The Enzymic Oxidation of Chlorogenic Acid and some Reactions of the Quinone Produced

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1. Partially purified preparations of tobacco-leaf *o*-diphenol oxidase (*o*-quinol–oxygen oxidoreductase; EC 1.10.3.1) oxidize chlorogenic acid to brown products, absorbing, on average, 1·6 atoms of oxygen/mol. oxidized, and evolving a little carbon dioxide. 2. The effect of benzenesulphinic acid on the oxidation suggests that the first stage is the formation of a quinone; the solution does not go brown, oxygen uptake is restricted to 1 atom/mol. oxidized, and a compound is produced whose composition corresponds to that of a sulphone of the quinone derived from chlorogenic acid. 3. Several other compounds that react with quinones affect the oxidation of chlorogenic acid. The colour of the products formed and the oxygen absorbed in their formation suggest that the quinone formed in the oxidation reacts with these compounds in the same way as do simpler quinones. 4. Some compounds that are often used to prevent the oxidation of polyphenols were tested to see if they act by inhibiting *o*-diphenol oxidase, by reacting with quinone intermediates, or both. 5. Ascorbate inhibits the enzyme and also reduces the quinone. 6. Potassium ethyl xanthate, diethyldithiocarbamate and cysteine inhibit the enzyme to different extents, and also react with the quinone. The nature of the reaction depends on the relative concentrations of inhibitor and chlorogenic acid. Excess of inhibitor prevents the solution from turning brown and restricts oxygen uptake to 1 atom/mol. of chlorogenic acid oxidized; smaller amounts do not prevent browning and slightly increase oxygen uptake. 7. 2-Mercaptobenzothiazole inhibits the enzyme, and also probably reacts with the quinone; inhibited enzyme is reactivated as if the inhibitor is removed as traces of quinone are produced. 8. Thioglycollate and polyvinylpyrrolidone inhibit the enzyme. Thioglycollate probably reduces the quinone to a small extent.

The intermediates formed when polyphenols* are oxidatively polymerized inactivate or bind some of the more labile enzymes and plant viruses. In particular, cucumber mosaic virus is inactivated in extracts of infected tobacco leaves during the oxidation of chlorogenic acid, and getting infective extracts requires extraction methods that prevent this oxidation (Harrison & Pierpoint, 1963; Pierpoint & Harrison, 1963). It is not known which of the oxidation products is the inactivator and which parts of the virus are sensitive. The first steps in answering these questions require knowledge of the mechanism by which chlorogenic acid is oxidized, and of the reactions that are likely to occur between intermediates in the process and components of leaf extracts.

If the oxidation follows a course similar to the oxidation of catechol by mushroom enzymes (Mallette, 1950), the initial step would be the formation of a quinone with the uptake of 1 mol. of oxygen/mol. of quinone formed. Non-enzymic reactions could then convert half of the quinone into hydroxyquinone and regenerate chlorogenic acid from the rest, and polymerization of the hydroxyquinone would produce the brown end products:

$$\text{Chlorogenic acid} + [O] \xrightarrow{\text{Enzyme}} \text{quinone} \quad (1)$$

$$\text{Quinone} + H_2O \rightarrow \text{hydroxychlorogenic acid} \quad (2)$$

$$\text{Quinone} + \text{hydroxychlorogenic acid} \rightarrow \text{hydroxyquinone} + \text{chlorogenic acid} \quad (3)$$

$$\text{Chlorogenic acid} + [O] \xrightarrow{\text{Enzyme}} \text{quinone} \quad (1a)$$

$$\text{Hydroxyquinone} \rightarrow \text{polymer} \quad (4)$$

* In the usual nomenclature of these phenols, the prefixes poly- and di- refer to the number of hydroxyl groups attached to the benzene ring. *o*-Quinol, as used in the systematic name for polyphenol oxidase recommended by the I.U.B. Commission on Enzymes, refers to any substance containing the *o*-dihydroxybenzene group.

The present paper supplements the incomplete evidence of Clayton (1959) for the formation of a quinone during the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase (*o*-quinol–oxygen oxidoreductase; EC 1.10.3.1). It presents evidence that this quinone reacts with a range of compounds containing nitrogen and sulphur much as do simpler quinones, and that these reactions may involve additional oxygen absorption. In addition, a number of substances that are commonly used to prevent the oxidation of polyphenols are shown to affect this process either by inhibiting the enzyme, by combining with the quinone intermediate, or both.

EXPERIMENTAL

o-Diphenol oxidase preparations. Leaves of *Nicotiana tabacum* (var. Xanthi-nc) had their mid-veins removed and were stored, generally overnight but occasionally for a few days, at −15°. Batches (20g.) of frozen material were disrupted in 200ml. of cold (−15°) acetone by treatment in an MSE Ato-Mix blender for 2min., and the protein precipitate was collected by filtration, washed with more cold acetone and dried over P_2O_5 in a vacuum desiccator. The acetone-dried powder was extracted for 30min. at 0° with 35 times its own weight of 0·1M-tris buffer, pH7·2, containing cysteine (0·05–0·1M); the residue, separated by centrifugation, was re-extracted with a quarter of the initial volume of buffered cysteine. The two extracts were combined and mixed with approx. 2·5 times their volume of cold (0–5°) saturated $(NH_4)_2SO_4$ solution. The precipitated protein was usually left overnight, then collected by centrifugation, dissolved in approx. 20ml. of 0·2M-tris buffer, pH7·0, and dialysed overnight against two 4l. lots of water. It was clarified by centrifugation, if necessary, before being stored at −15°.

This procedure, which is based on the first stages of the method of purification described by Clayton (1959), was carried out in the cold room (0–5°) as far as possible. It usually produced 20–30ml. of solution containing about 1mg. of protein N/ml. Sometimes this solution was slightly brown, indicating that, in spite of the precautions taken, there was some oxidation of leaf polyphenols during the preparation.

Manometric estimations. The action of *o*-diphenol oxidase was followed at 30° by conventional manometry under conditions that measure either initial enzyme activity, or the total amount of oxygen absorbed/mol. of substrate oxidized. Substances could therefore be tested as inhibitors of *o*-diphenol oxidase, and also for reaction with the quinone intermediates formed during the oxidation.

When enzyme activity was measured, each manometer flask generally contained enzyme preparation (0–90μg. of protein N), tris buffer, pH7 (200μmoles), chlorogenic acid (24μmoles) and water to 3ml.; changes, as when the pH was altered or tris buffer was replaced by phosphate buffer, are mentioned in the text. Carbon dioxide was absorbed by KOH in the centre wells, and the flasks were shaken at about 80 oscillations/min. In these conditions the enzyme was almost saturated with substrate; the Michaelis constant is about 2·7mM (Mrs P. Wrench, unpublished work). Oxygen uptake was approximately linear for 10min. after mixing enzyme and substrate. It was measured as a routine for 4 or 5min. when, over the range of protein concentrations used, it was proportional to the amount of enzyme preparation added. Substances tested as inhibitors were added, in solution at pH7, to the main compartments of the flasks. They were therefore incubated with enzyme during the 10min. equilibration period before substrate was tipped in from the side arms.

In the 5min. reaction period the oxygen uptake seldom exceeded 57μl., corresponding to the oxidation of 20% of the added chlorogenic acid to quinone. The bulk of the oxygen is therefore likely to be absorbed in this reaction rather than the oxidation of chlorogenic acid regenerated from the quinone by reactions (2) and (3). To confirm that this was so, most estimations were repeated in the presence of 33mM-benzenesulphinic acid. As shown below, this substance removes quinone from the reaction medium, so preventing its participation in further reactions. Enzyme activities determined in its presence were only slightly lower (5–7%) than those determined without it. Objections to the direct manometric determination of *o*-diphenol oxidase have been raised by Nelson & Dawson (1944); however, the 'chronometric' method of estimation, recommended by Nelson & Dawson (1944), is inappropriate for the tobacco-leaf oxidase, which is inhibited by ascorbate (Fig. 4).

When the total oxygen absorbed/mol. of substrate oxidized was being measured, each manometer flask contained 6μmoles of chlorogenic acid, which was oxidized to completion by an excess (0·2–2·0mg. of protein N) of enzyme preparation. The reaction was followed for 45–60min., but had usually stopped after 20min., when little or no chlorogenic acid could be detected chromatographically in the reaction medium. The enzyme present was at least twice, and more usually 4 times, that necessary to complete the oxidation in 20min. Such an excess made it possible to measure the total oxygen absorbed when chlorogenic acid was completely oxidized in the presence of enzyme inhibitors.

Examination of reaction products. Spectrophotometric examination of reaction media was carried out with either a Unicam SP.500 or an Optica SF4 recording spectrophotometer.

Chromatographic examination was done either on the reaction mixture itself or on ether extracts of it. When the mixture was used the reaction was carried out in dilute buffer or, after careful pH adjustment of the reactants, without buffer. The final solution was then spotted directly on to paper. Ether extracts were made by extracting the reaction medium twice, first after acidifying it to pH4 with N-HCl and again after adjusting the pH to 2. The two extracts were pooled and concentrated before application to paper. Chromatography was performed on sheets (approx. 23cm. × 57cm.) of Whatman 3MM filter paper, with as solvent the upper phase formed from butan-1-ol–acetic acid–water (4:1:5, by vol.). Irrigation was downwards and was continued for 10–22hr., depending on the separation required. After drying, the chromatograms were examined in daylight and in u.v. light. This was repeated after they had been exposed to NH_3 vapour. They were then either sprayed with or dipped into one of the following reagents. (1) $FeCl_3,6H_2O$ (1%, w/v) plus $K_3Fe(CN)_6$ (1%, w/v), which becomes blue with polyphenols and other reducing agents (Barton, Evans & Gardner, 1952). (2) Hoepfner's reagent

[$NaNO_2$ (5%, w/v) in 0·9N-acetic acid followed by N-NaOH], which gives a pink or yellow–orange colour with chlorogenic acid and related polyphenols (Roberts & Wood, 1951). (3) I_2 (0·1N) dissolved in KI (0·12N) to which is added NaN_3 (3%, w/v) immediately before use, this reagent being rapidly bleached by compounds containing C=S or SH groups and, less readily, by disulphides (Feigl, 1954; Chargaff, Levine & Green, 1948). However, results obtained with it were interpreted cautiously as chlorogenic acid and some of its derivatives also decolorize it fairly quickly; presumably I_2 reacts with the unsaturated linkage in their side chains. (4) 2,6-Dichloroquinonechloroimide (0·2%, w/v) in ethanol, which gives yellow–brown colours with some thiols and slowly produces a range of colours with other compounds (Smith & Birchenough, 1958). (5) Hydrated ammonium molybdate (2·5%, w/v) in 0·2N-HCl, which gives purple–blue colours with xanthate (Lederer & Lederer, 1957), but was also found to produce dirty yellow colours with chlorogenic acid and its derivatives. (6) Ninhydrin (0·2%, w/v) in acetone, followed by heating for a few minutes at 80°.

The sulphone formed from benzenesulphinic acid was separated from the reaction medium by a method similar to that used by Pugh & Raper (1927) to isolate *o*-dihydroxydiphenyl sulphone. The reaction medium from five manometer flasks was acidified to pH4 and extracted with five 20ml. lots of ether. This was repeated after further acidification to pH2·5. The pooled extracts were evaporated to dryness and the residue was treated with 40ml. of boiling benzene to dissolve any benzenesulphinic acid. The benzene treatment was repeated three times and the final white residue recrystallized from water and dried over P_2O_5. The product was analysed for C, H and S by Weiler and Strauss (Microanalytical Laboratory, Oxford). Its absorption spectrum (Fig. 2) was not altered when it was chromatographed in butanol–acetic acid–water and eluted from the paper with water.

Reagents. Tris buffer was a 0·2M solution of tris adjusted to pH7 with 2N-HCl. Phosphate buffer was similarly a 0·2M solution of NaH_2PO_4 adjusted to the required pH by conc. HCl or KOH. The chlorogenic acid was a commercial sample from Koch-Light Laboratories Ltd., Colnbrook, Bucks. It contained a minor component that fluoresced blue–grey in u.v. light and had R_F 0·2–0·3 in butanol–acetic acid–water. The contaminant was unaffected by tobacco-leaf extracts and is unlikely to have affected any of the reactions studied.

RESULTS

Oxidation of chlorogenic acid

The oxidation of chlorogenic acid to brown end products by excess of enzyme involved the absorption of 1·4–1·9 atoms of oxygen/mol. of chlorogenic acid oxidized. A little carbon dioxide (less than 0·15mol./mol.) was evolved, especially towards the end of the reaction.

Oxygen uptake during the oxidation of catechol by mushroom *o*-diphenol oxidase can be resolved into two stages at low pH values (Dawson & Nelson, 1938*b*). The first stage is the rapid enzymic oxidation of catechol to *o*-quinone; the second depends on the non-enzymic conversion of quinone into 4-hydroxyquinone, which is slow in acid conditions. The oxidation of chlorogenic acid by tobacco-leaf enzyme followed a slightly different course at pH4 than at pH7 (Fig. 1), but gave no unequivocal evidence that the oxidation occurred in two stages.

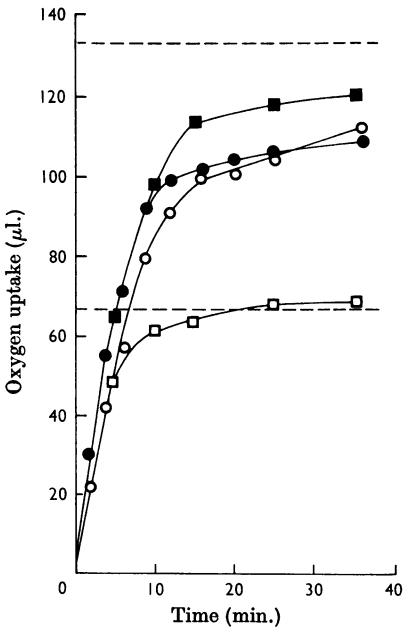


Fig. 1. Effect of pH and of benzenesulphinic acid on the oxidation of chlorogenic acid. In one experiment, chlorogenic acid (6μmoles) was oxidized by enzyme preparation (2mg. of protein N) in 125mM-phosphate buffer at pH7·6 (●) or pH4·5 (○). In the other experiment the oxidation (0·74mg. of protein N) occurred in tris buffer at pH7, with (□) or without (■) 4mM-benzenesulphinic acid. The broken lines show oxygen uptake corresponding to 1 and 2 atoms/mol. of chlorogenic acid oxidized.

The effect of benzenesulphinic acid on the oxidation gave evidence for the formation of quinones. This compound, which has previously been used in studies on the oxidation of monophenols (Pugh & Raper, 1927), reacts with quinones to produce sulphones (Hinsberg & Himmelschein, 1896) by the reaction:

$$C_6H_5 \cdot SO_2H + C_6H_4(O)_2 \rightarrow C_6H_5 \cdot SO_2 \cdot C_6H_3(OH)_2$$

When 3·3–33mM-benzenesulphinic acid was present during the oxidation of 3mM-chlorogenic acid by excess of enzyme, it restricted oxygen uptake to 1 atom/mol. of chlorogenic acid (Fig. 1) and prevented browning. Smaller amounts of benzenesulphinic acid were progressively less effective, but still appeared to combine with the first products of the reaction (Table 1). Benzenesulphinic acid had

Table 1. *Effect of benzenesulphinic acid on the oxidation of chlorogenic acid by tobacco-leaf* o-*diphenol oxidase*

The oxidation of 9 μmoles of chlorogenic acid was taken to completion by an excess of enzyme preparation (0·3 mg. of protein N in Expt. 1; 0·2 mg. of protein N in Expt. 2) in the presence of different amounts of benzenesulphinic acid.

Expt. no.	Concn. of chlorogenic acid (mM)	Concn. of benzenesulphinic acid (mM)	Oxygen uptake (atoms/mol. of chlorogenic acid oxidized)	Final colour of reaction mixture
1	3	0	1·5	Brown
	0	33·0	—	Colourless
	3	33·0	1·08	Yellow
	3	13·2	0·98	Yellow
	3	3·3	1·08	Yellow
	3	1·0	1·45	Orange–brown
2	3	0	1·59	Brown
	3	1·65	1·12	Pale brown
	3	0·99	1·33	Brown
	3	0·66	1·34	Brown
	3	0·33	1·50	Brown

little or no effect on *o*-diphenol oxidase as judged by its effect on the initial rate of oxidation. A small (5%) inhibition, occasionally observed with 33 mM-benzenesulphinic acid, can be explained in terms of a prevention of the secondary stages of the oxidation (reactions 2, 3 and 1a) which occur to a small extent in these estimations of initial reaction rate.

The compound formed between benzenesulphinic acid and the oxidation product of chlorogenic acid is yellow. After acidification it was readily extracted into ether, and on chromatography it migrated as a single spot (R_F about 0·74) with a characteristic yellow–green fluorescence in u.v. light. Exposure to ammonia intensified its yellow colour and also its fluorescence. Hoepfner's reagent turned it orange–yellow. These properties are similar to those of chlorogenic acid, which, however, migrated more slowly (R_F 0·57); they are unlike those of benzenesulphinic acid, which moved at a similar speed. It resembled both these compounds in becoming blue with the ferric chloride–ferricyanide spray, but differed in that it gave a blue colour also with ferric chloride. This compound was not formed when either chlorogenic acid or benzenesulphinic acid was omitted from the reaction medium, and only in trace amounts in the absence of enzyme.

By making use of its solubility in ether and insolubility in hot benzene, this compound was extracted from the reaction mixture and freed from most of its contaminants. A sample, recrystallized once from water, had the composition C, 51·7; H, 4·6; S, 6·2%; loss in weight on drying at 98°, 2·8%. The sulphone formed from benzenesulphinic acid and the *o*-quinone related to chlorogenic acid

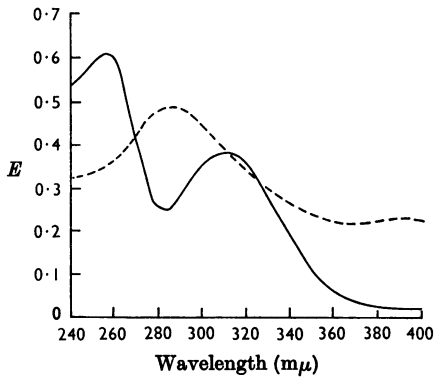


Fig. 2. Spectrum of the sulphone formed from benzenesulphinic acid during the oxidation of chlorogenic acid. The recrystallized material was dissolved (14·6 μg./ml.) in water (——) and N-NaOH was added to a final concentration of 2·5 mN (----).

($C_{22}H_{22}O_{11}S$; see Scheme 1) requires C, 53·5; H, 4·5; S, 6·5%; its monohydrate requires C, 51·6; H, 4·7; S, 6·3%; loss in weight on drying, 3·4%. The ultraviolet spectrum of this compound in water (Fig. 2) has peaks at 254 and 309 mμ. Assuming that it is the monohydrated sulphone, the values of ϵ at these wavelengths are about 21 000 and 13 400 respectively. The addition of alkali displaces both these peaks to longer wavelengths (Fig. 2) in a manner that depends on the concentration of alkali and which alters with time.

Toluenesulphinic acid affected the oxidation of chlorogenic acid in the same way as did benzene-

sulphinic acid. The sulphone formed moved a little slower on chromatography than did the sulphone of benzenesulphinic acid.

Effect of substances that react with quinones on the oxidation of chlorogenic acid

Many substances are known to react with quinones (Mason, 1955) and the effect of some of these on the oxidation of chlorogenic acid was studied.

Aniline. Aniline has previously been used to trap quinones formed during the oxidation of phenol by mushroom *o*-diphenol oxidase (Pugh & Raper, 1927). It inhibited the initial rate of tobacco-leaf *o*-diphenol oxidase by 20% at a concentration of 33mM. However, at a similar concentration, and in the presence of an excess of enzyme, it increased the oxygen absorbed by small amounts of chlorogenic acid to approx. 3atoms/mol. Red compounds appeared almost as soon as the reagents were mixed, and the final colour was a deep cherry-red. Larger amounts of aniline had the same effect except that the final products were dark brown and tended to be precipitated from solution. Aniline thus reacts with the quinone of chlorogenic acid as it does with simpler quinones (Pugh & Raper, 1927). It is likely that at least two molecules of aniline are substituted through their nitrogen atoms into the aromatic nucleus of the quinone (Scheme 1), and in the presence of more concentrated aniline the quinone groups of the dianilidoquinone combine with more aniline to give dark coloured anils.

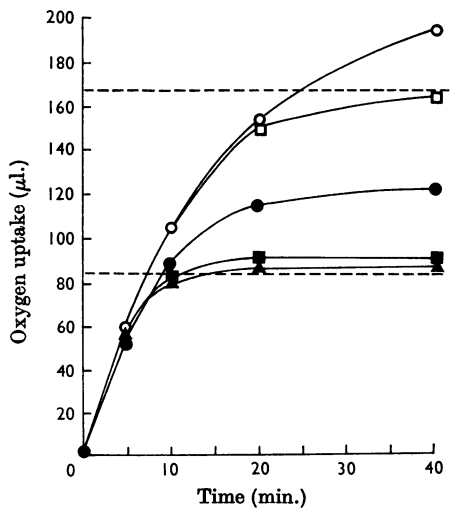


Fig. 3. Effect of nitrogen-containing substances on the oxidation of chlorogenic acid. Chlorogenic acid (7·5μmoles) was oxidized at pH8 by excess of enzyme preparation (400μg. of protein N) as described in the text (●). The nitrogen-containing substances (90μmoles), tested either singly or in combination, were: DL-proline (□); glycine (○); sodium barbiturate (▲); sodium barbiturate plus DL-proline (■); sodium barbiturate plus glycine (■). The broken lines at 84 and 168μl. show oxygen uptake corresponding to 1 and 2 atoms/mol. of chlorogenic acid oxidized.

Glycine. When glycine (30mM) was present during the oxidation of small amounts (3mM) of chlorogenic acid by an excess of enzyme, it affected both oxygen uptake and colour development (see also Hess, 1958). Red–brown colours developed immediately the reaction started and these were finally more intense than those obtained without glycine. The absorption of oxygen was prolonged beyond 30min., and the amount absorbed increased to well over 2atoms/mol. of substrate. Both effects were greater at pH8, and it is at this pH that the experiments described in Fig. 3 were conducted. The pigments formed from chlorogenic acid and glycine thus appear to resemble those found during the oxidation of catechol (James, Roberts, Beevers & de Kock, 1948; Jackson & Kendal, 1949). They probably contain one molecule of glycine linked to the aromatic nucleus by its nitrogen atom (Scheme 1), and the oxygen absorbed in excess of that required for their formation (2atoms/mol.) is probably due to their catalysing the oxidative deamination of additional glycine.

Amino acids with secondary amine groups. Proline and sarcosine (30mM) also stimulated oxygen uptake, but the final amount absorbed was approx. 2atoms/mol. of chlorogenic acid (Fig. 3). Deep-purple colours formed immediately the reagents were mixed. The coloured complexes (see Scheme 1) resembled those formed from these amino acids during the oxidation of catechol (Jackson & Kendal, 1949) in a number of respects. Thus they were reversibly decolorized by ascorbic acid, and irreversibly by hydrochloric acid. Hydrogen ions were liberated during their formation in unbuffered solution, and they were formed more rapidly when chlorogenic acid was oxidized at pH7 than when it was oxidized, at almost the same rate, at pH5.

Other amines. Two tertiary amines, triethanolamine and 2-dimethylaminoethanol (30mM), had no effect on the oxidation of chlorogenic acid, judged either by colour formation or oxygen uptake. A substitutable hydrogen on the amine nitrogen atom appears to be necessary for reaction with the enzymically produced quinone, just as Beevers & James (1948) found it necessary for reaction with the quinone produced during catechol oxidation. But it is evidently not the only requirement; several purines and pyrimidines with primary amine groups also did not affect the oxidation.

Heterocyclic nitrogen compounds. *o*-Quinones react readily with indoles that are unsubstituted in the 3-position, to give a series of highly coloured indol-3′-ylquinones (Bu'Lock & Harley-Mason, 1951). The presence of indole (10–20mM) during the oxidation of chlorogenic acid increased oxygen uptake by a small and rather variable amount (10–40%) provided that the pH was below 8. However, the final reaction mixture was brown, and there was no apparent formation of blue indolylquinones. Steric effects are known to limit the formation of these compounds (Bu'Lock & Harley-Mason, 1951), and they may prevent reaction between indole and the quinone produced from chlorogenic acid. Indoles substituted in the 3-position react with quinones to give a different type of product (Bu'Lock & Harley-Mason, 1951); Leopold & Plummer (1961) described the formation of red pigments when indol-3-ylacetic acid and chlorogenic acid were incubated with *o*-diphenol oxidase. In the present experiments also, a purple–red substance with a broad absorption peak between 520 and 530mμ was formed when the oxidation occurred at pH 5·5. The oxygen uptake corresponded to about 4 atoms of oxygen/mol. of chlorogenic acid, suggesting that more than one indole molecule was substituted into the quinone ring. Much less oxygen, about 2 atoms/mol. of chlorogenic acid, was absorbed when the reaction occurred at pH 7, and red compounds were not apparent until the reaction mixture was acidified.

Barbituric acid (30mM) affects chlorogenic acid oxidation just as benzenesulphinic acid does. It completely prevented the formation of brown end products and restricted oxygen uptake to 1 atom/mol. of chlorogenic acid (Fig. 3). In its presence, as in the presence of benzenesulphinic acid (13mM), the characteristic pigment and oxygen-absorption effects of glycine and proline were completely prevented (Fig. 3). Barbiturates, such as sodium barbitone and sodium phenobarbitone, in which the reactive methylene group is substituted, had no effect. It seems likely therefore that it is via this methylene group that one molecule of barbituric acid combines with one molecule of enzymically generated quinone.

Effect of inhibitors on the oxidation of chlorogenic acid

Several substances commonly used to prevent oxidation of polyphenols were tested to see whether they affect the oxidation of chlorogenic acid by inhibiting tobacco-leaf enzyme, by combining with quinone intermediates, or both.

Effects on the enzyme were measured under conditions in which the bulk of the oxygen uptake was from the enzymic conversion of added chlorogenic acid into quinone. Most were repeated in the presence of benzenesulphinic acid (33mM) to ensure that all the oxygen is absorbed in this reaction. Substances were tested for reaction with quinone by adding them to small amounts of chlorogenic acid, which were being completely oxidized by an excess of enzyme, and observing changes in oxygen uptake and browning of the solution. These changes are largest when the tested substances are present in quantities at least equimolar with chlorogenic acid (1–2mM). Inhibitors of *o*-diphenol oxidase can therefore be tested for reaction with quinones provided that at this concentration they do not inhibit the enzyme by more than about 80%. Of the substances tested, only 2-mercaptobenzothiazole was so inhibitory that it could not be tested in this way.

Ascorbate. This compound reduces quinones to phenolic compounds, and has been used in *o*-diphenol oxidase estimations to keep the quinone concentration low (Nelson & Dawson, 1944). However, it inhibits potato *o*-diphenol oxidase (Baruah & Swain, 1953).

Ascorbate also greatly decreased the initial rate at which tobacco-leaf *o*-diphenol oxidase oxidized chlorogenic acid; in the presence of benzenesulphinic acid, a concentration of 2mM halved the rate (Fig. 4). When small amounts of chlorogenic acid were oxidized by an excess of enzyme in the presence of partially inhibiting quantities of ascorbate, the total amount of oxygen absorbed was increased, and the appearance of brown end products was delayed. Both extra oxygen uptake (Table 2) and the delay in browning were proportional to the ascorbate added. The quinone derived

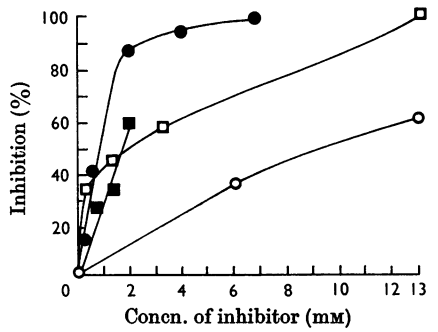


Fig. 4. Inhibition of tobacco-leaf *o*-diphenol oxidase. The effect of thioglycollate (●), ascorbate (□), cysteine (○) and polyvinylpyrrolidone (■) on the initial rate at which the enzyme preparation oxidized chlorogenic acid was determined as described in the Experimental section. Benzenesulphinic acid (33mM) was present in the reaction mixtures except in the experiment with polyvinylpyrrolidone (Kollidon 25; average mol.wt. 24500).

Table 2. *Increase in oxygen uptake when ascorbate is present during the oxidation of chlorogenic acid*

Chlorogenic acid (6 μmoles in Expt. 1; 3 μmoles in Expt. 2) was oxidized to completion by tobacco-leaf *o*-diphenol oxidase (0·88 mg. of protein N in Expt. 1; 0·46 mg. of protein N in Expt. 2) in the presence of different amounts of ascorbate or ascorbate plus benzenesulphinic acid. Neither of these substances gave appreciable oxygen uptake in the absence of chlorogenic acid.

Expt. no.	Benzenesulphinic acid added (μmoles)	Ascorbate added (μmoles)	Oxygen absorbed (μg.atoms)	Oxygen absorbed due to ascorbate (μg.atoms)
1		0	8·3	—
		2	10·8	2·5
		8	16·5	8·2
		12	22·5	14·2
		20	27·3	19·0
2	0	0	5·1	—
	40	0	3·0	—
	0	8	12·6	7·5
	40	8	10·0	7·0

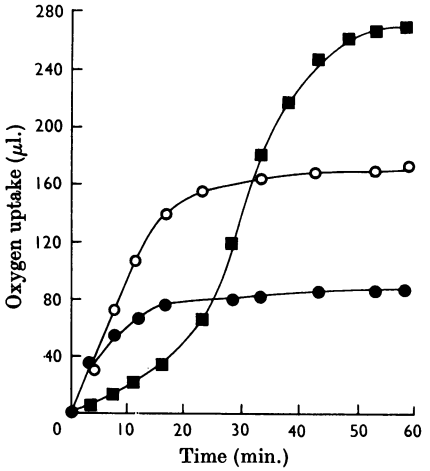


Fig. 5. Reactivation of *o*-diphenol oxidase partially inhibited by ascorbate. Chlorogenic acid (6 μmoles) was oxidized by tobacco-leaf extract (96 μg. of protein N) in the absence (●) and in the presence of 8 μmoles (○) and 20 μmoles (■) of ascorbate.

from chlorogenic acid is therefore, like other quinones, rapidly reduced by ascorbate (Scheme 1); it only accumulates and condenses to brown products when all ascorbate has been oxidized. The increased oxygen uptake caused by 8 μmoles of ascorbate was only slightly diminished by 40 μmoles of benzenesulphinic acid (Table 2), suggesting that the quinone reacts more readily with ascorbate than with benzenesulphinic acid. However, a larger (30-fold) excess of benzenesulphinic acid apparently prevents quinones removing ascorbate, for dilute solutions of ascorbate (1 mM) inhibit *o*-diphenol oxidase more in the presence of benzenesulphinic acid than in its absence. Removal of ascorbate by reaction with quinone probably explains why Farkas, Király & Solymosy (1960) failed to observe an inhibition of tobacco *o*-diphenol oxidase by ascorbate.

Another consequence of the quinone–ascorbate reaction is that *o*-diphenol oxidase, partially inhibited by ascorbate, was, in some conditions, reactivated during the oxidation of chlorogenic acid (Fig. 5). Reactivation of *o*-diphenol oxidases, partially inhibited by a number of other compounds, has previously been observed, but not always attributed to quinone–inhibitor reactions (see Wojtczak & Chmurzynska, 1960).

Potassium ethyl xanthate. This compound chelates copper and other metals and has been used to inhibit copper-containing oxidases (James, 1953; Hill & Mann, 1962). There was a small gas uptake when it was incubated with enzyme preparation. It was equivalent to 20 μl. of oxygen absorbed/hr./80 μmoles of xanthate, but was considerably more in crude leaf extracts. It seems not to be catalysed either by an enzyme or a metal, and it was corrected for in the following experiments. Potassium ethyl xanthate decreased the initial rate of oxidation of chlorogenic acid (Fig. 6), but concentrations less than 5 mM had proportionally more effect than larger ones. This does not depend on the quinone produced during the oxidation; when the oxidation occurred in benzenesulphinic acid, small amounts of xanthate were, proportionally, even more inhibitory (Fig. 6), probably because the benzenesulphinic acid prevented their removal by the reaction with quinone described below.

The complete oxidation of small amounts of chlorogenic acid (3mM) was affected by slightly larger amounts of potassium ethyl xanthate, much as it was by benzenesulphinic acid. The formation of brown pigments was prevented, and oxygen uptake was restricted to about 1 atom/mol. of chlorogenic acid (Fig. 7). This suggests that xanthate combines with the quinones formed during the oxidation (Scheme 1). However, xanthate also had another effect on the oxidation: amounts too small to prevent the formation of brown pigments increased oxygen uptake by 10–20 μl. (Fig. 7). This increase was roughly

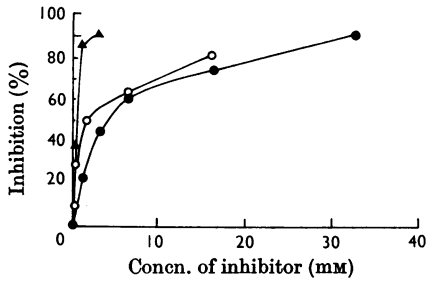


Fig. 6. Inhibition of tobacco-leaf *o*-diphenol oxidase by potassium ethyl xanthate and diethyldithiocarbamate. The rate at which chlorogenic acid (24 μmoles) was oxidized was measured over a 5min. period in the presence of potassium ethyl xanthate (●), potassium ethyl xanthate plus 33mM-benzenesulphinic acid (○), or diethyldithiocarbamate (▲).

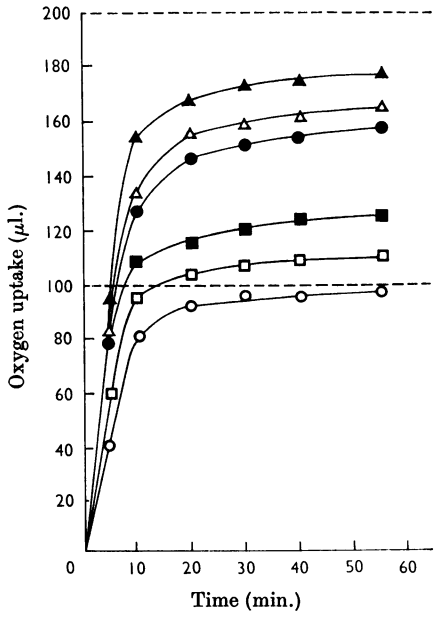


Fig. 7. Effect of potassium ethyl xanthate on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase. Chlorogenic acid (3mM) was oxidized to completion by an excess of enzyme preparation (about 1mg. of protein N) in the presence of potassium ethyl xanthate of the following concentrations; 0 (●); 0·33mM (△); 1·6mM (▲); 3·3mM (■); 6·6mM (□); 11·5mM (○); 33mM (□). The broken lines at 100 and 200 μl. show the oxygen uptake corresponding to the absorption of 1 and 2 atoms/mol. of chlorogenic acid oxidized.

Table 3. *Amount of potassium ethyl xanthate necessary to inhibit the oxidation of different amounts of chlorogenic acid*

Different amounts of chlorogenic acid were oxidized to completion by an excess of enzyme preparation (0·75mg. of protein N) in the presence of different amounts of xanthate.

Concn. of chlorogenic acid (mM)	Concn. of potassium ethyl xanthate (mM)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Colour of final reaction medium
1·0	0	1·95	Brown
1·0	0·6	1·60	Pale brown
1·0	1·0	1·30	Yellow
1·0	3·3	1·30	Yellow
2·0	0	1·50	Brown
2·0	1·0	1·65	Brown
2·0	1·3	1·60	Brown
2·0	2·0	1·20	Yellow
2·0	4·0	1·20	Yellow
3·0	0	1·50	Brown
3·0	2·0	1·56	Brown
3·0	2·5	1·20	Pale brown
3·0	3·0	1·14	Yellow
3·0	4·0	1·10	Yellow

proportional to the amount of xanthate present and it approximated to that expected if the SH groups of xanthate reduced the quinone to chlorogenic acid as does ascorbate. This reduction may also occur to a limited extent with larger amounts of xanthate; oxygen uptake in the presence of 33mM-xanthate was frequently (Fig. 7) a little more than 1 atom/mol. of chlorogenic acid.

The way in which the amount of xanthate necessary to prevent browning and to restrict oxygen uptake depended on the amount of chlorogenic acid oxidized (Table 3) is consistent with the idea that xanthate combines with quinone formed during the oxidation. As 1mM-, 2mM- and 3mM-xanthate respectively prevent browning of 1mM-, 2mM- and 3mM-chlorogenic acid solutions, it also seems that these reactants combine in a 1:1 ratio and have a high affinity for each other. Additional evidence of this affinity is that the increased oxygen absorption, and the colours formed when glycine, proline, sarcosine or aniline (30mM) is present during the oxidation of chlorogenic acid, are prevented by 13·3mM-xanthate.

Two products of the reaction between xanthate and the quinone were detected by chromatographing the reaction mixtures; no trace of either was seen if enzyme, xanthate or chlorogenic acid was omitted. Only one of these products could be extracted by ether from acidified solution, as could the sulphone derived from benzenesulphinic acid. This substance ran faster than chlorogenic acid as a diffuse spot with R_F about 0·8. It appeared as a dark indistinct shadow when viewed under u.v. light, but was a bright yellow in both visible and u.v. light after the chromatograms had been exposed to ammonia vapour. The other compound was detected when the reaction mixture was spotted directly on to chromatogram paper. In u.v. light it was a dull-yellow spot with R_F between 0·15 and 0·25. It too became bright yellow when exposed to ammonia vapour. Both compounds resembled chlorogenic acid in giving blue colours with the ferric chloride–ferricyanide reagent, dirty yellow colours with acid ammonium molybdate and yellow-brown colours with Hoepfner's reagent. The faster-moving one also resembled chlorogenic acid in that it gave a slowly developing (30min.) brown spot when sprayed with 2,6-dichloroquinone-chloroimide and did not bleach the iodine–azide reagent instantaneously. The slower-moving compound, on the other hand, reacted much more quickly with both these sprays, which suggests that it contains free SH or C=S groups.

Diethyldithiocarbamate. Diethyldithiocarbamate is a chelating agent that, although it chelates copper less specifically than does potassium ethyl xanthate (Hill & Mann, 1962), has often been used to inhibit copper-containing enzymes (James, 1953). It decreased the initial rate at which tobacco-leaf *o*-diphenol oxidase oxidized chlorogenic acid more than did potassium ethyl xanthate (Fig. 6). Although inhibition by 1mM-diethyldithiocarbamate was about 80% complete, it was possible, with large amounts of enzyme, to test this substance on complete oxidation of mM-chlorogenic acid. It affected this oxidation just as xanthate did (Table 4). Amounts (1·3–2mM) somewhat greater than those of chlorogenic acid restricted oxygen uptake to about 1 atom/mol. of chlorogenic acid, and kept the mixture yellow or very pale brown. Smaller amounts, which did not prevent browning, increased oxygen uptake beyond the control. This increase was usually 10–20μl., and sometimes more than would be expected from a reduction of the enzymically generated quinone by diethyldithiocarbamate.

A yellow material could be separated chromatographically from the reaction medium after the oxidation of chlorogenic acid in equimolar diethyldithiocarbamate. It had R_F between 0·4 and

Table 4. *Effect of diethyldithiocarbamate on the oxidation of chlorogenic acid by tobacco-leaf* o-*diphenol oxidase*

Chlorogenic acid was oxidized to completion by an excess of enzyme preparation (about 1mg. of protein N) in the presence of different amounts of diethyldithiocarboamate.

Concn. of diethyldithiocarbamate (mM)	Concn. of chlorogenic acid (mM)	Oxygen absorbed (μl.)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Colour of reaction mixture
0·0	1	63	1·88	Brown
2·0	1	33	1·00	Yellow
1·3	1	37	1·10	Yellow
1·0	1	53	1·60	Yellow
0·7	1	80	2·40	Brown
2·0	0	2	—	—

0·5, and appeared as a dark absorbing spot in u.v. light. Like chlorogenic acid its colour was intensified by ammonia vapour, and it gave a blue spot with the ferric chloride–ferricyanide reagent. It reacted only weakly with Hoepfner's reagent and very slowly if at all with the iodine–azide spray. It was not formed when chlorogenic acid, diethyldithiocarbamate or enzyme was omitted from the reaction mixture, and was less obvious when higher concentrations of diethyldithiocarbamate were used.

Cysteine. Cysteine has been frequently used as an inhibitor of polyphenol oxidations (e.g. Clayton, 1959). It combines through its sulphur atom with a number of quinones (Mason, 1955), and with intermediates in the oxidation of chlorogenic acid by apple extracts (Henze, 1956; Hulme, 1958; Walker, 1964). It was appreciably oxidized when incubated alone with tobacco-leaf *o*-diphenol oxidase, and oxygen absorptions measured in the experiments described below have been corrected for this.

Cysteine decreased the initial rate at which chlorogenic acid was oxidized by tobacco-leaf extracts (Fig. 4), although it was less effective than the other inhibitors tested. Its effects on the complete oxidation of small amounts (6 μmoles) of chlorogenic acid resemble those of potassium ethyl xanthate and diethyldithiocarbamate, and depend on the ratio of cysteine to chlorogenic acid. When this ratio is more than 1·5, oxygen uptake is restricted to 0·7–0·8 atom/mol. of chlorogenic acid, and the solution does not turn brown (Table 5). In these conditions cysteine probably condenses with the quinone via its SH group (Scheme 1); the oxygen uptake is probably 1 atom of oxygen/mol. of chlorogenic acid, but appears less because the correction for autoxidation of cysteine is over-estimated. When the ratio of cysteine to chlorogenic acid is less than 1·5, browning is not prevented but oxygen uptake is increased (Table 5). The reaction in these conditions may be a reduction of the quinone by the SH groups; but it may also be a reaction of the type between quinone and glycine (Scheme 1). This suggestion receives some support from the fact that cystine similarly increased oxygen consumption without affecting browning of the solution (Table 5).

Chromatographic analysis of solutions in which chlorogenic acid was oxidized in the presence of a fourfold excess of cysteine showed that the disappearance of chlorogenic acid was accompanied by the formation of a slow-moving material (R_F 0·18–0·25) that fluoresced whitish-yellow in u.v. light. It was yellow in both visible and u.v. light after exposure to ammonia fumes, and it gave a blue colour with the ferric chloride–ferricyanide reagent. As it also reacted with ninhydrin and the iodine–azide reagents, it almost certainly contains chlorogenic acid and cysteine groupings. It is probably the same compound as that formed when apple extracts oxidize chlorogenic acid and cysteine (Henze, 1956; Walker, 1964). However, in addition to this substance, small amounts of two other fluorescent compounds were formed, and at least one of them (R_F about 0·6) contained amino nitrogen and, probably, sulphur. There was also ninhydrin-positive material (R_F about 0·15) that

Table 5. *Effect of cysteine and cystine on the enzymic oxidation of chlorogenic acid*

Chlorogenic acid was oxidized to completion by enzyme preparation (57 μg. of protein N) in the presence of cysteine, cystine, or, as a control, benzenesulphinic acid. In the first experiment corrections were made for the autoxidation of cysteine; these certainly over-estimate the oxidation of cysteine that occurs in the presence of chlorogenic acid, and the values marked with an asterisk (*) are therefore low.

Concn. of chlorogenic acid (mM)	Concn. of benzene-sulphinic acid (mM)	Concn. of cysteine (mM)	Concn. of cystine (mM)	Oxygen absorbed (μl.)	Oxygen absorbed (atoms/mol. of chlorogenic acid oxidized)	Final colour of reaction mixture
2	0	0·0	—	93	1·40	Brown
2	0	9·0	—	47*	0·70	Yellow
2	0	3·0	—	102*	1·50	Pale brown
2	0	1·8	—	110*	1·65	Brown
2	0	0·6	—	104	1·55	Brown
2	33	0·0	—	66	1·00	Yellow
2	0	—	0·0	93	1·40	Brown
2	0	—	13·0	106	1·60	Brown
2	0	—	6·5	107	1·60	Brown
2	0	—	1·3	104	1·55	Brown
2	0	—	0·6	102	1·50	Brown
2	13	—	0·0	63	0·95	Yellow

was formed non-enzymically from cysteine and chlorogenic acid.

Thioglycollate. Thioglycollate inhibited the initial rate of oxidation of chlorogenic acid more than did xanthate (Fig. 4). Concentrations that did not completely inhibit the enzyme neither prevented the formation of brown pigments during the oxidation of small amounts of chlorogenic acid nor restricted oxygen absorption. After allowing for the small autoxidation of thioglycollate oxygen uptake was, in fact, increased by 10–30%. With less than 1·3mM-thioglycollate, this increase depended roughly on the thioglycollate concentration, and approximated to that expected if thioglycollate, like ascorbate, reduced the enzymically generated quinone back to chlorogenic acid. There was no indication that thioglycollate combines with the enzymically produced quinone as it does with simpler quinones (Mason, 1955). Walker (1964) also failed to find evidence for this reaction with the *o*-diphenol oxidase of apples.

2 - *Mercaptobenzothiazole.* 2 - Mercaptobenzothiazole has been observed by J. K. Palmer & J. B. Roberts (personal communication) to be a potent inhibitor of banana and mushroom *o*-diphenol oxidases, whose action at low concentrations is, however, to delay the onset of oxidation. A similar effect is observed on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase (Fig. 8). Concentrations less than 0·16mM delayed oxidation, which, however, once started, proceeded at approximately the same rate and to approximately the same extent as it did in the absence of inhibitor. The length of the delay was roughly proportional to the amount of inhibitor added, and it was extended indefinitely (more than 22-fold) by 33mM-benzenesulphinic acid. The delay was about twice as long at pH 6·8 as at pH 8·2 (Fig. 8).

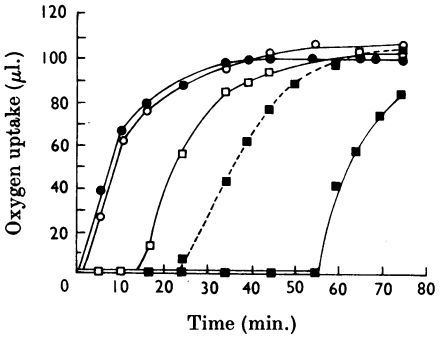


Fig. 8. Delay produced in the action of tobacco-leaf *o*-diphenol oxidase by 2-mercaptobenzothiazole. The enzyme (20μg. of protein N) oxidized chlorogenic acid (24μmoles) at pH 6·8, in the presence of 2-mercaptobenzothiazole of the following concentrations: 0 (●); 0·033mM (○); 0·066mM (□); 0·1mM (■). The broken line shows the course of the oxidation in the presence of 0·1mM-inhibitor at pH 8·2.

J. K. Palmer (personal communication) explains the recovery of inhibited banana *o*-diphenol oxidase as the oxidation of 2-mercaptobenzothiazole to the non-inhibitory disulphide by quinone produced as the oxidation proceeds slowly. It also seems likely that traces of quinone reverse the inhibition of the tobacco-leaf enzyme; the period of inhibition is prolonged by acid conditions, in which the rate of autoxidation of chlorogenic acid is low, as well as by benzenesulphinic acid, which combines with quinones. But the small amounts of mercaptobenzothiazole involved in the inhibition make it difficult to tell by manometric methods whether the quinone oxidizes it to disulphide, or reacts to form a thiazole–quinone compound.

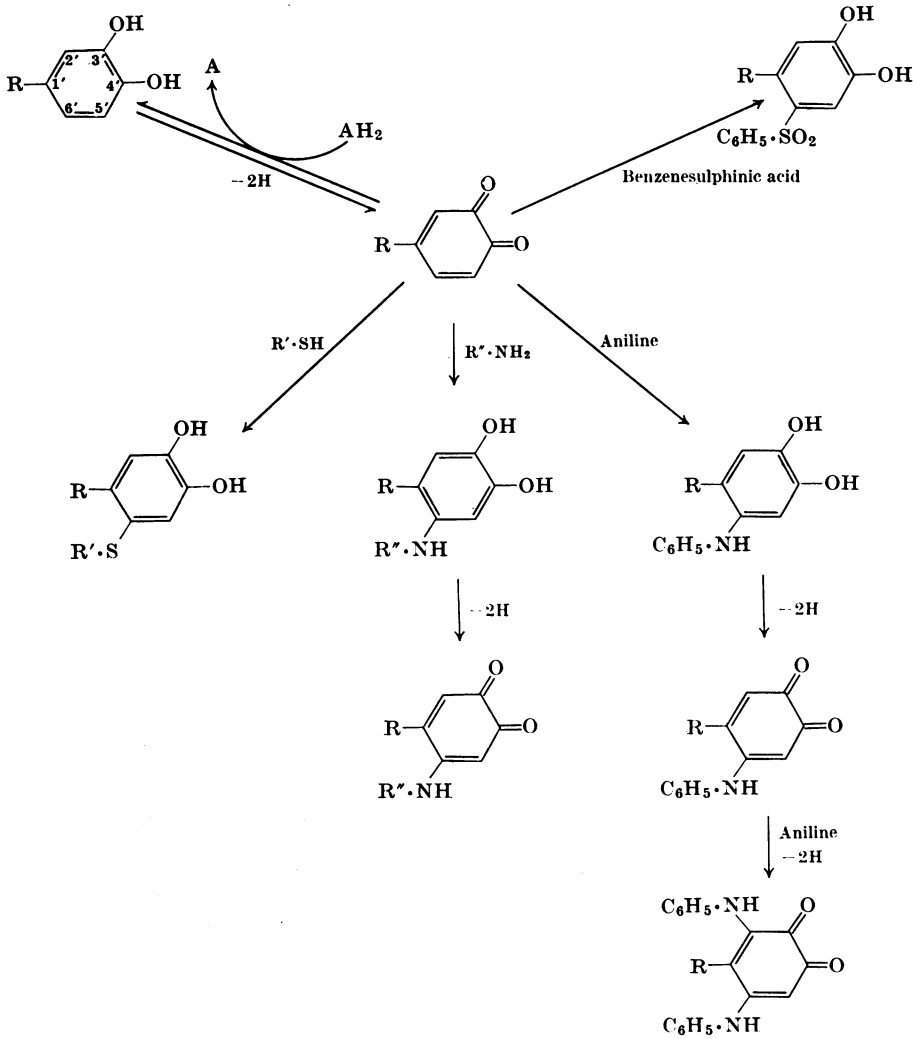
Polyvinylpyrrolidone. Polyvinylpyrrolidone has been used to preserve fruit mitochondria during their isolation from tissues rich in polyphenols (Jones & Hulme, 1961; Hulme, Jones & Wooltorton, 1964). There is some uncertainty as to how it acts, but recent evidence suggests that it inhibits apple *o*-diphenol oxidase (Harel, Mayer & Shain, 1964). In agreement with this suggestion is its inhibition of the initial rate of oxidation of chlorogenic acid by tobacco-leaf extracts (Fig. 4). Over the same concentration range it had no effect on the complete oxidation of small amounts of chlorogenic acid; it is therefore unlikely to act either by absorbing chlorogenic acid or the quinones derived from it.

DISCUSSION

The effect of benzenesulphinic acid on the oxidation of chlorogenic acid by tobacco-leaf *o*-diphenol oxidase leaves no doubt that the first step in the oxidation is the formation of a quinone with an oxygen uptake of 1 atom/mol. formed. The fate of the quinone is less certain. It may well undergo reactions (2)–(4), analogous to those postulated for β-naphthaquinone in aqueous solution (Fieser & Peters, 1931) and for *o*-benzoquinone formed during the oxidation of catechol by mushroom *o*-diphenol oxidase (Wagreich & Nelson, 1938; Mallette, 1950). The purple compound that appears when proline is present during the oxidation may indicate that a hydroxyquinone is formed (James *et al.* 1948). However, a more likely view is that this pigment is derived directly from the initial quinone rather than a hydroxyquinone (Jackson & Kendal, 1949; Hess, 1958).

Assuming this sequence of reactions to occur, there are two obvious differences from the reactions involved in the oxidation of catechol. First, the hydration of the quinone derived from chlorogenic

acid is much less sensitive to pH than is the hydration of *o*-benzoquinone; pH values below 6 failed to resolve the oxidation of chlorogenic acid into fast and slow components as they do for the oxidation of catechol (Dawson & Nelson, 1938*b*; Jackson & Kendal, 1949). Secondly, the reactions occur less completely, so that the final oxygen uptake is less than 2 atoms/mol. of chlorogenic acid and only approaches this value in dilute solutions of chlorogenic acid (Table 3). This may be because some of the enzymically produced quinone reacts with unchanged chlorogenic acid, especially when this is present in excess, and so prevents its oxidation. An analogous reaction occurs between *o*-benzoquinone and catechol (Dawson & Nelson, 1938*a*) although it apparently has little effect on the amount of oxygen consumed when catechol oxidation is followed manometrically. Alternatively, quinone may be removed from the reaction sequence by the type of quinone–quinone condensation that occurs in concentrated solutions of β-naphthaquinone (Fieser & Peters, 1931).



Scheme 1. Some of the probable reactions of the quinone derived from chlorogenic acid. R represents the quinic acid-containing portion of the chlorogenic acid molecule [·CH:CH·CO_2·$C_6H_7(OH)_3(CO_2H)$]; R′·SH represents thiols such as diethyldithiocarbamate, ethyl xanthate and cysteine; R″·NH_2 represents amino acids such as glycine, cystine and possibly cysteine; AH_2 represents reducing compounds such as ascorbate, thioglycollate and possibly the other thiols tested. Proline and sarcosine react like amino acids with primary amino groups. The possible formation of anil compounds from aniline is not shown.

The quinone derived from chlorogenic acid reacts very much like simpler quinones (see Mason, 1955) and is readily substituted by $-NH_2$, $>NH$, $>CH_2$ and $-SH$ groups. By analogy with the simpler quinones, the most likely position for substitution is the 6′-position of the quinone ring (see Scheme 1); the other likely position (1′) is already occupied by the quinic acid side chain. The proximity of this bulky side chain might be expected to hinder some substitutions at the 6′-position. It would explain the lack of reaction with indole, and possibly also with thioglycollate.

The compounds probably formed from the quinone are shown in Scheme 1. The products of the reaction between the quinone and benzenesulphinic acid or barbiturate, probably 6′-derivatives of chlorogenic acid, are stable and without much colour in neutral solution. The only oxygen absorbed in their formation (1 atom/mol.) is that necessary to form the quinone from chlorogenic acid. The corresponding derivatives of glycine and proline are, however, further oxidized to substituted quinones, absorbing an additional atom of oxygen/mol. and producing intense colours. This also happens to the aniline derivative; but the 6′-anilidoquinone is apparently further substituted by aniline, possibly in the 2′-position, and reoxidized to a dianilidoquinone. A similar dianilidoquinone is thought to be formed from at least one other substituted quinone (Mason, 1955). Disubstitution of the quinone nucleus by indol-3-ylacetic acid probably explains the large oxygen consumption that occurs in the presence of this compound.

The reactions between the quinone of chlorogenic acid and thiols do not involve the uptake of additional oxygen nor do they produce intense colours. The main products are therefore likely to be monosubstituted chlorogenic acid compounds (Scheme 1). But both manometric and chromatographic information suggests that additional reactions occur. The increased oxygen absorption, most noticeable with small concentrations of thiols, may be due to the SH groups reducing the quinone to chlorogenic acid, a reaction that may also occur with thioglycollate. Alternatively, it may be due to some of the monosubstituted dihydric phenol being oxidized by an excess of quinone and then further substituted by thiol. Although this seems less likely, it would explain the formation of two chromatographically distinct products from xanthate. The range of trace products formed from cysteine, however, may be because not only cysteine, but also cystine derived from it, react with quinone via amino groups. Glutathione, which would also be expected to react with quinones through its NH_2 and SH groups, gives two distinct products when it is present during the oxidation of catechol (Mason, 1955); a colourless one when glutathione is in excess, and a purple one when catechol is in excess. Glutathione added to apple tissue oxidizing chlorogenic acid gives rise to 'a whole series of fluorescent compounds' (Hulme, 1958).

All the sulphur-containing compounds tested, except thioglycollate, interfere with polyphenol oxidation by inhibiting the enzyme and also by combining with the quinone product. These two effects explain why xanthate can be used to prepare infective extracts from tobacco leaves infected with cucumber mosaic virus (Pierpoint & Harrison, 1963) at concentrations that do not completely inhibit the leaf *o*-diphenol oxidase. These two effects also explain the reactivation of *o*-diphenol oxidase inhibited by sulphur-containing compounds: traces of quinone react with, and so remove, the inhibitor. In the present work this reactivation has been observed with ascorbate, which is almost certainly oxidized by the quinone, and with 2-mercaptobenzothiazole, which is also probably oxidized (J. K. Palmer, personal communication). The reactivation of insect *o*-diphenol oxidase inhibited by thiourea is more likely to involve the formation of thiourea–quinone complexes (Wojtczak & Chmurzynska, 1960).

Diethyldithiocarbamate and potassium ethyl xanthate have been used as selective inhibitors to implicate copper-containing enzymes in metabolic processes of plants (see James, 1953). In addition to the recognized difficulties of this technique, two new ones appear if inhibitions *in vivo* of *o*-diphenol oxidase can be quickly reversed by accumulating quinones, and if the two inhibitors affect quinone-dependent as well as copper-dependent systems. Thus James & Boulter (1955) found that the respiratory system of 7-day-old barley roots is sensitive to diethyldithiocarbamate, and concluded that a copper-dependent enzyme, probably ascorbic oxidase, was the terminal oxidase. But quinones are now known to be involved in cytochrome electron-transport systems; the possibility that some of them are not fully substituted and so would react with diethyldithiocarbamate is an alternative explanation that ought to be borne in mind.

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