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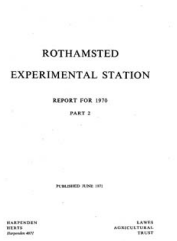

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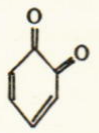
Formation and Behaviour of *o*-Quinones in Some Processes of Agricultural Importance

W. S. PIERPOINT

This review is concerned with a group of reactions that occur in living and decaying organisms and are thought to involve *o*-quinones. The reactions affect some agricultural and food-producing processes, and are relevant to problems studied in several departments at Rothamsted.

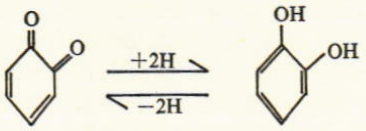
Quinones, so called because they derive from quinic acid a component of cinchona bark, are cyclic α-β unsaturated diketones. The two ketone groups are, usually, part of a six-membered ring of carbon atoms, and can occupy two positions relative to each other. These are the ortho- and para- positions and are exemplified in *o*-benzoquinone (I) and *p*-benzoquinone (II)



I

II

The nomenclature emphasises the aromatic compounds to which the quinones can be converted either by reduction or as a result of substitution into the six-carbon ring



The ease with which this conversion occurs differs from one quinone to another. It depends on attached groups that may stabilise either the quinone or the aromatic form, but usually goes more easily with *o*-quinones. Thus these compounds are the more reactive and the more powerful oxidising agents. *o*-Benzoquinone, for example, polymerises rapidly in water; it was not prepared till 1904, 66 years after *p*-benzoquinone was isolated, when Willstater devised a completely anhydrous method of oxidising catechol. This difference in stability between *o*- and *p*-quinones is reflected in the mode of occurrence and metabolic functions of the two types of quinones.

p-Quinones have been isolated from many living organisms. Some are widespread and, because of their reversible reducibility, may be essential cellular components. A family of isoprenoid substituted *p*-benzoquinones, aptly called ubiquinones, function as hydrogen carriers in the respiratory cytochrome systems of many, if not all, aerobic cells. Similarly the related plastoquinones are hydrogen carriers in photosynthesis, and phylloquinone, a derivative of *p*-naphthoquinone is a vitamin, K_1, in higher animals. Also, more than 200 *p*-quinones of more restricted distribution have been isolated from flowering plants, fungi, lichens and insects. They include derivatives of *p*-benzo, naphtho-,

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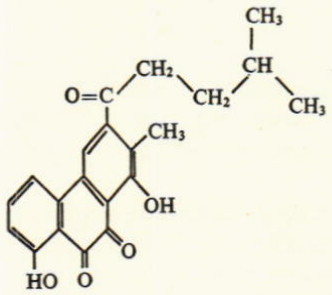
and anthroquinone, and some are peculiar to a given species. They are regarded as secondary metabolic products and, except for those with antibiotic properties, their metabolic significance is uncertain.

In contrast, only about half a dozen *o*-quinones have been isolated from organisms. They are, typically, red fungal pigments of limited interest in which the *o*-quinone structure is stabilised by fused benzene rings, as in piloquinone from *Streptomyces pilosus*.



The reactivity of *o*-quinones seems not only to make them less likely than *p*-quinones to occur as metabolic end products, but also less likely to be stable extractable components of metabolic pathways. In the reactions, metabolic or post-mortem, that concern us, they occur only as transient intermediates, whose formation has been demonstrated by trapping them with suitable reagents. In many reactions they have not been trapped; their formation is inferred from the known mechanisms of similar reactions.

Enzymic formation of *o*-quinones and their subsequent reactions

The reactions that produce *o*-quinones are the oxidation of phenols, generally *o*-dihydroxyphenols, by polyphenoloxidases. Suitable phenols are widespread in biological tissue, and plants characteristically contain derivatives of cinnamic acid, such as chlorogenic acid (Fig. 1), or flavanols such as epicatechin (Fig. 4). The polyphenoloxidases, copper-containing proteins, are also widespread if not strictly ubiquitous. Some of them oxidize only selected *o*-dihydroxyphenols, although the *o*-quinones produced may oxidize other compounds and, in the process, be reduced to the original phenol. Others, usually the tyrosinases, not only oxidize dihydroxyphenols, but convert monohydroxyphenols, such as tyrosine, to oxidizable dihydroxyphenols. Other systems, enzymic and non-enzymic, oxidize dihydroxyphenols but do not produce *o*-quinones. Reactions catalysed by the laccases, for example, proceed by single electron-removing steps and produce semi-quinones, whose subsequent behaviour differs from that of *o*-quinones.

Once formed, *o*-quinones react non-enzymically with many compounds. They may polymerize, be reduced, or suffer nucleophilic attack by substances possessing amino, thiol and 'activated' methylene groups. In all but the simplest or controlled systems therefore, they are liable to undergo a mixture of initial reactions. Even in simplified systems there are secondary reactions, often involving more quinone, whose course may critically depend on the concentration of quinone, relative amounts of other reactants and acidity. It is difficult therefore to summarise these reactions briefly, especially in a way applicable to more than one or two quinones. Reaction schemes in Fig. 1 and 2 can be regarded only as simplified summaries of the initial reactions that quinones undergo. They are based on reactions suggested for chlorogenoquinone, the *o*-quinone derived from the oxidation of chlorogenic acid by tobacco-leaf polyphenoloxidase. Their formulation

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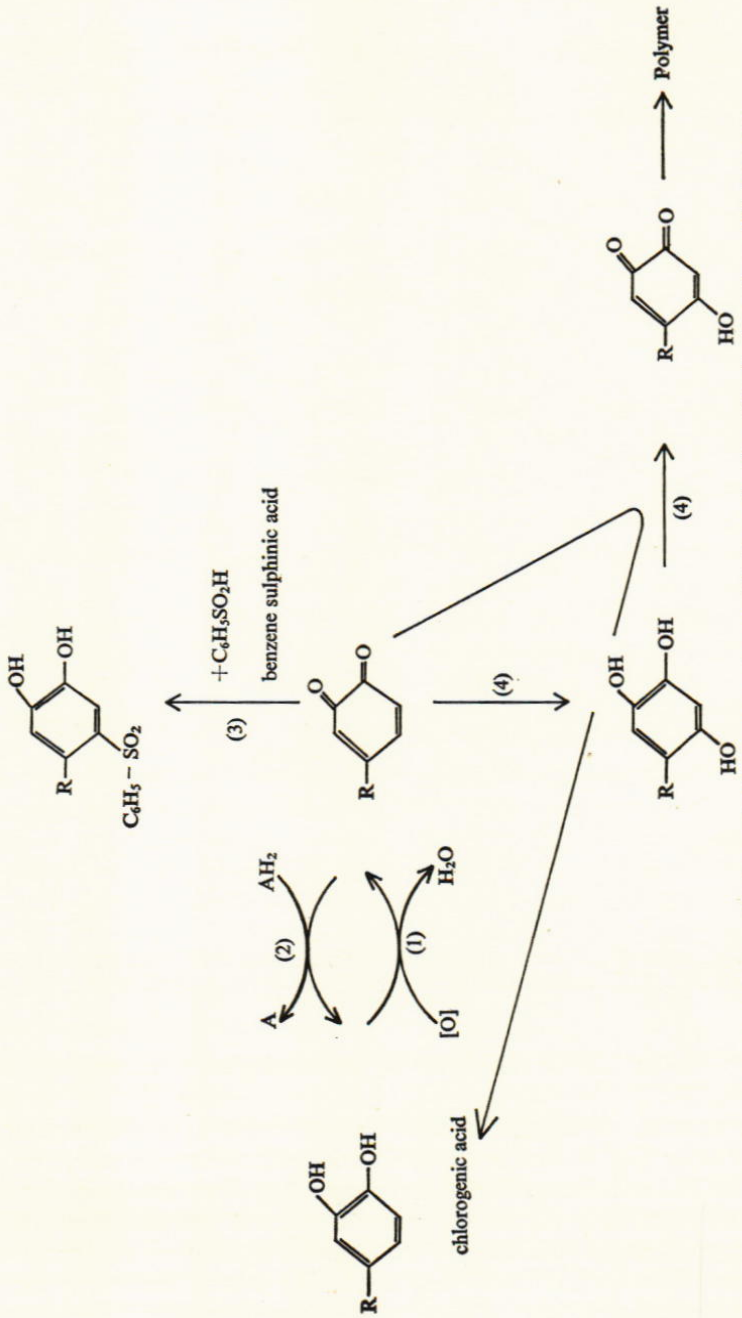

FIG. 1. The enzymic production of chlorogenoquinone (1), its reduction (2), its trapping with benzene sulphinic acid (3), and its polymerization (4). R is $C_6H_7(OH)_3(COOH)-O_2C-CH=CH-$.

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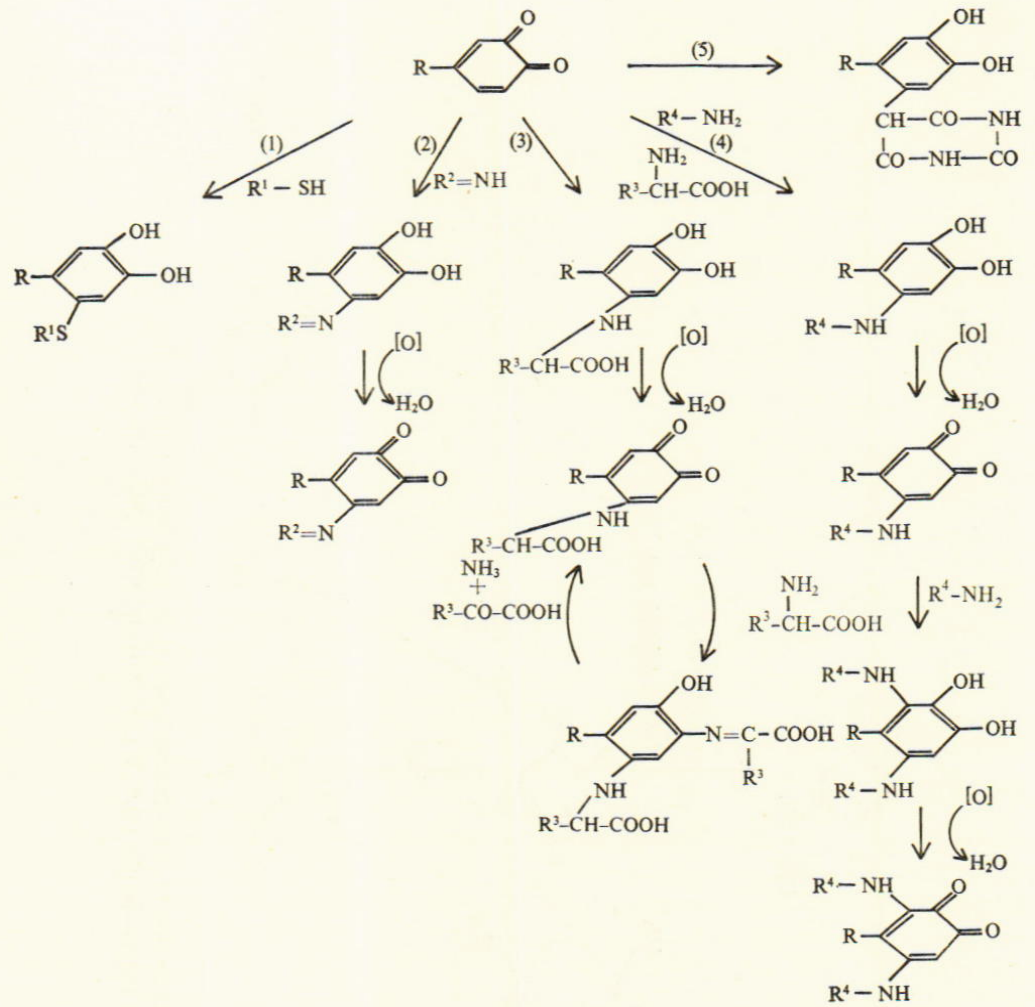

FIG. 2. Probable course of reactions of chlorogenoquinone with thiol compounds (1), amino acids with secondary amine groups (2), amino acids (3), amines (4), and the active methylene group of barbituric acid (5).

(Pierpoint, 1966; 1969a; 1969b) relies heavily on analogies with reactions of simpler quinones (Mason, 1955).

Enzymically-generated chlorogenoquinone is reconverted to chlorogenic acid by reducing agents (AH_2; Fig. 1) such as ascorbic acid, reduced coenzymes, and in part by —SH compounds. The reaction goes readily enough, but, for some quinones, is accelerated by an enzyme from peas. As the chlorogenic acid can be reoxidised, this provides a system for the continuous oxidation of AH_2 and for delaying the accumulation of quinone; the rates of both reactions follow a complex course because reducing agents partially inhibit polyphenoloxidases. The reaction of the quinone with benzene sulphinic acid has no biological significance, but it is included in Fig. 1 as an example of the trapping reactions used to demonstrate the formation of *o*-quinones *in vitro*. When the sulphinic acid is in excess, the oxygen absorbed is restricted to that involved in the formation of the quinone. The sulphones formed are stable enough to be isolated and identified. In

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the absence of reducing and trapping reagents, chlorogenoquinone polymerises to brown, poorly defined pigments. The reactions may (Fig. 1), by analogy with those suggested for *o*-benzoquinone (Wagreich & Nelson, 1938), involve water and an oxidation by more quinone. Similarly by analogy, the polymers may contain aromatic rings directly linked or joined to alkyl groups through ether bonds.

The reactions between chlorogenoquinone and amines, amino acids, thiol compounds and compounds with reactive methylene groups are formulated (Fig. 2) as if the primary point of attack was the 6′ position; this, *a priori*, seems probable, and in the reaction with benzene sulphinic acid almost certain. The mono-substituted products are aromatic and probably colourless: that derived from barbituric acid is stable, as are those from some thiol compounds unless there is a large excess of quinone. Other primary products, such as the aminohydroquinones, are further oxidized, probably by more chlorogenoquinone, to coloured quinoid forms. The reaction stops here with secondary amines such as proline, and the products are stable enough to be identified spectrophotometrically, even though not to be isolated. The quinone substituted with primary amines, e.g. aniline, however, is further substituted and oxidized to give a disubstituted quinone, and this may be repeated until all the available positions on the chlorogenoquinone are occupied. Disubstitution may occur with amino acids also (Fig. 2), but the course of this reaction is obscured by secondary reactions in which the monosubstituted quinone oxidatively deaminates excess amino acid. Trautner and Roberts (1950) describe an imaginative and feasible way in which this might occur.

Proteins have a range of chemical groups, amine, α-amino and thiol, potentially able to react with *o*-quinones. Although some of these will be 'buried' in the interior of the protein and consequently unreactive, exposed groups will probably be reactive because of their peptide bonding. Cross links may be formed between different parts of the protein, as reactive groups from different parts of the peptide chain attack either the same quinone molecule (e.g. –NH–Q–NH–) or different ends of a group of polymerizing quinone molecules ($-NH-Q-(Q)_n-Q-NH-$). Not only would this alter the biological properties of the protein but it may alter its gross physical properties. This, of course, is what happens when collagen and proteins of hides are converted to leather by treating them under denaturating conditions with chemical tanning-agents such as *p*-benzoquinone.

There is another possible course for the reaction between quinones and proteins. The polymers formed from some phenols, especially the flavanols, might have 'tannin-like' properties, and, as do the classical vegetable tannins, complex with and possibly precipitate proteins. These reactions would depend initially on hydrogen-bonding between phenolic groups and peptide bonds, and be reversed by detergents or alkali. However, they would become less reversible with time as *o*-quinone groups form in the phenol and react covalently with suitable groups on the proteins. The end products therefore may well be difficult to distinguish from those formed directly from proteins and polymerizing *o*-quinones.

Because of the reactivity of *o*-quinones, the coexistence of polyphenoloxidase and oxidizable *o*-dihydroxyphenols in a single cell is potentially suicidal. Clearly mechanisms are required to control the oxidations or prevent them. Necessary contact between these enzymes and their substrates, as for example when polyphenoloxidases hydroxylate monophenols, is probably controlled by the redox balance of the cell: an enzyme has been described that reduces *o*-quinones at the expense of reduced coenzymes. There are other ways of controlling these reactions or of preventing them occurring prematurely. Thus the oxidation of phenols to melanins in mammalian pigment cells is restricted to

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membrane-bound organelles, and the polyphenoloxidase of some insect tissues occurs as inactive pro-enzyme. Similarly the polyphenoloxidase of phenol-rich *Vicia faba* leaves exists in a masked condition (Kenten, 1957), and many plant phenols are converted to more stable esters and glycosides. Enzymes and substrates may also be separated in plant cells, although the evidence for this is not conclusive. Many of the claims that plant polyphenoloxidases are contained in specific cell components are open to doubt (Sanderson, 1964), and little is known of the intracellular location of polyphenols.

Whatever control mechanisms may operate in a plant cell, they are removed when the cell is injured. Phenolic compounds are then oxidized and *o*-quinones produced. These may react with cell constituents increasing the injury, or polymerize to brown or other dark coloured products. This accidental production of *o*-quinones is as relevant to our subject as their controlled metabolic production. When it occurs in part of a tissue as a result of a local injury we will call it adventitious, and when as a general reaction of a dying organism, post-mortem. These three types of reactions, metabolic, adventitious and post-mortem, somewhat arbitrarily separated, will serve as three convenient classes in which to consider some of the reactions involving *o*-quinones.

Metabolic processes involving *o*-quinones

Of the metabolic roles that have been suggested for *o*-quinones, some, such as a respiratory function, are doubtful, and others, including tropolone synthesis, are of limited interest. The most relevant of the remainder is their role in reinforcing wall structures. They seem not to be involved in the synthesis of lignin; although this synthesis involves the oxidative coupling of phenols, it requires peroxidases or laccases and the intermediates are semi-quinones. *o*-Quinones, however, are almost certainly involved in the sclerotinization of exoskeletons and egg cases of arthropods, and in the melanization of seedcoats, spore walls and pigment cells of many other organisms.

Fresh cuticles of many arthropods, especially insects, as well as the walls of egg capsules, are pale soft structures. They are mainly proteinaceous, although the exoskeletons contain also the polysaccharide chitin. Over a period of hours, they harden and darken into leathery, rubbery or horny membranes of great mechanical strength and chemical resistance. Pryor (1940) emphasised that, during this process, for which he coined the term sclerotinization, they accumulated much (20–40% dry weight) phenolic material, including *o*-dihydroxyphenols. He argued that the process was, in fact, a quinone-tanning; that *o*-quinones produced by the oxidation of the phenols cross-linked the protein chains into rigid lattice structures.

There is evidence from several insects to support this idea, and to indicate how the process is controlled. For instance, immediately before they pupate, the larvae of the blowfly (*Calliphora erythrocephala*) accumulate much *N*-acetyl dopamine (*N*-acetyl-3,4-dihydroxy-phenyl-β-ethylamine) in the blood. This is produced from tyrosine under the influence of the hormone ecdysone. Ecdysone also seems to activate a latent polyphenoloxidase located in or near the cuticle. Conditions are thus established for an *o*-quinone to form where it can diffuse into and sclerotinize the cuticle. The tanning of the egg capsule of the cockroach (*Blatta orientalis*) is similarly brought about by the quinone derived from 3,4-dihydroxybenzoic acid. This phenol is secreted as a 4-*O*-β-glucoside, along with polyphenoloxidase and the protein that forms the egg wall, by one of a pair of glands that discharge into the egg pouch. However, tanning starts only when a glucosidase is secreted by the other gland and hydrolyses the glucoside into an oxidizable dihydroxyphenol.

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The reactions between the quinones and the cuticular proteins are poorly understood because little is known of the untanned proteins themselves, many reactions are possible, and the products are difficult to analyse. The picture of the process is necessarily vague and based largely on inference from simpler systems. Usually, but not always, the proteins involved contain little S, and free amino groups become fewer during the reaction. The primary reactions probably involve terminal amino groups, and then lysine ϵ-amino groups, to give amino hydroquinones which are converted to coloured amino quinones. Quinone bridges linking protein amino groups, such as –NH–Q–NH– and –NH–Q–Q–Q–NH–, are then possible depending on the availability of substitutable positions in each quinone molecule. These bridges may also link protein to any unacetylated amino groups in chitin. How many such bridges exist, and which groups they predominantly connect, is unknown, but tanned cuticle is so rich in phenol that, if much of it exists as polymer, penetrating the interstices of the proteins, not many bridges would be needed to confer rigidity on both the protein and the chitin.

Another type of bridge has been suggested that may cross-link peptide chains in tanned insect cuticles. It is based on tyrosine residues in the proteins, which, if accessible, may be hydroxylated by insect polyphenoloxidases to *o*-diphenols and further oxidized to *o*-quinones. These may condense with each other, with nearby amino groups or with tanning quinone. This possibility has received little attention. But cuticle protein is rich in tyrosine, and much of it seems to be altered chemically during sclerotinization. More germane perhaps, biphenyl linkages, such as would be produced by the condensation of adjacent oxidized tyrosine residues, have been identified in resilin, a remarkably rubber-like protein that forms wing-hinges of some insects (Andersen, 1966).

Fragmentary evidence suggests that some cuticles and outer membranes of nematodes are also tanned by *o*-quinones. Phenols have been detected, chemically and histochemically, in the outer cuticular layers of both adults and larvae in several species (e.g. *Ascaris*). However, the pale colour of these cuticles, and their digestion by the proteolytic enzyme papain, argue against much tanning. There is more tanning in the egg-coat and cyst-wall of the potato cyst eelworm (*Heterodera rostochiensis*). Both contain 2–3% of phenols that are liberated on hydrolysis (Clarke, Cox & Shepherd, 1967; Clarke, 1968). Moreover the cyst-wall, in its formation from the body wall of the female, shows the colour and textural changes associated with sclerotinization, and possesses a polyphenoloxidase.

Sclerotinisation alters the properties of the cyst-wall, including its permeability to water, and undoubtedly contributes to the remarkable longevity (up to eight years) of these cysts in soil (A celebrated Shakesperean clown also attributed the preservation of tanned bodies in soil to their impermeability to water (*Hamlet*, Act V, Scene 1).) Besides this structural role, the phenols and quinones of the wall may well aid in hatching of the eggs; wall fragments double the hatchability of *H. rostochiensis* eggs (Shepherd & Cox, 1967), and synthetic quinones, including *o*-naphthoquinone, stimulate the hatching of *H. schachtii* eggs (Clarke & Shepherd, 1964).

A special case of cell wall reinforcement is when the phenolic component is a derivative of dihydroxyphenylalanine (dopa). The protein complex is then a dark pigment containing polymerized indole structures and known classically as melanin. Plant melanins occur typically in seed coats and the walls of fungal spores. They undoubtedly contribute to the durability of these bodies: the resistance of wall preparations from *Aspergillus*, *Rhizoctonia* and *Sclerotium* spp. to lysis by added enzymes or by soil bacteria is related to their melanin content (Kuo & Alexander, 1967). However, the melanins of plant tissues are less well characterized than those of animals. These, as the main pigments of

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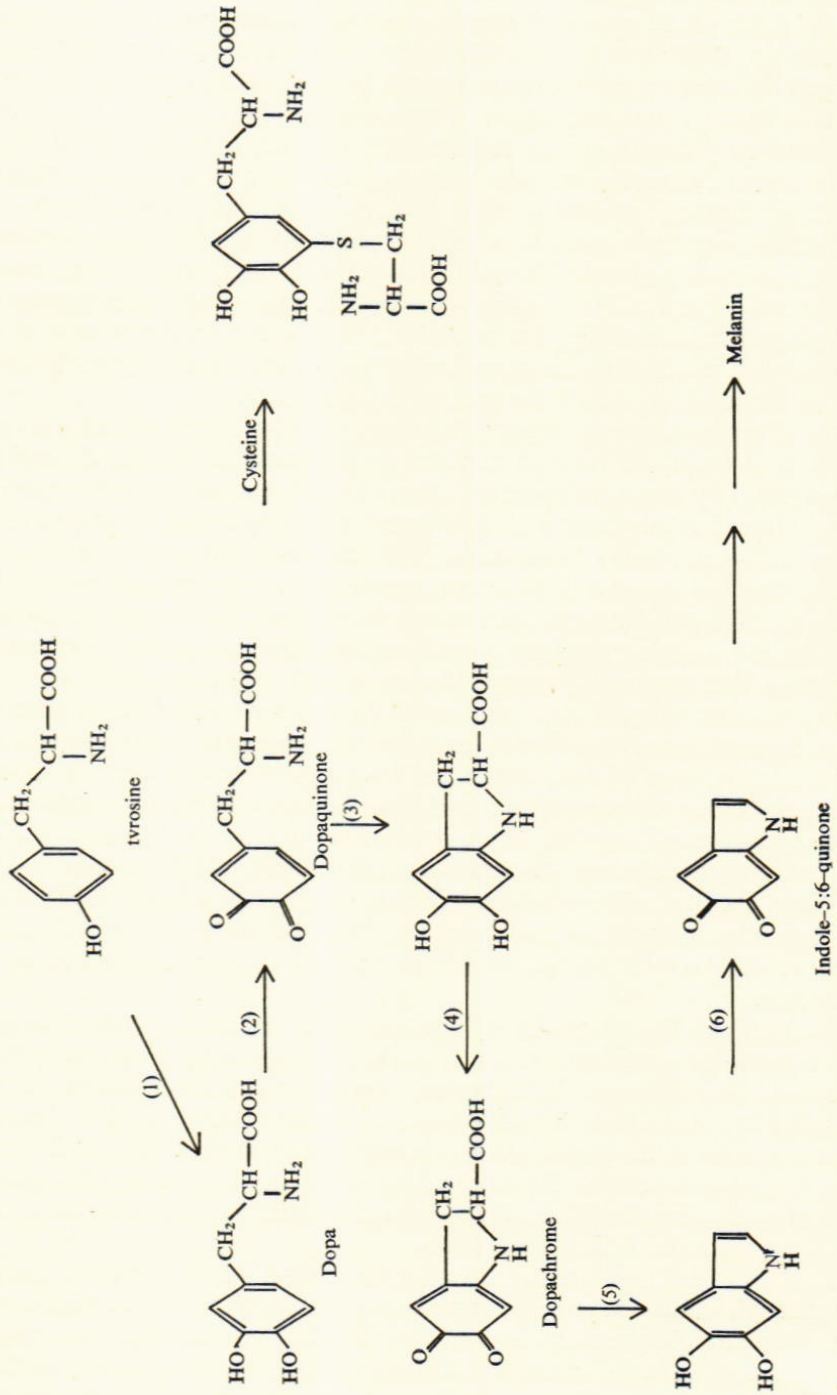

FIG. 3. The formation of melanin by the oxidation of tyrosine. Reactions (1) and (2), and possibly (4) and (6) require polyphenoloxidase. Also shown is the reaction of dopaquinone with cysteine which is believed (Nicolaus *et al.*, 1969) to give rise to a family of pigments which decorate red human hair and the feathers of New Hampshire chickens.

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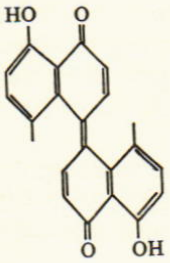
vertebrate hair and skin, have attracted much attention; they pose both intricate chemical problems and urgent social ones.

The first stages in melanin formation probably involve three oxidations of dihydroxyphenylalanine by polyphenoloxidase, and three distinct *o*-quinones (Raper, 1938). The first, dopaquinone, reacts intramolecularly (Fig. 3), the amino group of the side chain substituting into the quinone nucleus to produce a bi-cyclic compound. This is oxidized to the second quinone, dopachrome, which in turn, after decarboxylation, is oxidized to indole-5,6-quinone. There is little certainty about subsequent stages; indole-5,6-quinone alone polymerizes to black insoluble substances, but in natural melanins these are linked both to protein and to metals. The quinones probably form an irregular three-dimensional lattice in which each quinone unit has multiple links, and which is attached to proteins predominantly through –S– atoms. Some of the quinone units may be derived from dopaquinone or dopachrome; some of them may, as electron spin resonance studies suggest, be free-radical semi-quinones.

Wall strengthening polymers based on phenols and quinones other than dopa and dopachrome have been recognised in plant walls, especially those of fungi. The dark pigments of sunflower and melon seeds are, for example, probably derived from catechol (Nicolaus *et al.*, 1964): the black 'melanochitin' that is partly responsible for the nickname (King Alfred's Cakes) of the fungus *Daldinia*, is an extended *p*-naphthoquinone polymer:



It is probably cross-linked to unacetylated amino groups of chitin, and polyphenoloxidase is probably involved in its formation (Allport & Bu'Lock, 1958). Probably many other pigments, loosely called melanins because of their appearance and reaction in hi to-chemical tests, will prove also to be derived from phenols other than dopa, and not, strictly speaking, melanins at all. There seems a need for a new general term, analogous to sclerotins, to describe all such materials derived from plant cell walls by the interpolation of polymerizing phenols. The term melanin, as lignin, could then be restricted to polymers derived from one phenol.

Adventitious production of *o*-quinones

The antimicrobial properties of phenols and quinones have long been known and made use of, so it is not surprising that the phenols and *o*-quinones of plants should have been considered as part of the plant's defences against infecting organisms. This idea receives support from the increased phenol and polyphenoloxidase of diseased plants; it has the added attraction that differences between the phenols of different species and varieties of plants may partially account for one of the salient facts of plant pathology—the host specificity of many parasites. These ideas, and the comparative ease with which phenols having antimicrobial activity can be extracted from many plants, have led to an immense literature. However, there are as yet very few examples in which phenols or quinones are clearly and unequivocally responsible for resistance to disease.

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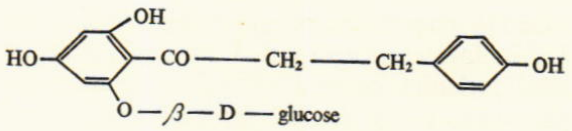
One such example is the resistance of onions to the smudge disease fungus *Colletotrichum circinans*. Spores of this fungus germinate on the skin of the onion, penetrate the dead outer scale leaves, and then grow into and parasitize the fleshy leaves of the bulb. Many resistant varieties of onion have red or yellow outer scale leaves, but their resistance depends on the *o*-dihydroxyphenols, catechol and protocatechuic acid, in the cells of the dead outer scales and not on the anthocyanin pigments. These phenols prevent infection by inhibiting the germination of spores. How they do this, and whether they need to be oxidized to quinones, is not known.

Other host-parasite interactions show no such simple relation between resistance and the presence or absence of a particular phenol. Resistance may be conferred by a larger concentration of a substance that occurs in all varieties, or the presence of a mechanism that must be triggered to expose the parasite to this substance. The first explanation has been invoked for the resistance of potatoes to the scab fungus (*Streptomyces scabies*) and to *Verticillium* wilt. Scab-resistant varieties grown in U.S.A. contain more chlorogenic acid than do susceptible ones, and it is mainly concentrated in the periderm of young, rapidly growing tubers, especially round the lenticels through which the fungus invades. Similarly, wilt-resistant varieties have more chlorogenic acid in the vascular tissue of underground stems and only lose their resistance as it disappears. Chlorogenic acid, in amounts less than those estimated to occur in potato sap, stops the *in vitro* growth of both *S. scabies* and *V. albo-atrum*, and the germination of spores of *V. albo-atrum*. Moreover, it does so more in conditions in which it is slowly oxidized. However, there are many difficulties in the way of accepting the idea that chlorogenic acid, or oxidizing chlorogenic acid released from damaged cells, controls the fungal invasion; surveys of European potatoes (e.g. McKee, 1958) show no relation between scab-resistance and phenol content.

Careful studies suggest that the phenol and polyphenoloxidase of young apple leaves confer some resistance against the apple-scab fungus, *Venturia inaequalis*. The main phenol in these leaves, which may account for 4–8% of their fresh weight, is phloridzin:



It is hydrolysed to the aglycone phloretin by a leaf glucosidase, and both these phenols are hydroxylated to *o*-diphenols and oxidized to *o*-quinones by polyphenoloxidase. The balance between these reactions is complex and depends on the pH of the leaf sap (Raa, 1968), but there is no appreciable difference between those occurring in the sap of scab-resistant and susceptible leaves. However, some intermediates of the oxidation, most probably *o*-quinones, are fungicidal; and there are reasons for thinking that they are formed and affect the growth of *V. inaequalis* only in resistant leaves.

The hyphae of scab fungus proliferate between the epidermis and cuticle of susceptible leaves without penetrating the host cells or, in the first stages, causing their collapse. By contrast, cells of resistant hosts rapidly collapse and die around the primary invading hyphae. This 'hypersensitive' reaction stops the growth of the fungi, possibly because it allows the production of *o*-quinones. This quinone-producing mechanism is thus an anti-infection device common to both susceptible and resistant leaves, but which is triggered off only in resistant ones. Proteins have been isolated from culture filtrates of *V. inaequalis* that do cause necrosis in resistant leaves. Curiously enough they seem to be melanoproteins.

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'Hypersensitive' reactions are important resistance mechanisms in many plants. Rapid collapse of cells is enough to prevent the growth of many parasites, but the concomitant oxidation of phenols to *o*-quinones and polymers may also discourage saprophytic organisms that would otherwise grow on dead tissue. Even when these compounds do not affect the pathogen directly, they may inhibit the 'tissue-macerating' enzymes it secretes to facilitate its spread.

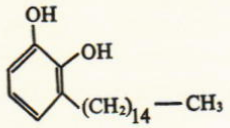
Although some macerating enzymes, especially polygalacturonases and others that depolymerise pectin are only slightly affected by simple polyphenols, they are strongly inhibited by oxidized chlorogenic acid and catechins, the substances that accumulate in browning tissue. This inhibition probably occurs naturally, and the resistance of apple varieties to the spread of the brown-rot fungus (*Sclerotinia fructigena*) from wounds, is proportional to the rate at which they discolour. Moreover resistance is decreased by painting the wounds with substances, such as glutathione, that prevent browning. Very little of the active polygalacturonase secreted by fungi can be recovered from the fungus-rotted tissue.

The fungi that produce the soft white rots of apples, in particular *Penicillium expansum*, seem to have a mechanism that prevents these inhibitory polymers from forming. They are thought to produce substances, some possibly derived from the phenols of the apple tissue, which inhibit polyphenoloxidase (Walker, 1969). Much pectin is broken down in these infections, and polygalacturonase can be isolated from the rotted tissue. In spite of this, *P. expansum* lesions spread slower than those of brown rots, which suggests that if *S. fructigena* develops an anti-browning mechanism it might be even more aggressive.

Infection by viruses can also affect the metabolism of phenolic compounds by plants. Indeed, chemical tests for phenols have often been suggested as methods of diagnosing some infections, although these have not proved satisfactory (e.g. Holden, 1957). It has also been suggested that the oxidation of phenols restricts the intercellular movement of viruses, much as it is thought to do for *V. inaequalis*.

Necrotic lesions are often associated with the localisation of virus in a leaf. They have been regarded as hypersensitive responses in which a metabolic upset has allowed the oxidation of phenols, and this has killed the cells and stopped virus movement. Farkas, Kiraly & Solymosy (1960) showed that infections producing local necrotic rather than systemic symptoms, greatly increase the polyphenoloxidase of leaves, and that infiltrating leaves of *Nicotiana glutinosa* with the reducing agent ascorbic acid decreased the number of lesions produced by tobacco mosaic virus, without appreciably affecting the multiplication of the virus. But, however good the evidence that phenols are involved in lesion formation, it is unlikely that the necrosis prevents the movement of virus: Bawden (1964) describes many instances where it does not, and also some where virus is localised without necrosis.

A different type of defence mechanism, the chemical warfare waged against animals by poison ivy and poison oak, probably involves *o*-quinones. The active substances produced by poison ivy (*Rhus toxicodendron radicans*) are 3-pentadecyl catechol and related compounds with ethylenic links in the side chain:



These are primary skin irritants, but more important they act as haptens, and produce allergic responses. The most likely mechanism for their conversion into antigens is that

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once inside the animal, they are oxidized to *o*-quinones which react with serum proteins: such quinone–protein complexes have been made *in vitro* and their antigenicity demonstrated. *Rhus vernicifera*, the Japanese lac tree, produces the same or similar phenols, but uses them less aggressively; they are in the latex, and when exposed to air are oxidized by a latex enzyme to hard insoluble polymers. These probably protect the wounds of damaged trees: in manufactured lacquers they both protect and decorate furniture.

Post-mortem production of *o*-quinones

I. Possible role in the formation of humus. As vegetable matter decays in the field, and its cellular structure disintegrates, most of its *o*-dihydroxyphenols will be oxidized. The resulting quinones will polymerize and, in doing so, combine with other cell constituents, especially proteins and amino acids. The products are likely to be heterogenous and to include some of the partial structures already indicated. As they are added to the top layers of the soil, their structure will be modified by oxidative and hydrolytic activities of microorganisms. There are, however, some reasons for thinking that quinone-protein polymers are not quickly degraded as are proteins (Bremner & Shaw, 1957); they are more resistant than proteins to many hydrolytic enzymes. This has encouraged the idea that polymerized quinones, combined with proteins and amino acids, survive in the soil and contribute to its relatively stable humus component.

Opinion on the chemical structure of these humic materials is not unanimous. The highest common factor of many opinions is that they are a mixture of amorphous three-dimensional polymeric acids of high molecular weights, with aromatic, partly quinoid structures, and with a range of molecular sizes. They are unexpectedly rich in amino acids, which account for as much as a third to a half of soil nitrogen (Bremner, 1955) and which *in situ* are probably linked by peptide bonds. These amino acids, in addition to other compounds that can be removed by hydrolysis in 6N HCl, 'turn over' in soil much faster than does the non-hydrolysable aromatic nucleus; the average age of the hydrolysable components of Broadbalk top soil, sampled from the unmanured plot in 1881, is 510 years compared to 2560 for the non-hydrolysable fraction (Dr. D. S. Jenkinson, personal communication). Some of these properties are, at least, consistent with the idea that humic materials are derived from heteropolymers of proteins and quinones. Model compounds prepared by polymerizing *o*- and *p*-quinones in the presence of proteins such as casein (Ladd & Butler, 1966) show an encouraging resemblance to humic acids. They behave similarly during chromatography on columns of Sephadex, and, on acid hydrolysis, produce amino acids, ammonia and unreleased N. Moreover, their amino acids, as those of humic acid, are slowly released by some fungal proteases: it would be interesting to know whether the polymers remaining after losing their amino acids can combine with, or otherwise 'pick up', fresh protein or quinone-proteins, as soil humus seems to do.

If quinones are involved in the genesis of humic materials, they need not only be those derived from plant *o*-dihydroxyphenols; soil microorganisms may produce and oxidize their own phenols. Swaby and Ladd (1963) and Flaig (1960) outlined two distinct ways in which this could occur. The phenols may be synthesised intracellularly from carbohydrate substrates, polymerize with proteins as the cells decay, and be released into the soil only as the cell walls are lysed. Alternatively, the reactions may be extracellular, at least in part, as when the microorganisms hydrolyse and oxidize plant lignins. Lignins are themselves polymerized phenols derived from coniferyl alcohol and related compounds, but most of their hydroxyl groups are methylated. However, as they decompose

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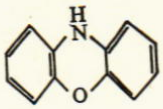
in soil they lose their methoxy groups and concomitantly bind N. Flaig argued that the demethylation is hydrolysis by fungal enzymes, and that N is bound when *o*-dihydroxyphenols are produced from the polymer and oxidized to quinones that react with ammonia, amino acids or proteins. Some of these reactions have been reconstructed *in vitro*, and suggest that the products include, in addition to structures already mentioned, amino phenols or amino quinones that may polymerize to heterocyclic aromatic structures such as phenoxazines:



But how far such structures contribute to soil organic matter is unknown.

About a tenth to a sixth of the organic sulphur of soil is present as α-amino acids, which, on acid hydrolysis, yield methionine, methionine sulphoxide, cystine and cysteic acid. They probably occur in peptide form, and possibly in quinone-protein heteropolymers. If this is so, there is the additional possibility that some soil sulphur exists as cysteine linked to a quinone nucleus through its S (Fig. 2), in addition to, or instead of, its N. Little is known of the stability of these S–C bonds, although some of those in melanin resist acid hydrolysis (Nicolaus *et al.*, 1964). This possibility is worth consideration therefore, even if very little of the S in a plant is present as –SH able to combine with quinones: some protein –SH groups have great affinity for quinones, and stable compounds accumulate in soil even though added in small amounts.

2. Formation of *o*-quinones during the preparation and storing of food materials. Cellular injuries inflicted during the harvesting, processing and storage of plant materials cause oxidation of any phenols present and the polymerization of *o*-quinones. These 'enzymic' browning reactions (so called to distinguish them from non-enzymic ones, such as those that occur on heating proteins and sugars) adversely affect the value of most fruits and vegetables, spoil their colour and flavour and, by the reaction in Fig. 1, decrease their vitamin C content. The phenols most usually implicated are caffeic and chlorogenic acids as these are most readily oxidized, although oxidized flavanols are reported to produce most of the brown discoloration in apples and pears. Browning may occur in canned, frozen and even dehydrated materials, and much effort has gone into devising methods of preventing it. These include quick heating or blanching, which is designed to inactivate enzymes, excluding air, and adding substances such as sulphite or ascorbate that inhibit polyphenoloxidases or combine with quinones.

Reactions of *o*-quinones with proteins in plant protein concentrates may decrease their nutritional value. The biologically-measured nutritional value of many such feeding stuffs is less than that predicted from their known amino acid contents; some amino acids seem unavailable to animals although they are liberated by the hydrolysis in 6N HCl that precedes amino acid estimations. Lysine is often biologically unavailable in this manner, which is important because it is one of the amino acids deficient in many diets. Probably its ϵ-NH_2 is involved in linkages stable to enzymic digestion but broken by acid hydrolysis, and some of these linkages may involve *o*-quinones. The biological value and *in vivo* digestibility of casein are decreased by reaction with caffeoquinone and chlorogenoquinone (Horigome & Kandatsu, 1968): and lysine–quinone bonds, as those between other amino acids and quinones (Ladd & Butler, 1966), are probably partially hydrolysed by acid.

The nutritional value of plant protein concentrates is often also limited by methionine,

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which is both scarce and 'unavailable'. However, it is less probable than with lysine that this is because it reacts with *o*-quinones. *o*-Quinones may oxidize it to methionine sulphoxide but this would not necessarily decrease its biological usefulness. Reactions that might do so by oxidizing it to a sulphone or forming S^+–quinone bonds have been postulated, but preliminary efforts to demonstrate them with chlorogenoquinone and methionine have failed.

Bound quinones are most likely to occur in proteins extracted from tissues rich in phenols. They may well affect the quality of leaf-protein concentrates that have been advocated (Pirie, 1969) as dietary supplements. Feeding tests show that the nutritive value of these preparations is usually good but varies with the species and age of the leaves that have been extracted. Poorer samples tend to have more of their lysine ϵ-NH_2 unreactive towards nitrous acid (Dr. R. L. M. Synge, personal communication), suggesting that part of the loss in nutritive value may reflect reactions with quinones: there is ample opportunity for these reactions during the large scale extraction. The *in vitro* digestion of these leaf protein preparations with proteolytic enzymes has been used (e.g. Byers, 1967) to judge their biological value. These tests also are likely to be affected by protein-bound quinones: the susceptibility of dried pasture plants to pepsin depends on the *o*-dihydroxyphenol and polyphenoloxidase content of fresh leaves (Horigome & Kandatsu, 1968). However, these tests, especially those that use plant proteolytic enzymes, are not necessarily affected by bound quinones in the same way as is biological digestibility. Thus papain and ficin, in contrast to carboxypeptidase and trypsin, are not inhibited by polymerized quinones, but in some conditions stimulated (Ladd & Butler, 1969).

Not all the phenolic oxidations and *o*-quinone reactions in agricultural products are deleterious: the processing and desired characteristics of beverages and stimulants such as tea, cocoa, cider and tobacco depend on them. This is most evident with black tea. Although the main reason for drinking tea is ostensibly the stimulation derived from its caffeine, most of the expertise that goes into producing acceptable tea is concerned with its phenolic content. Agricultural aspects are designed to produce leaves rich in both phenols and polyphenoloxidase: the critical conditions of fermentation are designed to allow the enzyme to oxidize the phenols, allow the *o*-quinones produced to condense to coloured polymers and to stop the process at that point. These polymers confer on brewed tea its colour, most of its palate, and some of its odour. When their formation is prevented, as in green teas, the brew is, by comparison, pale and insipid.

Because of the many types of phenols in the leaves of the tea plant, and the many possible interactions of the corresponding *o*-quinones, it could reasonably have been expected that the chemical reactions are enormously complex. However, E. A. H. Roberts pointed out: (1) that the main difference between fermented and unfermented leaf lay in the disappearance of epigallocatechin (Fig. 4) and its gallate ester; (2) that compounds resembling the main soluble condensed phenols in brewed tea can be produced *in vitro* by oxidizing these two substances; (3) that the condensed phenols are probably dimers of these catechins uncombined with amino acids.

Fig. 4 shows a scheme by which one of the main groups of tea pigments, the theaflavins, is probably formed. It differs a little from the scheme of Roberts, and it envisages the oxidation of both epigallocatechin and catechin (Takino *et al.*, 1964). The theaflavin formed by the condensation of the catechin *o*-quinones has a seven-membered tropolone ring. Either one or both of the hydroxyl groups on the O-containing rings may be esterified with gallic acid, and these esters probably form a larger proportion of tea theaflavins than does the unesterified compound. The other main group of tea pigments, the redder,

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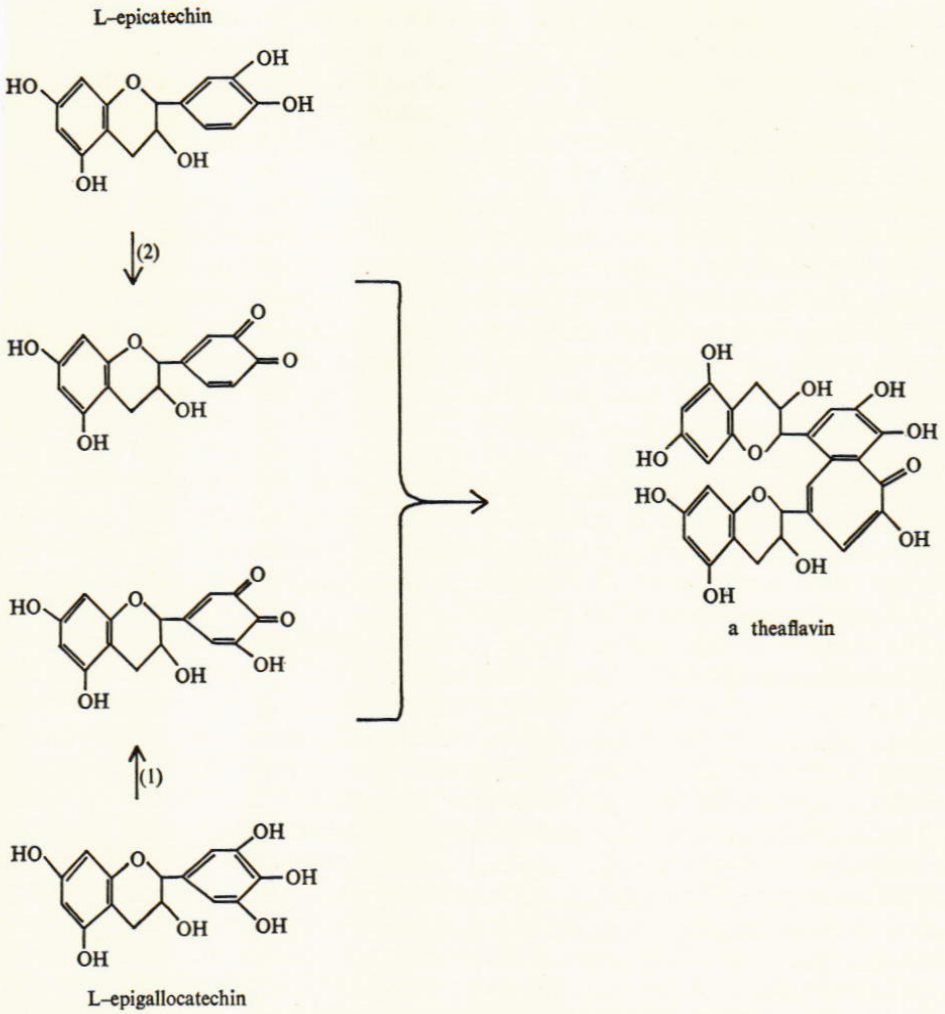

FIG. 4. Reaction scheme for the formation of theaflavins, orange coloured astringent pigments, during the fermentation of tea (after Takino *et al.*, 1964). Reactions (1) and (2) are catalysed by polyphenoloxidase.

less astringent thearubigins, are less well characterised, but they probably contain polymerized catechin groups, some in the oxidized *o*-quinone form.

Although the main pigments of brewed tea seem not to contain N, reactions between *o*-quinones and nitrogenous compounds occur during fermentation and may contribute to the quality of the manufactured product. Thus, the aldehydes, especially phenylacetaldehyde, which are partly responsible for the aroma of tea are probably produced by the quinone-catalysed oxidative deamination of amino acids.

3. Formation of *o*-quinones in the laboratory. A third, rather more specialised, environment in which plants may die, and where the *post mortem* formation of *o*-quinones occur, is the laboratory. Whether the vegetable matter is ground, homogenised, disintegrated or macerated, provided it contains polyphenoloxidase and oxidizable phenols, and conditions do not completely and immediately inactivate the enzyme, some *o*-quinone will

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be formed. Even when the enzyme is inactivated, some *o*-quinone may be produced in extracts alkaline enough (pH > 8) for the phenols to auto-oxidize. When enough has been formed to remove most of the reducing substances, the extract will probably brown. The brown materials often complicate the estimation and isolation of other substances in these extracts: when the substances in question react with *o*-quinones there is an extra hazard not always fully appreciated.

These reactions may produce new species of comparatively small compounds, such as quinone-peptides, which complicate the difficult task of characterising the non-protein N fraction of leaves. They may remove substances that are being searched for, and, less expectedly, they may artificially produce them. Both these last effects are possible with the growth substance indol-3-yl acetic acid (IAA). In slightly acid solution, *o*-quinones react with IAA to give inactive compounds of unknown constitution: those from chlorogenoquinone are intensely red. By contrast, in slightly alkaline solution *o*-quinones produce IAA by reaction with tryptophan. The reaction is an example of the catalytic deamination of amino acids by *o*-quinones (Trautner & Roberts, 1950) already mentioned, followed by the spontaneous decarboxylation of the keto acid produced. It is questionable whether IAA is produced this way physiologically, but its production complicated efforts to detect a protein-bound and slowly released form of IAA in extracts of French Bean leaves, where the *o*-quinones that catalyse it are derived from novel esters of caffeic acid (Wheeler & King, 1968).

Many enzymes are more active in plant extracts prepared by methods minimising the oxidation of phenols, probably because they react with and are inactivated by *o*-quinones. A few such enzymes, for example sucrose synthetase from sugar cane and phosphorylase from potatoes, are sensitive to synthetic quinones and are inactivated *in vitro* when added polyphenols are oxidized by polyphenoloxidase. This type of inactivation is, initially at least, distinguishable from that caused by preformed tannins or quinone polymers with tannin-like properties. It has been variously attributed to the reaction of *o*-quinones with amino or sulphydryl groups of the protein or with a prosthetic group such as pyridoxal phosphate. Sometimes, as with sucrose synthetase, the reaction seems partly reversed by –SH reagents, implying that the quinone oxidizes protein –SH to –S–S– instead of forming an –S–hydroquinone adduct, but none of the inhibiting reactions has yet been studied with the precision that protein chemistry currently allows. Perhaps this is why this type of inactivation is often overlooked, or when recognised causes surprise. Some enzyme studies demand that it is not overlooked; if it is, attempts to measure enzymes in different physiological states of a tissue may simply measure differences in the amounts of inhibitory *o*-quinones different extracts produce.

By a rare piece of biochemical justice, polyphenoloxidases themselves react with and are affected by the *o*-quinones they produce. Purified preparations from various sources contain fractions whose colour suggests that they contain some bound quinone material, which increases as they are allowed to oxidize an appropriate substrate. Reaction with quinones probably explains the progressive inactivation of polyphenoloxidases during an oxidation: 'reaction inactivation' is decreased when the oxidation produces a substituted *o*-quinone less likely to react with proteins.

The extraction of cell organelles from plant tissues is also complicated by enzymically produced *o*-quinones, and requires conditions that avoid phenol oxidation. This is so for extracting the mitochondria of sweet potato tubers (*Ipomoea batatas*), the chloroplasts of sugar cane leaves (*Saccharum officinale*), and various plant viruses. All, once extracted, are sensitive to added phenols plus polyphenoloxidase, and most of them to synthetic *o*-quinones. Rather surprisingly more is known about the inactivation of the viruses.

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Thus the inactivation of cucumber mosaic virus, by oxidizing chlorogenic acid, is not caused by the oxidation of phenolic residues in the virus or by the polymerized products of the oxidation, but involves an intermediate of the oxidation that probably reacts with the protein rather than the nucleic acid of the virus (Harrison & Pierpoint, 1963; Pierpoint & Harrison, 1963). Prune dwarf virus reacts with synthetic *o*-benzoquinone, and the uninfected particles retain the morphological and serological properties of the virus (Hampton & Fulton, 1961). Tulare apple mosaic virus reacts not only with *o*-benzoquinone but also with such fully substituted derivatives as tetrachlorobenzoquinone. Mink (1965) argued that the inactivation is therefore caused by the oxidation of a viral group rather than by adduct formation. However, he also showed that the tetra substituted derivatives form adducts with the virus, and not only inactivate it but progressively modify its sedimentation, spectral and serological properties. Possibly the amino and thiol groups of the protein displace the halogen atoms from the substituted quinones.

Only a minority of known plant viruses seem to be inactivated by the *o*-quinones formed in leaf extracts. This may mean that it is advantageous for a virus to be insensitive to these compounds, or that the sensitive ones have been overlooked. However, the fact that viruses are not inactivated does not necessarily mean that they do not react with *o*-quinones, and there is evidence that southern bean mosaic virus reacts with *o*-quinones without losing its infectivity. This apparent *tolerance* to *o*-quinones contrasts with the *resistance* of some strains of tobacco mosaic virus. Neither intact TMV, nor its depolymerized protein, can be induced to react with chlorogenoquinone, although each protein subunit contains one thiol and two amino groups. This emphasises that, because of the differences in molecular architecture, the amino and sulphydryl groups of different proteins differ in accessibility to, and affinity for, *o*-quinones, as they do for other reagents.

Table 1 lists five principle ways of preventing *o*-quinones forming in plant extracts, although it is not always easy to judge why a technique is effective. Polyvinylpyrrolidone, for example, was initially used in enzymic extraction because it absorbs tannins strongly, but it absorbs simple *o*-dihydroxyphenols much less strongly and owes some of its efficacy to inhibiting polyphenoloxidase. Similarly DIECA, a powerful oxidase inhibitor, also combines with *o*-quinones, and conversely, the reducing agent ascorbate also inhibits polyphenoloxidase. Cysteine, depending on the conditions, combines with quinones, reduces them to phenols and inhibits the oxidase.

All the procedures listed have been effective in protecting some particular plant component from *o*-quinones. None is generally useful. The reducing agents, for example, are rapidly oxidized by some tissue extracts and DIECA and potassium ethyl xanthate break down in acid extracts. None of the enzyme inhibitors is specific for polyphenoloxidase; those that chelate metals affect other metal-dependent enzymes, and metabisulphite, although very effective for some purposes, affects pyridoxal phosphate and probably enzymes containing this cofactor. Disrupting tissues in an N_2 atmosphere is cumbersome; although it can be done much more conveniently in a specially designed press (e.g. Pirie, 1961) than in a glove box, it may still involve the difficulty of working up the resulting extract anaerobically. The choice of the most appropriate method, or combination of methods, for preventing *o*-quinones being produced, depends very much on the tissue used, the phenolic systems it contains, and the component that is to be recovered. It is very much a matter for trial and error.

There is a final caution: the phenol content of frozen leaves stored between 0 and −15°C slowly decreases, and the possibility exists that *o*-quinones are formed, and react with some leaf components even in these conditions. This is probably also true for unfrozen

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TABLE 1

Methods of preventing o-quinones forming in plant extracts

Principle	Technique	Example
Exclude oxygen	Disrupt tissue in N_2 atmosphere	Extraction of bulk leaf protein
Remove polyphenols	Extract tissue with solvents	Washing acetone powders of leaves
	Absorb phenols onto polymers: polyvinylpyrrolidone (PVP), insoluble PVP (polyclar AT), polyethylene glycol, albumin	Extraction of enzymes from apple fruit and leaves of peppermint (*Mentha piperita*, L.)
Inhibit polyphenoloxidase	Extract tissue in: trichloroacetic acid	Extraction of amino acids from leaves
	Extract tissue in: sodium diethyldithiocarbamate (DIECA), potassium ethylxanthate, thioglycollate, metabisulphite	Extraction of viruses from leaves of tobacco (*Nicotiana tabaccum*) and enzymes from tubers of potatoes (*Solanum tuberosum*)
Reduce quinones	Extract tissue in ascorbate	,, ,, ,, ,, ,,
Trap quinones	Extract tissue in: cysteine, benzene sulphinic acid	Extraction of enzymes, including polyphenoloxidase, from acetone powders

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but senescing tissue. Even though such tissue is then extracted so as to prevent further *o*-quinone formation, these components will have been modified. Some plant enzymes that can be resolved electrophoretically into several active components may owe some of their multiplicity to this reaction: it is important to distinguish such artifacts, if they occur, from physiologically separate isozymes.

Summary

o-Quinones are often produced as transient intermediates when *o*-dihydroxyphenols are enzymically oxidized. They polymerize rapidly to compounds whose complexity depends on the *o*-quinone involved and the presence of substances containing amino or sulphydryl groups with which they react. Some of the heteropolymers they form, insect cuticles, melanins and possibly humus, are very complex: because of the range of repeating units and different linkages they contain, it is not certain whether they have a regular recognisable molecular structure or resemble, in Freundenberg's phrase, 'chemical compost heaps'.

o-Quinones are formed and polymerize *in vivo* during the hardening of arthropod cuticles (sclerotinisation) and the hardening of plant seed and spore walls (melanisation). They are also formed adventiously, as when plants are invaded by microorganisms, and either mechanically or chemically may help restrict the multiplication or spread of the pathogen. Their formation during the harvesting, storing or processing of plant materials affects, for good or ill, the quality of the product, and the polymers they produce during the decay of vegetable matter may be the parent substances of soil humic matter.

Unless precautions are taken, *o*-quinones can form in plant extracts made in the laboratory. Their subsequent reactions may complicate or even prevent the extraction and estimation of organelles, enzymes, viruses and metabolites.

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