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o-Quinones Formed in Plant Extracts

THEIR REACTIONS WITH AMINO ACIDS AND PEPTIDES

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1. The reactions of amino acids and peptides with the *o*-quinones produced by the enzymic oxidation of chlorogenic acid and caffeic acid have been studied manometrically and spectrophotometrically. 2. Amino acids, except lysine and cysteine, react primarily through their α-amino groups to give red or brown products. These reactions, which compete with the polymerization of the quinones, are followed by secondary reactions that may absorb oxygen and give products with other colours. 3. The ϵ-amino group of lysine reacts with the *o*-quinones in a similar way. The thiol group of cysteine reacts with the quinones, without absorbing oxygen, giving colourless products. 4. Peptides containing cysteine react with the *o*-quinones through their thiol group. 5. Other peptides, such as glycyl-leucine and leucylglycine, react primarily through their α-amino group and the overall reaction resembles that of the *N*-terminal amino acid except that it is quicker. 6. With some peptides, the secondary reactions differ from those that occur between the *o*-quinones and the *N*-terminal amino acids. The colours produced from carnosine resemble those produced from histidine rather than those from β-alanine, and the reactions of prolylalanine with *o*-quinones are more complex than those of proline.

Chlorogenic acid, caffeic acid and related *o*-diphenols of plant tissues are rapidly oxidized by *o*-diphenol oxidases (EC 1.10.3.1) in tissue extracts. The first products of these oxidations are highly reactive *o*-quinones, which, although they cannot be isolated, can be trapped quantitatively by added benzenesulphinic acid (Pierpoint, 1966). These quinones, for which the trivial names chlorogenoquinone and caffeoquinone are suggested, normally polymerize to give complex brown products of high molecular weight. They can also react with the proteins of the plant extracts, modifying the properties of these and inactivating some enzymes (Alberghina, 1964; Anderson & Rowan, 1966), and plant viruses (Pierpoint & Harrison, 1963; Mink, 1965).

Speculations about these quinone–protein interactions have often been made; by analogy with the reactions of amino acids and simpler quinones they are thought to involve the oxidation of protein thiol groups, or the formation of quinone adducts with protein thiol or amino groups, or both. Alberghina (1964) found no simple correlation between the susceptibility of some enzymes to inactivation by chlorogenoquinone and *p*-hydroxymercuribenzoate and so stressed the importance of reactions between quinones and protein amino groups; on the other hand Mink (1965) suggested that the primary inactivating reaction between chlorogenoquinone and Tulare apple mosaic virus was the oxidation of a thiol group. There are, as yet, no detailed studies of the chemical modifications that these natural *o*-quinones induce in plant proteins.

This paper describes the reaction of enzymically generated chlorogenoquinone and caffeoquinone with amino acids and simple peptides, extending earlier information (Pierpoint, 1966; Haider, Frederick & Flaig, 1965). It was hoped that the colours produced and oxygen absorbed in these reactions would be characteristic enough to help in identifying the amino acid residues in proteins that reacted with these quinones. This study closely parallels that of the reactions between amino acids and melanogenic quinones by Mason & Peterson (1965). Its relevance to the reactions of chlorogenoquinone with protein is shown in the accompanying paper (Pierpoint, 1969).

EXPERIMENTAL

o-Diphenol oxidase. Preparations of the enzyme were made from frozen leaves of *Nicotiana tabacum* (var. Xanthi-nc) essentially by the first three stages of the procedure of Clayton (1959). It was usually found necessary to concentrate the preparations after the ethanol and dialysis treatment. Sometimes this was done by freeze-drying, but more often by precipitating again with

$(NH_4)_2SO_4$ (550g./l.), extracting the precipitate four or five times with a minimum volume of tris buffer (0·02M; pH7), and dialysing. The resulting brown solutions (about 1ml./g. of acetone-dried powder) had oxidase activity such that 0·2–0·3ml. oxidized chlorogenic acid at its maximal rate in the enzyme assay, and 0·02–0·04ml. produced half the maximal rate.

Manometric estimations. Amino acids and their derivatives were made to react with chlorogenoquinone or caffeoquinone in manometric flasks. Beside the amino acid derivative (100μmoles), the reaction mixture (3ml.) contained chlorogenic acid or caffeic acid (6μmoles), an excess of *o*-diphenol oxidase (0·1–0·2ml.) and either tris (200μmoles; pH7 or 7·8) or sodium phosphate–potassium phosphate (200μmoles; pH5) buffer. The reaction was started by tipping the phenolic acid from the side arm of the manometer flask, and following the progress of the reaction for at least an hour at 30°. Any CO_2 produced during the reaction was absorbed by KOH in the centre wells of the manometer flasks. *o*-Diphenol oxidase activity was measured manometrically as the initial rate (4min.) of oxidation of chlorogenic acid (24μmoles) in the presence of benzenesulphinic acid (100μmoles), to restrict the reaction to a single oxygen-consuming stage (Pierpoint, 1966).

Spectrophotometric examinations. At a time (2–3hr.) after the start of the reaction, the reaction mixtures were diluted with a suitable amount (1–3vol.) of water and examined on an Optica SF4 recording spectrophotometer. Sometimes the examination was made earlier (1hr.) or later (up to 16hr.) to record fading or slowly developing colours.

Reagents. Tris buffer was a 0·2M solution of tris adjusted to pH7 with 2M-HCl. Phosphate buffers were made from a stock solution of M-NaH_2PO_4–M-KOH, which was diluted and adjusted to the required pH by the addition of conc. HCl. The chlorogenic acid was a commercial sample described by Pierpoint (1966). Caffeic acid was bought from Koch–Light Laboratories Ltd. and purified by dissolving it in hot water (20vol.), boiling for a few moments with a little charcoal, filtering and acidifying the cooling filtrate with a few drops of M-HCl. The dried crystalline product was shown by paper chromatography (in 5% acetic acid) and by n.m.r. analysis (by Dr N. Janes) to consist of the *trans*-isomer of caffeic acid with little or no *cis*-form.

RESULTS

Oxidation of chlorogenic acid and caffeic acid. Preparations of *o*-diphenol oxidase from tobacco leaves oxidize chlorogenic acid quickly to greenish compounds that change to brown end products during 10–15min. They oxidize caffeic acid immediately to red–brown compounds, and although these colours also fade, the end products are redder than those from chlorogenic acid.

Oxygen uptake with caffeic acid (2mM) is a little slower than with chlorogenic acid, as the enzyme has a smaller affinity (Michaelis constant 6–9mM) for caffeic acid. The effect of benzenesulphinic acid (Fig. 1) suggested that the first stage in the oxidation is, as with chlorogenic acid, the formation of an *o*-quinone:

$$\text{Caffeic acid} + [O] \rightarrow o\text{-quinone} + H_2O$$

The subsequent polymerization of the quinone absorbed another 0·35–0·95 atom of oxygen, depending on the caffeic acid concentration. No trace of esculetin, which is formed from the oxidation of *cis*-caffeic acid (Satô, 1967), could be detected chromatographically at any stage of the oxidation.

Reactions between quinones and amino acids primarily involving α-amino groups. The neutral amino acids α-alanine, β-alanine and leucine had little effect on the oxidation of phenolic acids; instant production of brown colours from chlorogenic acid and bright-red ones from caffeic acid was the most obvious sign that they reacted with the quinones. The colours quickly faded to browns and red–browns similar to those formed without amino acids, although the products formed by oxidizing

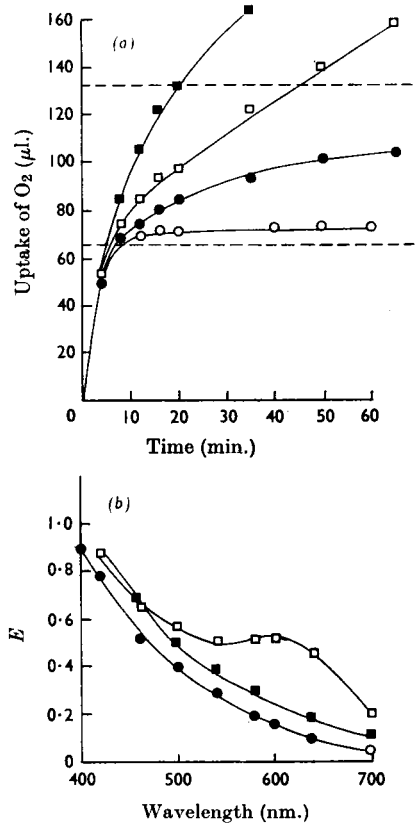


Fig. 1. Oxidation of caffeic acid and the effect of glycine derivatives on this. Caffeic acid (6μmoles) was oxidized, by an excess of enzyme at pH7, alone (●) or in the presence of benzenesulphinic acid (○), glycine (□) or methyl glycine (■). Broken lines (*a*) correspond to the uptake of 1 and 2 atoms of oxygen/molecule of caffeic acid present. Reaction mixtures were diluted with 3vol. of water about 10hr. after the start of the reaction and their spectra measured (*b*).

Table 1. *Effect of amino acids on the enzymic oxidation of caffeic acid and chlorogenic acid*

The oxidations were followed manometrically for an hour and the 'extra' oxygen uptake stimulated by the amino acid was found by comparison with controls without amino acids. The stable colours of the reaction mixtures, produced after an hour or more, were also compared spectrophotometrically with these controls after suitable dilution.

		Effect on oxidation of chlorogenic acid		Effect on oxidation of caffeic acid	
Amino acid	pH	'Extra' uptake of O_2 (μl.)	Final colour	'Extra' uptake of O_2 (μl.)	Final colour
DL-Methionine	7·0	10	No difference	20	Darker and redder; absorbs more between 500 and 700 nm.
	7·8	32	Darker; peak at 700 nm.		
Serine	7·0			22	Redder; absorption shoulder at 620 nm.
	7·8	40	Little darker		
DL-Threonine	7·0			20	Darker and redder; absorbs more between 450 and 600 nm.
	7·8	66	Darker; slight absorption shoulder 680–740 nm.		
Aspartate	7·0			16	Very slightly darker
	7·8	17	Darker; shoulder 680–700 nm.		
Asparagine	7·0	7	No difference	20	Slightly darker; higher absorption 450–700 nm.
	7·8	31	Darker; shoulder 680–700 nm.		
Glutamate	7·0			0	Slightly redder; absorbs a little more between 480 and 580 nm.
	7·8	12	Darker; absorption shoulder around 680 nm.		
L-Glutamine	7·0			28	Dark blue–green; high absorption between 450 and 700 nm. Shoulder near 640 nm.
	7·8	32	Green–brown; peak at 660–700 nm.		
Arginine	7·0	40	Dark brown; large absorption peak at 700 nm.	30	Dark green–brown; small peak at 620 nm.
L-Homoarginine	7·0	16	Darker; slight peak at 700 nm.	—	—
	7·8	32			
L-Tyrosine	7·0			10	Very slightly darker
	7·8	8	Darker; slight peak at 700–720 nm.		
Histidine	7·0			30	Yellow–brown; high absorption below 450 nm.; slight shoulder 680–700 nm.
	7·8	54	Slightly green; high absorption below 450 nm.; slight peak near 700 nm.		
Proline	7·0	20	Purple–brown; higher absorption 450–600 nm.	8	Red; large absorption peak 510–530 nm.
	7·8	33	Red–brown; higher absorption 450–600 nm.	31	Purple–red; absorption peak 510–530 nm.
Hydroxyproline	7·0			12	Red–purple; peak 530–540 nm.
	7·8	25	Red–brown; higher absorption 450–600 nm.	30	Purple; absorption peak 520–540 nm.
DL-Tryptophan	7·0	24	Pink–brown; peaks at 510–550 nm.	25	Slightly darker; shoulder at 560 nm.
	7·8	44	Intense brown–red; large peaks at 510 nm. and 550 nm.	54	Red–brown; absorption peaks 530 nm and 565 nm.

caffeic acid in the presence of leucine were redder and had a stronger absorption between 450 and 550 nm. (see Fig. 4), whereas those formed from oxidizing chlorogenic acid at pH 7·8 in the presence of either α- or β-alanine had a small absorption band at 680–700 nm. There seems to be no reaction between the enzymically produced *o*-quinones and *N*-acetyl derivatives of these amino acids.

Glycine also had only a small effect on the stable colours formed from chlorogenic acid, but it increased the amount of oxygen absorbed in the reaction (Pierpoint, 1966). It similarly increased oxygen absorption with caffeic acid, but it had a more characteristic effect on the colours produced (Fig. 1). The intense reds, which formed instantaneously, quickly faded to browns, but they darkened

over the course of a few hours to dark blues, which absorbed strongly at 580–600nm. The effects are smaller when less glycine is added, or when the oxidation is done at a lower pH. The secondary reactions leading to the formation of the 600nm. absorption band require the presence of a free carboxyl group; they do not occur with methyl glycine, although this ester increases the oxygen uptake (Fig. 1).

The effect of some other amino acids on the oxidation of chlorogenic and caffeic acids is summarized in Table 1. The amount of oxygen absorbed in excess of that involved in the oxidation of phenol alone depends on the amino acid. Thus the hydroxy acids stimulate large oxygen absorptions during the oxidation of chlorogenic acid or caffeic acid, whereas glutamate has little effect on either. Moreover, the order of effectiveness of amino acids on the oxidation of caffeic acid is like that on the oxidation of catechol (Haider *et al.* 1965). Glutamine and asparagine are more active than glutamate and aspartate, perhaps illustrating the adverse effect of free carboxyl groups on the oxygen-absorbing reactions that is seen by comparing the activity of glycine and methyl glycine (Fig. 1). The total oxygen absorbed in the presence of proline, especially at pH 7·8, corresponded closely to 2 atoms/molecule of phenolic acid oxidized.

The colours produced during the oxidations are usually more characteristic of the phenol being oxidized than of the amino acid present. With chlorogenic acid, brown colours develop very quickly, darken during the next few hours and often have a distinct absorption band near 700nm. With caffeic acid the initial bright reds fade to red-browns, but the final solutions absorb more light over the region 450–700nm. than do the products of caffeic acid alone. Histidine is an exception to this generalization; it produced more yellowish products from both phenolic acids, although the absorption spectra showed no characteristic bands between 400 and 800nm. Proline also gave distinctive colours, purple-reds, which characterize the reactions between secondary amines and *o*-quinones (Jackson

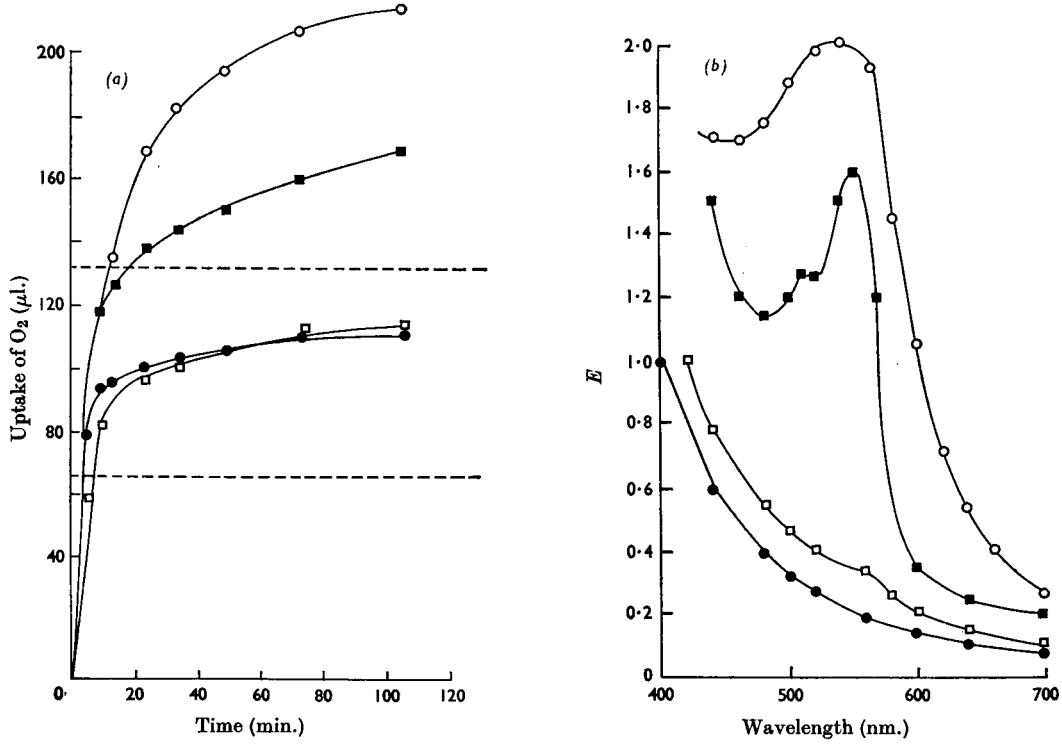


Fig. 2. Effect of tryptophan and indol-3-ylacetic acid on the oxidation of chlorogenic acid. The oxidation of chlorogenic acid (6 μmoles) by an excess of enzyme followed much the same course at pH 5·5, 7·0 and 7·8 (●) (*a*). Tryptophan (100 μmoles) stimulated oxygen uptake at pH 7·8 (■) but not at pH 5·5 (□); conversely indol-3-ylacetic acid (100 μmoles) stimulated oxygen uptake at pH 5·5 (○) but not at pH 7·8 (●). Broken lines (*a*) correspond to the uptake of 1 and 2 atoms of oxygen/molecule of caffeic acid. Reaction mixtures were diluted with 3 vol. of water before their absorption spectra were measured (*b*).

& Kendal, 1949). The colour produced from proline and caffeic acid had a pronounced absorption maximum at about 510–540nm.: the corresponding colour from chlorogenic acid was much less stable and within an hour had faded to a reddish brown.

Tryptophan is the only other amino acid in Table 1 that gave characteristic colours with the quinones. It produced intense and stable reds, which are more obvious with chlorogenoquinone than caffeoquinone (Fig. 2). These reactions can be clearly distinguished by their pH-dependence from the reactions of 3-substituted indole compounds with chlorogenoquinone, even though these (e.g. indol-3-ylacetic acid) also give purple–reds and absorb oxygen (Fig. 2).

With all the amino acids so far listed, the α-amino group is likely to be involved in the primary reaction with quinones. *N*-Acetyl derivatives of histidine, tyrosine, tryptophan, proline, arginine, glutamine and glycine did not, apparently, react; neither did imidazole acetate nor acetamide. The side chains of these amino acids are therefore only likely to be involved in secondary reactions after the reaction of the α-amino groups with quinones.

Reactions between quinones and amino acids containing ε-amino and thiol groups. Evidence that the ε-amino group of lysine reacts with quinones is given in Fig. 3, which shows that *N*-α-benzyloxycarbonyl-L-lysine affects both the absorption of oxygen and the spectra produced during the oxidation of caffeic acid. The effect on both processes is less than that produced by lysine. α-Acetyl-DL-lysine also reacts with the quinones: the spectrum of the red–brown product with caffeoquinone is almost identical with that shown in Fig. 3 for the α-benzyloxycarbonyl compound.

The thiol group of cysteine seems to have a greater affinity than the α-amino group for *o*-quinones; excess of cysteine reacts with chlorogenoquinone, without absorbing oxygen, to produce colourless products (Pierpoint, 1966). Cysteine reacts with caffeoquinone in a similar manner. *N*-Acetylcysteine also reacts with both quinones much as cysteine does, and, because of its slower autoxidation, is clearly seen to restrict the oxygen absorbed in the oxidation of the phenolic acids to the one atom involved in the formation of the quinones.

Reactions between quinones and peptides. It would be expected that the primary reaction between peptides and enzymically generated quinones would involve the terminal amino groups, and that the overall reactions would resemble those of the terminal amino acids. Use was made of the characteristic reaction of glycine with caffeoquinone to show that this is true with some dipeptides. Thus glycyl-L-leucine (Fig. 4) and glycyl-DL-alanine, like glycine itself, stimulate uptake of oxygen during the oxidation of caffeic acid, and produce blue colours with absorption bands near 620nm. In contrast, DL-leucylglycine (Fig. 4) and DL-alanylglycine stimulate very little extra oxygen absorption, and give red–brown products as do leucine and alanine. The only observable difference between reactions of the peptides and the terminal amino acids is that the peptides react quicker.

With some peptides the course of the reaction with quinones, and especially the final colour, differed from that produced by the terminal amino acid. This was seen with glycylglycine, which stimulated about three times as much 'extra' oxygen absorption during the oxidation of caffeic acid as did glycine, but which gave a much smaller absorption band at 600nm. Both these effects may result from removing a carboxyl group from the proximity of the α-amino group, as they also occur with the methyl ester of glycine (Fig. 1), and they are larger with ethyl glycylglycine than with

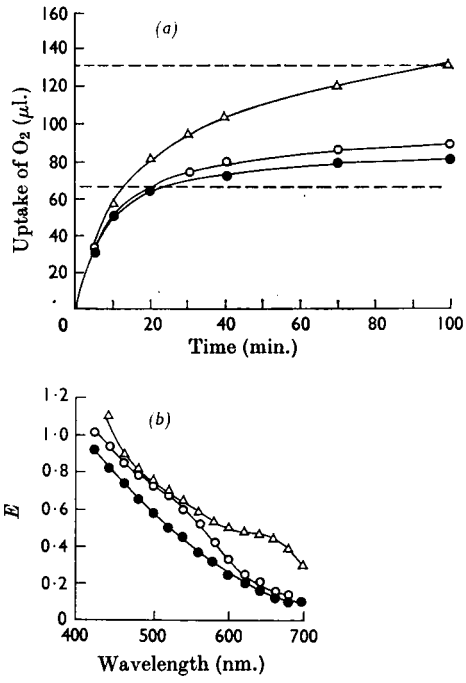


Fig. 3. Effect of lysine derivatives on the oxidation of caffeic acid. Caffeic acid (6 μmoles) was oxidized at pH 7 by an excess of enzyme, alone (●), in the presence of 100 μmoles of L-lysine (△) and in the presence of 100 μmoles of *N*-α-benzyloxycarbonyl-L-lysine (○). Broken lines (*a*) correspond to the uptake of 1 and 2 atoms of oxygen/molecule of caffeic acid. Reaction mixtures were diluted with an equal volume of water before their absorption spectra were measured relative to water (*b*).

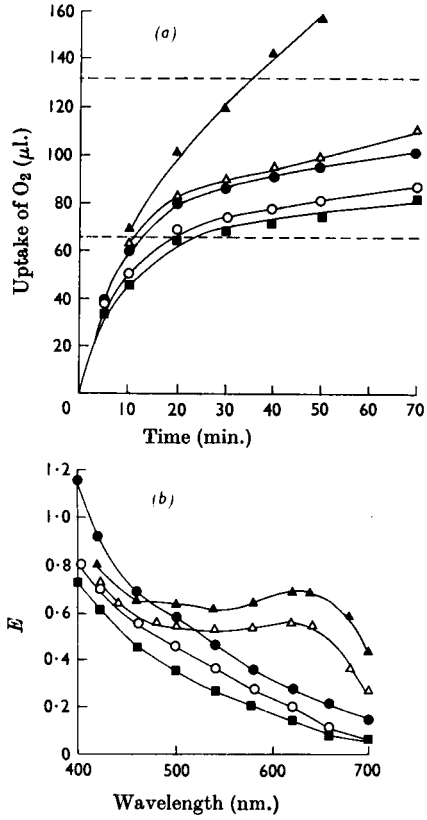


Fig. 4. Effect of peptides of glycine and leucine on the oxidation of caffeic acid. Caffeic acid (6 µmoles) was oxidized, at pH 7, alone (■) or in the presence of 100 µmoles of L-leucine (○), glycine (△), DL-leucylglycine (●) or glycyl-L-leucine (▲). Broken lines (*a*) correspond to the uptake of 1 and 2 atoms of oxygen/molecule of caffeic acid. Reaction mixtures were diluted with 2 vol. of water and left on the bench overnight before the spectra were measured (*b*)

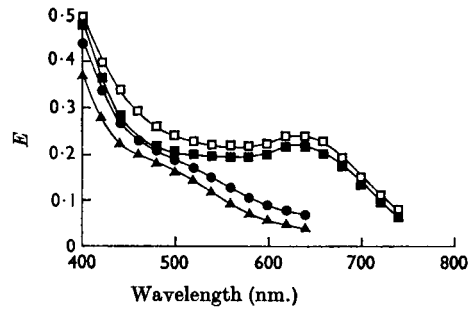


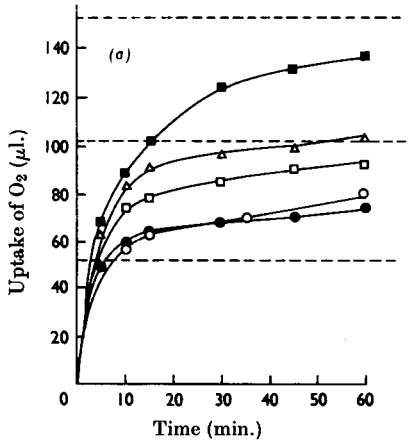
Fig. 5. Colours produced during the reaction of caffeoquinone with carnosine. Caffeoquinone was produced by adding potassium ferricyanide (3 µmoles) to caffeic acid (3 µmoles in 0·2 M-tris, pH 7) in an evacuated Thunberg tube. The absorption spectrum of the reaction mixture (▲) was compared with those produced in the presence of 50 µmoles of β-alanine (●), L-histidine (□) and L-carnosine (■) after about 2 hr. at room temperature.

glycylglycine. Both effects also occur with ethyl esters of diglycyl- and triglycyl-glycine.

The reactions of L-carnosine (β-alanyl-L-histidine) with the quinones are also not those expected of the *N*-terminal amino acid. Although the initial reactions resemble those of β-alanine, and give brown reaction mixtures that absorb little oxygen, the final colours suggest that the secondary reactions are affected by the *N*-bound histidine. This was more apparent in the reaction with caffeoquinone than with chlorogenoquinone, and was clearly seen when the amino acids reacted *in vacuo*, with caffeoquinone generated from caffeic acid by the addition of ferricyanide. After 2 or 3 hr. both carnosine and histidine had produced green–blue products (absorption maxima near 640 nm.) in contrast with the red–brown ones produced by β-alanine (Fig. 5).

Another striking example of the way the reaction of a peptide may differ from that of the terminal amino acid is seen with peptides of proline and alanine. Prolylalanine reacts with enzymically produced chlorogenoquinone much as proline does. It gives a rapidly fading purple, and increases, at pH 7·8, the oxygen absorbed to about 2 atoms/molecule of chlorogenic acid oxidized; similarly alanylproline reacts like alanine. However, the reaction of these peptides during the oxidation of caffeic acid is more complex (Fig. 6). In particular, no stable purple colour is formed from prolylalanine, as it is from proline, and almost 3 atoms of oxygen are absorbed in the reaction. The peptide-linked alanine seems to have a qualitative rather than a quantitative effect on the reaction of the terminal proline.

Reaction between quinones and glutathione. The primary reaction between chlorogenoquinone and a thiol-containing peptide is likely to involve the thiol group rather than the amino group. Thus the reactions of glutathione with caffeoquinone and chlorogenoquinone resemble those of cysteine. Glutathione is only slightly less effective than cysteine in preventing the development of brown products from chlorogenoquinone; this suggests that its thiol group has a smaller affinity for the quinone than that of cysteine. However, thiol-containing amino acids react with quinones in a number of ways (Mason & Peterson, 1965; Pierpoint, 1966), and one of the alternative reactions, for example one that involves the amino group, may be more effective with glutathione than with cysteine.



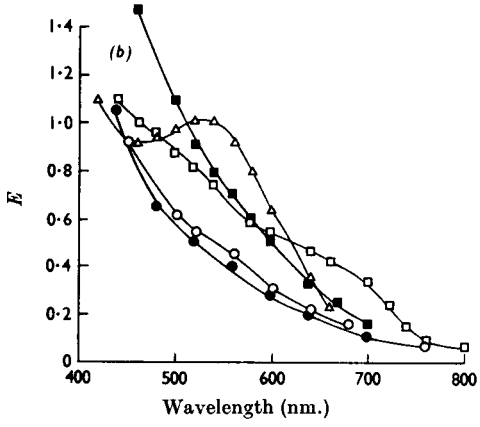
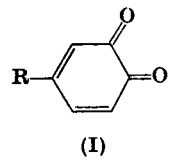


Fig. 6. Effect of proline derivatives on the oxidation of caffeic acid. Caffeic acid (4·6 μmoles) was oxidized, at pH 7, by an excess of enzyme, alone (●) or with 100 μmoles of DL-alanine (○), L-hydroxyproline (△), L-alanyl-L-proline (□) or L-prolyl-L-alanine (■). Broken lines (*a*) correspond to the uptake of 1, 2 and 3 atoms of oxygen/molecule of caffeic acid. Reaction mixtures were diluted with an equal volume of water before the spectra were measured relative to water (*b*).



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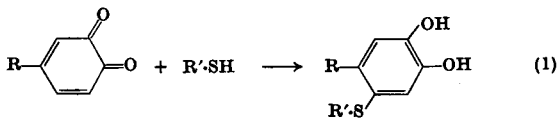

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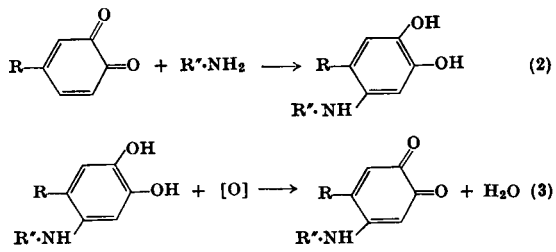

(2)

(3)

DISCUSSION

By combining the new evidence with previous information on the reaction of amino acids with *o*-quinones (Mason, 1955*a*), the reactions of amino acids with enzymically generated chlorogenoquinone and caffeoquinone (I) can be summarized:

(*a*) Cysteine reacts primarily through its thiol group; the reaction does not involve oxygen and it gives a colourless fairly stable product (reaction 1). Other reactions are also likely to occur, especially when there is a large excess of quinone. They may involve the simple oxidation of thiol to S·S, the oxidation of the product of reaction (1) to a quinone derivative, or the reaction of the quinone with the α-amino group. However, any reaction with the α-amino group of cysteine appears to be less important with chlorogenoquinone than with either *o*-benzoquinone (Mason & Peterson, 1965) or 1,2-naphthaquinone (Rees & Pirie, 1967).

(*b*) The other amino acids primarily react with quinones through their amino groups, giving monosubstituted phenols that may be oxidized, probably non-enzymically (Mason, 1955*b*), to coloured quinone derivatives (reactions 2 and 3).

These reactions go nearly to completion only with proline and hydroxyproline, amino acids with secondary amine groups, and the products, especially those formed from caffeoquinone, have a similar colour and stability to those formed from simpler quinones (Jackson & Kendal, 1949).

With most of the other amino acids, reactions (2) and (3) compete less successfully with the polymerization of the *o*-quinones, and are followed by ill-defined secondary reactions. Some of these are responsible for a prolonged absorption of oxygen; they may involve a cyclic sequence of reactions similar to those by which *o*-benzoquinone is thought to oxidize amino acids (Trautner & Roberts, 1951), although this has been questioned (Haider *et al.* 1965). Other secondary reactions cause the slow colour changes that take place during an hour or so,

and they can sometimes be clearly distinguished from the oxygen-absorbing ones: those that form blue colours from caffeoquinone and glycine do not occur with methyl glycine, whereas those that increase oxygen absorption do (Fig. 1).

The primary reactions of chlorogenoquinone and caffeoquinone with peptides and proteins are likely to involve only the thiol, the terminal α-amino and the lysine ϵ-amino groups. Peptide nitrogen and the nitrogen-containing side chains of histidine, tryptophan and arginine seem not to react. The same conclusion was reached for the reaction of simpler and more stable *o*-quinones with proteins (e.g. Mason, 1955*a*), although some unstable melanogenic quinones probably react only with protein thiol groups (Mason & Peterson, 1965).

To judge from the speed with which the initial colour changes occur, chlorogenoquinone and caffeoquinone react faster with the terminal amino groups of peptides than they do with the free amino acids. This is also true of *o*-benzoquinone and has been called the 'peptide effect' (Mason, 1955*b*). One consequence of this is that, with peptides, reactions (2) and (3) compete more successfully with the polymerization of the quinones. As quinone polymerization absorbs only 0·2–0·8 atom of oxygen/molecule of quinone, this would probably increase the amount of oxygen absorbed, and would also increase the amount of substituted quinone available for secondary reactions. These considerations may explain why more oxygen is absorbed in the presence of glycyl-leucine than of glycine (Fig. 4). However, they do not explain the differences between the amount of oxygen absorbed in the reactions of caffeoquinone with prolylalanine and with proline: with proline, reactions (2) and (3) probably go nearly to completion, and seem not to be followed by further oxygen-absorbing reactions. It is possible that the peptide-bound proline, unlike proline, disubstitutes into the quinone nucleus, a reaction that occurs between the terminal proline of salmine and *o*-benzoquinone (Mason & Peterson, 1955) and that may occur between the ethyl ester of hydroxyproline and *o*-benzoquinone (Jackson & Kendal, 1949). Whatever the mechanism of the reactions, it seems important that the terminal amino acid of a protein may not only react faster than the free amino acid with an *o*-quinone, but it may react in a more complicated manner.

In the reactions written, substitution into the quinone ring of chlorogenoquinone is assumed to occur at the 6′ position. This assumption was previously made (Pierpoint, 1966) by analogy with the substitution pattern of simpler *o*-quinones. In contrast, Bariana, Krupey, Scarpati, Freedman & Sehon (1965) assumed that chlorogenoquinone reacted with protein amino groups through the 5′ position, because the Mannich reaction, which they assumed occurred at the 5′ position, blocked this reaction. There is no direct evidence to decide between these alternative views. However, the sulphone previously shown to be formed from the reaction of chlorogenoquinone and benzenesulphinic acid (Pierpoint, 1966), has been isolated and examined by nuclear magnetic resonance; the results, given in the Appendix to this paper, are consistent with the assumed nature of the sulphone and show that the phenolic nucleus is substituted at the 6′ position.

REFERENCES

Alberghina, F. A. M. (1964). *Life Sci.* **3**, 49.

Anderson, J. W. & Rowan, K. S. (1966). *Phytochemistry*, **6**, 1047.

Bariani, D. S., Krupey, J., Scarpati, L. M., Freedman, S. D. & Sehon, A. H. (1965). *Nature, Lond.*, **207**, 1155.

Clayton, R. A. (1959). *Arch. Biochem. Biophys.* **81**, 404.

Haider, K., Frederick, L. R. & Flaig, W. (1965). *Plant & Soil*, **22**, 49.

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

Mason, H. S. (1955*a*). *Advanc. Enzymol.* **16**, 105.

Mason, H. S. (1955*b*). *Nature, Lond.*, **175**, 771.

Mason, H. S. & Peterson, E. W. (1955). *J. biol. Chem.* **212**, 485.

Mason, H. S. & Peterson, E. W. (1965). *Biochim. biophys. Acta*, **111**, 134.

Mink, G. I. (1965). *Virology*, **26**, 700.

Pierpoint, W. S. (1966). *Biochem. J.* **98**, 567.

Pierpoint, W. S. (1969). *Biochem. J.* **112**, 619.

Pierpoint, W. S. & Harrison, B. D. (1963). *J. gen. Microbiol.* **32**, 429.

Rees, J. R. & Pirie, A. (1967). *Biochem. J.* **102**, 853.

Satô, M. (1967). *Phytochemistry*, **6**, 1363.

Trautner, E. M. & Roberts, E. A. H. (1951). *Aust. J. sci. Res. Ser.* B, **3**, 356.