an ester of a second aminoacid to give the corresponding peptide derivatives. No studies of the peptide reaction under anhydrous conditions were reported. This was of interest in view of the recent work of Emery and Gold[2] on the reactivities of mixed anhydrides in which they stress not only the importance of steric hindrance and charge-distribution in the molecular anhydride, but also the importance of the polarity of the solvent on the acylation ratio obtained when the anhydride reacts with an amine. According to these authors, under anhydrous conditions nucleophilic attack in the molecular anhydride will occur at that carbonyl carbon atom which has the lowest electron density and, other things being equal, which is less hindered sterically. Under aqueous conditions, however, these considerations become invalid, possibly due to ionization of the anhydride, and a marked change or even a reversal of the acylation ratio is observed. Predictions concerning the reaction in polar solvents, therefore, are uncertain. Under anhydrous conditions, however, the anhydride forming acid, the acylation reaction of which is to be suppressed, should be one in which the combined operation of an electronic and a steric effect would lessen the probability of nucleophilic attack at this part of the anhydride molecule and favor substitution at the

(1) T. Wieland and R. Sehring, *Ann.*, **519**, 122 (1950).

(2) A. R. Emery and V. Gold, *J. Chem. Soc.*, 1443, 1447, 1455 (1950).