THE FATE OF PHENOLIC POLLUTANTS IN AQUATIC PLANTS

Jeremy William Spouge

Thesis submitted to the University of Kent at Canterbury in part fulfilment of the requirements for the degree of Doctor of Philosophy

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Abstract of a thesis submitted to the University of Kent at Canterbury in part fulfillment of the requirements for the degree of Doctor of Philosophy.

1. Uptake and metabolism of phenol by higher plants has been investigated. Studies based on reports of the removal of phenol from water by aquatic plants showed that the metabolism of phenol by microorganisms in soil-free cultures was considerable.
2. Subsequent use of [^{14}C]phenol demonstrated that a substantial part of the radioactivity thus supplied to the roots and rhizomes of *Phragmites communis* specimens entered the plants and was rapidly distributed throughout the tissues. The rate and extent of loss of phenol from such plant cultures greatly exceeded that in control (plant-free) cultures, confirming that the presence of specimens of this species was responsible for a major proportion of the phenol loss.
3. Both ethanol-soluble and insoluble fractions of the radioactive material absorbed by *P.communis* specimens were found, the latter predominating, and more radioactive material was usually found in insoluble residues of excised tissue pieces of *P.communis*, *Mentha aquatica*, *Typha latifolia*, *Iris pseudacorus*, and *Iris foetidissima* than in the respective ethanolic extracts, suggesting that immobilization of phenol was a common response to this toxic compound.
4. Incubation of cell-free extracts of *M.aquatica*, *P.communis* *T.latifolia* and *I.pseudacorus* with phenol yielded catechol, which was isolated from preparations of the first named species and identified by chromatography and spectroscopy. *P.communis* preparations contained lesser amounts which could be identified by chromatography, and trace amounts appeared in preparations of the other two species. The ability to hydroxylate monophenolic compounds was confirmed by the isolation from reaction mixtures of plant extracts containing *o*-cresol and *p*-coumarate of the appropriate *o*-diphenols, 4-methylcatechol and caffeate.
5. When excised tissues of *P.communis* were treated with [^{14}C]phenol, ethanol extracts of the tissues contained both radioactive catechol and phenol-β-glucoside, which were identified by chromatography and repeated recrystallization with authentic materials. Two routes of detoxication of phenol were thus demonstrated in *P.communis*; glucosylation, which masks the reactive hydroxyl of the molecule, and conversion to catechol, which may itself be converted to ethanol-insoluble polymeric or protein-bound products.
6. The activity of phenolases responsible for diphenol oxidation was found in most tissues of the plant species examined and the formation of complex products from catechol in the presence of *P.communis* preparations was suspected. Preparations of tissues from all the plant species possessed O_2-consuming activities stimulated by monophenols, including phenol, but the identity of the enzyme(s) responsible for catechol formation remained obscure.
7. Cell-free preparations and excised tissues of plants failed to produce significant amounts of $^{14}CO_2$ from [^{14}C]phenol. There was no evidence of the formation of immediate ring-fission products in the presence of *P.communis* extracts, and whole specimens of this species did not release $^{14}CO_2$ into the phyllosphere when [^{14}C]phenol was supplied to the roots.
8. Conjugation and immobilization of applied phenol are important means of detoxication in *P.communis* and probably in *M.aquatica* *T.latifolia*, *I.pseudacorus* and *I.foetidissima*, all of which lacked the ability to cleave the aromatic ring.

SECTION 1. INTRODUCTION

1.1. Higher plants in the treatment of wastewater.

Emergent and floating wetland and aquatic plants currently attract considerable interest for their functions in removing mineral ions (nutrient stripping) and other organic pollutant materials from fresh water. The individual properties of the species considered (which are named in the discussions concerning them) as well as those of their habitat account for their high productivity (Keefe, 1972; Westlake, 1963) and concomitant pollutant removal.

The abundant soil water of wetlands retains in solution the mineral elements required for plant growth, and the continued availability of water may permit growth in wetlands when terrestrial systems are subject to drought (Keefe, 1972). Keefe has also suggested that the growth season of temperate wetlands is extended, perhaps by the temperature buffering potential of water bodies. The concentration of nutrient elements is replenished by flowing or tidal waters, the biomass productivity of which is higher than that of still-water environments (Schelske and Odum, 1961) under similar conditions. Buttery *et al.* (1965) observed in a streamside marsh dominated by *Phragmites communis* and *Glyceria maxima* that soil solution concentrations of phosphate and nitrate were greater by up to an order of magnitude at the stream margins of the marsh than at the landward boundary, probably as the result of particle deposition in flowing and tidal waters (Gorham and Pearsall, 1956; Blum, 1969; Ranwell, 1964a, 1964b; Keefe, 1972).

The growth form of many marshland plants, bearing many narrow leaves and long photosynthetic stems, presents a very large area of energy-absorbing plant surfaces, so that incident solar radiation is efficiently trapped. This photosynthetic area, expressed as its ratio to unit area of ground surface is called the leaf area index

(LAI). Keefe (1972) suggested that marsh plants might have an LAI of 15, whereas Pearsall (1954) has estimated LAI of some crop plants as 2-4, and Zelitch (1971), reviewing factors important in plant productivity, gives estimates of up to 13 for the LAI of some agricultural crops.

The high productivity of wetland ecosystems has been reviewed by Keefe (1972), and annual harvestable biomass or annual standing crop and total productivity of wetland plants have been estimated for several species of plants. The latter quantity is difficult to estimate, requiring excavation of the subterranean and subaquatic parts of emergent plants, and is comparatively rarely given. Harvestable yield of essential nutrients contained in the tissues of plants can be calculated for species of interest in nutrient-stripping schemes. Table 1.1. shows productivity and harvest yield data for several species of emergent and floating aquatic and wetland plants which have been examined with reference to their nutrient-stripping capabilities.

Westlake (1963), reviewing productivity of green plants in terrestrial, aquatic, and wetland habitats, noted that the data of Seidel (1959) for the productivity of *Schoenoplectus lacustris* were exceeded only by tropical rainforest, aquaculture, and reedswamp systems, and show *S. lacustris* reedswamp to be the most productive system found in cool temperate climatic conditions.

Such data as these suggest that the harvestable mineral nutrient yields from such plant species will be large, and provide the rationale for the use of emergent and floating aquatic plants in nutrient-stripping projects, and as agents of water quality improvement. Table 1.2. presents estimates of nutrient yields from some plant species.

Table 1.1. Summary of harvestable biomass and total annual production data for some species of wetland and aquatic plants of potential value in nutrient-stripping and water purification

Maximum and minimum biomass or production figures were drawn from Keefe's (1972) review or from the other references given below.

Plant species	Aerial biomass [g(dry)/m^2]	Aerial net production [g(dry)/m^2/year]	Country	References
Phragmites communis	497 - 3650	2695	England, Sweden	Keefe (1972)
Schoenoplectus lacustris	1000	6400	Germany	Seidel (1959)
Typha latifolia	60 - 1527	684 - 1358	England, U.S.A.	Keefe (1972)
Eichhornia crassipes *	1276 - 1478		U.S.A.	Keefe (1972)
		15400	U.S.A.	Wolverton and McDonald (1979)
Lemna minor *	25.2	1141	Laboratory culture	Harvey and Fox (1973)

* All data are for aerial parts of the plants, except for the two species shown where whole, floating, plants can readily be harvested. The maximum figure for *E.crassipes* was obtained by frequent cropping.

Table 1.2 Estimates of mineral nutrient yields harvestable from some wetland and aquatic plant species

The data presented below were derived from those of Keefe's (1972) review, by calculation from maximum and minimum production and elemental composition figures, or were given by the other authors cited.

Plant species	Yield of nutrients (g/m^2/year)			Basis on which the estimate was calculated	References
	N	P	K		
Phragmites communis	17.5-96.2	0.8-4.6		Annual production	Keefe (1972)
		6.3	36.3	Biomass	Seidel (1966; 1976)
Schoenoplectus lacustris		6.7	34.6	Biomass	Seidel (1966; 1976)
Typha latifolia	6.2-48.9	0.9-4.1		Annual production	Keefe (1972)
	263	40.3	457	Continuous cropping (estimate)	Boyd (1970a)
Eichhornia crassipes	198	32.2	318.8	Continuous cropping (estimate)	Boyd (1970a)
	159.1	36.4		Estimate only	Wolverton *et al.* (1976)
Lemna minor	52.4	9.1	22.1	Continuous cropping of laboratory culture	Harvey and Fox (1973)

In addition to their demonstrably high productivity, wetland plants exhibit responses to the presence of high concentrations of mineral nutrients which could be of value. Boyd (1970b) observed a positive correlation between mineral nutrient concentration in plant tissues and the concentrations in the surrounding water. Harper and Daniel (1934) suggested that *Typha* species absorbed P in excess of the quantity immediately required for growth, when large supplies of the element were available. Gerloff and Krombholz (1966) also observed excess consumption of nutrient minerals by freshwater plants, finding that productivity correlated with internal nutrient concentrations below critical threshold concentrations, but in excess of the threshold, production did not increase, and the excess nutrient content represented "luxury consumption". In an area of marshland receiving sewage-treatment effluents, Grant and Patrick (1970) found that the nutrient uptake of plants exceeded that in other areas of the marsh, confirming the response to elevated mineral nutrient concentration.

1.2. Uptake of phenol by green plants considered as wastewater treatment agents.

European investigations of the beneficial effect of plants on polluted waters have been concentrated in the main on *Schoenoplectus lacustris*, which is widely distributed in central and northern Europe, and is highly tolerant of polluted conditions. Seidel (1952; 1959; 1974a) has made extensive studies of the morphology, growth habit, reproduction, and ecology of *S. lacustris*, and has pursued investigations of the nutrient-stripping and pollutant-absorbing capabilities of this plant to the logical conclusion of large-scale practical applications. Early studies of Seidel (1963) showed that mature plants of *S. lacustris*, grown in water or mineral nutrient

medium containing 10mg/l phenol (about 0.11mM), removed the phenol entirely from 5l of medium in 5h. Several other species of wetland plants, *Sparganium simplex*, *Sparganium erectum*, *Alisma plantago-aquatica*, *Schoenoplectus maritimus*, and *Typha angustifolia* were comparatively intolerant of phenol from 10-1000mg/l, or were rapidly killed, but a species of *Juncus* was also able to reduce a similar phenol content in water without sustaining damage. When plants of *S. lacustris* were harvested and analysed, phenol was found to be distributed throughout the tissues, though it predominated in the extensive rhizome system. Over the concentration range 5-30mg/l, phenol actually appeared to encourage plant growth, as plants cultured in phenol for 97 days were heavier (fresh weight) than those cultured in medium alone. The possibility that phenol was serving as a carbon source for the plant tissues received attention in subsequent communications from Seidel and coworkers. That phenol removal was associated with vigorous metabolic activity was suggested by the observation that rapid phenol removal from the culture medium took place during the April to October growth period of *S. lacustris* specimens (Seidel, 1965). Some process of adaptation to the presence of phenol was noted in this and a later communication of Seidel (1966), when short-lived toxicity symptoms resulting from exposure to phenol were observed. The plant specimens subsequently recovered, and removed phenol from their medium at a substantial rate. After exposure to phenol for 3 years, plants of 300g fresh weight had removed 35g phenol from their culture media, with no permanent damage. In comparative data, it was suggested that *S. lacustris* was very much more tolerant of phenol than terrestrial plants. Phenol, in these experiments, was only one of seventeen organic compounds which were removed from water by specimens of *S. lacustris* (Seidel, 1966). Several phenolics, among them *p*-cresol, resorcinol, pyrogallol,

hydroquinone (quinol),*p*-benzoquinone, guaiacol, phloroglucinol, and *p*-chlorophenol, were reduced in concentration or removed from water.

Although *S.lacustris* had such a remarkable effect on the concentration of phenol in its medium, other wetland species have received comparatively little attention. Phenol (10-20mg/l) was toxic to several species (Seidel, 1963) but *Juncus maritimus* and *J.effusus* (Seidel 1973a) removed phenol from water at a rate comparable with that of *S.lacustris*, and the former, when exposed to pentachlorophenol, removed 3-4mg/l/day of the compound from cultures for each 100g fresh plant tissue. The greatest rate of removal of pentachlorophenol in these specimens was achieved in the presence of NaCl, and with discontinuous exposure to the phenol. Seidel (1974b) reported that the uptake of pentachlorophenol with *J.effusus* was more effective than with *J.maritimus*, *Bolboschoenus maritimus*, *S.lacustris*, and *Schoenoplectus tabernaemontani*.

In experiments with *S.lacustris*, the removal of phenol was connected in some way with the growth form of the plant. Seidel (1963; 1966) suggested that extension of the shoots from a basal meristem, and the possession of aerenchymatous shoots, properties shared with other rushes, were factors determining the efficiency with which this species absorbed phenol. *S.lacustris* was also capable of modifications at tissue level in response to phenol exposure. Changes were observed in the distribution of stomata, and small processes arose from the epidermal cells of plants exposed to high concentrations of phenol (Seidel, 1966).

All the plants so far considered are of emergent habit, but *Eichhornia crassipes*, considered as a nutrient-stripping crop in the United States, can also remove phenol from culture media. Specimens of this floating aquatic species of dry weight 2.75g removed phenol entirely from solutions initially containing 25-100mg/l within 72h (Wolverton,

1975; Wolverton and McKown, 1976). It is perhaps significant that this species, like *S. lacustris*, contains much aerenchymatous tissue.

1.3. Fate of phenol applied to plants.

Wolverton and McKown (1976) reported that when phenol had been removed from the culture media of specimens of *E. crassipes*, none could be detected within the plant tissues. As ^{14}C-labelled phenol was used in these experiments, it is surprising that no attempt was made to trace the material but Seidel *et al.* (1967) showed that unidentified compounds were produced when [^{14}C]phenol was injected into the basal meristem of *S. lacustris* plants, or infused into rhizomes. Phenol itself had previously been found to be dispersed throughout the plant (Seidel, 1963), and was released as the unaltered volatilised compound from aerial parts when applied to the roots and rhizome in liquid cultures (Seidel and Kickuth, 1965). Kickuth (1970) described the results of experiments in which [^{14}C] phenol was applied as a pulse-label to the peeled, methanol-surface-sterilized rhizomes of *S. lacustris* plants. After 18h exposure, and further 24h incubation, a small proportion (8%) of applied radioactivity was recovered from the plant tissues, of which significant fractions were picolinic acid, alanine, and unaltered phenol. Salicylate was similarly metabolized, to picolinate and another, unidentified, pyridyl compound. The large proportion of radioactivity which remained unaccounted for was probably lost as $^{14}CO_2$ as a result of complete degradation of phenol. Kickuth (1970) proposed that phenol and salicylate were respectively *ortho*-hydroxylated or decarboxylated to yield catechol, which underwent *meta*-ring-fission, forming 2-hydroxymuconate semialdehyde, which, on condensation with ammonia, yielded the pyridyl compounds, or which was degraded entirely to CO_2, but no assays for the essential enzymes of these pathways were done.

Other than these examples, there is little information on the fate of phenol applied to aquatic or wetland plants, but consideration of the uptake and metabolism of other aromatic compounds by higher plants and microorganisms suggests that metabolic processes able to accommodate exogenous phenol are widespread.

Uptake of low molecular weight phenolic compounds by plants; effects of phenolic compounds on plant cells.

Seidel (1963; 1966), Seidel and Kickuth (1965) and Kickuth (1970) have shown that phenol penetrates the tissues of whole plants; indeed, it seems improbable that such a compound can be excluded from plant cells. Weakly acidic or basic compounds pass cell membranes in undissociated form (Simon and Beevers, 1952; Collander, 1959), and have uptake rates which can be predicted by methods described by Collander (1959), who discussed experiments with *Chara ceratophylla* and *Nitella mucronata* where the permeability of a number of compounds bore a simple relationship with their water-lipid partition coefficients and molecular size.

Ionization, however, complicates the prediction of permeability of weakly acidic compounds to cells, and Wright and Diamond (1969) demonstrated that uptake of small, comparatively lipid-insoluble molecules to cells of rabbit gall-bladder was more pronounced than might be expected from water-lipid partition coefficients, and was little affected by pH, which significantly alters the ratio of ionized and unionized molecules of weak acids. A molecular size-related uptake path, characterised by local concentration of polar moeities in the cell membrane, was proposed to account for the uptake of ionized molecules. Jackson *et al.* (1970), working with excised barley roots, similarly found that uptake of organic acids was greater than expected at pH values conducive to ionization.

Phenol itself, examined as one of 206 compounds by Wright and Diamond (1969) had a low "reflexion coefficient" - a term expressing the tendency of the compound to be excluded from membranes - and was rapidly taken up by rabbit gall-bladder cells. In general, when considering the very widespread importance of phenolic compounds in the environment and within plants, the uptake of phenols by plant cells is comparatively neglected (Glass and Bohm, 1971). The latter workers investigated the uptake of [^{14}C]quinol and its monoglucoside, [^{14}C]arbutin, by barley (*Hordeum vulgare*) roots. The two compounds were chosen on the basis of their natural occurrence in plants (Karrer, 1958) in the case of arbutin, and the occurrence in some wastewaters of hydroquinone (Water Research Centre, 1980). Arbutin was apparently taken up by an active process, while the aglycone was passively absorbed. Quinol was nevertheless concentrated within the plant tissues, but in this case the concentration gradient required to allow continued absorption of the compound was probably maintained by its metabolism within the tissue.

Because phenolic acids may be of significance in allelopathic plant interactions (Glass, 1973; 1976), the phytotoxity of several such compounds has been examined. Phenolic acids inhibited uptake of phosphate (Glass, 1973; 1975), they depolarized plant cell membranes (Glass and Dunlop, 1974), inhibited potassium absorption (Glass, 1974), and caused leakiness, which effects were probably attributable to interaction of the comparatively non-polar compounds with the cell membrane.

Metabolism of phenols by higher plants.

Having been absorbed by plant cells, three major metabolic routes might accommodate exogenously-supplied phenol. Oxidative metabolism of phenolic compounds can readily be demonstrated in many

plant tissues; the activity of polyphenol oxidase enzymes hydroxylates monophenolic compounds and further oxidises the resulting diphenols, forming reactive quinones. Ring fission of several *o*-diphenols, produced perhaps from monophenols by hydroxylating enzymes, has recently been shown in plant tissues. Finally, it is very likely that simple phenols in plant tissues become converted to glycosidic conjugates, in a process analogous to animal tissue detoxication processes.

These three systems can result in the eventual destruction, immobilization, or detoxication of phenolic compounds.

Ring-fission of phenol, catechol, and related compounds.

Higher plant aromatic ring-fission reactions have been sought in many species, using as models the well characterised aromatic metabolic sequences of microorganisms. The probable similarity of the products of plant and microbial aromatic biodegradation, and the comparative ease with which competent microbial populations can be selected make it important to determine that observed activity is due unequivocally to the plant tissues. Ellis and Towers (1970) expressed similar reservations concerning the interpretation of results purporting to show the degradation of aromatic compounds in whole plant tissue preparations not unequivocally axenic.

Plant ring-fission of aromatics may start with non-phenolic precursors as the initial substrate. When benzene and toluene were detected in green avocado fruits (Jansen, 1963; 1964; Jansen and Wallace, 1965), Jansen and Olson (1969) applied vapourized [^{14}C]benzene and [^{14}C]toluene to fruits, and trapped $^{14}CO_2$ from the airstream passed over them. A substantial amount of the radioactivity applied was retained in non-volatile form in the tissues; at least some oxidation or cleavage of the aromatic ring must have occurred to allow the

subsequent formation of immobile materials, and $^{14}CO_2$ was presumably generated after a ring-fission process. Durmishidze and Ugrekhelidze (1969), in studies of hydrocarbon metabolism by higher plants, applied [^{14}C]benzene to the $HgCl_2$-sterilised roots of tea plants, and trapped $^{14}CO_2$ in a chamber separately enclosing the aerial parts of the plant. Similar results were obtained when [^{14}C]benzene was introduced into the xylem of the stem. Again, when directly injected, a proportion of the applied radioactivity was shown to be immobilized in non-volatile ethanol-insoluble form, and as a result of both treatments, a large number of extractable non-aromatic plant constituents were found to be ^{14}C-labelled. Other plants were also capable of metabolism of benzene, and Durmishidze *et al.* (1969) subsequently incubated homogenates of tea and grape-vine leaves with [^{14}C]benzene and [^{14}C]-phenol, and reported that benzene was converted to phenol, and that the products of ring-fission could be isolated chromatographically.

Surveys of aromatic ring-fission ability in plants have often been made, without extensive characterisation of the pathways, relying for the most part on detection of $^{14}CO_2$ production by experimental plant materials supplied with labelled aromatic substrates. Craigie *et al.* (1965) supplied [^{14}C]phloroglucinol to nine species of marine algae in axenic culture, and found that six species produced $^{14}CO_2$ from the substrate. When preconditioned with non-radioactive phloroglucinol, eight species released $^{14}CO_2$ on application of the radioactive material, indicating that some inducible process might be responsible for the production of $^{14}CO_2$. Using algae as experimental plants, Ellis (1977) also observed that phenol was degraded by five freshwater species in axenic culture, one of which required induction with non-radioactive substrate, and others of which showed depressed levels of activity against phenol after previous exposure, suggesting that although some algae were competent to metabolise phenol, the

compound was nevertheless toxic on prolonged exposure. Catechol was readily degraded to CO_2 by all six species studied; in one case the ability to convert phenol to catechol may therefore have been lacking.

Degradation of the aromatic ring by more highly organised plants than algae has been successfully shown in tissue and cell cultures, which, like axenic cultures of algae, can exclude the effects of microorganisms. It should be borne in mind that the responses of a plant species in comparatively disorganised cultures need not reflect identical metabolism in the differentiated tissues of a whole plant subject to normal physiological control, so studies with intact plants or undamaged but excised tissues are still required.

As the result of a survey of twenty species from eleven families of plants, Ellis (1971) concluded that catechol ring-fission activity was widely distributed in the Plant Kingdom. All released $^{14}CO_2$ from [^{14}C]catechol when the substrate was applied to tissue cultures; fifteen cultures posessed the ability to decarboxylate salicylate, and traces of $^{14}CO_2$ were released as a result of the fission of ring-labelled salicylate by thirteen species. The species most active against catechol (in tissue culture) were *Malus coronaria*, *Phaseolus angularis*, and *Phaseolus aureus*. Catechol degradation by *P.aureus* was also reported by Berlin *et al.* (1971), who also found that *Glycine max* possessed the activity. *P.aureus* tissue was analysed further by Ellis (1971), when substantial conversion of catechol to its β-*D*-glucoside was found, together with a large (50%) fraction not recoverable in ethanolic extracts, suggesting that not only did the tissue degrade catechol, but also conjugated it, and rendered it immobile as the result of phenolase activity. Ellis (1973) further surveyed plant species for aromatic degradation activities, and extended the studies to other compounds. Of ten plant species of

eight families in cell culture, all degraded catechol, and phenolic natural products like tyrosine, dihydroxyphenylalanine (DOPA) and homogentisate.

Degradation of natural products with substituents to the aromatic ring in addition to, or other than, the catecholic *o*-dihydroxyl moiety may also occur in plants. Ellis and Towers (1970) supplied ^{14}C-labelled tyrosine, phenylalanine, tryptophan, cinnamate, salicylate, and benzoate to tissue cultures of *Ruta graveolens* and *Melilotus alba*, and detected the release of $^{14}CO_2$ from phenylalanine and tryptophan by both species. Cinnamate was degraded only by *M.alba* tissue, and neither culture attacked the ring of benzoate, salicylate, or tyrosine. Protocatechuate is well-known as a ring-fission substrate of microorganisms, and its catabolism has also been shown in some plant materials. Berlin *et al.* (1971), using cell cultures of *P.aureus* and *G.max*, found that the former released $^{14}CO_2$ from protocatechuate, *p*-hydroxybenzoate, vanillate, caffeate, and several other aromatic compounds. Protocatechuate and caffeate were rapidly attacked, suggesting that they were, or were similar to, naturally occurring substrates. *G.max* cultures had a similar range of activity, but only *P.aureus* was capable of the degradation of benzoate, at a very low rate.

Studies of the aromatic metabolism of *Tecoma stans*, using compounds other than catechol, have provided some firm evidence for the existence of enzymes capable of aromatic ring-fission in plants. Sharma *et al.* (1972) reported the oxygenative ring-cleavage of the catecholic 2,3-dihydroxybenzoic (pyrocatechuic) acid in this species, the isolated product being 2,6-dioxa-3,7-dioxobicyclo(3:3:0)octane-8-carboxylic acid. Such a compound could have been formed by the rearrangement through one of two isomeric muconolactones of the

initial product of a pyrocatechuate-2,3-dioxygenase, 3-carboxy-*cis,cis*-muconate. The enzyme responsible for pyrocatechuate ring-fission in *T.stans* was subsequently isolated from soluble and chloroplast fractions of leaves by Sharma and Vaidyanathan (1975a; 1975b), but the formation of α-carboxymuconate could not be demonstrated other than by spectral observations of reaction mixtures. The enzyme was comparatively specific for pyrocatechuate, but showed activity with 2,3-dihydroxy-*p*-toluate and 2,3-dihydroxy-*p*-cumate, and was inactive against catechol, and 3,4-dihydroxylated aromatic compounds such as DOPA, 4-methylcatechol, and protocatechuate. The enzyme showed several properties characteristic of a copper-protein, unlike microbial pyrocatechases (Hayaishi and Nozaki, 1969), which are non-haem Fe containing oxygenases.

Subsequent examination of *T.stans* has shown that the species also possesses protocatechuate-3,4-dioxygenase. The enzyme, isolated only from soluble fractions of tissue by Mohan *et al.* (1979), was purified almost to homogeneity, and was absolutely specific for protocatechuate. The product, 3-carboxy-*cis,cis*-muconate, was identified and isolated, but the enzyme, although performing a function similar to those from microbial sources, showed several differences from highly characterised fungal and bacterial preparations.

Evidence of aromatic ring-fission of tyrosine, and the related phenylalanine and DOPA, while obtained from $^{14}CO_2$ release experiments, has also been inferred indirectly from the study of the biosynthesis of some plant pigments and other secondary products. Several pathways for the biodegradation of tyrosine exist (Ellis, 1974), possibly *via* homogentisate (Ellis, 1973), *via* *p*-coumarate (Ellis, 1974), and hence caffeate and protocatechuate (Ellis, 1977; Berlin *et al.*, 1971), or after *o*-hydroxylation to DOPA, by ring-fission and subsequent recyclization to the heterocyclic compounds betalamic, stizolobic, and

stizolobinic acids (Miller *et al.*, 1968; Impellizzeri and Piatelli, 1972; Senoh *et al.*, 1964; Senoh and Sakan, 1966). Biosynthetic studies of such compounds were incomplete when reviewed by Ellis (1974), but betalamic and stizolobic acids might be formed after 4,5-ring-fission of DOPA, and stizolobinic acid after 2,3-fission.

Pathways of aromatic catabolism which lead to recyclization of the ring-fission product to yield heterocyclic molecules bear an obvious similarity to the suggestions of Kickuth (1970), that phenol, after hydroxylation and ring-fission in *S. lacustris*, cyclizes to 2-picolinate; this process was described in microorganisms by Dagley *et al.* (1960; 1965) and Dagley and Wood (1965), and occurs non-enzymically in the presence of NH_4^+.

Evidence that ring-fission of aromatic compounds by microbial-type specific ring-fission oxygenases is not universal was provided by Prasad and Ellis (1978) who characterised catechol ring-cleavage activity in cell cultures of the soybean *G.max*, finding that peroxidase was the enzyme responsible.

Horseradish peroxidase, cell cultures, and culture filtrates augmented with H_2O_2 all stimulated the release of $^{14}CO_2$ from [^{14}C] catechol. The process was not absolutely dependent on the presence of O_2, and H_2O_2 could be substituted for O_2 in anaerobic cultures to permit ring-fission. Compounds expected from catechol ring-fission by typical catechol oxygenases were apparently not formed by the ring-fission reactions operating in these soybean cultures.

Plants, like microorganisms, can be shown to possess both *ortho* and *meta* aromatic ring-fission mechanisms, with some evidence that specificities of the enzymes responsible differ. Kickuth (1970) suggested that phenol ring-fission, *via* catechol, took place *via* a *meta*-route, while both phenol and naturally occurring aromatic acids are degraded in other species by *ortho*-fission. One of the

ortho-fission enzymes of *T.stans* accommodated substrate analogues (though not catechol), suggesting a generalised ring-fission pathway, but there is no evidence of the existence in plants of the tangential and specific substrate-induced catabolic pathways found in microorganisms. It is not possible at present, therefore, to predict the likely route of ring-fission of phenol in a plant species.

The polyphenol oxidase complex of enzymes.

Polyphenol oxidases are probably the most extensively investigated enzymes of aromatic metabolism in plants and many microorganisms, and their almost ubiquitous occurrence, and wide range of activities and substrate specificities suggests that they might function to detoxicate exogenous phenol and other aromatic compounds.

Nomenclature of these enzymes presents problems, as observed by recent reviewers (Mayer and Harel, 1979), who chose to discuss phenol oxidising activities under the general title of polyphenol oxidase. Until recently, formal nomenclature differentiated usefully between laccase [*p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)], and catechol oxidase [*o*-diphenol: oxygen oxidoreductase (EC 1.10.3.1)] but revision of nomenclature (International Union of Biochemistry, 1973) now has both major activities defined as monophenol monooxygenase (EC 1.14.18.1), characterised by a single type reaction. The latest recommendation again differentiates the activities, however (International Union of Biochemistry, 1979). Mason's (1955) early description of the catalysis of phenol oxidising reactions as functions of the "phenolase complex" had much to commend it. Laccase is a useful name for enzymes oxidising *p*-diphenols, as their activities

towards monophenols and diphenols are not implicitly excluded, and catechol oxidase is likewise a useful label for the appropriate activity. These names, and the terms cresolase and tyrosinase, were coined by early workers to describe the activity of enzymes against common laboratory substrates and are still widely used.

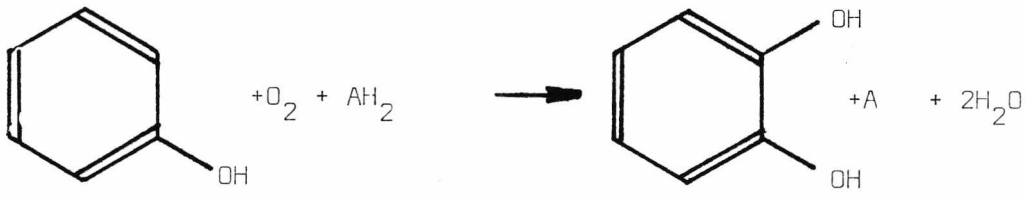
The descriptive terminologies of various authors will be used where appropriate in this work, with the understanding that all refer to some activity of formally-named monophenol monooxygenase, and polyphenol oxidase is used as the general name, after Mayer and Harel (1979).

Catechol oxidase (*o*-diphenol oxidase) has two distinct activities, although these are generally found to be inseparable. The enzyme is capable, in the presence of suitable coreactants, of the *o*-hydroxylation of monophenols, and the conversion of the resulting, or other *o*-diphenols to *o*-quinones without an additional reactant (Mason, 1955; Brooks and Dawson, 1966; Malmstrom and Ryden, 1968; Vanneste and Zuberbuhler, 1974; Mayer and Harel, 1979). The reactions, summarised in Fig. 1.1, are very different; the *o*-hydroxylation activity, with reference to the terminology of Hayaishi (1968; 1974) is that of an external monooxygenase, inserting one atom of molecular oxygen into the substrate and requiring an electron donor or reductant to consume the other. The quinone-generating activity is that of an oxidase, in which protons from the substrate molecule are used to reduce molecular oxygen to water. Mason *et al.* (1955) using $^{18}O_2$ and $H_2{}^{18}O$ showed that an atom derived from molecular oxygen and not from water was indeed present in the 4,5-dimethylcatechol produced by polyphenol oxidase activity against 3,4-dimethylphenol.

Some purified preparations of polyphenol oxidase exhibit a

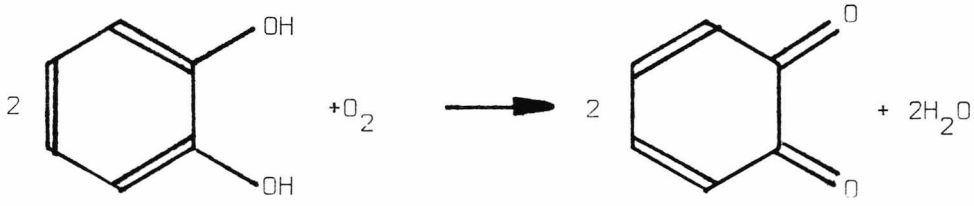
Figure 1.1 Summarized reactions of polyphenol oxidase

I Monophenol hydroxylating activity



Monophenol Acceptor *o*-Diphenol

II Diphenol oxidising activity



o-Quinone

prolonged lag before oxidation of monophenolic substrates commences (Malmstrom and Ryden, 1968), unless catalytic quantities of a diphenol are added. Ascorbate and other electron donors may also function in this manner, and modern concepts of activity of this enzyme suggest that monophenol hydroxylation and diphenol oxidation are stoicheiometrically coupled, the diphenol serving as the cofactor. Vanneste and Zuberbuhler (1974) discussed evidence in support of this concept. It is probable, if this is indeed the case, that the lag period observed when monophenols alone are incubated with polyphenol oxidases persists until sufficient monophenol has been autooxidized to a diphenol capable of serving as the cosubstrate. Kendal (1949) observed that 3,4-dimethylphenol, which on complete oxidation by polyphenol oxidase yields a particularly stable quinone, required a very long period of incubation before generation of the catechol by polyphenol oxidase occurred. In this model of monophenol hydroxylating activity, additional reductants such as ascorbic acid will function by reducing *o*-quinones to diphenols to serve in turn as the required cosubstrate. McIntyre and Vaughan (1975) and Butt (1979) have recently discussed this model for the mechanism of polyphenol oxidases, Vanneste and Zuberbuhler (1974) have reviewed several possible mechanisms, and Mayer and Harel (1979) have recently reviewed most aspects of polyphenol oxidase biochemistry, without reaching firm conclusions on the mechanism of the reactions it catalyses.

Hydroxylation of monophenolic substrates by polyphenol oxidases

It has been argued (Kim and Tchen, 1962; Dressler and Dawson, 1960) that *o*-diphenols produced by the activity of polyphenol oxidase are not normally released from the enzyme, and are further oxidised to quinones. Isolation of the products of monophenol

hydroxylation has nevertheless been reported. Fling *et al.* (1963) observed that a highly purified *Neurospora crassa* tyrosinase, while incapable of the oxidation of catechol, catalysed the oxidation of DOPA to DOPA-quinone, and hydroxylated tyrosine. DOPA accumulated in reaction mixtures containing tyrosine and this enzyme.

Using plant phenol oxidase preparations, hydroxylation of monophenols can be demonstrated readily. Using preparations from *Beta vulgaris* (spinach beet) leaves, Vaughan and Butt (1967; 1968; 1969a; 1969b; 1970; 1972) isolated caffeate chromatographically from reaction mixtures of *p*-coumarate and enzyme preparations. Stafford and Dresler (1972) detected caffeate chromatographically after hydroxylation of *p*-coumarate in the presence of an enzyme from *Sorghum vulgare* although if considered as a polyphenol oxidase, certain properties of the *S.vulgare* enzyme were atypical. The *B.vulgaris* and *S.vulgare* enzymes both required cofactors to catalyse the *o*-hydroxylation of *p*-coumarate; the former was active in the presence of either ascorbate, tetrahydrofolate, NADH or NADPH, or the synthetic 6,7-dimethyl - 5,6,7,8-tetrahydropteridine, the last named being the most effective. The *S.vulgare* enzyme required ascorbate, or 2-amino-4-hydroxy-6,7-dimethylpteridine, but NADH and NADPH would function less efficiently. Both enzymes were sensitive to copper reagents, and retained some diphenol oxidase activity.

Stafford (1974) discussed the sorghum and spinach enzyme investigations with reference to the long-standing controversy over separability of monophenol and diphenol activities of polyphenol oxidase. It was suggested that the requirement of the plant hydroxylases for NADH or another reductant might indicate the presence of enzymes separate or modified from polyphenol oxidase activity. For the spinach enzyme, a model of activity has been proposed which incorporates

the evidence that diphenol oxidation and monophenol oxygenation proceeded simultaneously (McIntyre and Vaughan, 1975). In sorghum preparations, the double activity in constant ratio was not observed (Stafford and Dresler, 1972; Stafford and Bliss, 1973) suggesting that a comparatively specific *p*-coumarate hydroxylase may coexist with a polyphenol oxidase of broader specificity, accounting for variability observed in the ratio of monophenol and diphenol activities. Very many attempts have been made to separate monophenol hydroxylating and diphenol oxidising activities from polyphenol oxidases, with varying degrees of success. The usual result is to obtain fractions with different ratios of monophenol and diphenol activity, or with variations in substrate specificity. Much work of this nature was reviewed by Mayer and Harel (1979).

In general then, it is difficult to demonstrate that plants possess specific monophenol hydroxylating enzymes separate from polyphenol oxidase activity, and it must be assumed that phenol itself will be acted upon by the peripheral activity of an enzyme of low specificity. Hydroxylation of such substrates as *p*-cresol and tyrosine is commonly reported; the use of phenol as an experimental substrate of polyphenol oxidase, however, is rare. In one reported instance of its use, (Taneja and Sachar, 1974), phenol was unaffected by enzymes isolated from germinating wheat seeds, as was *p*-cresol, but tyrosine was oxidised. There is no evidence that the well-defined hydroxylating activity of sorghum and spinach-beet enzymes is of sufficiently broad specificity to accommodate phenol itself, but the attack on many monophenolic substrates, such as *p*-cresol, by bacterial and fungal hydroxylases and plant polyphenol oxidases suggests that enzymes with the ability to carry out this reaction might be found in plants, or perhaps evolve in those exposed for long periods to pollutant phenols.

Glucosylation of phenols by plants.

The process of conjugation of a toxic material, in which the physiologically active group of the molecule is rendered inert or more hydrophilic, is found throughout both Plant and Animal Kingdoms, though some major biochemical differences exist.

The major detoxication pathways for phenolic compounds in plants lead to formation of a glycoside of the applied compound (Harborne, 1977), whereas in higher animals the major detoxication route involves conversion of phenols to phenolic ethereal sulphates or glucuronides (Williams, 1964). Most simple feeding experiments have shown that glycosylation of phenols occurs rapidly on entry of phenolic compounds into plant tissues, and, in general, plant materials seem highly adapted to metabolism of phenols in this manner. Most major organs of plants (roots, leaves, or seeds in most quoted examples) apparently possess the requisite enzymes to carry out glycosylation. As almost all low molecular weight phenols in living plant cells occur in conjugated form (Harborne, 1964), it is not surprising that the glycosylation mechanism is frequently observed.

Phenolic glycosides consist of a phenolic compound (the aglycone) and a carbohydrate moiety (the glycone) and are chemically ether derivatives, though ester compounds may be formed with carboxylated phenols. Harborne (1977) gives the example of glucosylation of *p*-coumaric acid, which may be found as both *p*-coumaric acid-β-*D*-glucoside, the ether glucoside formed by conjugation of the phenolic hydroxyl group of the molecule, and as *p*-coumaroylglucose, the ester glucoside formed by conjugation of the carboxylic acid. In plant tissues, *D*-glucose is the most common glycone of naturally-occurring glycosides, and the glycosidic link is usually β with respect to the sugar (Hopkinson, 1969). The pyranose form of the sugar predominates

in plant glycosides (Miller, 1973).

The synthesis of glucosides of applied phenols in plant tissues requires a source of glucose, and in several early investigations it was found that glucose alone was not sufficient. In dialyzed extracts of broadbean (*Vicia faba*) and pine (*Pinus sylvestris*) seeds, Miwa *et al.* (1957) observed that glucosylation of phenols took place only when the extracts were supplemented with ATP and "yeast nucleotide". Glucose-1-phosphate, synthesized as an intermediate, was thought to be the glucose donor, but subsequent investigations showed that uridinediphosphate glucose (UDPG) was the actual glucose donor species. Tabone (1955) and Jacobelli *et al.* (1958) obtained the ether glucoside of anthranilic acid from extracts of lentils supplied with the aromatic and UDPG, and Cardini and Leloir (1957) and Yamaha and Cardini (1960a) clearly demonstrated the involvement of UDPG in glucoside synthesis. Extracts of wheatgerm supplemented with UDPG catalyzed the formation of glucosides of quinol and catechol, the formation of the former, arbutin, proceeding at a greater rate, but phenol itself was not glucosylated. Monoglucosides thus formed could be further elaborated by the activity of another, presumably separate, enzyme of wheatgerm to corresponding gentiobiosides (Yamaha and Cardini, 1960b). Phenol-β-*D*-glucoside, supplied with UDPG, was the optimal substrate of the latter enzyme, which was not stimulated by free monosaccharides, disaccharides or polysaccharides supplied as glucose donors. Quinol gentiobioside was found (Conchie *et al.*, 1961) to be further glucosylated in the presence of wheatgerm extract and UDPG to a trisaccharide glycoside.

UDPG occurs widely in plant tissues (Ginsberg *et al.*, 1956) and has been isolated by Cardini *et al.* (1950; 1955), Caputto *et al.* (1949), and Cardini and Leloir (1957), but Trivelloni *et al.* (1962) have shown that adenosinediphosphate-glucose was also an effective glucose

donor for the synthesis of arbutin by wheatgerm enzyme, yielding glucosides identical to those formed in the presence of UDPG.

The formation of glucosides from exogenous phenolic compounds applied to plant tissues has often been demonstrated, but phenol itself is not often considered as a substrate. However, Nystrom *et al.* (1959) supplied [^{14}C]glucose and phenol to leaves of wheat and barley, and successfully isolated radioactive phenol-β-*D*-glucoside from leaf extracts.

In contrast with these results, Pridham (1958) found that although broadbean shoots were capable of the glucosylation of catechol, quinol, resorcinol, and phloroglucinol, phenol was not demonstrably glucosylated.

An indication of the widespread occurrence of the ability to glucosylate applied phenols was later achieved by Pridham (1964) who supplied quinol and resorcinol to specimens and tissue pieces of 55 species of plants. The ability to form β-*D*-glucosides of the phenols was apparent in twenty Angiosperms, five Gymnosperms, nine Bryophytes (eight mosses and one liverwort) and three ferns. The activity of ferns was confirmed by Glass and Bohm (1970), who examined twenty-nine species from twenty-one genera and found all capable of the glucosylation of quinol and catechol. Of the Angiosperm species examined, *Elodea canadensis*, *Lemna minor*, and *Utricularia vulgaris*, all aquatic plants, were unable to glucosylate phenols. Pridham (1964) observed that algae were incapable of glucosylation. The ten algae examined, both organised and unicellular species, were all incapable of glucosylation, as were *Aspergillus niger* and *Psalliota campestris*, two fungi examined.

The functions in plants of the phenol glucosylation system have been the subjects of discussion. Early workers viewed the glucosides of phenols as metabolic end products or "waste products", safely stored in the vacuole until the dissociation of the plant tissue. In view of the energy and carbon demand of such a disposal method, it has also been suggested that the glucosides provide readily mobilized reserves of carbohydrate (or conversely perhaps, of phenolics for flavonoid or lignin biosynthesis). Hopkinson (1969) observed that both these hypotheses were difficult to prove, although it is quite possible that glucosides are consumed in periods of starvation.

The glucosylation of phenolic hydroxyl and acidic moieties effectively protects these groups against further reaction until the glucosidic linkage is hydrolysed. Formation of the ester glucoside of cinnamic acids, for example, may serve to neutralize the acid groups of the compounds, and glucosylation of phenolic hydroxyl groups prevents the activity of polyphenol oxidase.

The detoxication function of glucoside formation is probably the most easily established, especially in cell and tissue cultures and excised tissues, but although plants in both terrestrial and aquatic environments are undoubtedly exposed to a multitude of phenolic compounds, the function of the reaction in Nature is unclear.

1.4 Environmental aspects of simple phenolic compounds

Phenol as a pollutant chemical

Major producers of effluents containing phenols are the coking, (coal carbonization), petroleum, and petrochemical industries. Phenols have also been reported as components of the effluent from dyestuffs and wood distillation plants (Water Quality Criteria Data Book, 1970), and phenols and wood-derived phenolic acids are

characteristic products of paper-making processes (Hunter, 1971).

The carbonization of coal typically produces 0.23m^3 of contaminated water/tonne of coal processed, and in 1972, 30 x 10^6t of coal were carbonized in the United Kingdom (Catchpole and Stafford, 1977). Coking or coal gasification for town gas production has declined markedly in the U.K., but the processes envisaged to generate synthetic gases and petrochemical feedstocks from coal to replace petroleum and natural gas also generate phenolic wastes, and the production of metallurgical coke and coal-derived chemicals continues.

Analyses of coking plant effluents (British Coke Research Association, 1962; Barker and Hollingsworth, 1958), more recently reviewed and supplemented by Catchpole and Stafford (1977) and Wheatstone (1977) show that these large volumes of contaminated water (liquor) contain phenol, catechol, resorcinol, all three isomeric cresols, the six isomeric xylenols, three ethylphenols, and various other alkyl-substituted diphenols.

Barker and Hollingsworth (1958; 1959) found that gasification process effluents contained high concentrations of phenol, up to about 3.8g/1 in some stages of the process, and coke-oven effluents contained rather less, up to 2.2g/1, a figure closely matched by later data of Cooper and Wheatstone (1973), who found 2.3g/1 of phenol in ammoniacal liquor. The liquor from a Lurgi coal-gasification process, one method of generating synthetic gases and chemical feedstock from coal, contained 1.23-1.34g/1 of phenol. Of the coke-oven liquors reported by Wheatstone, (1977) to contain 1.35 1.1, and 0.97g/1 of total phenols, phenol itself comprised 0.89, 0.73 and 0.71g/1.

Most phenolic effluents are treated before discharge by digestion, oxidation in ponds or ditches, or in trickling filters, often with efficiencies as high as 90-95% reduction in Biological

Oxygen Demand and phenols content (Ashmore *et al.*, 1967), but Stafford and Callely (1973), investigating a waste-tip percolation and lagoon oxidation method of purification, fed from a Chesterfield, Derbyshire, coking plant having an ammoniacal liquor effluent which contained up to 1.5g/l of total phenols, found that parts of the lagoon where the total phenols concentration always exceeded 1g/l were entirely free of microbial life. Such passive treatment systems are clearly inappropriate for concentrated liquors, and risk escape of highly toxic phenolic liquors to natural waters.

The aromatic content of crude oils (Freegarde *et al.*, 1971) can approach 14% in low boiling-range fractions, and could conceivably be liable to photooxidation to hydroxylated compounds (Freegarde *et al.*, 1971; Dean, 1968), but the petroleum refining and petrochemical industries are probably the main source of oil-derived phenolic compounds.

Catalytic "cracking" of hydrocarbons can yield aqueous scrubbing effluents containing a spectrum of phenols very similar to those found in coal-carbonization and gasification effluents, but most phenols from this process are reclaimed. Phenols concentration in various plant effluents may be of the order of a few hundred mg/l, but the phenols content of "phenolic spent caustic liquor", the scrubber effluent from catalytic cracking, may be 8g/l, after some reclamation of phenols. Diluted effluent water from petroleum processing plants is of the order of 10-25mg/l with respect to phenols (Beychok, 1967).

McKinney, (1963) and Davis, (1967) have discussed the treatment of petroleum industry wastes. From a survey of twelve refineries in the United States, McKinney (1963) gave typical average effluent production as 1100gal/min (5000l/min), containing 27mg/l of total phenols. Gravity separators remove most oil from aqueous refinery wastes, and most phenols with it, and further treatment by digestion

and oxidation in ponds or in trickling filters results in an effluent usually containing phenols at concentrations of the order of 10mg/1. Davis (1967) has described functioning treatment plants for petroleum industry effluents capable of 99.5% reduction in phenols content of the aqueous effluent, from, for example, 285mg/1 to 1.4mg/1, with some effluents reduced to 0.15mg/1 total phenols.

Other than by accidental release, therefore, the major phenol-producing industrial processes discharge large volumes of low-level wastes, which may nevertheless still be toxic, and are in any case highly undesirable in waters which are subsequently drawn for human consumption. Some international and national water quality standards suggest that the concentration of phenols in water to be used for human consumption should be, at most, 1-2µg/1 (World Health Organisation, 1961; World Health Organisation, 1963; United States Public Health Service, 1963). Phenols in water, when present alone, cause unpleasant tastes at 50-100µg/1, and, if chlorinated, at about 5µg/1 (Burtschell *et al.*, 1959; Beychok, 1967).

Phenol concentrations present in natural waters are generally of a low order, even when arising from industrial discharges, but severe phenol pollution is occasionally recorded. Water from the Rivers Cherwell and Mole, in Essex, when analysed by the Water Research Centre (1980), contained, respectively, less than 3µg/1 and less than 3ng/1 phenol. Analyses of water from rivers in Germany (Kunte and Slemrova, 1975), showed phenol to be present in concentrations from 0.24-1.4µg/1. Leachate from landfills, however, contained significantly larger concentrations of phenol, from 2-6mg/1 (Water Research Centre, 1980). Other phenols, such as catechol, quinol, and resorcinol, may be detected at similar concentrations. The major survey at the Water Research Centre (1980) gives many analyses of phenol itself and many other phenolic compounds in effluents and water-bodies in the ng/1-mg/1 ranges.

Simple aromatic compounds present in the environment as a result of natural processes.

Many simple aromatic compounds, often regarded as common plant constituents, have been implicated in the phenomenon of allelopathy (Rice, 1974). Although simple phenols are not commonly implicated in allelopathic interactions, quinol is one of the toxins of chaparral vegetation of the United States (Rice,1974). Phenolic and aromatic acids are more commonly suspected as the toxic agents in such plant interactions (Rice, 1974), and are released in comparatively large amounts by the decay of plant materials (Rice, 1974; Guenzi and McCalla, 1966a, 1966b; Borner, 1960; Guenzi *et al.*, 1967). Whitehead (1964) analysed various soils for their content of phenolic acids, and found concentrations of the order of 10-100μM in the soil solution.

In aquatic environments, release of phenolic materials from decaying plant material is also apparent. Studies of rice soils have shown that aromatic acids are present in the soil solution. Chandramohan *et al.* (1973) found *p*-hydroxybenzoate, vanillate and *p*-coumarate among other phenolic compounds in rice soils and observed that 100μM coumarate was toxic to rice seedlings.

In view of the general occurrence of aromatic or phenolic compounds of undoubted phytotoxicity (Glass, 1976; Borner, 1960; Guenzi *et al.*, 1967) in soils, both terrestrial and aquatic, some detoxicative ability in plants may be expected. Seidel (1966) has observed that the tolerance of aquatic plants to phenol is somewhat higher than that of terrestrial species, even though Pridham (1964) has shown that a number of truly aquatic plant species lacked the ability to glucosylate simple phenols, a characteristic detoxication mechanism. Levin (1971), reviewing the phenolic constituents of plants with respect to their interactions with microorganisms and

insects, drew attention to some contrasts between the secondary constituents of aquatic and terrestrial species; the former, in general, do not produce the spectrum of phenolic compounds found in terrestrial plants. Such differences might be reflected in the major metabolic processes involving phenols.

1.5 Aspects of plant and soil microbiology of importance in the investigation of phenol degradation by plants.

Plants in their natural environment are associated with very large numbers of microorganisms, which inhabit (i) the soil or other medium around plants, (ii) the root zone (rhizosphere), (iii) the root surface (rhizoplane), and (iv) aerial surfaces (the phylloplane). Unless grown from sterilized material, laboratory or any other cultures of plants will be associated with populations of microorganisms derived from the soil from which they were collected, or from their own seeds, or will become associated with organisms from the laboratory or other environment.

Microorganisms associated with plant surfaces.

The primary character of the rhizosphere is that this zone of root influence contains a greater number of microorganisms exhibiting a greater range and extent of activities than root-free soil. Katznelson (1965) regards rhizosphere soil as that which adheres to roots after loosesoil is shaken off. Counts of microorganisms in rhizosphere soil may be as high as 10^9/g of soil, with larger populations of eubacteria, fungi, and actinomycetes than in plant-free soil. The conditions of the rhizosphere favour the predominance of Gram-negative non-sporing rod-shaped bacteria, and this effect is yet more pronounced in the rhizoplane. Species of *Pseudomonas* are particularly common, and members of the genera *Arthrobacter*,

Flavobacterium, *Mycobacterium*, *Cellulomonas*, *Micrococcus*, *Achromobacter*, and *Mycoplana* also occur in large numbers. Fungi are commonly represented by *Fusarium*, *Cylindrocarpon*, *Mucor*, *Rhizopus*, *Penicillium*, and *Aspergillus*, which occur frequently in soil (Burges, 1965; Katznelson, 1965). Many of these fungal species were isolated from washed roots, and are therefore almost certainly rhizoplane, as well as rhizosphere, organisms. The major inoculum of fungi growing on root surfaces is from the soil (Pugh, 1974), although this is probably augmented by an inoculum from seeds. Actinomycetes are widely distributed, occurring commonly in soils (Goodfellow and Cross, 1974) and have been observed attached to plant roots by Williams *et al.* (1970; 1971) who detected *Streptomyces* on rootlets of *Pinus*.

Bacteria of the phylloplane, as in the rhizosphere and rhizoplane, are predominantly Gram-negative, and the major genera are *Erwinia*, *Pseudomonas*, *Flavobacterium*, and *Xanthomonas* (Blakeman and Brodie, 1976). Goodfellow *et al.* (1976), Austin *et al.* (1978), and Dickinson *et al.* (1975) have made numerical taxonomic studies of phylloplane bacteria of *Lolium perenne*, confirming the frequent occurrence of these genera on a graminaceous plant. Bacteria corresponding with marker strains of *Pseudomonas fluorescens*, *Ps.putida* and *Ps.testosteroni* were found, among other Pseudomonads. Estimates of population size of one species, the very common *Erwinia herbicola*, on leaves of graminaceous and leguminous plants range from 10^5-10^9 colonies/g fresh weight of leaf tissue (Last and Warren, 1972). The macerated foliage of such plants yielded 10^5-10^7 colonies/g fresh weight of tissue.

A list of fungal genera isolated from plant leaf surfaces was presented by Dickinson (1976), which included many of those already shown on plant roots.

Although, as Dickinson (1976) noted, the majority of studies of the phylloplane have been carried out on agricultural crop plants, Apinis *et al.* (1972) examined the mycoflora of leaves of *P.communis*, and Pugh and Mulder (1971) that of *T.latifolia*. The latter study showed that although young emerging leaves were populated in the main by bacteria, fungal colonizers such as *Aureobasidium pullulans* occur with increasing frequency as the plant matures. *Alternaria*, *Cephalosporium*, and *Cladosporium* species were also generally well represented. Roots and rhizomes of *T.latifolia* were very sparsely populated with fungi, of a limited range of species. *Cladosporium herbarum* was a prominent colonizer of leaves, roots, leaf sheaths, and partially decayed litter.

Colonization of leaves of *P.communis* by fungi (Apinis *et al.*, 1972) was related to the age of the leaf, and members of the fungal population showed seasonal variability in their occurrence, but a wide range of species was represented, many of which were examined by Cain *et al.* (1968) in a survey of aromatic catabolism by fungi. Forty-nine species were recorded from six habitats, and *Alternaria*, *Cephalosporium*, *Cladosporium*, *Diplorhinotrichum*, and *Phoma* species were common early colonizers. The influence of habitat on the fungal colonization of *P.communis* was strong; only six species were reported from all six sites. Saprophytes and colonizers of senescent and dead leaves were similarly diverse (Apinis *et al.*, 1972).

Last and Deighton (1965) considered that bacteria and yeast-like fungi outnumbered filamentous fungi, and drew attention to the effects of the methods used in the isolation of microbial colonists of leaves. Pugh and Buckley (1971), using several techniques, including surface sterilization, to characterize the closely associated phylloplane mycoflora of *Acer pseudoplatanus* leaves found that *Aureobasidium* and *Sporobolomyces* were common.

In aquatic debris, a major component of the substrate of *P. communis*, microbial populations may be rather dissimilar to those of terrestrial soils. Freshwater aquatic fungi are found (Jones, 1974), and fungal and actinomycete activity may be more significant than that of bacteria in litter decay in freshwater environments (Willoughby, 1974). Litter from a freshwater system examined by Willoughby (1974) bore *Alternaria*, *Candida*, *Cladosporium*, *Fusarium*, *Penicillium*, *Mucor*, *Trichoderma*, and *Phoma* species.

Populations of actinomycetes in waterlogged or peat soils, and in *Juncus* litter, were apparently small (Goodfellow and Cross, 1974; Latter and Cragg, 1967); otherwise information on the importance of actinomycetes in aquatic litter deposits was sparse (Goodfellow and Cross, 1974). Thermophilic actinomycetes have, though, been found in moderate numbers in lake margin litter and peat, and lake muds. It is probable that a large part of such isolates is composed of spores of terrestrial organisms washed into aquatic deposits from surrounding land, and therefore having little influence on the materials and plants of the aquatic environment.

Microorganisms associated with internal surfaces of plant tissues.

Microorganisms which are essentially inseparable from plant tissues may possess biochemical properties which can be confused with those of the host plant tissue. In order to study the aromatic metabolism of plant tissues therefore, the presence and activity of internal microbial populations must be eliminated or satisfactorily accounted for.

A plant-microorganism association of major importance is the establishment of mycorrhizae. Endomycorrhizae are, if not ubiquitous, very widespread among higher plants, and ectomycorrhizae too are found frequently. This subject has been reviewed extensively

(Mosse, 1973; Gerdemann, 1968; Meyer, 1974), but the existence of mycorrhizae in wetland and aquatic plants is rarely reported. Indeed, in surveys and reviews for mycorrhizal associations, Gerdemann (1975), and Khan (1974) found none in wetland species, and Read *et al.* (1976) found that infection of several marsh species was rare, and when present, was of low intensity. These observations were made in plants from central and eastern England, and though in studies of plants from Denmark, Sondergaard and Laegaard (1977) found some evidence of the development of endomycorrhizae in eleven species of twelve, *P. communis* was infected only at a low level.

Many phytopathogens and non-pathogenic internal colonizers are members of genera which dissimilate phenols, both natural and industrial. They may originate in the germinating seed, for Mundt and Hinkle (1976) found many genera of bacteria in isolates from surface-sterilized seed of twenty-seven plant species. These bacteria, including *Pseudomonas*, *Acinetobacter*, *Alcaligenes*, *Brevibacterium*, *Micrococcus*, and *Nocardia* species, conceivably entered seeds *via* the vascular system of the developing ovule, the pollen tube during fertilization, and fissures in the seed coat. The results obtained by Mundt and Hinkle (1976) suggested that infection of seeds arose by chance, and although the frequency and intensity of infection was apparently modified by structural and physiological factors, no particular characteristic could prevent infection entirely except the presence of the hard, waxy seed-coat in, for example, seeds of mimosa or yellowwood. It seems likely therefore that even plants raised from surface-sterilized seed may bear a bacterial inoculum.

Bacteria have been found inside the living cells of some plants (Menely and Stanghellini, 1975), but in the absence of pathogenic conditions, such a phenomenon is probably rare; internal colonization of intercellular spaces of tissues is more likely. Healthy tissues

of several species have yielded isolates of *Pseudomonas*, *Erwinia*, *Bacillus*, and *Xanthomonas* species (Menely and Stanghellini, 1972; Samish *et al.*, 1961; Hollis, 1951). As certain of these may be plant pathogens, some authors suspected that such colonizers are organisms of latent pathogenicity (Hayward, 1974), or represent inapparent or symptomless infections (Gaumann, 1950). Transmission of microorganisms about plants can be systemic; in large plants, isolates of micro-organisms can be made from sites several metres distant from any apparent pathological lesion, the organisms remaining latent in phloem and cambium tissues (Gardner and Kado, 1973; Cameron, 1970). Isolates of *Bacillus megaterium* and *Enterobacter cloacae* from healthy potato tubers were of particular interest, as invasion of the tuber was suspected to be *via* the vascular tissue, and stem samples of potato plants frequently contained bacteria (Hollis, 1951).

Fungal colonization of healthy tissue seems less common, but Kommedahl *et al.* (1979) reported that most of the corn (*Zea mays*) stems examined contained non-pathogenic infections of five *Fusarium* species. Abawi and Lorbeer (1972) found infections of *Fusarium oxysporum* in *Oxalis corniculata*. The fungus, pathogenic in other plants, had no effect on this host. Verhoeff (1974) reviewed the latent or symptomless infection of plants by fungi.

Differentiation between internal and external microbial colonists of plants may be difficult. Leben (1965; 1968; 1971; 1972) and Darbyshire and Greaves (1970; 1971) suggested that bacteria of the phylloplane, especially in buds, and rhizoplane are so closely associated with the plant tissues as to be effectively internal. Bacteria were present on the epidermis in the depressions formed at the junctions of adjacent epidermal cells, and on roots, bacteria were embedded in a discontinuous matrix of about 5µm thickness, closely adpressed to epidermal cell walls.

Hayward (1974) observed that bacteria within plant tissues often resembled the saprophytes of the rhizosphere or phylloplane of the host plant, and *Pseudomonas* species associated with *Pisum sativum* plants were among those found in the root surface matrix material (Darbyshire and Greaves, 1970; 1971). Mechanisms for the internal colonization of leaves were explored by Hildebrand *et al.* (1980), who infiltrated leaves with *Pseudomonas* suspensions, and found that the organisms were trapped in waterfilms and cell interstices within the leaf tissues. Barnes (1965) has also commented on the occurrence of bacteria within intercellular leaf spaces.

Metabolism of phenol by microorganisms.

Both bacteria and fungi possess the ability to degrade and modify aromatic compounds, and several of the species associated with plants in this natural environment have well defined activities against phenol. Some of these microorganisms have been used in laboratory investigations of aromatic biodegradation. The body of information accumulated from studies of microbial aromatic metabolism is used as the model for plant aromatic biodegradation (Kickuth, 1970; Durmishidze and Ugrekhelidze, 1969; Durmishidze *et al.*, 1969).

The unsubstituted aromatic ring, and, to a lesser extent, alkyl-substituted aromatic ring structures, are stable resonance forms, inert to attack by fission-catalyzing enzymes (Dagley, 1975). Monophenols are likewise electronically unsuitable for most enzymically catalyzed fission reactions, but *ortho*-diphenols are ring-fission substrates. Such compounds are cleaved by intra- or extradiol attack, the processes usually described as, respectively, *ortho* and *meta* ring-fission (Fig. 1.2). The essential first stage of phenol metabolism then, is the generation of catechol (Fig. 1.2); this reaction is mediated by aromatic hydroxylases, more properly

Figure 1.2 The reactions of phenol degradation by microorganisms

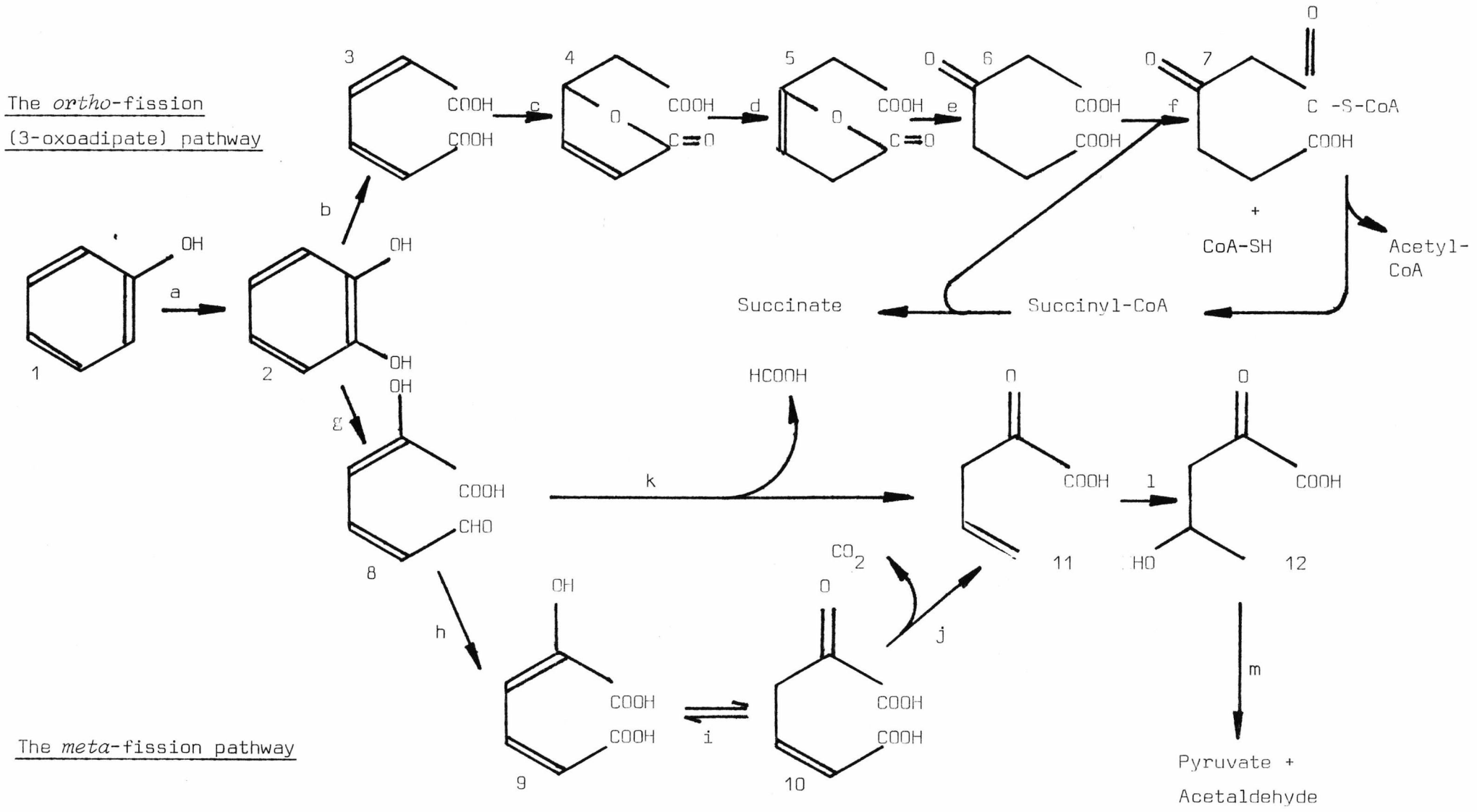
The scheme of reactions is taken from Dagley *et al.* (1960), Ornston (1971), Stanier and Ornston (1973), Dagley and Nicholson (1970), and Williams and Murray (1974).

Key (1) Phenol, (2) Catechol, (3) *cis, cis*-Muconate, (4) (+)Muconolactone, (5) 3-Oxoadipate enol-lactone, (6) 3-Oxoadipate, (7) 3-Oxoadipyl-coenzyme A;

(8) 2-Hydroxymuconate semialdehyde, (9) 4-Oxalocrotonate (enol form), (10) 4-Oxalocrotonate (keto form), (11) 2-Oxopent-4-enoate, (12) 4-Hydroxy-2-oxovalerate;

(a) Phenol hydroxylase - see text, (b) Catechol-1,2-oxygenase, (c) *cis, cis*-Muconate lactonizing enzyme, (d) Muconolactone isomerase, (e) 3-Oxoadipate enol-lactone hydrolase, (f) 3-Oxoadipate-CoA transferase;

(g) Catechol-2,3-oxygenase, (k) 2-Hydroxymuconate semialdehyde hydrolase, (h) 2-Hydroxy-muconate semialdehyde dehydrogenase, (i) 4-Oxalocrotonate tautomerase, (j) 4-Oxalocrotonate decarboxylase, (l) 2-Oxopent-4-enoate hydratase, (m) 4-Hydroxy-2-oxovalerate aldolase.


The *ortho*-fission
(3-oxoadipate) pathway
OH
a
b
OH
OH
1
2
3
COOH
COOH
c
4
O
COOH
C=O
d
5
O
COOH
C=O
e
O
6
COOH
COOH
f
O
7
C -S-CoA
COOH
+
CoA-SH
Acetyl-
CoA
Succinate
Succinyl-CoA
g
OH
COOH
CHO
8
HCOOH
k
O
COOH
11
l
O
COOH
HO
12
CO2
h
OH
COOH
COOH
9
i
O
COOH
COOH
10
j
m
Pyruvate +
Acetaldehyde
The *meta*-fission pathway

monooxygenases, which insert one atom of molecular oxygen into the substrate molecule and reduce the other to water with NAD(P)H. Most well characterized microbial hydroxylases, such as orcinol hydroxylase (Ohta and Ribbons, 1970; Ohta *et al.*, 1975), salicylate hydroxylase (Takemori *et al.*, 1969; White-Stevens and Kamin, 1972), and *p*-hydroxybenzoate hydroxylases (Hesp *et al.*, 1969; Howell *et al.*, 1972) are flavoproteins, though there is some evidence that polyphenol oxidase-like enzymes may be responsible for phenol or other aromatic hydroxylations in some bacteria (Nakagawa and Takeda, 1962; Jayasankar and Bhat, 1966; Seidman *et al.*, 1969; Nambudiri *et al.*, 1972).

The bacterial enzymes responsible for the *o*-hydroxylation of phenol itself are often relatively non-specific, accommodating other monophenols like the cresols, and often *m*-diphenols (Nakagawa and Takeda, 1962; Chapman, 1972; Ribbons, 1966; 1970; Buswell, 1975). The fungal flavoprotein phenol hydroxylase isolated by Neujahr and Gaal (1973) from *Trichosporon cutaneum* was also non-specific.

The ring-fission of catechol is catalyzed by one of two dioxygenases, catechol-1,2-oxygenase and catechol-2,3-oxygenase, which, respectively, promote *ortho* and *meta*-fission (Fig. 1.2). These enzymes are specific for their mode of ring-fission. In bacteria which degrade phenol, either pathway may operate, but not both, and in general, a particular microbial strain or species employs only one of the pathways for dissimilation of the primary substrate (Stanier and Ornston, 1973). Unlike those of the *ortho*-fission pathway, the enzymes of the *meta*-fission pathway are relatively tolerant of substituted compounds and are often induced by alkyl-substituted phenols, and Ornston (1971) suggested that the *meta*-fission pathway is a generalized one for the dissimilation of

a variety of aromatic substrates. Phenol itself induced the enzymes of *meta*-fission in strainsof *Ps.putida* (Feist and Hegeman, 1969a; 1969b) and is also degraded by *meta*-fission in *Alcaligenes eutrophus* (Johnson and Stanier, 1971), *Micrococcus varians* (Jayasankar and Bhat, 1966) and *Ps.aeruginosa* (Ribbons, 1966; 1970). Growth of *Ps.putida* strains with phenol does not necessarily induce the enzymes of *meta*-fission however; the great majority of the strains examined by Fiest and Hegeman (1969a; 1969b) dissimilated phenol *via* the *ortho* route, and of the Pseudomonads, Stanier and Ornston (1973) listed nine species in addition to *Ps.putida* which can dissimilate aromatic compounds in this way.

In the fungi too, aromatic catabolism is widespread. Cain *et al.* (1968) found that extracts of *Fusarium, Aspergillus, Penicillium, Cylindrocephalum, Phoma, Cephalosporium, Polystictus, Mucor, Debaromyces, Aureobasidium,* and *Candida* species all exhibited catechol-1,2-oxygenase activity when grown on appropriate inducer substrates. Representatives of many genera utilized several monophenolic compounds as growth substrates, those related to lignin constituents often giving particularly high growth yields, and *Fusarium oxysporum* utilized phenol as the sole carbon and energy source. Utilization of phenols was generally more restricted than that of benzoic acids.

A very wide range of monophenols was oxidized by the soil yeasts *Trichosporon cutaneum* and *Candida tropicalis* (Neujahr and Gaal, 1973; Gaal and Neujahr, 1979). Phenol itself was dissimilated by *ortho*-fission in these species. Henderson (1961a; 1961b) and Gross *et al.*, (1956) established that catechol and protocatechuate, derived from lignin-related compounds (Henderson, 1961a; 1961b) were dissimilated in the same way, and it seems that *meta*-fission of aromatics is rare in fungi (Cain *et al.*, 1968).

1.6 The aim of the study

The intention of the work performed in this study was to demonstrate the fate of phenol applied to common wetland plant species. The likelihood of exposure of aquatic plants to phenol has been considered, especially with reference to the use of such plants in water pollution control systems. The studies of Seidel and coworkers have suggested that phenol, a toxic compound, and a possible model for other contaminants of water, is metabolized in green plants; this study extends the investigation to another plant species and attempts to account for the earlier observations.

It was anticipated that phenol would be detoxicated on entry to a plant by (i) glycosylation to a less toxic, more readily mobile compound, (ii) by degradation, or (iii) by immobilization. Enzymes responsible for glycosylation, oxidative ring-fission, hydroxylation, and oxidative polymerization of low molecular weight phenolics were investigated, and uptake of [U-^{14}C]phenol and its removal from experimental culture media were demonstrated. Microbiological influences on these processes in particular were considered, as they had been overlooked in other investigations.

Many species of higher plants have been named in the foregoing discussions, but consideration of the flora of wetlands of the United Kingdom suggested that the cosmopolitan species *P.communis*, being widespread and hardy, would be worthy of study. The response of *S.lacustris* to phenol has been investigated in sufficient detail by Seidel's group to suggest that the plant is capable of phenol degradation, but no such information exists for *P.communis*. Previous studies (Seidel and Happel, 1975a; 1975b) have suggested that this species is useful in water-purification plantations (though not necessarily as a scrubber of organic pollutants); in such plantations exposure

to phenols may be problematic unless the plants have some means of disposal of, or resistance to, phenolic compounds. The widespread distribution of *P.communis* implies that some populations of the species will almost certainly be exposed to phenolic pollutants, and therefore an appreciation of the response of the plant to such materials will be of value.

In addition to *P.communis*, several other species were chosen for comparative study on the basis of reports by other workers. *E.crassipes* and *Lemna minor* (Harvey and Fox, 1973) have both been considered as nutrient stripping crop species, in which circumstances their exposure to polluted water is likely, and the response of both to simple phenols has previously been demonstrated, though in little detail. *Mentha aquatica*, *Iris pseudacorus*, and *A.plantago-aquatica* have been examined before with respect to their response to polluted conditions (Seidel, 1963; 1966; 1971; 1973b).

Various preparations of wheatgerm, broadbean (*Vicia faba*), and barley (*Hordeum vulgare*), have been used in investigations of the phenol detoxication reaction by glucosylation, and were used in experiments to test assay systems for this reaction.

Other plant species discussed in Sections 2, 3, 4, and 5, and not already named, were chosen to diversify the initial range of this study. *Iris foetidissima*, for example, was chosen as a terrestrial relative of *I.pseudacorus*, in which contrasting responses to phenol might be shown.

SECTION 2 MATERIALS AND METHODS

2.1. COLLECTION AND SUPPLY OF PLANT MATERIAL

Phragmites communis Trin. and *Lemna minor* L. were collected in April 1976 and November 1975 respectively, from sites within the Stodmarsh National Nature Reserve (Stodmarsh, near Canterbury, Kent) with the permission and assistance of the Warden. The reserve comprises areas of shallow open water, and extensive *P.communis* reedmarsh, some parts of which are permanently flooded, the remainder being flooded annually during the winter. *P.communis* specimens were taken from a site within the summer-dry reedbeds (National Grid Reference TR 231 619, Ordnance Survey Sheet 179, 1:50 000 First Series) and *L.minor* from the surface of a drainage canal (NGR TR 206 611, Ordnance Survey Sheet 179, 1:50 000 First Series). Mature seed of *P.communis* was taken from plants at the collection site described, in October 1975, and stored at room temperature in paper envelopes until required.

Specimens of *Typha latifolia* L., *Typha minima*, *Eichhornia crassipes* (Mart) Solms., *Butomus umbellatus* L., *Alisma plantago-aquatica* L., *Iris pseudacorus* L., and *Mentha aquatica* L. were purchased from the London Aquatic Supply Co. Ltd., Enfield, Middlesex. Specimens of *Iris foetidissima* L. were obtained by dividing mature plants of nursery origin grown by Dr R.B. Cain, University of Kent, Canterbury. *Eriophorum vaginatum* L. specimens were collected from Slaithwaite Moor, a peaty moorland site near Huddersfield, West Yorkshire (National Grid Reference SE 045 148, Ordnance Survey Sheet 110, 1:50 000 Second Series). Limited numbers of specimens of a plant tentatively identified as a *Schoenoplectus* species were taken from Stodmarsh, at a site close to the collection point of *P.communis*. Identifiable specimens of other *Schoenoplectus* species were not

readily available locally, so an ornamental variety, *Schoenoplectus tabernaemontani* var. *albescens* was purchased from the London Aquatic Supply Co.

The identity of all species was confirmed by reference to Clapham *et al.* (1962), except in the case of *E.crassipes*, which was drawn and described by Penfound and Earle (1948). Further descriptions of *P.communis* were found in Haslam (1972).

2.2. CULTURE OF PLANT MATERIAL

2.2.1 Culture of *Phragmites communis* from seed

Mature seed of *P.communis* was shaken from collected inflorescences of the plant. The germination rate of seed sown in moist sand or on moistened filter papers in covered vessels at 15-25°C (night and day temperatures respectively) in a greenhouse was 53% of a sample of 2000 seeds in 5 days. The seed failed to germinate if it was water-saturated, or exposed to constant temperatures of 15 or 20°C, but did not require stratification. Such results confirm previous observations. Haslam (1975) showed that samples of seed from two British populations of *P.communis* required a day temperature above 20°C and night temperature at least 10°C below this for maximal performance. Growth of seedlings in half-strength Hoagland nutrient medium (Table 2.1) was slow, and performance of seedlings known to be derived from the same inflorescence was highly variable. Seedlings for experiments were selected for similar height and developmental state. The small plants were maintained in soil-free culture in half-strength Hoagland medium, supported in 250ml plastic beakers in autoclaved quartz sand until 15cm in height, at which stage groups of seedlings were secured with cotton-wool plugs in the necks of 250ml conical flasks containing

Table 2.1. Hoagland medium as modified by Johnson *et al.* (1957)

The medium components as presented were of half the concentration originally used by Johnson *et al.* (1957).

Macronutrient components	Concentration in finished medium (mM)
KNO_3	3.0
$Ca(NO_3)_2.4H_2O$	2.0
$NH_4H_2PO_4$	1.0
$MgSO_4.7H_2O$	0.5

Micronutrient components	Concentration in finished medium (μM)	
KCl	25.00	single stock solution
H_3BO_3	12.50	
$MnSO_4.H_2O$	1.00	
$ZnSO_4.7H_2O$	1.00	
$CuSO_4.5H_2O$	0.25	
H_2MoO_4 (85% MoO_3)	0.25	
FeEDTA	10.00	

The medium was adjusted to pH6.5 by the addition of 1M NaOH or 1M HCl as required. When required sterile, $NH_4H_2PO_4$ solution was made up and sterilized in half the final volume of the medium, and all other components in the remainder. The two parts were mixed when cool, after autoclaving at 15lb/in^2 for 20 min. All materials were of Analytical grade where available.

nutrient medium. The medium in all cultures was sterilized before use by autoclaving (15 lb/in^2 for 20 min).

Sterile plants were raised from lots of 100 seeds, sterilized before germination in 5ml of calcium hypochlorite reagent (Wilson, 1915) in previously sterilized vials. The sterilant was prepared by suspending 71g solid calcium hypochlorite in 900ml water, with stirring, for 10min. The insoluble material was removed by sedimentation and the hazy supernatant quickly filtered and made up to 1l. Seeds were suspended in the sterilant for 30min and washed in five changes of sterile distilled water. The germination of treated seeds on moistened, sterilized filter papers was 40-50%. Sterility was examined by macerating 5 lots of 100 seedlings (2cm high) in separate 5ml volumes of buffered physiological saline solution, from which 1ml of the macerate supernatant was spread on the surface of Oxoid nutrient agar plates (Oxoid Ltd., London SE1). The plates were incubated at 25°C for 72h; an average of one such plate in five showed the presence of contaminating organisms, indicating a sterility rate of at least 80% in 100 plant lots.

2.2.2. Culture of mature plants of *Phragmites communis*

Whole plants of *P. communis* were collected in April 1976 from a site which had been harvested for reed in the Autumn of 1975, and was therefore clear of hardened reed stems and much litter.

The subterranean parts of the plant were excavated in soil blocks of about 30cm x 30cm in surface area and 40-50cm in depth. Emergent shoots were present at a density of 255/m^2, and unemerged shoots at over 400/m^2, the total area examined being 3m^2. The soil profile of the area from which the samples were taken consisted of

a litter layer 10-20cm in depth, surmounting an horizon (20-30cm) of degraded organic material, becoming progressively richer in mineral material with depth. Below 30-40cm from the surface lay a substratum of dense, reduced, clay. Few, large, rhizomes of *P. communis* were present in the clay horizon; most were present in the organic horizon above it. pH Values of the litter layer, the intermediate horizon, and the clay were, respectively, 6.5, 6.4, and 5.6. These values fell within the rangesnoted by Haslam (1972), of pH 3.6-8.6 for the surface of the substrate material of stands of *P. communis*, and the more restricted range, from pH 5.5 to 7.5, in mature stands.

Adhering soil and litter was washed from the plants, and samples of rhizome selected for culture in the greenhouse and in outside tanks.

Emergent shoots of *P. communis* arise from vertical rhizomes, which develop in early autumn from the advancing horizontal rhizomes. Most aerial shoot buds are the apical buds of rhizomes formed from axillary branches of the horizontal rhizomes, but the terminal, apical, bud can also emerge to form a shoot. In winter, shoot initials lie dormant near the soil surface. The aerial-dormant phase lasts for about four months, and emergence of shoots may occur, if frost does not damage them, in February, but emergence is usually delayed until the warmer months of March to May. Further, later, emergence is also common. Dormancy may be broken by mechanical injury to the rhizome, allowing emergence of shoots if the temperature at the surface is high enough (Haslam, 1969a; 1969b; 1972).

Samples of plant material detached from horozontal rhizomes, consisting of rhizome pieces of at least 2-3 internodes in length,

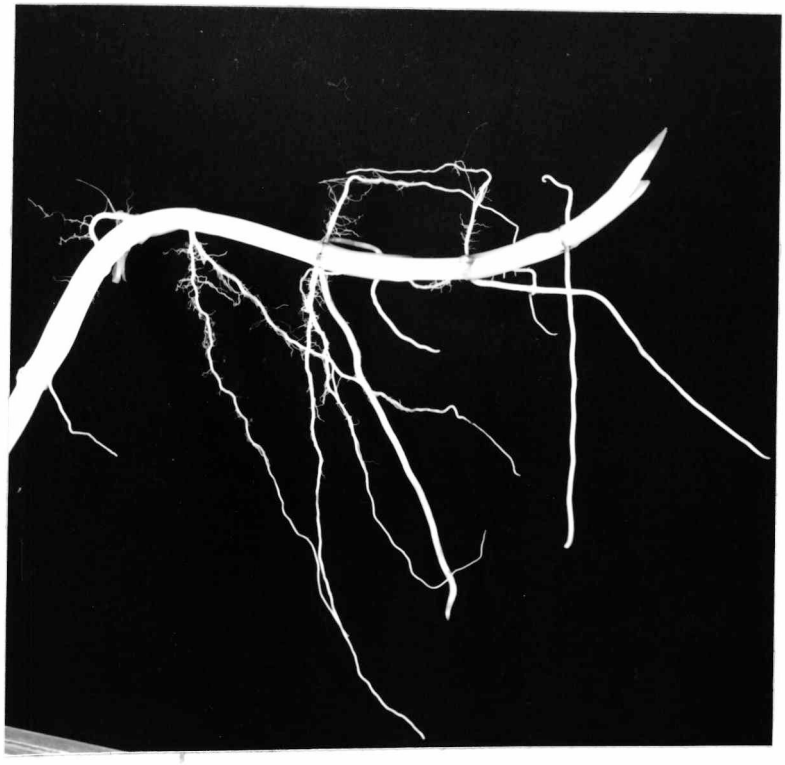
and bearing unemerged aerial shoot buds developed into vigorous plants in the greenhouse. Emergence occurred 5-10 days after potting samples in John Innes No.3 compost (T.Denne and Sons, Wincheap, Canterbury, Kent), watered daily, or placing them in liquid nutrient medium. For culture in liquid medium, the terminal buds of rhizome pieces were anchored with cotton-wool bungs in the necks of 250 or 500ml conical flasks, the volume of medium being sufficient to bathe the entire root mass and rhizomes except the apical internode. The half-strength Hoagland medium (Table 2.1) used in these cultures was sterile, replenished daily with sterile distilled water and changed weekly. All culture flasks were previously sterilized, and were wrapped with foil to exclude light. The temperature in the greenhouse was maintained within the limits of 15°C and 25°C at night and day respectively, and plants subjected to excessive variations in temperature were replaced from stocks held in culture tanks. Lighting was augmented at night by 60-80W warm white fluorescent lamps 1m above the bench.

Cultured material was replenished at regular intervals from stocks held in outside tanks, from which two long harvests of pre-emergent shoots could be made annually. One of these consisted of later developed summer shoots, harvested in April, May and June, and the other was of autumn-formed overwintering shoots, which could be harvested in the winter months and cultured before natural emergence in March and April. Haslam (1969a; 1969b) showed that the size (diameter) of the initial axillary bud producing a shoot-bearing rhizome was related to the stem-base diameter and the eventual height of the emergent shoot, so ascending rhizome pieces of both harvests were chosen to be of similar size, within the range 7-10mm in diameter. By this method, stocks of uniform emergent plants were

obtained. Plate 2.1 shows a typical *P.communis* shoot initial.

Plants from both compost pots and cultures in liquid nutrient medium were grown for 12 weeks, after which time they were 60-70cm in height, and bore seven to ten leaves, excluding the developing leaf at the apex. As Buttery and Lambert (1965) considered that stands of *P.communis* at Surlingham Broad, Norfolk, were, by virtue of their mode of vegetative propagation, essentially clonal, it was judged that the cultured material taken from Stodmarsh would be effectively physiologically homogeneous. Limitations of time and growth facilities presented the desirable course of producing cloned *P.communis* from a single parent tissue specimen.

The culture tank facility consisted of a series of three 100l plastic tanks, set up as shown in Fig. 2.1 in a sheltered position outside the laboratory and greenhouse. After washing the system for 24h with tapwater, concentrated nutrient solutions were added to the circulating water to yield the concentrations of half-strength Hoagland medium (Table 2.1). The medium was replaced annually. Plant material extracted by washing from soil blocks was introduced to the sand layer at a density of about 100 pre-emergent shoots per tank, corresponding to a population density of 370 shoots/m^2. Emergence of the shoots after planting in April 1976 took 7-14 days, following which the aerial parts of the cultured population developed normally. Emergence in this, the first growth season, continued from the last week of April to the last week of May 1976; such plants attained maturity and flowered from the second week of August 1976. Hardened reeds were cut in December 1976 and January 1977, and emergence of new shoots commenced in March 1977, continuing until May. Growth and development in the second and third growing seasons closely resembled the first. Hardened stems were cleared again



10cm

Plate 2.1 Typical shoot initial of *Phragmites communis*. The specimen, taken from outside tank cultures (Section 2.2.2) was an overwintering shoot, lying dormant 1-2cm below the surface of the sand, and was part of a harvest taken for propagation in the greenhouse and laboratory in April 1978. It was harvested, washed free of sand, and photographed at once with no further preparation.

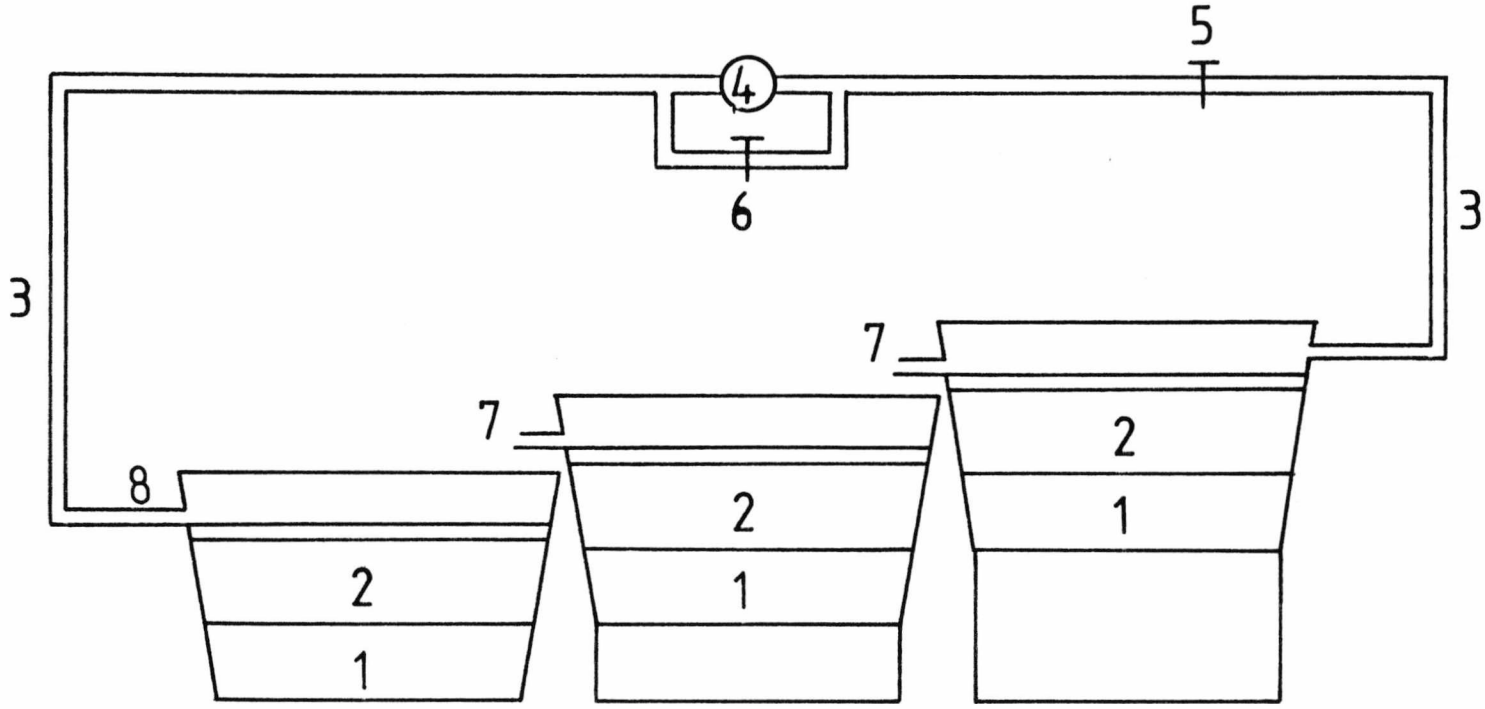


Figure 2.1 Schematic diagram of apparatus for outside culture of *Phragmites communis*.

The three 100l plastic tanks contained coarse flint gravel of 4cm mesh (1), surmounted by 30cm of washed horticultural silver sand (2), the inert filling occupying the 100l nominal capacity of the vessels. Nutrient medium (175l total volume of half-strength Hoagland medium) was circulated through plastic tubing (3) by a centrifugal pump (4), and covered the inert filling, which had a void volume of about 40%, to a depth of 5-10cm. Suitable adjustment of the flow value (5) and the feedback loop valve (6) gave a circulation rate of 2l/min and prevented the pump from running dry. The passage of the medium through the overflows (7) into the next tank in line aerated the medium, and a 100µm strainer at the outflow (8) prevented the ingress of plant and sand debris to the pump.

between second and third seasons. The density of shoots at maturity in the second season (August 1977) was 110 shoots/m^2, within the range reported by Haslam (1969a) for a lightly frosted population at Icklingham, Suffolk. Density in the third growth season was not calculated, as two tanks had by then been disturbed for the collection of rhizome pieces.

2.2.3. Culture of other rooted plant species

Plants of *T.latifolia*, *T.minima*, *E.vaginatum*, *I.pseudacorus*, *I.foetidissima*, *M.aquatica*, *B.umbellatus*, *S.tabernaemontani* var. *albescens*, *A.plantago-aquatica*, and *Schoenoplectus* sp. were separated into viable units of rhizomes where applicable and cultured in the greenhouse by both methods described for *P.communis*. Ondok (1972) also describes the propagation of *S.lacustris* from rhizome pieces in sand-supported hydroponic culture. *M.aquatica* was subcultured from a single parent specimen by placing stem pieces bearing four leaves and two stripped nodes in compost or liquid medium; the two stripped nodes were bathed by medium or covered by the compost and developed roots in 2-3 days. All other species were cultured from original stocks of specimens of equal size and development. A minimum of 10 plants of each species was maintained for two growing seasons in the greenhouse. *M.aquatica* could be reproduced vegetatively throughout the year; the other species were subcultured from March to September in each year. *T.latifolia*, *T.minima*, *I.pseudacorus* and *I.foetidissima* could be subcultured by methods described for *P.communis*, taking rhizome pieces bearing pre-emergent shoots. All species were grown for 12 weeks before harvest for experiments. The conditions in the greenhouse were as for *P.communis*, and all specimens in soil compost were watered daily.

Culture in saturated compost or soil was abandoned, as nutrient leached from the pots into the surrounding water supported heavy growths of algae in the warm greenhouse conditions.

2.2.4 Culture of *Eichhornia crassipes*

Commercially supplied specimens of *E.crassipes* were cultured in 20l of half-strength Hoagland medium (Table 2.1) in a plastic tank in the greenhouse. Juvenile plants arising vegetatively from the original stock were detached and transferred to a constant temperature room, where they were grown at 22 ± 1°C, 18h photoperiod, in 8l of half-strength Hoagland medium in plastic trays. The generation time of the plant, from detachment from parents to the formation of the next generation of viable detached plants was 15 days. Juvenile plants were detached from the parent plants after 5 days' growth of the 'stolons' bearing them. The doubling time of the population (estimated as number of plants) was 8 days. Mature plants produced up to 3 offspring, and plants for experiment were taken 9 days after detachment from the parent. The biology of *E.crassipes* has been described by Penfound and Earle (1948), and Chadwick and Obeid (1966).

E.crassipes could not be maintained in the greenhouse over winter because a sufficiently high minimum temperature could not be achieved, so stocks were repurchased when required in the spring.

2.2.5. Culture of *Lemna minor*

Collected samples of *L.minor* were separated into individual leaves by shaking or teasing, and sterilized by floating them for 4min on the surface of a solution of commercial sodium hypochlorite (Fisons Scientific Apparatus, Loughborough, Leicestershire) diluted to 30% of its original concentration. After such treatment, a small

cluster of cells at the proximal end of the leaves remained unbleached, and new plants arose from this tissue. Treated leaves were transferred singly and aseptically through 5 changes of sterile distilled water to 25ml of sterilized medium (Table 2.2) contained in 100ml flat bottles, stoppered with cotton wool. Cultures were incubated at 22 ± 1°C under constant illumination for 48h. After this initial period, the regenerating plants were exposed to an 18h photoperiod. Of three axenic cultures successfully established from single leaves, those two showing the highest growth rates were selected, and stock cultures of the strains were maintained in 250ml volumes of medium, subcultured every ten days. Sterility was checked at the beginning of each subculture growth period and when samples were taken for experiments, by macerating 10 plants in 10ml of sterile buffered physiological saline solution and spreading 1ml of the macerate supernatant on the surface of nutrient agar plates. Plates were examined for colonies after 72h incubation at 25°C. The medium of cultures was checked by plating 0.1ml samples directly. The growth rates of *L.minor* cultures were estimated by counting leaves, which method generally correlated well with dry weight methods.

2.2.6. Growth of plant specimens in the constant temperature room

At least one generation of all vegetatively produced plant specimens was grown in a constant temperature room at 22 ± 1°C with an 18h photoperiod. The specimens were illuminated by an array of eight 60-80W warm white fluorescent tubes 1m above the bench, *E.crassipes* was cultured routinely under these conditions after detachment of the first generation of vegetatively produced specimens from greenhouse stock. Seedlings of *P.communis* and all stocks of *L.minor* were grown and maintained in the constant temperature room, and in all experiments in which plants

Table 2.2. Culture medium for *Lemna minor*.

Macronutrient components	Concentration in finished medium (mM)
KCl	1.0
$Ca(NO_3)_2$	0.4
$MgSO_4$	0.2
NaCl	0.1
Tris phosphate, monobasic	0.5
Sucrose (anhydrous)	6.0

Micronutrient components

In 1l of finished medium, 1ml of the following single concentrated stock was included:

Disodium EDTA	2205mg/l
MoO_3	170mg/l
H_3BO_3	550mg/l
$ZnCl_2$	300mg/l
$MnCl_2.2H_2O$	180mg/l
$FeCl_3$	250mg/l
$CoCl_2.6H_2O$	15mg/l
$CaCl_2.2H_2O$	0.25mg/l

The medium was adjusted to pH7.0 and sterilized when complete by autoclaving. Sucrose was present as an indicator of microbial contamination, and was omitted from test media using phenol.

were exposed to phenol, these conditions were used unless stated otherwise in the text.

The temperature of the medium in closed vessels containing *L.minor* was necessarily higher (at 25°C) than the controlled air temperature and the vessels containing the root and rhizome parts of other plant species were foil-wrapped to exclude light and to prevent the heating effect.

2.3. MICROBIOLOGICAL METHODS

2.3.1. Isolation of phenol-degrading microorganisms from plant tissue surfaces

Samples of leaf, stem, rhizome, and root tissues were cut, using sterilized instruments, from replicates of plant specimens which were to be exposed to phenol. Duplicate sections or discs of each specimen (1cm in length or diameter) were rolled or blotted (Potter, 1910) onto the surface of mineral salts agar containing (g/l) $(NH_4)_2SO_4$, 0.50; KH_2PO_4, 0.50; $MgSO_4$, 0.05; agar (Oxoid CM3, Oxoid Ltd, London SE1), 12; and 1ml/l of Barnett and Ingram (1955) micronutrient solution. The medium was adjusted to pH7.2 and sterilized by autoclaving. Filter-sterilized phenol was added to 1mM concentration after cooling to about 50°C. After incubation at 25°C for 72h, the plates were scored for the appearance of microbial colonies.

2.3.2. Isolation of phenol-degrading microorganisms from surface-sterilized plant tissues.

Samples of tissues as already described (Section 2.3.1) were cut from plant specimens and quickly weighed. The samples were sterilized by exposure for 30min to acidified mercuric chloride

sterilant (Vincent, 1970) (0.1% w/v $HgCl_2$, with the addition of 0.5ml of 1M HCl per 100ml of solution) following 1-2s immersion in 95% (v/v) aq. ethanol as a wetting agent. After washing by transfer through six changes of sterile distilled water, the samples were macerated in known volumes of buffered physiological saline solution (8.00g/l NaCl, 0.34g/l KH_2PO_4, 1.21g/l K_2HPO_4, pH7.3, sterilized by autoclaving) or homogenized in the same medium in a sterile Virtis blender (Virtis Research Equipment, Gardiner, New York). The homogenates were allowed to sediment to remove large particles, and 1ml aliquots of the supernatant, serially diluted to 1 in 10^6, were spread on nutrient agar, nutrient agar plus 1mM phenol, and on phenol-mineral salts agar (Section 2.4.1). Phenol-mineral salts agar plates were replica-plated to further plates of the same medium with sterile velvet pads (Lederberg and Lederberg 1952) after 72h incubation, to eliminate organisms which may have been growing at the expense of plant constituents in the spread macerate. All plates were scored after 72h incubation at 25°C, and the number of viable organisms (colonies) per gram fresh weight of plant tissues calculated.

For counts of microorganisms in the culture media of sterile and non-sterile plants exposed to phenol, duplicate 1ml aliquots of such plant culture media were serially diluted to 1 in 10^6, and the dilutions plated on nutrient agar, nutrient agar plus 1mM phenol, and 1mM phenol-mineral salts agar by the drop-count method of Miles and Misra (1938). Colony counts were made after incubation at 25°C for 72h.

2.3.3 Phenol die-away experiments

Phenol disappearance was examined in die-away tests run in three different media, containing phenol at appropriate concentrations

described in the text: (i) River water collected in previously sterilized containers from a drainage canal adjoining the *P. communis* collection site, filtered through Whatman No.1 papers and used unsterilized; (ii) Half-strength Hoagland medium as used in plant cultures, used sterile and non-sterile; (iii) Organisation for Economic Co-operation and Development (OECD, 1971) Standard Test medium, the components of which are shown in Table (2.3). Duplicate 1l volumes of each medium were incubated at 25°C in the dark, and were shaken at 60 rev/min on a rotary shaker. Samples were taken aseptically for colorimetric determination of phenol. Inocula for sterile media were prepared from 100g soil samples taken in sterile containers from the root zone of *P. communis* plants at the collection site and in soil compost greenhouse pots. The greenhouse pots contained 12-week-old plants. The soil samples were suspended with vigorous shaking in 1l of tapwater, dechlorinated by standing at room temperature for 48h, and the supernatants, after 30min sedimentation, were filtered through Whatman No.1 papers, the first 200ml of filtrate being discarded. 2ml of the hazy filtrates were used as inocula.

2.3.4 Preparation of plant material for microscopy

Samples of root and rhizome tissues of five *P. communis* specimens from pots and from soil-free cultures were washed in sterile distilled water. Short sections of root and thick hand sections of rhizome were stained in lactophenol - cotton blue for 10-30min, washed in distilled water, mounted in 50% (v/v) aq.glycerol, and examined at once by light microscopy for evidence of blue-stained fungal mycelia. It was found unnecessary to clear specimens unless they were to be examined for internal

Table 2.3. OECD (1971) Standard Test Medium for die-away experiments

Constituent	Concentration in finished medium (mg/l)
KH_2PO_4	8.50
K_2HPO_4	21.75
$Na_2HPO_4.2H_2O$	33.40
NH_4Cl	1.70

The following constituents were added separately, in the order in which they are shown, with mixing before each subsequent addition.

Constituent	Concentration in finished medium (mg/l)
$MgSO_4 \cdot 7H_2O$	22.50
$CaCl_2$	27.50
$FeCl_3 \cdot 6H_2O$	0.25

The pH of the medium was 7.20 $\pm$ 0.05 without further adjustment. When the medium was required sterile $MgSO_4$, $CaCl_2$, and $FeCl_3$ were added to cooled, autoclaved medium with filter-sterilization.

fungal growth, for which samples were cleared for 24-48h in KOH before staining.

For survey of the external and internal structures of plant tissues for visible contamination by microorganisms by scanning electron microscopy, tissue samples cut from plants were washed and fixed for 24h at room temperature in a solution of 2.5% (v/v) glutaraldehyde and 10mM $CaCl_2$ in 85mM cacodylate buffer (pH7.2). The specimens were washed free of fixative and dried by successive suspension for 3h in 25% (v/v) aq. acetone, 3h in 50% (v/v) aq. acetone, and 24h in anhydrous acetone, with three changes of the last. After the final change of acetone, the specimens were dried with liquid CO_2 in a critical-point drier (Polaron Equipment Ltd, Middlesex), trimmed to expose fresh internal surfaces, mounted with colloidal silver, and gold-coated in a Polaron splutter-coater. Prepared specimens were examined on a Cambridge Stereoscan S.60 scanning electron microscope (Cambridge Instrument Co., Cambridge).

2.4. PURIFICATION OF COMPOUNDS TO BE USED AS SUBSTRATES AND CHROMATOGRAPHIC STANDARDS.

Phenol was purified from reagent grade material by redistillation under O_2-free dry N_2, the fraction distilling at 181-183°C being collected. Material prepared thus had melting point 42°C (uncorrected), was chromatographically homogeneous in all solvent systems used (Section 2.5) but had pink-coloured crystals, and appeared, on drying in a desiccator over silica-gel desiccant, to contain water. Redistilled phenol was therefore recrystallized three times from warm benzene. The benzene was evaporated under O_2-free dry N_2 to promote crystallization, and traces of the solvent were removed from

the purified material by drying *in vacuo* overnight, after which no further weight loss occurred. The recrystallized phenol (m.p. 42°C, uncorrected) was used as the substrate for biological experiments, and as a chromatographic standard.

Catechol was initially purified by sublimation onto a cold-finger condenser under O_2-free dry N_2, but the collected catechol had both plate-like and needle-like crystals, was grey-brown in colour, and was not chromatographically homogeneous. In most solvent systems used, a di- or polyphenolic, relatively immobile, product was detected. Catechol for experimental use was therefore purified by thrice recrystallizing resublimed material from hot benzene under O_2-free N_2. The final product, dried overnight *in vacuo*, consisted of white crystals (m.p. 104-105°C, uncorrected).

Phenyl-β-*D*-glucopyranoside (phenyl-β-*D*-glucoside) was purchased from Sigma Chemical Co., Poole, Dorset. As supplied, the material was chromatographically homogeneous, contained no phenol (estimated by the 4-aminophenazone method) and no free glucose (estimated by glucose oxidase by the method of Lloyd and Whelan, 1969). Upon hydrolysis with commercial β-glucosidase (Sigma, almond emulsin) the only phenolic compound detected by chromatography was phenol, which was released in stoicheiometric yield. The material was used as supplied for enzyme and chromatographic experiments, but was recrystallized three times from hot water before use in isotope-trapping experiments.

Arbutin (quinol-β-*D*-glucopyranoside), purchased from Sigma, was similarly hydrolyzed and found by chromatography to contain only quinol as the aglycone.

All other phenolic and other substrates were of usual commercial quality; their provenance is shown where appropriate.

2.5. CHROMATOGRAPHY

Paper chromatography was performed in the descending mode on Whatman No.1 paper, developed with 2 solvents: butan-1-ol/acetic acid/water (4:1:5, upper phase used) and butan-1-ol/benzene/acetic acid/water (2:2:2:1, single phase), all proportions by volume.

Phenolic compounds were located by spraying air-dried chromatograms with a 2%(w/v) solution of 2,6-dichloroquinone chloroimide (Gibbs' reagent) in chloroform. Di- and polyphenolic compounds gave a brown, red, or purple colour reaction with this reagent; monophenols developed blue colourations on the application of an overspray of 10%(w/v) aq. Na_2CO_3 solution. The colour reaction of di- and polyphenols changed on addition of the alkali, giving colours characteristic of the compounds present. Phenyl-β-*D*-glucoside cannot be detected by application of these reagents, but was hydrolyzed on the paper by spraying with 10ml per sheet of a solution of 3mg (10-15 units) of commercial β-glucosidase (Sigma) in 10ml 0.2M disodium hydrogen orthophosphate — 0.1M citric acid buffer (pH5.25). Chromatograms were incubated at room temperature until dry, and Gibbs' reagent spray was then used to detect the phenol released by hydrolysis. Arbutin reacted on chromatograms as a monophenolic compound if detected before enzymic hydrolysis, but developed the brown colour reaction of quinol if located after treatment with enzyme.

Thin-layer chromatography (t.l.c) was carried out on both cellulose and silica-gel plates. Cellulose plates were either prepared in the laboratory from cellulose powder (Type 100 from Sigma), with a coating thickness of 0.50 or 0.25mm, or were obtained commercially with 0.16mm layer thickness (Kodak Ltd, Kirby, Liverpool). Precoated plates were routinely cut into 5 x 20cm or 10 x 10cm pieces.

Silica-gel t.l.c. plates of 0.1mm layer thickness were purchased from Kodak.

Solvents used on cellulose thin-layer plates were (1) benzene/methanol/acetic acid (45:8:4); (2) 6% aq. acetic acid; (3) benzene/acetic acid/water (6:7:3, upper phase); (4) benzene/acetic acid/water, (10:4:1, monophasic); (5) butan-1-ol/ethanol/water (40:11:19); and (6) *tert*-butanol/acetic acid/water (3:1:1), all proportions by volume.

Solvents used to develop silica-gel plates were (7) chloroform/acetic acid (9:1); (8) benzene/ethyl acetate (55:45); (9) benzene/ethanol (10:1), all proportions by volume. The plates were not activated before use,

The methods of detection of phenolic and glucosidic compounds were essentially as described for paper chromatograms.

2.6 ESTIMATION OF SUBSTRATES AND PRODUCTS

2.6.1 Estimation of phenol

Concentrations of phenol in aqueous solution were estimated colorimetrically with 4-aminophenazone [4-(dimethylamino)-1,2-dihydro-1,5-dimethyl-2-phenyl-3H-pyrazol-3-one)] (British Drug Houses Ltd., Poole, Dorset). The method was modified from that of Snell and Snell (1953). Similar methods were described and discussed by Allen *et al.* (1974), with special reference to analyses of natural and waste waters.

To 5ml of a sample containing between 0.05µmol and 0.5µmol phenol was added 0.5ml of 6M aq. NH_3 solution, 0.25ml of 3% (w/v) 4-aminophenazone solution, and 0.5ml of 10% (w/v) potassium ferricyanide solution, with mixing after each addition. The absorbance of the mixture at 510nm was determined within 15min of the final addition.

Blanks contained no phenol. The absorbance of the reagent mixture was linear with respect to phenol concentration over the range described, which, with suitable sample dilution and use of an appropriate spectrophotometer with scale expansion facility, could accommodate estimations of phenol from 1μM upwards.

Protein extracts and plant homogenates had no significant effect on the method within the ranges of protein concentration encountered. The phenolic glucosides and mono- and diphenols discussed elsewhere caused no significant interference in the estimation of phenol.

2.6.2. Estimation of Catechols

The method for estimation of catechols was derived from that of Nair and Vaidyanathan (1964). To 0.5ml of a solution containing up to 1μmol of catechol was added 0.5ml of 10%(w/v) trichloroacetic acid, 1ml of 10%(w/v) sodium tungstate, 0.5ml of 0.5M HCl, and 1ml of 0.5%(w/v) $NaNO_2$ solutions. The reaction mixture was shaken after each addition. When the solution had stood for 5min at room temperature after the last addition, 2ml of 0.5M NaOH was finally added, with immediate mixing, and the absorbance at 510nm determined within 15min.

The assay as described is group-specific for *ortho*-diphenols, and was used to estimate catechol and 4-methylcatechol. The absorbance of the coloured product in the latter case was determined at 495nm.

Phenol, *p*-cresol, and quinol did not react in this assay, and did not affect the absorption of the diphenol product.

2.6.3. Estimation of *p*-Hydroxycinnamic (*p*-coumaric) acid;

The method of Vaughan and Butt (1969b) was used to estimate

concentrations of *p*-coumaric acid. Samples of 0.3ml, containing up to 0.5µmol of *p*-coumaric acid, were diluted to 3.3ml with distilled water, then 1ml of $HgSO_4$ reagent (30g of $HgSO_4$ dissolved in 200ml of 2.5M H_2SO_4, filtered before use) was added with shaking, and the mixture heated on a boiling water-bath for 10min. After cooling at room temperature for 60min, 0.8ml of 0.5%(w/v) $NaNO_2$ was added, and the mixture shaken and allowed to stand for a further 20min. The absorbance of the product was determined at 490nm. Caffeic (3,4-dihydroxycinnamic) acid did not react in this assay.

When the method was applied to incubation mixtures containing some plant extracts, a precipitate formed on heating. This was removed by centrifugation, the supernatant being decanted and retained. The pellet was resuspended in a mixture of 1ml of distilled water and 1ml of $HgSO_4$ reagent, and heated for 10min. After centrifuging the sample again, the supernatant was added to the first for analysis.

2.6.4. Estimation of Caffeic acid.

Concentrations of caffeic acid in solutions were determined by the method of Vaughan and Butt (1969b). Samples of 2ml, containing up to 1µmol of caffeic acid at pH 3.2-3.4 (adjusted with 1M NaOH or 1M HCl) were treated with 2ml of 5%(w/v) aq. acetic acid and 2ml of 0.5%(w/v) $NaNO_2$ solution. After standing at room temperature for 5min, the absorbance of the red product was determined at 525nm.

2.7. PREPARATION OF CELL-FREE PLANT TISSUE HOMOGENATES.

Plant tissues were cut from roots, rhizomes, or leaves, weighed, washed well in sterile distilled water, cut into small segments, and quickly chilled in 3-4 volumes of previously cooled 0.1M

potassium phosphate buffer, pH7.0. The tissue was homogenized, with the addition of 1-2g of quartz sand, with a cold pestle and mortar. The homogenate supernatant was strained through four layers of washed cotton muslin, and the residue re-extracted with a further 1-2 vol. of buffer. After straining, the two homogenate supernatants were pooled and centrifuged at 1000*g*. The temperature was maintained at 0-4°C throughout. The 1000*g* supernatant served as the source of enzymes (crude extract).

Fractionation of crude extract by centrifugation is described where appropriate in the text.

The protein content of extracts was determined by the method of Lowry *et al.* (1951) after dilution of the samples in 1M KOH. The standard protein used for calibration was bovine serum albumen (Sigma, Fraction V), dried to constant weight in a desiccator.

2.8 ENZYME ASSAY METHODS

2.8.1 Spectrophotometric assay of Monophenol, dihydroxyphenylalanine: oxygen oxidoreductase (EC.1.14.18.1) (Monophenol monooxygenase; polyphenol oxidase).

The 1ml assay volume in 1cm light-path quartz cuvettes contained, in 100µmol potassium phosphate buffer, pH7.0; 0.1, 1.0, or 10.0µmol substrate, and 25-250µl of cell-free extract. The reaction was started by addition of substrate to the cuvette. Assays were performed at 25°C in a Pye Unicam SP1800 double-beam spectrophotometer with a Unicam SP45 chart recorder. Where changes in the absorption spectrum were complex or unknown, the absorption spectrum of the incubation mixture was determined by scanning from 210 to 700nm at regular intervals using the fast scan mode (50nm/min). If the absorption maximum of the product was known, kinetics of the

reaction were observed at a single wavelength only. Results are expressed as rate of change of absorbance per unit time, ΔA/h or ΔA/min at the appropriate wavelengths, representing the appearance of reaction products.

2.8.2 Polarographic assay of Polyphenol oxidase:

Assays were carried out in 1 or 2ml volumes in the chamber of a Clark type oxygen electrode (Rank Brothers, Bottisham, Cambridge), stirred at a constant rate throughout. Oxygen consumption was monitored on a Perkin-Elmer PE165 chart recorder. Oxygen content of the solution at 25°C was taken from Chappel (1964) as 0.237μmol O_2/ml.

Substrate solutions (kept at incubation temperature unless unstable) and enzyme preparations were introduced into temperature equilibrated buffer (100mM potassium phosphate, pH7.0) in the chamber with microsyringes. Reactions were started by introduction of the phenolic substrate after stabilization of the instrument baseline or measurement of the endogenous O_2 consumption rate.

When attempting to determine kinetic constants for enzymic activities, O_2 concentration in the buffer was reduced when necessary by passing N_2 through a fine needle into the chamber until the O_2 concentration of the buffer, as measured by the recorder, reached the approximate level required. Experiments were then started immediately.

2.8.3. Spectrophotometric assay for β-*D*-Glucoside glucohydrolase (EC.3.2.1.21) (β-glucosidase).

β-Glucosidase activity was assayed by spectrophotometric determination of the activity of the enzyme against *p*-nitrophenyl-β-*D*-glucoside (Sigma). The extinction coefficient of the aglycone,

p-nitrophenol, at 400nm was taken as 18300 at pH10.2 (Duerksen and Halvorsen, 1958) and determined as 10000 at pH7.0 in 100mM potassium phosphate buffer. Halvorsen (1966) gives ε 9600 at pH6.8. These values were confirmed by experiment using recrystallized *p*-nitrophenol.

In assays to screen for the presence of β-glucosidase activity in extracts the 1ml assay volume contained up to 0.25ml of cell-free extract and 10μmol of substrate in 100μmol of potassium phosphate buffer, pH7.0. Change of A_{400} was determined after single 1h incubations at 25°C, the reaction being initiated by addition of the substrate.

For quantitative determinations of enzyme activity, a discontinuous assay method was used. Incubation mixtures (1ml) in the desired buffers contained up to 0.5ml of cell-free extract and appropriate concentrations of substrate. After starting the reaction by addition of substrate, 0.1ml samples were withdrawn at intervals and mixed with 0.9ml of 0.4M glycine buffer, pH10.2, which stopped the reaction. The A_{400} was determined, and the time course of the reaction determined graphically. Descriptions of the buffers and substrate concentrations used are given in the text.

2.8.4 Assays for glucosylating activity (UDPglucosyltransferase: UDPglucose; phenol β-*D*-glucosyltransferase) (EC.2.4.1.35)

This activity can be assayed in two ways. The formation of UDP (uridinediphosphate) may be determined by the addition to reaction mixtures of phosphoenolpyruvate (PEP) and pyruvate kinase (PK). In the presence of potassium and magnesium ions, UDP and PEP are converted to uridinetriphosphate and pyruvate, which latter may be estimated colorimetrically as its 2,4-dinitrophenylhydrazine derivative. Such methods were employed by Yamaha and Cardini

(1960a; 1960b) and were similar to those of Cabib and Leloir (1958).

The formation of pyruvate may also be assayed by its reduction to lactate in the presence of NADH and lactate dehydrogenase (LDH), when the decrease in absorbance at 340nm is measured. Such an assay may be performed continuously, or may be used to give an end-point result of pyruvate concentration in suitably inactivated samples (Breckenridge and Crawford, 1960; Passoneau and Rottenberg, 1973; Grassl, 1974).

Alternatively, the concentration of uridinediphosphateglucose (UDPG) remaining after the activity of glucose-transferring enzymes may be assayed with the specific UDPG-dehydrogenase (Keppler and Decker, 1974). NAD is converted to NADH in this system, and the appearance of the latter is determined spectrophotometrically. This assay is performed in inactivated and usually in deproteinized incubation mixtures containing UDPG.

Wheatgerm (from Sigma and purchased locally) and broadbean seed (*Vicia faba* var. *Aquadulce* and var. *Longpod*, both from Suttons Seeds Ltd., Torquay, Devon, purchased locally) were prepared, respectively, by the methods of Yamaha and Cardini (1960a; 1960b) and Pridham and Saltmarsh (1963).

For wheatgerm, 100g of the material was suspended in 300ml of 50mM potassium phosphate buffer, pH7.0, and the resulting slurry was stirred slowly at 2°C for 2h. After centrifugation at 16,000*g* for 20min, the clear supernatant was fractionated by the addition of solid ammonium sulphate, that fraction obtained by precipitation between 20 and 50% saturation being retained. The pellet collected by centrifugation at 16,000*g* for 20min was dissolved in 50mM potassium phosphate buffer, pH7.0, 1mM with respect to EDTA and cysteine, in a total

volume of 102ml. This extract, made and kept at 2°C throughout, contained about 100mg/ml of protein, measured by the method of Lowry *et al.* (1951), and corresponded with fraction 1 of Yamaha and Cardini (1960a; 1960b). No further purification was attempted.

For broadbean preparations, 17.5g of dry seeds, with the testas removed, were macerated with 52.5ml of ice-cold 50mM sodium phosphate buffer, pH7.0, in a blender. The slurry was allowed to stand for 2h at 2°C, and centrifuged at 16,000*g* for 20min at 2°C to yield a clear supernatant, which was dialyzed against 50mM sodium phosphate buffer, pH7.0, at 5°C for 16h. This extract, containing about 100mg/ml protein, was used as the source of enzyme.

For other plant tissues, the method of Section 2.7 was followed, and all extracts, including those of broadbean and wheatgerm, were made with both sodium and potassium phosphate buffers, pH7.0, with and without the addition of EDTA and cysteine to 1mM concentration. Wheatgerm and broadbean extracts were also made without the ammonium sulphate precipitation and dialysis steps.

For assay of glucosylating activity by derivatization of pyruvate, incubation mixtures (total volume 0.1ml) contained (µmol), 0.1-0.5 phenolic substrate, 0.1-0.5 UDPG (Sigma), 0.1 EDTA, 0.1 cysteine, 20 Tris-maleate buffer, pH6.8, and up to 50µl of enzyme preparation. After incubation at 37°C or 25°C for periods up to 60min, the reaction mixture was inactivated by heating at 100°C for 3min. To the cooled sample was added 2.5µmol KCl, 2.5µmol $MgSO_4$, 20µmol Tris-maleate buffer, pH6.8, 1µmol PEP (Sigma), and 1 unit of PK (Sigma, Type II, from rabbit muscle), to a total volume of 0.2ml. After incubation at 37°C for 15min, 0.3ml of 0.1%(w/v) 2,4-dinitrophenylhydrazine solution was added, with mixing, and incubation was continued for 5min. 0.4ml of 10M NaOH was then added, and the mixture was finally diluted with 2.2ml of 95%(v/v) aq. ethanol. The absorbance of the pyruvate

2,4-dinitrophenylhydrazone was determined at 520nm, using as blanks assays which contained no phenolic substrate or no UDPG. Test assays prepared with known quantities of UDP (Sigma) or pyruvate (Sigma) yielded stoicheiometric results.

For assays of UDP formation by coupling PK and LDH systems, assay mixtures were designed after Grassl (1974) for an end point assay, and after Passoneau and Rottenberg (1973) for the kinetic assay.

Assays by the former method contained, in a total volume of 1ml, 100µmol of Tris-HCl or Tris-maleate buffer, pH7.5, 0.2µmol of NADH, 2µmol of PEP, 5µmol of $MgSO_4$ 10µmol of KCl, 20 units of LDH (Sigma Type II, from rabbit muscle), and 20 units of PK.

To this was added 0.1ml of heat inactivated reaction mixture containing plant extract, phenolic substrate, and UDPG, as already described. The decrease in absorbance at 340nm was monitored after incubation for 15min, the extinction coefficient of NADH being taken as 6220 (Dawson *et al.*, 1969). Assays were made at 37°C.

Continuous assay mixtures contained, in total volumes of 1ml, 0.2% bovine serum albumen as a stabilizer, and (µmol) 100 Tris-HCl buffer, pH7.5, 10 hydrazine - HCl, pH7.5, 50 KCl, 5 $MgCl_2$, 1 EDTA, 1PEP, 0.2NADH, 0.1-5 phenolic substrate, 0.1-5 UDPG, 20 units of PK and 30 units of LDH, and up to 100µl of plant extracts to initiate the reaction. Blank assays contained no phenolic substrate, and absorbance at 340nm was monitored continuously. Assays were made at 25°C.

Assays for UDPG with the specific dehydrogenase contained, in a total volume of 1ml (µmol), 500 glycine buffer, pH8.7, 6 NAD, 8 EDTA, 0.1 unit of UDPG dehydrogenase (Sigma) and a sample of plant extract incubation mixture containing up to 0.1µmol UDPG. After incubation at 37°C for 30min, the increase in absorbance at 340nm

was measured, using as blanks samples which contained no UDPG. Plant extract incubation mixtures of 0.1ml were inactivated by heating (100°C for 3min) before assay, and after centrifugation on a bench-top semi-micro centrifuge, required no further deproteinization.

2.8.5 Spectrophotometric detection of phenol ring-fission products.

The *meta*-fission product of catechol, 2-hydroxymuconate semialdehyde (ε, 44,000 at pH7.5, λmax 375nm; Nozaki, 1970) and its *ortho*-fission product, *cis,cis*-muconate (ε 17,000, at pH7.5, λmax 260nm) show substantial spectral differences from catechol itself at these wavelengths (Nakazawa and Nakazawa, 1970); hence the detection of these products in reaction mixtures of plant extracts and the appropriate phenols should be simple.

In a total volume of 1ml (3ml), assay mixtures contained 0.1-10μmol (0.3-30μmol) of phenolic substrate, 100μmol (300μmol) of potassium phosphate buffer, pH7.0, and 25-250μl (25-750ul) of cell-free plant extracts, and were incubated at 25°C. The reaction was initiated by the addition of the substrate, which was omitted from blank assays. Absorbance at either 375 or 260nm was monitored continuously.

2.9 RADIOCHEMICAL METHODS

Uniformly labelled [U-^{14}C] phenol was supplied by The Radiochemical Centre, Amersham. Its specific radioactivity was 58Ci/mol.

The radioactivity of plant extracts, tissues, and other samples was determined by scintillation counting on either Packard (Packard Tri-Carb 3375 or 3003 scintillation spectrometers, Packard Instruments Ltd, Caversham, Berkshire) or Beckman (Model LS-200B, Beckman-RIIC Ltd, Glenrothes, Fife) instruments. Corrections for quenching were

obtained either by using the Automatic External Standard systems attached to these instruments, or by internal standardization with [1-^{14}C] *n*-hexadecane (The Radiochemical Centre) of known specific radioactivity. The counting medium used throughout was a commercial phase-combining scintillation fluid (PCS, Hopkins and Williams Ltd, Romford, Essex) capable of dispersing aqueous solutions. Where whole tissues or extraction residues were to be measured for radioactivity, they were digested overnight in a commercial tissue solubilizer ("Soluene", Packard) before addition of scintillation fluid. Samples were dispersed in either 2ml of scintillation fluid contained in small-volume vial inserts, or in 10 or 20ml of fluid in standard 2.5cm diam. plastic vials. There was no significant difference in counting efficiency between the methods used.

Thin layer chromatograms containing radioactive materials were made on 4 x 20cm strips or cut to this size from 20 x 20cm plates after development with enzyme spray and chromogenic reagents. The strips were passed through a Packard Model 7200 Radiochromatogram Scanner at low speed (5 or 10min/cm, time constant 100, collimator slitwidth 2mm) to detect the position of radioactive compounds. The machine was operated in the 2π mode, using a 98% argon: 2% isobutane gas mixture at a flow rate of 100ml/min.

SECTION 3 RESULTS

3.1. INTRODUCTION: DEGRADATION OF PHENOL IN THE ENVIRONMENT

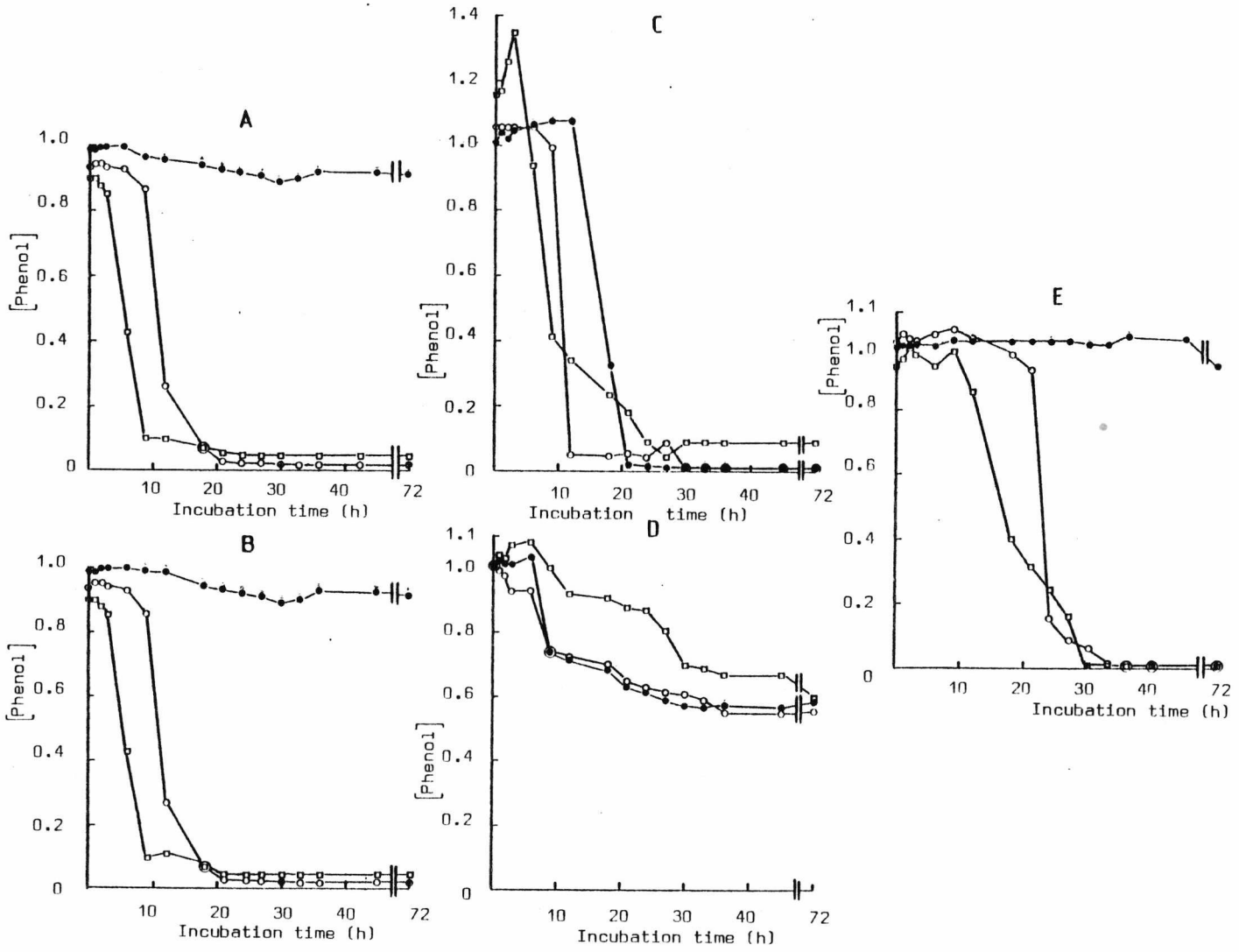
Phenol is commonly reported as a pollutant of water, is readily degraded by many microorganisms, and is not regarded as a recalcitrant organic pollutant. Several experiments were performed to assess the ease with which phenol might be degraded in natural conditions, including those in which the plant species examined grew, using modifications of the standard 'dieaway' test of the Organization for Economic Cooperation and Development (OECD, 1971) with a variety of inocula. This method was designed to assess the biodegradability of organic compounds under conditions which imitate those of natural waters, but with the closer control and repeatability obtainable by the use of defined media and laboratory conditions.

Phenol was added to the media shown in Fig.3.1at concentrations of 1mM, 100μM, and 10μM. As analysis for phenol was carried out by the colorimetric 4-aminophenazone method, experiments requiring the estimation of very low (less than 1μM for example) concentrations were not carried out. The media and the production of inocula are described in Section 2.4.3. The choice of media and inocula was intended to show the degradation of phenol by microorganisms associated with greenhouse plant specimens, the microflora of the immediate natural environment of *Phragmites communis*, and the adventitious aerial inoculum of the laboratory, in synthetic and natural media.

In four experiments, phenol at 100 and 10μM was shown to be rapidly degraded (Fig 3.1) by the natural microflora of river water and by soil inocula from the greenhouse and the *P. communis* collection site. In uninoculated but nonsterile Hoagland medium, degradation was slower, following a time-course like that shown by plant-free

Figure 3.1 Phenol dieaway experiments

Loss of phenol from sterilized OECD medium, (A) inoculated with greenhouse plant culture soil, (B) inoculated with soil from the collection site of *Phragmites communis*; and from half-strength Hoagland medium, inoculated (C) with plant culture soil and initially sterile, and (D) uninoculated but initially non-sterile; and (E) from raw, uninoculated river water. The initial concentrations of phenol were 1mM (●), 100μM (o), and 10μM (□). Further experimental details are given in the text and in Section 2.4.3.


A
B
C
D
E
[Phenol]
Incubation time (h)
0
0.2
0.4
0.6
0.8
1.0
1.1
1.2
1.4
10
20
30
40
72

control cultures in the same medium in Section 3.3.4; the lag period and extent of degradation after 72h were very similar. The maximum extent of degradation in the uninoculated dieaway test was about 40% for both 10μM and 100μM phenol after 72h, as it was in the subsequent experiments of Section 3.3.4.

Phenol, when initially present at 1mM concentration was degraded slowly or not at all in 3 days in both OECD (1971) medium and in river water, whether inoculated or not, and was significantly degraded only in Hoagland medium, where it was degraded entirely by a greenhouse soil inoculum.

Degradation of phenol in media with soil inocula from either source was essentially complete after 20h incubation, except when 1mM phenol was used; it was some 10h slower in river water. Every culture showed a lag phase before loss of phenol commenced, characteristic of the induction of metabolic processes in microorganisms and the development of a sufficiently large population to affect the concentration of phenol significantly.

Greenhouse soil was presumed to be the source of phenol-degrading organisms found to be associated with plants in other experiments, and in the case of *P. communis*, it is likely that the microflora of its collection site contributed to that of the greenhouse. Both inocula gave rise to microorganisms capable of the rapid and complete degradation of phenol in defined mineral media. By the criteria of the OECD (1971) test, phenol, in the standard medium, was readily biodegradable, and organisms capable of this activity have been shown to exist in all the environments considered, although higher (1mM) concentration of phenol were apparently toxic, or inhibitory to microbial growth. This exception is important when highly concentrated phenolic wastes are considered, implying that when phenol in excess of 1mM (about 100ppm) is released into water, substantial dilution must occur before degradation commences.

3.2. TOXICITY OF PHENOL TO PLANTS

In order that suitable concentrations of phenol might be chosen for later experiments, two plant species were tested for their response to phenol in liquid culture media.

3.2.1. Toxicity of phenol to *Lemna minor*

The growth rate of *L.minor* in defined medium was measured in axenic cultures containing phenol at various concentrations, and the effect of phenol on the calculated doubling time and exponential growth rate constant k of cultures at pH7.0 is shown in Fig.3.3. The growth rate of *L.minor* in basic liquid medium containing no phenol is shown in Fig.3.2. The effect of pH on phenol toxicity was investigated separately; the data of Fig.3.2 were obtained at the pH giving optimal growth of the plant in basic medium, and results shown in Figs.3.3 and 3.4 were derived from experiments in a wide-range buffer. The change of buffered medium had no effect on the growth of *L.minor* at pH8.0 (Figs. 3.4 and 3.2), but a different subculture of the plant, having a higher growth rate, was used for experiments recorded in Fig.3.3.

The effect of phenol on *L.minor* at constant pH was to restrict its growth. Phenol exerted slight toxicity at 10μM, but showed its greatest effect at concentrations of 100μM and above. The threshold for marked reduction of growth rate of *L.minor* was 100μM (Fig.3.3).

Fig.3.4 shows the effect of pH on growth of *L.minor* at a constant initial phenol concentration in the threshold toxicity range (100μM). The depression of growth, shown by reduction in mean exponential growth rate was generally greater at lower pH. Phenol (pK_a 10) therefore became more toxic as the molecule was progressively

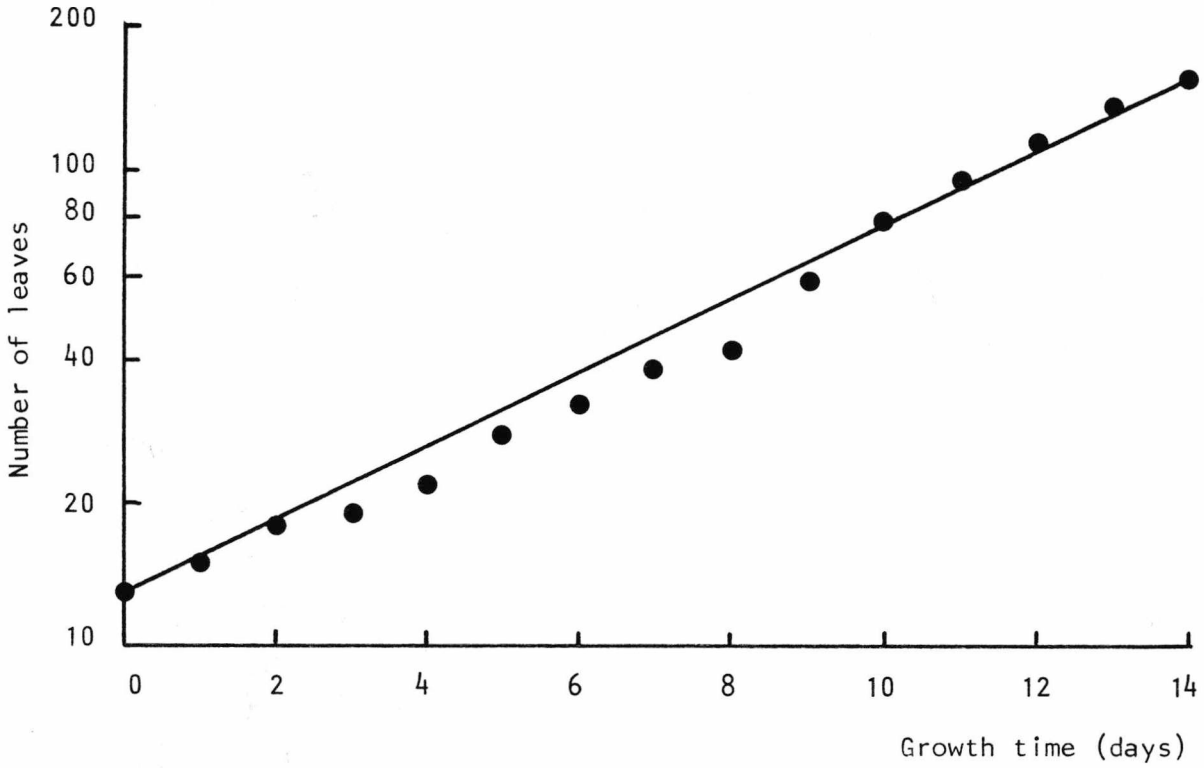


Figure 3.2 Growth of *Lemna minor* in culture at pH8.0.

An inoculum of 13 leaves of the plant was grown for 14 days in 1l of medium (Section 2.2.5, Table 2.2) containing no phenol, and maintained under the constant temperature room conditions described in Section 2.2.6.

The exponential growth rate constant k and the doubling time $T_{\frac{1}{2}}$ of the plant culture, calculated by the use of the formulae*, in which N_o and N_t respectively represent the number of leaves counted at the beginning of growth and at the end of the growth period, were, respectively, 0.26 day^{-1} and 3.85 days.

* $$k = \frac{\log_{10} N_t - \log_{10} N_o}{0.301t} \quad \text{and} \quad T_{\frac{1}{2}} = 1/k$$

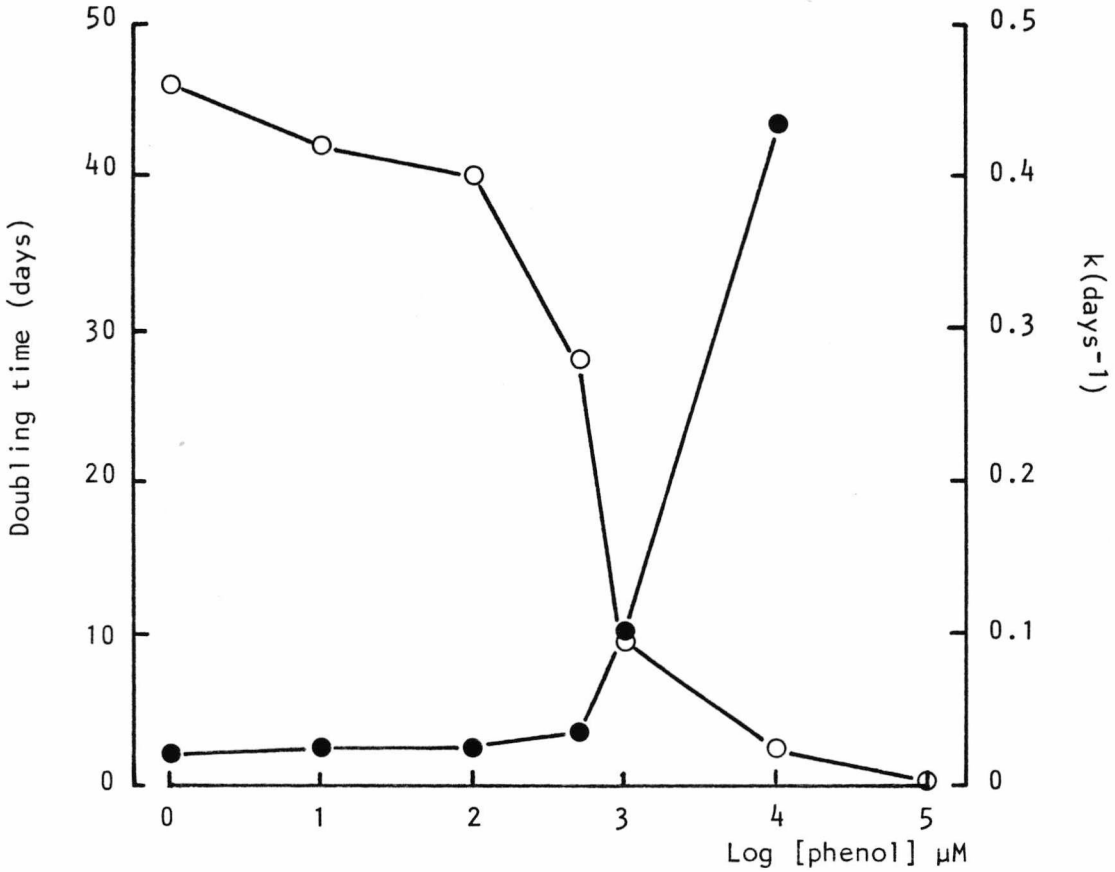


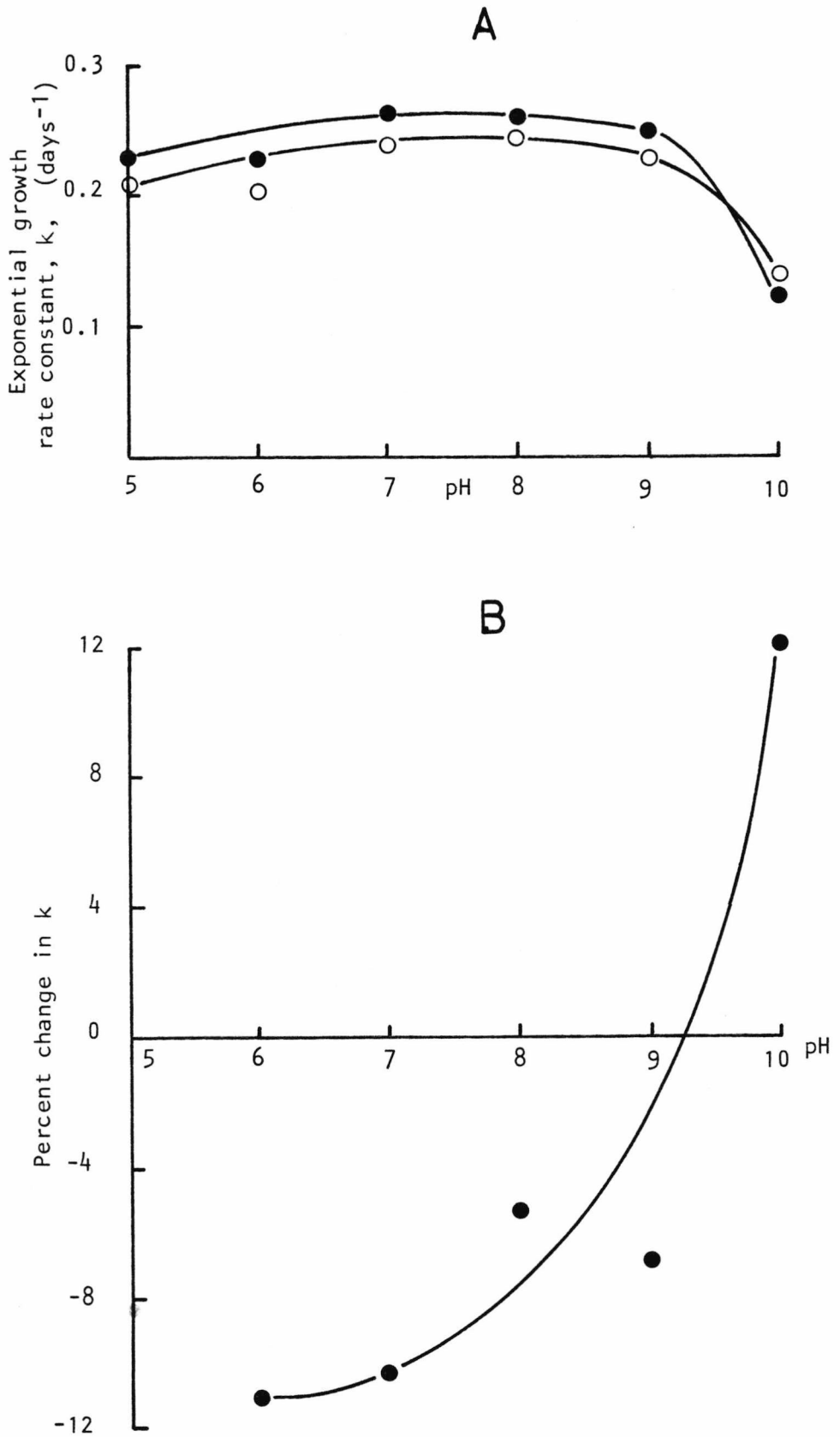
Figure 3.3 Effect of phenol on growth of *Lemna minor*

Inocula of 2 leaves of the plant were grown for 14 days in 50ml of medium made up in the buffer of Teorell and Stenhagen (1938), at pH7.0, containing phenol at initial concentrations of 1µM to 0.1M, under the constant temperature room conditions previously described (Section 2.2.5). Exponential growth rate constant, k, (o) and doubling time (●) are shown separately at each phenol concentration. Growth rate constant and doubling time in phenol-free control cultures were, respectively, 0.46 day^{-1} and 2.17 days.

Figure 3.4 Effect of pH on the toxicity of phenol to *Lemna minor*

Inocula of 10-20 leaves of the plant were grown in medium made up in the wide-range buffer of Teorell and Stenhagen (1938), containing 100μM phenol. Control cultures in the same buffers contained no phenol, and all were grown under the condition of Section 2.2.5.(A) Effect of pH on the exponential growth rate constant k in the presence (o) and absence (●) of phenol;
(B) Percentage change in k resulting from the addition of phenol;

$$\text{i.e. } \frac{k_{\text{control}} - k_{\text{phenol}}}{k_{\text{control}}} \times 100$$


A
Exponential growth rate constant, k, (days^{-1})
0.3
0.2
0.1
5
6
7
pH
8
9
10
B
Percent change in k
12
8
4
0
-4
-8
-12
5
6
7
8
9
10
pH

less dissociated at lower pH values, but phenol at pH10.0 stimulated *L.minor* growth markedly. In the absence of phenol, extremes of pH alone (pH values greater than 8 or less than 7) restricted the plant's growth (Fig.3.4A), while the effect of phenol was to lower the growth rate at most pH values, especially at lower pH.

A further toxic effect of phenol on *L.minor* was noted. While pH and low concentrations of phenol had no effect on the mean dry weight of *L.minor* leaves, phenol concentrations of 0.5mM and 1mM increased the dry weight of leaves from 0.16mg/leaf in control cultures to, respectively, 0.22mg/leaf and 0.18mg/leaf. Entire cultures of the plant were removed from the medium, blotted dry, counted, and dried to constant weight at 105°C for this determination, which was carried out on triplicate cultures. The control, 0.5mM phenol and 1mM phenol cultures contained, after growth, 175, 30, and 5 leaves respectively, corresponding with 28.0, 6.0, and 0.9mg dry tissue in total. The maximum deviation of individual results from the mean was 10.4%, suggesting that the result obtained from 0.5mM cultures was significant.

Absorption of phenol by the plant tissues to equilibrium with the medium (Section 3.3.3) cannot account for this weight gain, and there was no evidence in subsequent experiments (Section 3.3.3) that *L.minor* concentrated or incorporated phenol to the degree required to effect this weight gain.

3.2.2. Toxicity of phenol to *Phragmites communis*

Seedlings of *P.communis*, raised from surface sterilized seed were exposed to phenol at the threshold concentration (100μM) found for *L.minor* (Section 3.2.1). Seedlings, of mean fresh weight 75.9mg [samples of 10, 56 days grown, Section (3.3.2)] were treated with phenol for 7

days, after which period their mean fresh weight was 125mg, an increase of 49.1mg (64.7%). This fresh weight gain was not significantly different from that of control plants (fresh weight gain 46.8mg, 61.7%). Phenol at 100μM was therefore regarded as a suitable non-toxic concentration for application to seedlings and mature plants.

The effect of phenol on germination of *P. communis* was also examined, as this is a convenient toxicity index, obtained by other workers, and seed material has also received attention as a source of phenol-conjugating and oxidising enzymes.

The results of Fig.3.5 show that phenol of a concentration greater than 50μM in the water bathing seeds of *P. communis* restricted their germination, and 50% germination inhibition was achieved by 1mM phenol. These results suggested that germination of *P. communis* was more sensitive to phenol than was vegetative growth of the plant.

3.3. UPTAKE OF PHENOL BY PLANTS

3.3.1. Experiments to investigate the loss of phenol from the culture media of twelve species of plants

Preliminary experiments showed (Table 3.1) that phenol at 10 and 100μM initial concentration was rapidly lost from non-sterile culture solutions whether they contained plant material or not. In all cultures containing phenol at an initial concentration of 10μM, in plant cultures or in controls, no phenol remained after 48h incubation. Indeed, the presence of specimens of *Phragmites communis*, *Eriophorum vaginatum*, *Butomus umbellatus*, *Alisma plantago-aquatica*, and *Schoenoplectus tabernaemontani* var. *albescens* in cultures of initial phenol concentration 0.1mM seemed to decrease the rate of loss of phenol from the media, and the same five species,

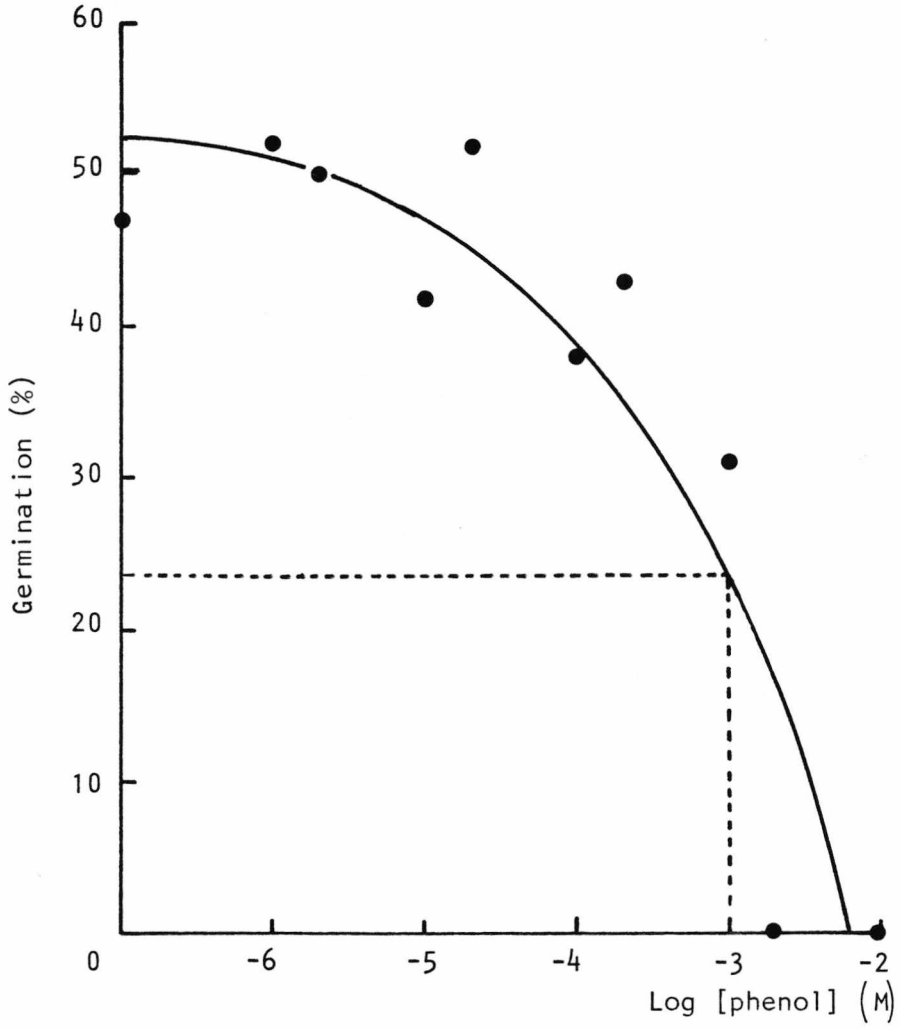


Figure 3.5 Effect of phenol on the germination of *Phragmites communis* seed.

Triplicate samples of 100 seeds of *P.communis* were germinated separately at 15-25°C (night and day temperatures respectively) on the surface of a double layer of filter-paper lining the bases of covered 9cm petri dishes. The paper was moistened with 4ml of water or with each phenol solution of the concentrations shown. The germination rate (percentage of seeds germinated) was counted after 5 days incubation, after which time no further change took place. The data presented are the means of triplicate results, and the concentration of phenol required to inhibit the germination of *P.communis* seed by 50% of the control (phenol-free) was estimated by interpolation as 1mM.

Table 3.1. Disappearance of phenol from the culture media of twelve species of plants.

Duplicate 12-week-old specimens of each species were incubated for 96h in separate volumes of half-strength Hoagland medium containing 0.01, 0.1, or 1mM phenol, under constant temperature room conditions (Section 2.2.6). Specimens of all species except *Eichhornia crassipes* were supported with cotton-wool bungs in flasks containing the specified volumes of medium; *E. crassipes* specimens were floated on medium in covered 1l beakers. Phenol concentration after 48 and 96h was determined by the 4-aminophenazone method, and results are expressed as percentagesof the concentration of phenol initially present, in order to eliminate difficulties in comparison caused by variation in starting concentrations of phenol after dilution from concentrated stock solution. Concentrations of phenol remaining after 48h only are given.

(Table 3.1)

Culture Plant species and controls	Volume of medium (ml)	Phenol remaining after 48h (% of starting concentration) Starting concentration of Phenol (mM)		
		0.01	0.1	1.0
Control	50	0	0	0
Control	125	0	1.5	0
Control	250	0	1.5	0
Control	500	0	0	0
Control	2000	0	0	0
Phragmites communis	250	0	25.0	86.0
Typha latifolia	2000	0	0	0
Typha minima	125	0	0	22.7
Eriophorum vaginatum	125	0	23.8	23.4
Iris pseudacorus	125	0	0	0
Iris foetidissima	125	0	0	0
Mentha aquatica	50	0	0	1.5
Butomus umbellatus	250	0	7.9	29.0
Alisma plantago-aquatica	250	0	7.7	13.5
Schoenoplectus tabernaemontani var. *albescens*	250	0	23.1	14.7
Schoenoplectus sp.	250	0	0	0
Eichhornia crassipes	500	0	0	13.5

with the addition of *Typha minima*, *Mentha aquatica*, and *Eichhornia crassipes*, showed a similar effect in cultures initially containing 1mM phenol.

The nature of the results obtained from these (non-sterile) experiments suggested that the volatility of phenol, even at low concentration, and the influence of microbial contamination in cultures of whole plants and control cultures could not be neglected.

3.3.2. Loss of phenol from root-sterile and non-root-sterile cultures of *Phragmites communis* seedlings

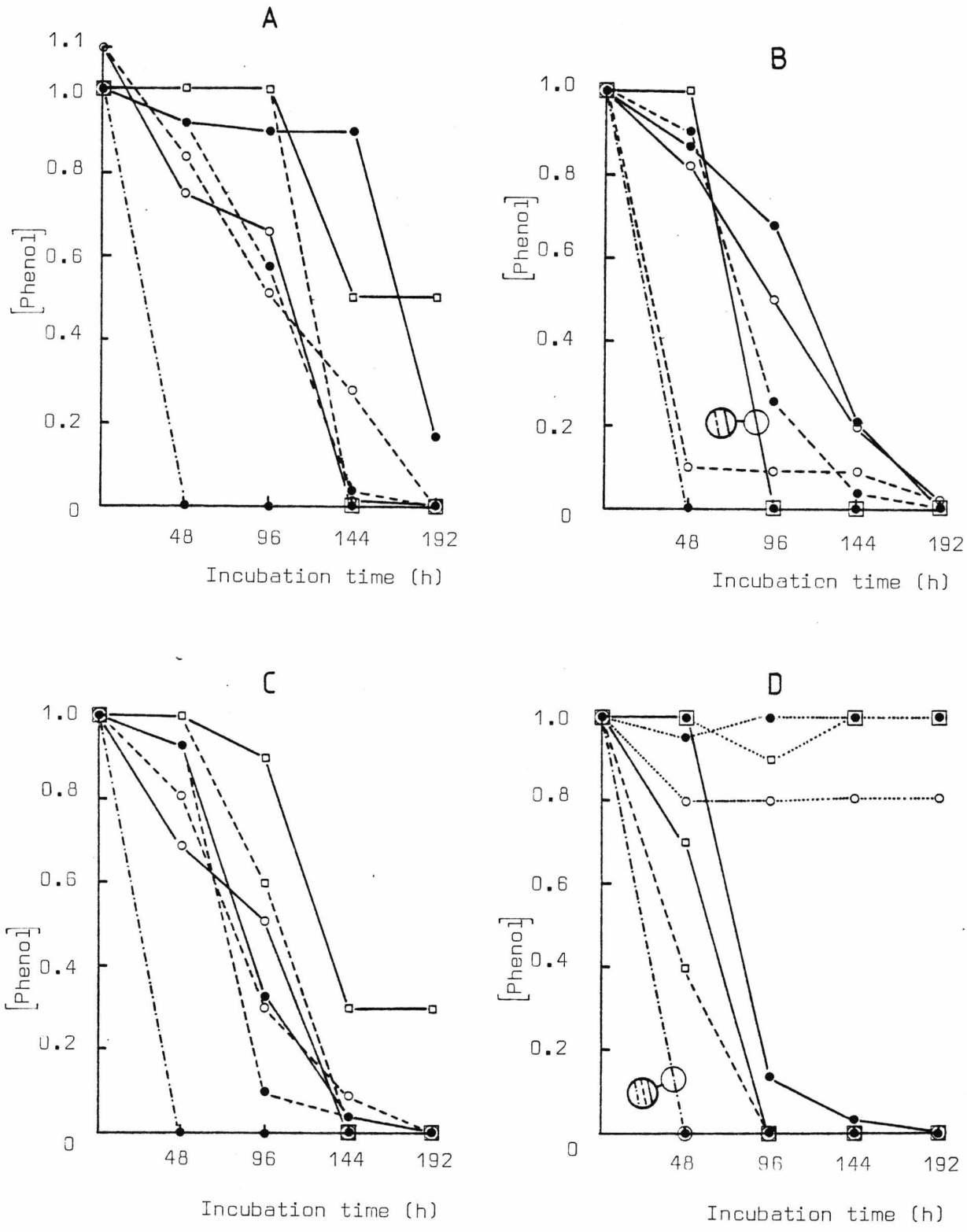
In an effort to avoid the loss of phenol from plant cultures, tentatively ascribed, at this stage, to the activity of microorganisms, whole plants of *Phragmites communis* were sterilized with H_2O_2, acidified $HgCl_2$ sterilant, or sodium hypochlorite, before treatment with phenol, but all three reagents damaged the plants and were ineffective. *P.communis* seedlings were similarly treated, but were also damaged, so sterile plants were raised from seed and tested for sterility as described in Section 2.2.1 . Plants were grown to provide four age groups, of 56, 63, 70, and 84 days, and ten samples of each age group were taken for fresh weight determination, to enable the number of plants to be adjusted to yield about 1g of fresh tissue per culture.

The results for cultures of *P.communis* seedlings which retained sterility are shown separately from those which lost sterility in Fig. 3.6 . All cultures which lost sterility did so before 48h incubation, suggesting that the contaminating organisms were introduced with the plant specimens, or were associated with them. In every case, however, at least one replicate culture of three of each

Figure 3.6 Removal of phenol from cultures by root-sterile and non-sterile seedlings of *Phragmites communis*.

Loss of phenol from cultures of initial phenol concentration 1mM (●), 100µM (○), and 10µM (□), was followed in sterile (—) and non-sterile (---) cultures of age (A) 84 days (3 plants, 0.959g), (B) 70 days (6 plants, 1.169g), (C) 63 days (9 plants, 1.107g), (D) 56 days (10 plants, 0.76g), and in non-sterile control cultures of similar groups of seedlings initially containing 1mM phenol (·-·) established in unsterilized medium, and in sterile cultures not containing seedlings (····).

All cultures were in 250ml of half-strength Hoagland medium, incubated at 22 ± 1°C in the constant temperature room. Samples were removed for colorimetric phenol analysis and for sterility testing (1ml, spread undiluted on nutrient agar, incubated for 72h at 25°C and scored positive or negative for contamination) with aseptic precautions.


A
B
C
D
[Phenol]
1.1
1.0
0.8
0.6
0.4
0.2
0
48
96
144
192
Incubation time (h)

age group of plants at each phenol concentration remained sterile. Sterile control cultures containing no plant material also remained sterile, and lost no phenol (Fig.3.6), but loss of phenol from 'positive control' non-root-sterile cultures, deliberately established from non-sterile plant material without axenic precautions, was very rapid (Fig. 3.6).

In cultures initially containing the highest (1mM) phenol concentration, cultures of plants of all age groups lost phenol more slowly when sterility was retained than when it was lost, but in cultures at lower phenol concentration, the result was less clear. The presence of plants in such sterile cultures was nevertheless associated with a phenol loss not resulting from its microbial degradation. The most rapid loss of phenol in cultures remaining sterile was from those containing 56-day-old plants, and was probably related to the larger root surface area for uptake or binding of phenol, relative to the fresh weight of the plant samples.

Rate of loss of phenol from 1mM initial concentration appeared to decrease with the age of the plants in sterile cultures; the maximum rates of decrease of phenol concentration in these cultures, estimated over a 48h period, and corrected for fresh weight of plant material were 0.016, 0.008, 0.012, and 0.023mM/h/g in cultures of plants of 84, 70, 63, and 56 days old respectively, these data partially confirming the observations.

The fresh weight of plant specimens exposed to all phenol concentrations was found at the end of the 192h period of these experiments. The results, given in Table 3.2 , confirmed those of Section 3.2.2 by showing that in no case did phenol significantly restrict the growth of the cultures.

Table 3.2. Effect of phenol on the growth of *Phragmites communis* seedlings

The fresh weight of plant specimens used to obtain the results shown in Fig.3.6. was determined, and compared with the fresh weight of similar plants which had not been treated with phenol. The results given are for seedlings in cultures which remained sterile, and are the mean weights of 3 plants of age 84 days, 6 of age 70 days, 9 of age 63 days, and 10 of age 56 days.

Age of Seedlings	Fresh wt. before incubation	Fresh weight after 192h incubation of seedlings exposed to phenol concentration shown (g)			
(days)	(g)	0	10μM	100μM	1mM
56	0.0762	0.131	0.133	0.135	0.130
63	0.1230	0.211	0.209	0.213	0.211
70	0.1948	0.259	0.255	0.256	0.257
84	0.3197	0.457	0.457	0.453	0.456

3.3.3. Uptake of phenol by *Lemna minor* in axenic cultures

Sterile cultures of *P.communis* in phenol media proved difficult to maintain, so in cultures of *L.minor* in phenol-containing media (Section 3.2.1), phenol concentration was measured periodically. In no case was the concentration of phenol reduced over the 14 day growth period of the plants.

In order to test whether sufficient plant material was present to affect phenol concentration significantly, 2 mass cultures were set up with sufficiently large inocula of *L.minor* to generate about 1000 leaves after 14 days' culture in 100ml of medium containing 10μmol phenol. Duplicate cultures were incubated under constant temperature room conditions and phenol concentration was determined at the beginning and end of the 14 day incubation period. After incubation, the cultures contained about 600 *L.minor* leaves, of total fresh weight 11.3g, dry weight 1.01g, and the concentration of phenol was reduced from 100μM to 87μM, a reduction of 13%, but this reduction corresponds closely with the effective 10.2% 'dilution' of the medium by the presence of the plant material, assuming that it may be represented by an equal volume of water or medium (Hancock, 1969).

L.minor therefore absorbed phenol to approximate equilibrium with the medium, and no effect of metabolism of phenol by this plant in axenic culture could be demonstrated. The discrepancy between the absorbing effect of the plant material and the measured loss of phenol (2.8%) is within the experimental error of sampling and phenol determination.

3.3.4. Loss of [U-^{14}C]phenol from cultures of *Phragmites communis*

Colorimetric estimation of phenol in culture media in the previous experiments became unreliable at low concentration, and gave no information on the fate of phenol applied to plant roots. Incorporation of [U-^{14}C]phenol in the culture media of plant specimens was intended to permit accurate determinations of phenol concentration, and to confirm the entry of phenol or phenol-derived materials into plant specimens.

Specimens of *P. communis* (12 weeks old), produced from rhizome pieces in the greenhouse, were transferred to 250ml of sterile half-strength Hoagland medium containing [U-^{14}C]phenol at 1µM, 5µM, 10µM, 50µM and 100µM final concentration and about 1.35nCi/ml (3000d.p.m/ml). The phenol concentration of the medium was confirmed colorimetrically at the start of incubation of the cultures. Five replicate plant cultures and duplicate control cultures containing no plant specimens at each phenol concentration were incubated in the greenhouse at 15-25 °C (night and day temperatures respectively). Radioactivity of a sample of the medium was determined initially and at intervals thereafter by liquid scintillation counting. After 120h incubation, three replicate plant specimens exposed to each phenol concentration were sacrificed to determine the distribution of radioactive material absorbed by them. The remaining two specimens were washed well in sterile distilled water and transferred to fresh 250ml volumes of medium containing the same concentrations of [U-^{14}C]phenol. Incubation of these specimens was continued for 40h, with periodic measurement of radioactivity remaining in the media, to show the effect on phenol concentration of plants pre-conditioned by exposure to it. The fresh weight of all plant specimens was found before and after incubation.

Results, given in Fig.3.7.,show that phenol, measured as radioactivity in the medium, decreased in concentration faster and to a lower limit in the presence of plants than in their absence. The loss of radioactivity showed a lag phase of 7h in plant cultures containing both 100μM and 50μM phenol, but plant cultures containing 10μM phenol or less showed no such lag. Control cultures also showed a lag before loss of radioactivity commenced in media initially containing 100μM, 50μM, 10μM, and 1μM phenol, the duration of this phase reaching 20h in cultures containing 1μM phenol. Loss of radioactivity from the initial total content of 307-318 nCi in each culture continued for 50 to 60h in plant cultures, after which time 33 - 78 nCi remained. In control cultures, loss of radioactivity ceased after 30 - 40h incubation, when 124 - 164 nCi remained.

When plant specimens were exposed for the second time to [U-^{14}C] phenol, the lag phase in loss of radioactivity from the cultures was entirely abolished, and the rate of loss of phenol was greater than in cultures containing plants not previously exposed to phenol. Loss of radioactivity from cultures containing twice-exposed plants continued for a shorter time, from 30 - 40h, but attained a similar lower limit, of 41 - 82 nCi in culture media.

The initial rates of loss of radioactivity, determined as the maximum rate after the lag period from plots of radioactivity remaining versus time (Fig.3.7) and calculated to represent rates of loss of phenol from cultures are shown in Table 3.3. The maximum initial rate of loss of phenol from cultures, calculated on the basis of entire cultures (250ml volume) and with respect to the fresh weight of plant tissue, was related to the concentration of phenol initially applied (Table 3.3).

When corrected for the loss of phenol from control cultures, a

Figure 3.7 Loss of [U-^{14}C] phenol from cultures of mature plants of *Phragmites communis*.

Loss of [U-^{14}C] phenol was monitored by scintillation counting at the times shown in the figures, (●) in plant cultures and (o) in controls with no plants. Initial phenol concentrations were (A) 100μM, (B) 50μM, (C) 10μM, (D) 5μM, and (E), 1μM; the initial total radioactivity was similar in each instance to ensure accuracy of measurement at low phenol concentration. The arrow indicates the time of transfer of two plant specimens to fresh [U-^{14}C]phenol-containing medium. The error bars indicate ± Standard Deviation about the mean of five replicate results. Further experimental details are given in the text.

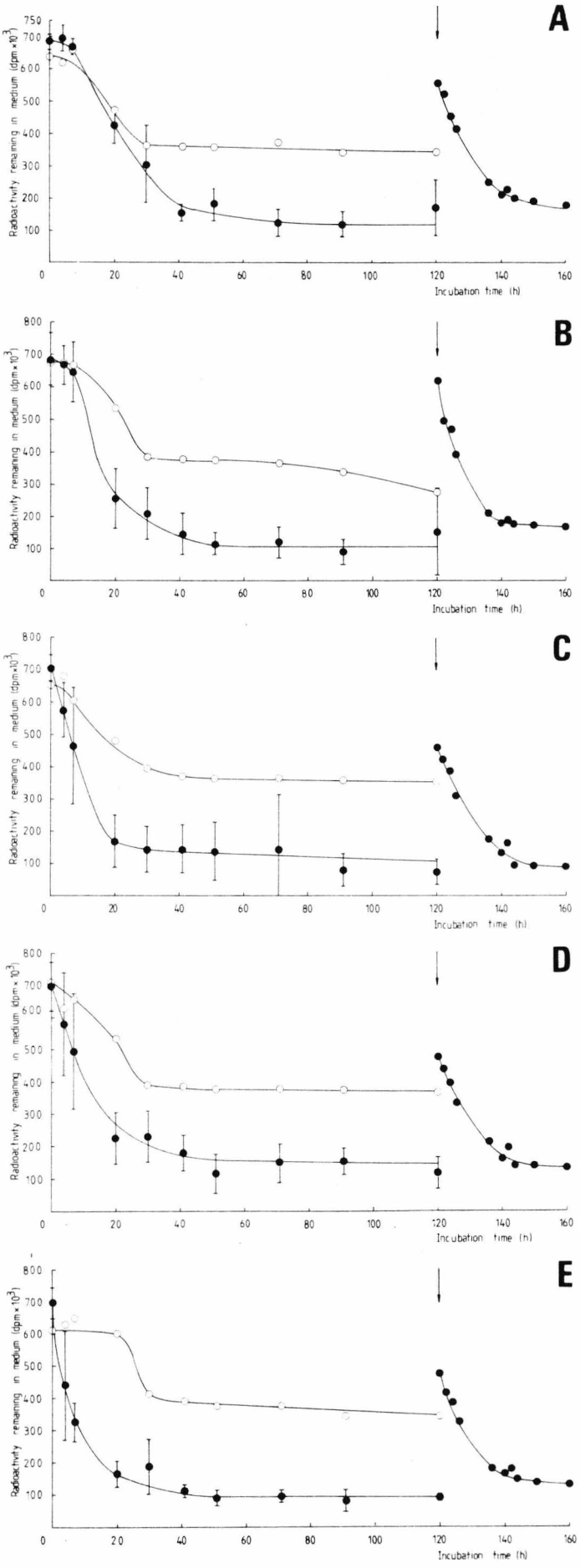

A
B
C
D
E
Radioactivity remaining in medium (dpm × 10³)
Incubation time (h)
0
20
40
60
80
100
120
140
160
100
200
300
400
500
600
700
800

Table 3.3 Loss of [U-^{14}C] phenol from cultures of *Phragmites communis* and from control cultures containing no plant specimens. Rate of loss of [U^{14}C] phenol derived from Figure 3.7. Experimental details are given in the text.

Initial concentration of [U-^{14}C] phenol (μM)	Rate of loss of [U-^{14}C] phenol from cultures, (nmol/h)			Net rate of loss of [U-^{14}C] phenol (nmol/h) (plant culture -control)	Mean fresh weight of plant tissue in culture (g)		Rate of loss of [U-^{14}C] phenol corrected for fresh weight of plant tissue in cultures, (nmol/h/g)			
	1st exposure of plants	2nd exposure of plants	Control cultures	1st exposure	1st exposure of plants (5 replicates)	2nd exposure of plants (2 replicates)	1st exposure of plants	2nd exposure of plants	1st exposure -control	2nd exposure -control
100	816.5	1256.7	561.7	254.8	3.645	4.045	224.0	310.7	69.9	171.8
50	536.4	1468.2	277.1	259.3	4.932	7.795	108.8	188.4	52.6	152.8
10	137.0	123.3	53.2	83.8	6.278	8.885	21.8	13.9	13.3	7.9
5	52.3	52.0	32.1	20.2	4.414	6.825	11.9	7.6	4.6	2.9
1	33.2	15.6	8.1	25.1	5.592	7.670	5.9	2.0	4.5	1.0

similar but non-linear relationship was observed (Fig.3.8). The partial loss of the relationship on correction suggests that the plant cultures are best regarded as single systems with respect to their loss of [U-^{14}C]phenol. the plant nevertheless being the most significant component.

Colorimetric determination of phenol at the end of the incubation periods showed none to be present. The remaining radioactivity, did not, therefore, represent unaltered phenol. The presence of microbial cell mass and phenol metabolites in such media was explored in subsequent experiments.

Although the media used in these experiments were sterilized before use, and cultures were made in previously sterilized glassware, loss of phenol from control cultures (without plants) was still observed. As it was probable that at least part of this loss was the result of infection by phenol-degrading microorganisms, all the media of experiments described in this section were sampled after 120h incubation for determination of the number of microorganisms present.

The results (Table 3.4) show that the counts of bacteria present were highly variable. Numbers of organisms detected on nutrient agar increased by nearly two orders of magnitude with decreasing phenol concentration in media containing plant specimens, but a similar inverse proportionality cannot be shown in control cultures. A similar relationship occurred in numbers of organisms grown on nutrient agar plus phenol and on phenol-mineral salts agar inoculated from plant cultures. The number of microorganisms detected on the latter from plant culture media containing 1μM phenol was lower than this relationship should suggest, but the concentration of phenol in such media was probably insufficient to support extensive microbial

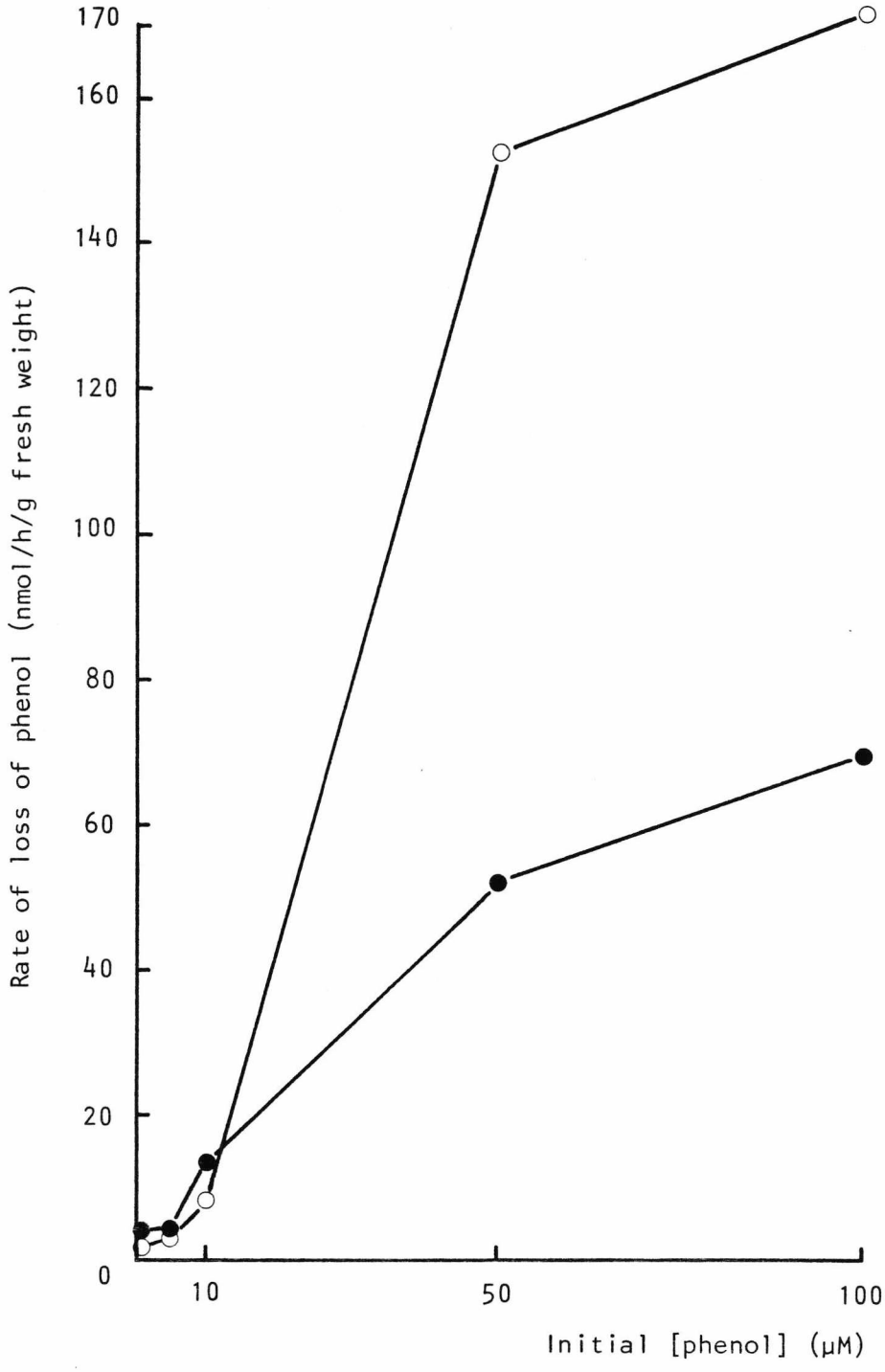


Figure 3.8 Loss of phenol from cultures of *Phragmites communis*.

Rate of loss of phenol from plant cultures exposed once (●) and twice (o) to [^{14}C] phenol, corrected for fresh weight of plant specimens and for loss rate from control cultures. Data from Table 3.3. Experimental details are given in the text.

Table 3.4. Evaluation of microbial populations in cultures of *Phragmites communis* and control cultures containing no plant specimens.

The viable count of organisms present, determined on the 3 agar media shown were measured in 1ml samples from plant cultures (Exptl.) and control cultures. Results for the former are the average of 5 replicates, and the latter the average of 2. The method is described in Section 2.3. The data presented are from cultures exposed once to phenol.

Initial concentration of phenol in media (μM)	Microbial counts/ml ($\times 10^{-6}$)					
	Medium on which count determined					
	Nutrient agar		Nutrient agar + 1mM phenol		1mM phenol-mineral salts agar	
	Exptl.	Control	Exptl.	Control	Exptl.	Control
100	1.3	58.3	0.4	39.0	0.2	10.4
50	1.6	3.4	0.5	2.7	0.6	2.0
10	9.4	51.4	2.6	9.2	8.1	46.4
5	90.8	13.9	21.6	18.6	40.8	10.8
1	79.3	27.9	23.6	11.7	8.6	16.8

growth. It was expected that phenol in the plant culture media would support larger populations of microorganisms at higher concentrations, but this was not so; the results suggest that both the presence of the plant and higher phenol concentrations restricted growth of microorganisms degrading phenol, those tolerant of it, and those not utilizing phenol as a carbon-source. A large increase in numbers of organisms present in plant culture media, detected on all three microbiological media, was observed between phenol concentrations of 10 and 5μM, suggesting that the threshold phenol concentration restricting microbial growth lay between these values. The presence of the plant in cultures containing phenol at a greater concentration than 5μM was apparently restrictive, as the numbers of organisms present in media without plants were from 3 to 50 times greater than those found in plant culture media of the same phenol concentration.

The lack of systematic relationship between microbial counts and phenol concentration in control media is ascribed to the adventitious nature of the contamination of such cultures by microorganisms.

Mycelial and yeast-like fungi did not appear on any of the neutral agar media used. Selection of fungi on more suitable media is described in Section 4.3.

The presence of bacteria capable of the dissimilation of phenol in plant cultures was clearly shown in these experiments, and such contamination, unavoidable unless sterile plant material was used, was judged sufficient to account for the losses of [$U^{14}C$]phenol not attributable to plant material. Taken in conjunction with the results of the major experiments of this section however, the generally smaller number of microorganisms in plant cultures of 100, 50, and 10μM phenol concentration shows clearly that in these cultures at least, phenol removal by the plant was significant.

3.3.5. Fate of [U-^{14}C]phenol administered to *Phragmites communis* specimens

Experiments in which [U-^{14}C]phenol was applied to the roots and rhizomes of *Phragmites communis* specimens showed that the plants enhanced the loss of radioactivity from the media. Three specimens exposed to phenol in these experiments (Section 3.3.4) were sacrificed after a single exposure so that a direct measure of the radioactivity absorbed by plant tissues might be made, and the fate of such radioactive material after the comparatively long (120h) incubation period determined.

The plant specimens were washed free of external [U-^{14}C]phenol-containing media with distilled water, blotted dry, and quickly separated into root, rhizome, stem, and leaf parts. After the fresh weight of each major tissue had been determined, they were immediately cut into pieces of 1cm or less, and plunged into boiling 80% (v/v) aq. ethanol. The material was extracted for 14h at 80°C and the ethanolic extract decanted and filtered hot. The solid residue was washed with 10ml aliquots of hot 80% (v/v) aq. ethanol until the material and washings were colourless, indicating efficient extraction of hot-ethanol-soluble components, of which chlorophyll is part. Root and rhizome tissues received three washes, which had been effective in the case of stem tissue. The ethanolic extracts and washings from each tissue were pooled and made up to known volume when cool, and the radioactivity of duplicate 1ml samples was determined. Residues on the filter papers were added back to the appropriate extraction residues, and the whole air dried (48h, 20°C) until visibly dry. The material was then dried *in vacuo* over a silica-gel desiccant until friable, when it was ground to an homogeneous fibrous powder. The powders were weighed and duplicate 10mg samples

of each were hydrated overnight at 20°C in 0.1ml distilled water in glass scintillation counter vials. To the hydrated material was added 0.9ml of a commercial tissue dispersing agent, and the mixture was allowed to digest for 24h at 20°C. After addition of scintillant, radioactivity of the digests was determined and corrected to d.p.m.

The results (Table 3.5) show the radioactivity recovered from, or determined in, the three replicate media and pooled extracts and residues of the three plants exposed to each phenol concentration (Section 3.3.4).

Total recovery of radioactivity supplied to the plants was of the order of 80%, with one anomalous exception in the case of plants exposed to 5μM phenol, where it was only 31%.

The results confirm that plant tissues were responsible for the major part of the phenol loss. The largest proportion of recovered radioactivity, in all cases, was found in the ethanol-insoluble residues of the root tissues of plant specimens. Root tissues also contained the largest amounts of radioactivity recoverable in ethanol extracts. In total, root tissues contained about 50% of the radioactivity applied to the plants, and rhizomes from 10 to 30% of the recovered radioactivity. Stem tissues contained from 3 to 9%, and leaf tissues 3 to 5% of the radioactivity. As radioactive phenol was applied to the plants *via* the roots and rhizome, it is not surprising that this is reflected in the higher quantities of radioactive material recoverable from them, but it is clear that little mobilization of phenol-derived material to aerial plant parts took place within the time-course of the experiment.

The distribution of radioactivity between the four major plant tissue parts showed no obvious relationship with the concentration of phenol to which the plants were exposed, but, when radioactivity recovery was converted by calculation to quantities of "phenol", and

Table 3.5. Recovery and distribution of radioactivity applied as [^{14}C]phenol to specimens of *Phragmites communis*

Radioactivity in culture media before and after incubation for 120h, and radioactivity recovered from ethanol extracts and from insoluble residues of plant tissues are given as d.p.m. and expressed in parentheses as percentages of that initially applied. Determinations of radioactivity in plant culture media are the average of results from the three appropriate plant cultures, and the results for radioactivity recovery are derived as the average per plant from pooled extracts and residues of three specimens. Experimental details of plant culture are given in Section 3.3.4, and details of extraction and measurement procedures are given in the accompanying text. Recovery as "phenol" was calculated from the specific radioactivity of phenol in each of the culture media at the start of incubation, total recovery of radioactivity being corrected first for the fresh weight (average of 3) of the plant tissue parts.

(Table 3.5)

Initial concentration of phenol applied to plants (μM)	Radioactivity initially applied (d.p.m)	Radioactivity lost from medium after 120h (d.p.m)	Plant tissue parts	Radioactivity recovered in ethanol extract (d.p.m)
100	698917	540417	Root Rhizome Stem Leaf Whole plant	25725 (3.68) 17542 (2.51) 8379 (1.20) 7533 (1.08) 59179 (8.47)
50	729083	518833	Root Rhizome Stem Leaf Whole plant	15730 (2.16) 9520 (1.31) 6300 (0.86) 7455 (1.02) 39005 (5.35)
10	702250	612417	Root Rhizome Stem Leaf Whole plant	12577 (1.79) 11249 (1.60) 6194 (0.88) 5539 (0.79) 35559 (5.06)
5	703750	558083	Root Rhizome Stem Leaf Whole plant	12437 (1.77) 11748 (1.67) 5231 (0.74) 5273 (0.75) 34689 (4.93)
1	672583	569083	Root Rhizome Stem Leaf Whole plant	12240 (1.82) 7933 (1.18) 8717 (1.30) 4080 (0.61) 32970 (4.90)

Radioactivity recovered in residue after extraction (d.p.m.)	Sum of radio-activity in plant tissues (d.p.m.)	Ratio of recovery of radio-activity, residues extracts	Fresh weight of plant tissue parts (g)	Recovery as "phenol" corrected for fresh weight (µmol/g)
319653 (45.74)	345378 (49.42)	12.4	0.96	12.9
156413 (22.38)	173955 (24.89)	8.9	1.21	5.1
12538 (1.79)	20917 (2.99)	1.5	0.94	0.8
16500 (2.36)	24033 (3.44)	2.2	0.38	2.3
505103 (72.27)	564282 (80.74)	8.5	3.50	5.8
285507 (39.16)	301237 (41.32)	18.2	0.82	6.3
118910 (16.31)	128430 (17.62)	12.5	1.11	2.0
60133 (8.25)	66433 (9.11)	9.6	0.77	1.5
26400 (3.62)	33855 (4.64)	3.5	0.32	1.8
490950 (67.34)	529955 (72.69)	12.6	3.02	3.0
307560 (43.80)	320137 (45.59)	24.4	1.14	1.0
190960 (27.19)	202209 (28.79)	17.0	2.13	0.3
20900 (2.98)	27094 (3.86)	3.4	0.81	0.1
12240 (1.74)	17779 (2.53)	2.2	0.46	0.1
531660 (75.71)	567219 (80.77)	15.0	4.54	0.4
104360 (14.83)	116797 (16.60)	8.4	0.54	0.4
56780 (8.07)	68528 (9.74)	4.8	1.32	0.1
13860 (1.97)	19091 (2.71)	2.7	0.53	0.06
7650 (1.09)	12923 (1.84)	1.5	0.41	0.06
182650 (25.95)	217339 (30.88)	5.3	2.81	0.1
376153 (55.93)	388393 (57.75)	31.1	1.48	0.1
97020 (14.42)	104953 (15.60)	12.5	1.42	0.03
23650 (3.52)	32367 (4.82)	2.8	0.74	0.02
19100 (2.84)	23180 (3.45)	4.7	0.56	0.02
515923 (76.71)	548893 (81.61)	15.6	4.21	0.05

corrected for the fresh weight of the plant tissues, recovery showed a relationship with the concentration of phenol initially applied. The plant did not therefore exclude phenol at higher concentrations, as greater *amounts* of phenol-derived radioactivity were recovered from plant tissues exposed to higher concentrations of radioactive phenol. These results help to confirm the suggestions of Section 3.3.4 that the rate of phenol loss from plant culture media was related in some manner to concentration, the *rate* of phenol loss being greater when more phenol was initially present.

The ratio of radioactivity recoveries in insoluble residues to that in ethanolic extracts showed very great variability between the four major tissue parts. As a convenient method of expressing the differences between the amounts of radioactivity recovered in these two fractions, the results suggest that the root tissues were more active in immobilizing [U-^{14}C]phenol-derived radioactivity than other plant parts.

In conjunction with information obtained on the distribution of radioactivity within whole plants, some interpretation of the fate of radioactivity derived from [U-^{14}C]phenol may be attempted. The greater part of the material remained in root tissues, which appeared to be active in immobilizing it, as comparatively little was found elsewhere in the plant. The lower ratios of insoluble residue to ethanol extract radioactivity in aerial parts of the plant suggest either that these tissues were less active in immobilizing unchanged radioactive material transported from the roots, or that translocated material was not as amenable to reactions producing insoluble products.

The account of radioactivity applied to plants, and measured in media and recovered from plants after incubation was subject to some

uncertainty. Recoveries of radioactivity from plants exposed to 100μM, 50μM, 10μM, 5μM, and 1μM phenol were respectively 80.74%, 72.69%, 80.77%, 30.88%, and 81.61% of that applied, but losses from the media, in the same order of presentation, were 77.32%, 71.16%, 87.20%, 79.30%, and 84.61%. Only in the latter three media was radioactivity unaccounted for, whereas losses caused by microbial metabolism or by possible conversion of [U-^{14}C]phenol to $^{14}CO_2$ by plants were expected in all cultures, and the presence of phenol-degrading microorganisms in all cultures was confirmed (Section 3.3.4). A subsequent experiment was intended to determine whether any unaccountable loss was attributable to the plants, and whether errors were present in the estimation of radioactivity accounts described here.

No phenol was detected colorimetrically in plant cultures after 120h incubation, although radioactive material was certainly present in all the media. The radioactivity remaining in media initially containing 100μM, 50μM, 10μM, 5μM, and 1μM phenol was respectively 22.68%, 28.84%, 12.79%, 20.70%, and 15.39% of that initially supplied. This may have been present as material absorbed onto plant debris, as microbial cell mass, or as phenol at a concentration below the detection limit of the colorimetric method, and an attempt to determine this was made in a subsequent experiment.

The ethanolic extracts from plant tissue parts, after determination of their content of radioactive material, were concentrated under reduced pressure at 30-35°C in a rotary film evaporator. Samples of each concentrate were spotted, with authentic materials, on paper chromatograms, which were developed in the descending mode in solvents (1) and (2) (Section 2.5). After development and compound visualization, no materials corresponding in R_F. with phenol [R_F. solvent (1), 0.92, (2) 0.97], catechol [R_F. (1) 0.80, (2) 0.84],

or phenyl-β-*D*-glucoside [R_F. (1) 0.74, (2) 0.52] were found. Authentic phenol was highly mobile in both solvents, and separated well from the two putative metabolites, and should have been detected readily if present (detection limit 1-10µg, corresponding to approximately 10-100nmol phenol). No significant quantities of radioactivity were found in paper sections cut from the zones of chromatograms corresponding with the positions of authentic materials, except in one instance in which, in solvent (1), an R_F. zone of 0.92-0.94 yielded radioactive material (170d.p.m). The extract giving this result was from root tissue, exposed initially to 100µM of labelled phenol. This result, indicating the possible presence of unaltered phenol, could not be confirmed in other solvents, or with extracts from any other tissue. Co-chromatography of samples of ethanol extract with radioactive material failed to improve results. It was concluded that the low level of radioactivity recoverable in ethanolic extracts of plant tissue was dispersed through such a variety of compounds that reliable detection of any one compound was impossible.

3.3.6 Loss of [U-^{14}C]phenol from cultures of *Phragmites communis* as volatile or gaseous products : Are $^{14}CO_2$ and [^{14}C]phenol released from intact-plant cultures?

In some of the experiments already described, significant fractions of radioactivity applied as [U-^{14}C]phenol to specimens of *P.communis* remained unaccounted for.

Seidel and Kickuth (1965) have shown that phenol vapour was released unaltered from the aerial parts of *Schoenoplectus lacustris* when it was applied in solution to the roots; it was conceivable therefore that some applied phenol in my experiments was also lost

by volatility, or was being converted to other gaseous metabolites.

Single specimens of *P.communis* (90-100cm high) of 12 weeks' growth were enclosed in the two-chambered apparatus shown schematically in Fig.3.9, in which the aerial and normally subterranean parts of the plants were isolated from each other and from the atmosphere. The root chamber contained 800ml of half-strength Hoagland solution, with the addition of [U-^{14}C]phenol to give a final concentration of 100µM and 0.6nCi/ml. The root chamber and medium were sterilized by autoclaving, and [U-^{14}C]phenol was added separately after filter-sterilization. Immediately after the plant was sealed into the apparatus, it was purged with CO_2-free air at 1l/min, passed through three KOH (2M) traps and one splash trap and split to provide separate airflows for the root and leaf chambers. Each inlet air line passed through a sterilizing filter. The exhaust air from each chamber passed through a further sterilizing line filter, to isolate the chamber from contamination, and through three KOH (2M) traps. The plant was kept in darkness at 22°C for the 72h duration of the experiment. The culture medium was sampled for radioactivity at the beginning of the experiment and at intervals thereafter through the sterile sample port, and each KOH trap was sampled for radioactivity at the same time. Four samples of 1ml were withdrawn from each alkali trap, of these, two were mixed at once with 10ml of scintillation fluid and their radioactivity determined. These would measure $^{14}CO_2$ and trapped [^{14}C]phenol vapour. The two remaining samples were diluted to 5ml, and acidified with an excess (3ml) of 1M HCl. The acid aqueous solutions were extracted three times with 10ml volumes of diethyl ether, the ether layers of the extraction were pooled, concentrated by evaporation, and finally made up to 2ml. The radioactivity of the concentrated extracts was determined, to show the presence of trapped [^{14}C]phenol vapour, but not $^{14}CO_2$, which

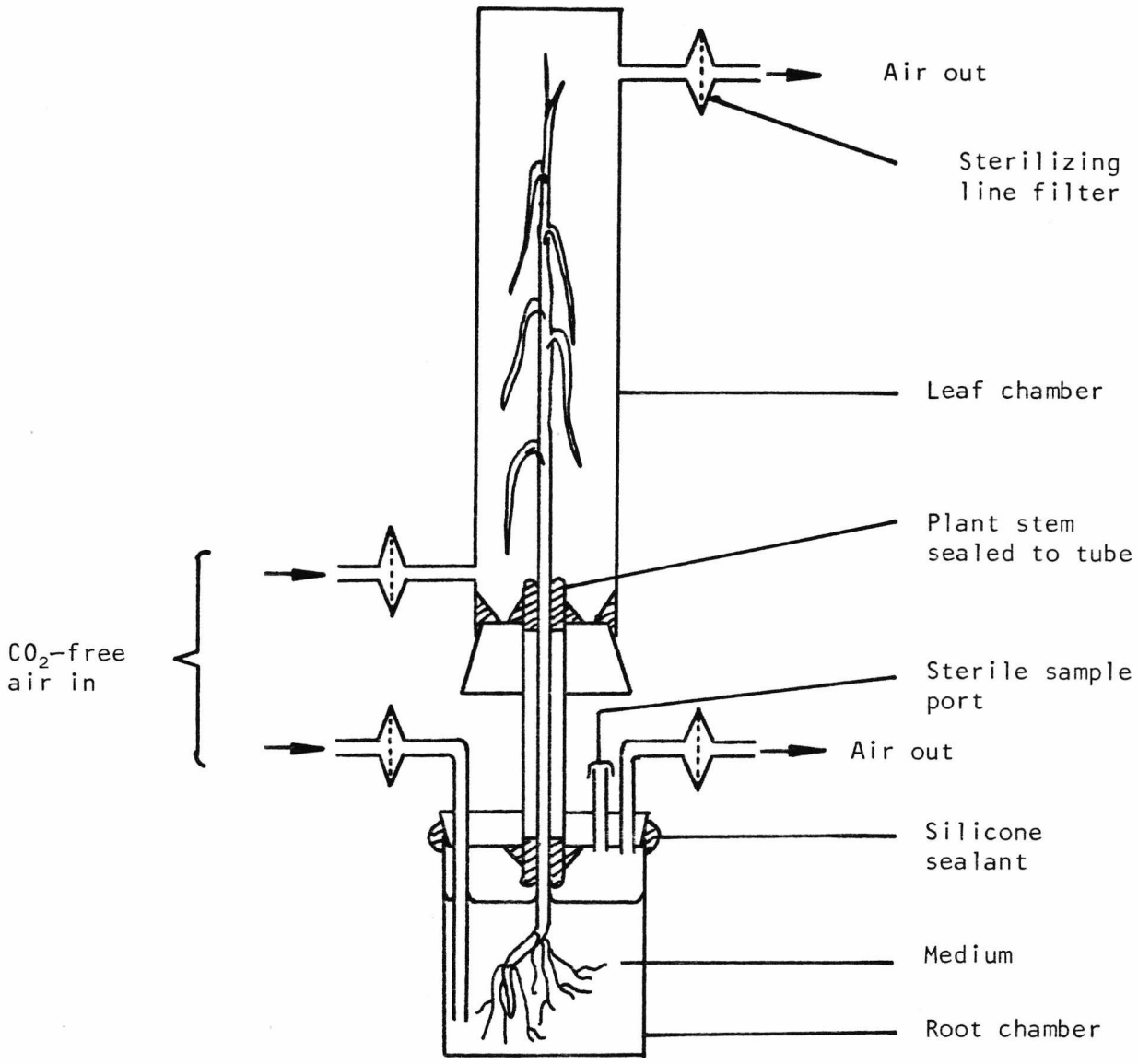


Figure 3.9 Apparatus to enclose separately the aerial and subterranean parts of specimens of *Phragmites communis* for exposure to [U-^{14}C] phenol with $^{14}CO_2$ trapping.

Plant specimens were 90-100cm in height, and bore 9 leaves. Experimental details are given in the text.

would be driven off by the acidification step. No radioactive material was found in the ether extracts of samples from any trap, however, although 1nCi of authentic [U-^{14}C]phenol, added to trial samples of 1ml of 2M KOH, could be recovered in 90% yield by this method.

The entire experiment was replicated, and the results presented are the average of 2 determinations. The details of the radioactivity recovered are summarized in Table 3.6, which shows that very little [U-^{14}C]phenol remained in the medium, but none of it could be recovered as vapour. $^{14}CO_2$ however, accounted for 21% of the radioactivity applied after 72h incubation, but was found exclusively in alkali traps attached to the root chamber.

The plant material from each experiment was cut into leaf, stem, rhizome, and root parts and processed as already described (Section 3.3.5) and ethanol extracts and residues from both experiments combined. The radioactivity of each tissue and fraction was determined and accounted. No residual phenol or its putative metabolites were detected by chromatography.

Recovery of radioactivity from the plant tissues was 67% of that applied (Table 3.6). Of the 9.7% of original radioactivity still remaining in the medium, a very small proportion was recovered on a 0.22μm filter. This fraction of the recovered radioactivity probably included microbial cell mass or plant debris but represented only 0.82% of that initially applied, (8.46% of the radioactivity remaining in the medium).

The microbial viable counts of the medium at the end of the experiment were (per ml) 35,000, 49,000, and 26,000 for total count, (determined on nutrient agar), phenol-tolerant (nutrient agar plus 1mM phenol) and phenol-utilizing bacteria (1mM phenol-mineral salts agar) respectively. These numbers are between one and two orders

Table 3.6. Recovery account of radioactivity applied as [U-^{14}C] phenol to specimens of *Phragmites communis* in $^{14}CO_2$-trapping experiments.

Experimental details are given in the text.

	Radioactivity (d.p.m.)	Radioactivity as % of applied
Radioactivity applied, [U-^{14}C] phenol	1072000	100.00
Radioactivity remaining in medium after 72h	104000	9.70
Radioactivity filtered from medium at 72h	8800	0.82
Radioactivity recovered from plant tissues	721755	67.33
Radioactivity recovered as $^{14}CO_2$	223156	20.82
Radioactivity recovered as phenol vapour	0	0
Unaccounted for	23089	2.15

of magnitude smaller than those found in previous experiments (Table 3.4), but might account for the amount of $^{14}CO_2$ evolved from phenol.

Although the average total fresh weight of the plant specimens used in this experiment was over five times greater than that of plants in other experiments, the total radioactivity recovered from the larger plants was not proportionally higher. The distribution of radioactivity in the plant tissue parts did show a similar pattern to that in earlier experiments, although the amount of radioactivity recovered per gram fresh weight of tissue was less (Table 3.7).

The release of radioactivity as $^{14}CO_2$ from the root chamber of the apparatus appeared to follow the rate of loss of radioactivity from the medium closely (Fig.3.10). As in other experiments loss of [U^{14}C]phenol showed a lag, of some 12h, but then accelerated to a maximum phenol loss rate of 3.36µmol/h for the entire culture.

Although quantitative comparisons between these and other experiments in which specimens of *P.communis* were exposed to radioactive phenol are poor, unequivocal evidence that [U^{14}C]phenol was converted to $^{14}CO_2$ in the root zone was obtained. There was no evidence that phenol applied to the plant *via* the roots was translocated and volatilized, or that other gaseous metabolic products were released from the aerial parts of the plant. The $^{14}CO_2$ released from the root zone might be a product of microbial breakdown of [U^{14}C]phenol, but bacterial numbers in the medium were small, and metabolism of phenol by the roots and rhizome of the plant cannot therefore be excluded.

The radioactivity remaining in the medium after incubation did not represent a concentration of phenol detectable by colorimetric analysis, so the medium was further analysed. After acidification (pH 1-2) by the addition of HCl , the media of both replicate experiments

Table 3.7. Recovery and distribution of radioactivity applied as [U-^{14}C]phenol to specimens of *Phragmites communis*

Radioactivity recovered from ethanol extracts of and insoluble residues from tissues of plants used in enclosed chambers with $^{14}CO_2$ trapping. Initial phenol concentration was 100μM, and experimental details are given in the text. Figures given in parentheses in columns 4, 5, and 6 express the recoveries of radioactivity as percentagesof that applied to the plants.

Radioactivity initially applied (d.p.m.)	Plant tissue parts	Fresh weight of plant tissue parts (g)	Radioactivity recovered in plant residues, after extraction (d.p.m.)	Radioactivity recovered in ethanol extracts (d.p.m.)	Sum of radioactivity recovered from plant tissues (d.p.m.)	Ratio of recovery residues extracts
1072000	Root (1)	4.77	396464 (36.98)	27288 (2.53)	423752 (39.53)	14.52
	Rhizome (2)	7.73	180450 (16.83)	19152 (1.79)	199602 (18.62)	9.42
	Stem (3)	4.20	34899 (3.26)	18270 (1.70)	53169 (4.96)	1.91
	Leaf (4)	1.67	36172 (3.37)	9060 (0.85)	45232 (4.22)	3.99
	Whole plant (Sum of 1-4)	18.37	647985 (60.45)	73770 (6.88)	721755 (67.33)	8.78

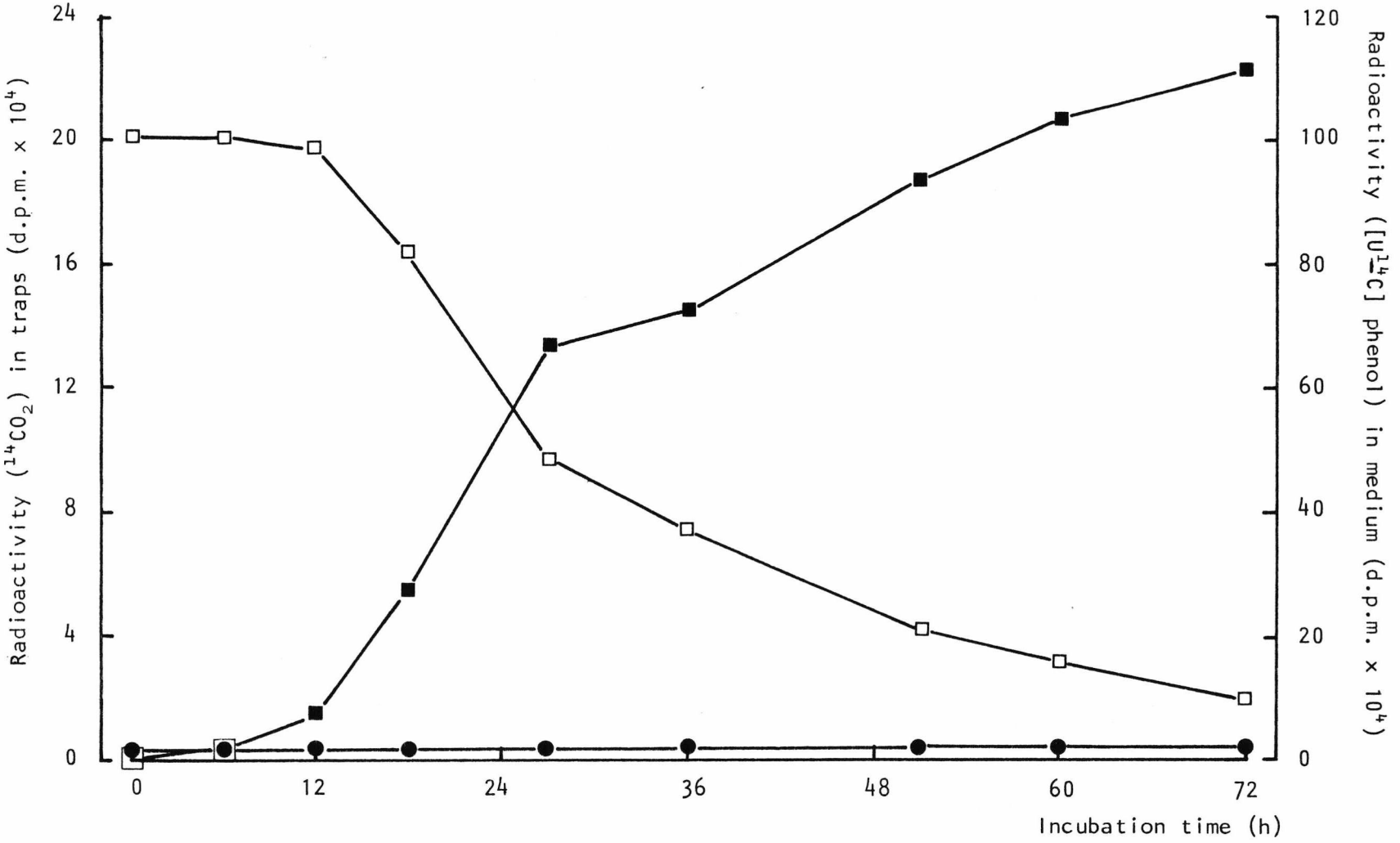


Figure 3.10 Radioactivity recovered as $^{14}CO_2$ from specimens of *Phragmites communis* exposed to [U-^{14}C] phenol
Radioactivity recovered as $^{14}CO_2$ in leaf chamber traps (●) and in root chamber traps (■), and ([U-^{14}C] phenol in the medium (□). Experimental details are given in the text.

were extracted three times with 100ml volumes of diethyl ether, which were pooled, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure at 30-40°C to known volume (5ml). Aliquots of 1ml of the concentrate were chromatographed on paper, using solvents (1) and (2) (Section 2), but showed no phenol or phenyl-β-*D*-glucoside. A brown spot of R_F 0.81 and 0.85 in solvents (1) and (2) respectively was however detected. This corresponded with the position of authentic catechol on such chromatograms, and radioactivity associated with the spot (detected by scintillation counting of paper sections) confirmed its derivation from [U-^{14}C]phenol, though it represented only 0.13% of the radioactivity remaining in the medium. Analysis of the entire chromatograms, after sectioning, by scintillation counting, showed that no other discrete radioactive zone was present, and nor was any other simple phenolic detectable by use of the chromogenic reagent. Many other water and ether-soluble radioactive compounds as well as the extractable catechol must be present in the medium after incubation.

While complete dissimilation of phenol to CO_2 by plant tissues has not been demonstrated unequivocally, the root *zone* of the plant certainly has this activity, and the detection of [^{14}C]catechol is a good indicator of the early steps in oxidative metabolism of [^{14}C]phenol, either by plant tissues or by microorganisms.

3.3.7 Distribution of radioactivity from [^{14}C]phenol in specimens of *Phragmites communis* shown by autoradiography.

Because [U-^{14}C]phenol itself was not evolved from, or detected in, the leaves and stems of plants in the previous study, experiments were set up to show the rate of progress of [U-^{14}C]phenol-derived radioactivity from roots to the leaves of *P.communis* specimens; previous experiments had shown that a substantial proportion of

radioactivity applied to the roots of plants was detectable in all tissues within 24h of application.

The paired plant specimen silhouettes and autoradiographs (Fig.3.11) show that phenol was strongly absorbed by those plant parts in contact with it. Translocation of radioactive material from the roots and rhizome, along the stems of the specimens was apparent after only 4h exposure to [U-^{14}C]phenol. By 18h radioactive material had reached the uppermost leaf tips.

Although no estimate can be made from these experiments of metabolism of phenol in *P.communis*, the movement of radioactive material from roots and rhizomes to leaf tissues was clearly confirmed. The rate of movement of radioactive material in the plant specimen exposed to [U-^{14}C]phenol for 4h, calculated by measurement of the radioactive trace from the uppermost point of immersion in radioactive medium, was 3.7cm/h.

Figure 3.11 Autoradiographs of *Phragmites communis* specimens exposed to [U^{14}C] phenol.

Plant specimens (12 weeks old), were placed in 250ml half-strength Hoagland medium, containing 100µM [U^{14}C] phenol (12.5µCi/flask).

The level of the liquid medium is marked on the stem of the plant on the figures.

After incubation in constant light at 22 ± 1°C for the times shown, one plant specimen was removed from the medium, washed for 1h in distilled water to remove adsorbed phenol, blotted dry, and pressed dry (1 week) between sheets of blotting paper. When preserved, the specimens were mounted on 35 x 3.5cm cardboard strips, and placed in direct contact with 35 x 3.5cm strips of x-ray film (Kodak Ltd, Kirby, Liverpool) in light-proof envelopes. After exposure for 14 days, the films were developed and contact-printed, and matched as shown with silhouette images of the plant specimens obtained on a curved-bed xerox photocopier. The paired images were reduced for presentation by photography.


55cm
0cm

SECTION 4 RESULTS

EXPERIMENTS WITH EXCISED PLANT TISSUES

4.1. THE UPTAKE OF [U-^{14}C]PHENOL BY EXCISED PLANT TISSUES

Clear indications that radioactive phenol was absorbed and metabolised by plant tissues was shown in Section 3, but the use of whole plants and long incubation periods required much material and lengthy processing, which prevented exploration of the early phases of phenol metabolism and rendered experiments liable to significant microbial contributions. For these reasons, comparisons of the uptake by individual plant tissues was examined by exposing small excised samples of root, leaf, rhizome, and stem tissues of selected species to [U-^{14}C] phenol. Root pieces were of 1cm length, taken from roots of 1-2mm diameter; rhizomes of plants posessing them, and stems where used, were cut into 1mm thick transverse sections; leaves were cut into 5mm discs with a very sharp punch, to prevent bruising of tissue adjacent to the cut.

The tissue samples were washed for a total of 1h, with gentle shaking, in five changes of sterile distilled water, to remove the contents of damaged cells, and samples of about 100mg (fresh weight) transferred to 1ml of water containing [U-^{14}C]phenol (1µmol, about 10^4 d.p.m) after lightly blotting them dry. Tissues were incubated for 24h at 22°C in darkness, with radioactivity measurements made before and after incubation. After incubation, the samples were removed, drained of medium, and washed in five changes of sterile distilled water to remove unabsorbed radioactive material. The tissues were again blotted dry, and extracted for 2h (after which time leaf discs were colourless) in 2ml of boiling 80% (v/v) aq. ethanol. The residues were removed by filtration, and washed three times with hot 80% (v/v) aq. ethanol, the washings being added to the appropriate

extracts. Pooled extracts were adjusted to known volumes and their radioactivity determined. The residues were digested overnight (16h, 20°C) in tissue dissociating agent before determination of radioactivity. The results presented are the mean of five replicate experiments performed with tissues from each plant species.

Ethanol extracts of the replicates of each tissue were subsequently pooled and concentrated under reduced pressure at 30-40°C for chromatography on paper in solvents (1) and (2) (Section 2.5).

Results of radioactivity determinations are shown in Table 4.1 . Loss of radioactivity from the media varied widely with species, as did recovery from the tissue pieces. In all samples, recovery of radioactivity (in total) was greater than would be expected if the tissues had reached equilibrium with the medium; i.e. assuming that tissues represented a weight-equivalent volume of medium (Hancock, 1969). This was largely accounted for by the immobilization of phenol-derived radioactivity, observed in the ethanol-insoluble residues of the tissues. Conversely, radioactivity recovered in the ethanol extracts was, in several cases, of similar order to, or less than, that which might be expected if tissues were in equilibrium with the medium, suggesting that phenol might continue to enter tissues as a result of immobilization. In most samples, a greater proportion of recovered radioactivity was found in insoluble materials. Roots of *I.foetidissima* and leaf samples of *M.aquatica* were notable exceptions. In general, the ratio of insoluble residue to ethanol extractable radioactivity in root and rhizome tissues exceeded that in aerial tissue parts. Root tissues of both *Iris* species however, showed the converse. The ratio of insoluble to extractable radioactivity was not expected to follow this pattern, as shown for *P.communis* in Section 3.3.5 , as all tissue pieces had cut edges, not bearing epidermal cells or cuticle.

Table 4.1. Uptake and recovery of radioactivity derived from [U-^{14}C] phenol by excised plant tissues.

Experimental procedures are given in the text.

Plant species	Tissue part	Radioactivity initially applied (d.p.m)	Radioactivity recovered in medium (d.p.m)	Total Radioactivity recovered from tissues (d.p.m)
Iris pseudacorus	Root	12534	7952	1767
	Rhizome		5018	3241
	Leaf		3604	9044
Iris foetidissima	Root	12534	7920	1938
	Rhizome		2673	10260
	Leaf		4372	8020
Mentha aquatica	Root	12534	4728	5717
	Stem		4582	*
	Leaf		1688	4360
Typha latifolia	Root	12534	1472	4958
	Rhizome		198	6185
	Leaf		4170	3580
Phragmites communis	Root	12534	2546	3890
	Rhizome		5441	3545
	Stem		832	7084
	Leaf		2114	3876

*The extract of *M.aquatica* was lost.

Total radioactivity recovered from tissues % of that applied	Radioactivity in ethanol extracts (d.p.m)	Radioactivity in tissue residues (d.p.m)	Ratio of recovery in residues extracts	Total radioactivity recovered in tissues per gram fresh wt (d.p.m/g)
14.1	735	1032	1.40	15839
25.9	942	2299	2.44	22185
72.2	3216	5828	1.81	60153
15.5	1069	869	0.81	14074
81.9	3061	7199	2.35	99505
64.0	3275	4745	1.45	72936
45.6	710	5007	7.05	41177
*	*	3322	*	*
34.8	3350	1010	0.30	41304
39.6	576	4382	0.61	36789
49.3	861	5324	6.18	32841
28.6	998	2582	2.59	19317
31.0	827	3063	3.70	40939
28.3	586	2959	5.05	21469
56.5	1719	5365	3.12	72604
30.9	1676	2200	1.31	67940

Table 4.1 also shows the uptake of radioactivity by the tissues on a constant fresh weight basis. Major differences between the radioactivity content of tissues of different species, and different tissues of the same species, were apparent, reflecting probable differences in the sum of their absorptive and metabolic properties. Results for the two *Iris* species show that both rhizome and leaf tissues accumulated more radioactive material than roots. In *P. communis*, the aerial parts were apparently able to accumulate more radioactive material on direct exposure to [U-^{14}C]phenol than roots and rhizome, but the converse was true of *T. latifolia*. In *M. aquatica*, which absorbed nearly equal amounts of radioactivity in leaf and root tissues, the root tissues nevertheless contained sevenfold more radioactivity in the insoluble fraction than was present in soluble form, while leaf tissue contained over three times more extractable radioactivity than was present in the insoluble residue. In this species, alone of the five examined, the cut edges of all tissue samples were blackened after incubation, indicating the presence, despite washing, of sufficient polyphenol oxidase to metabolize phenol. A control experiment performed with tissues of this plant showed that no blackening occurred when phenol was omitted from the medium, and hence that endogenous aromatic substrates were not responsible for this observation. Unwashed tissue samples, in the absence of phenol, did darken slightly after short incubation periods in air.

A similar series of experiments was subsequently carried out with root, rhizome and leaf tissues of *P. communis*, and, because of the high accumulation of radioactive material by its rhizome tissue in other experiments, the same three tissues of *T. latifolia*, in larger volumes of medium and with provision for $^{14}CO_2$ trapping.

Tissue samples were placed in sterilized medium (5ml, containing 5µmol [U-^{14}C]phenol; 0.1µCi), in previously sterilized sealed vials, each, in addition, holding a small tube (3cm x 0.8cm diam. Durham tubes) containing 1ml of hyamine hydroxide (1M solution in methanol) as a trapping agent for $^{14}CO_2$. The experiment was replicated five fold, and medium was assayed for radioactivity before and after incubation. Entire plant tissue sections were digested without previous ethanolic extraction for radioactivity determinations. Control incubations contained no plant tissue samples.

The recovery of radioactivity in the hyamine hydroxide traps of (no-plant) controls, after 24h incubation at 22°C in darkness, was (mean of 5 replicates) 0.29 ± 0.11 (SD)% of that applied, this value representing 366 d.p.m. per culture. In no tissue incubation was a recovery of $^{14}CO_2$ significantly greater than this found, although after 24h in each incubation other than the controls, phenol-degrading microorganisms were detected. The recovery of radioactivity from the plant tissue pieces was similar to that already described.

This experiment was subsequently repeated, under identical conditions, with root, rhizome, and leaf tissues of *P. communis* in the presence of 50µg/ml streptomycin, added to the medium after sterilization. Only the radioactivity of the $^{14}CO_2$ traps was measured after incubation, but results were very similar to those of the other experiments, namely 0.3 ± 0.12 (SD)% of the applied radioactivity being recovered in the hyamine hydroxide traps of (no-plant) controls, with no significant difference between these and plant tissue incubation.

All plant species examined were shown to be capable of the accumulation of radioactivity from [U-^{14}C]phenol, but chromatography of the concentrated ethanolic extracts did not show the presence of identifiable metabolites of phenol, or of phenol itself (detection

limit of phenol by chromatography was 1-10µg - about 0.1µmol) and release of $^{14}CO_2$ as a product of phenol metabolism by the plant tissue preparations was unsignificant.

The ineffectiveness of streptomycin in incubations of plant tissue pieces was surprising; antibiotic-treated and untreated incubation yielded very similar amounts of radioactivity in $^{14}CO_2$ traps. Numbers of microorganisms in the media were low, ranging from 1-4 x 10^3/ml after plating on 1mM phenol-mineral salts ager, and consideration of results of Section 3.3.4. suggested that the 1mM concentration of phenol used in these experiments was sufficient to restrict the development of the contaminating microflora for the period of incubation.

4.2. ISOLATION AND IDENTIFICATION OF METABOLITES OF PHENOL FROM TISSUES OF *Phragmites communis*

The uptake of considerable quantities of radioactive material, derived from [U-^{14}C]phenol, by excised tissues of *Phragmites communis*, and negligible recovery of $^{14}CO_2$ in the experiments in which radioactive phenol was supplied to tissue samples prompted a search for other metabolites of phenol in these tissues. Experiments of shorter duration were therefore attempted with the intention of detecting early metabolites of phenol.

Samples (2g each) of 1cm diam. leaf discs, 1cm lengths of excised roots (1-2mm diam), and 1mm thick transverse sections of 1cm diam. rhizome were taken from specimens of *P. communis* produced by growth in hydroponic culture. The tissues were taken from five plants, and the pieces randomized. Each sample was washed for a total of 1h in five changes of sterile 0.1M potassium phosphate buffer, pH7.0, and duplicate 1g samples of each tissue were transferred for incubation to

flasks containing 10ml of buffer and [U-^{14}C]phenol (10µmol, 0.5µCi). The incubation volume was doubled for root tissue. Duplicate incubations containing no plant tissues served as controls. The flasks were stoppered and incubatedwith gentle shaking at 22°C in darkness for 5h, after which duplicate aliquots of medium were taken for determination of radioactivity. The tissues were washed well with distilled water, and divided into two parts. Approximately 0.1g of each tissue was lightly blotted dry, weighed, and macerated in scintillation fluid for direct determination of recoverable radioactivity. The larger portion of each was extracted with 50ml of boiling 80% (v/v) aq. ethanol for 2h, when leaf tissues were colourless. The residues after filtration were washed with three changes of 5ml of boiling 80% (v/v) aq. ethanol, the washings and extracts combined, and their radioactivity determined. The extracted residues were dried and prepared for radioactivity determination as already described (Section 3.3.5). An account of radioactivity lost from the media and recovered in plant tissues is given in Table 4.2 .

When compared with the data of previous experiments, in which tissue pieces were exposed to [U-^{14}C]phenol for much longer periods, the uptake of radioactive material was of course lower. Leaf, rhizome, and root tissues in the present experiment contained respectively 1.48, 1.43, and 0.78 µmol of phenol/gram fresh weight of tissue (calculated from the specific radioactivity of phenol applied) after 5h incubation. A separate experiment performed under identical conditions, and using tissues prepared at the same time from the same plants, showed that radioactivity loss from the medium was essentially linear with respect to time in all tissues.

In incubations of leaf, root, and rhizome tissue, 17.0, 8.3, and 9.0% respectively applied radioactivity was lost during the 5h incubation period (Figure 4.1).

Table 4.2. Recovery of radioactivity applied to tissue samples of *Phragmites communis*.

Results are the mean of those from two incubations of each tissue of *P. communis*. Samples of 1g (fresh weight) were incubated in 10ml of 0.1M potassium phosphate buffer, pH7.0, containing 10µmol [$U^{14}C$] phenol (0.5µCi) for 5h at 22°C in darkness. Other experimental details are given in the text. Figures are d.p.m. unless otherwise stated, and the figures in parentheses record the radioactivity lost or recovered as a percentage of that originally applied.

Tissue part	Leaf	Root	Rhizome
Radioactivity applied	1343500	2695800	1345600
Radioactivity lost from medium	212900 (15.9)	192000 (7.1)	107500 (8.0)
Radioactivity recovered in fresh tissue	198869 (14.8)	192408 (7.1)	104930 (7.8)
Radioactivity recovered in ethanol extract	173037 (12.9)	163774 (6.1)	100750 (7.5)
Radioactivity recovered in extracted residue	18797 (1.4)	28188 (1.0)	18427 (1.4)
Total radioactivity recovered from extracted tissues	191834 (14.3)	191962 (7.1)	119177 (8.9)
Ratio of recovery in residues/extracts	0.11	0.17	0.18

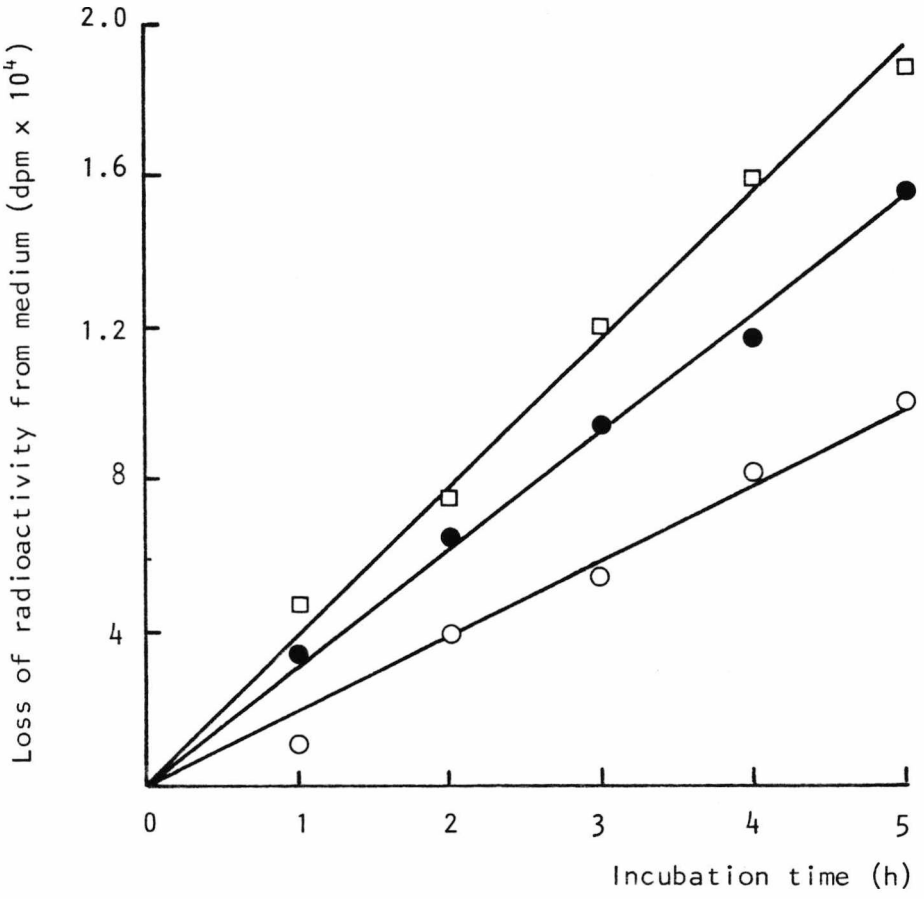


Figure 4.1 Loss of radioactivity supplied as [U-^{14}C] phenol from the medium of incubations of tissues of *Phragmites communis*.

Duplicate samples (1g) of root, rhizome, and leaf tissues were suspended in 10ml of 0.1M potassium phosphate buffer, pH7.0 (20ml for roots) containing 10µmol phenol and 0.05µCi of radioactivity (20µmol and 0.1µCi respectively for roots) and incubated at 22°C in darkness for 5h with gentle shaking. Radioactivity of the media was measured initially and at intervals thereafter by sampling (100µl) in duplicate, and the results presented are the mean of four results. Radioactivity remaining in root (●), rhizome (o) and leaf (□) incubations is shown.

Table 4.2 shows that in two cases, in root and leaf tissues, more radioactivity was absorbed than was to be expected if the tissue had attained equilibrium with the medium, but rhizome tissue appeared to exclude phenol. The larger part of the absorbed radioactivity found in processed plant tissues (after 5h incubation) was located in the ethanol-soluble fraction. This is in contrast with previous results, from experiments using plants and plant tissues incubated for 24 and 120h, in which most absorbed radioactivity was immobilized in ethanol-insoluble extracted residues. Total recovery of radioactivity corresponded closely with that lost from the medium; the sums of recoveries from ethanol extracts and residues of leaf, rhizome, and root tissues were respectively 90.1, 110.9, and 100% of the loss from the medium, and recovery from directly counted fresh tissue samples corresponded closely with these results, confirming the efficacy of the extraction and measuring techniques.

After completion of the incubations and determinations of radioactivity, both tissue extracts and the remaining incubation media were examined for products of phenol metabolism. No product of [U-^{14}C]phenol was found in any incubation medium after chromatography of both aqueous and diethyl ether-extracted preparations, though unaltered [U-^{14}C]phenol was readily reisolated.

Products of [U-^{14}C]phenol in tissue samples. After determination of radioactivity, the two ethanol extracts of each plant tissue were pooled and evaporated to dryness under reduced pressure at 30-40°C. The gummy residue was re-extracted three times with 10ml aliquots of hot (60°C) water. These extracts were pooled, evaporated to dryness as above, and the residue extracted thrice with 10ml volumes of cold methanol. The pooled methanol extracts were concentrated

under O_2-free dry N_2 to 10ml, their radioactivity determined, and the remainder used for chromatographic isolation of the products of phenol metabolism by the plant tissues.

All three tissues showed three major compounds to be present. Phenol (identified by its R_F. in five solvents on both silica-gel and cellulose t.l.c. plates) was always found. A further compound, tentatively identified as catechol, gave a brown, diphenolic, colour reaction with Gibbs' reagent (Section 2.5), and had R_F. values (0.75, 0.81. 0.43, 0.46, and 0.29 in solvents 1, 2, 3, 7, and 9 respectively) corresponding with authentic catechol. This compound was weakly radioactive. Phenyl-β-*D*-glucoside(R_Fs in solvents 1, 2 7, and 9 respectively 0.23, 0.97, 0.09, and 0.02) was not detected by its blue, monophenolic colour reaction with Gibbs' reagent after enzymic hydrolysis on t.l.c. plates, but a radioactive compound was located in the appropriate positions, corresponding with authentic material.

Methanol extracts of plant tissues were banded on preparative cellulose thin layer plates prepared in the laboratory, and after running in solvent (1), bands corresponding with authentic phenol, catechol, and phenyl-β-*D*-glucoside were scraped from the backing sheet and eluted in methanol. The methanol eluates were filtered and reduced to 10ml, and their radioactivity determined by scintillation counting. Distribution of radioactivity between [U-^{14}C]phenol and the suspected products is shown in Table 4.3. The R_F. value of authentic phenol in solvent (1) was 0.91, of catechol was 0.72, and of phenyl-β-*D*-glucoside was 0.30, giving sufficient separation for good preliminary purification. The largest part of the radioactivity applied was recovered from preparative t.l.c. as the material corresponding with phenyl-β-*D*-glucoside (Table 4.3), and very little as unaltered phenol,

Table 4.3. Recovery of radioactivity in metabolic products of [U-^{14}C] phenol applied to excised tissues of *Phragmites communis*

The table shows the recovery of radioactivity in pooled extracts of duplicate samples of plant tissues after ethanol extraction, purification by transfer to methanol, and after preparative chromatography to yield 2 major putative metabolites. All results shown are the average of two determinations of the radioactivity of each extract and eluate. Experimental procedures are described in the text. *The extract of leaf tissue applied to preparative t.l.c. plates was augmented with similar extract from a replicate experiment as much material was consumed in preliminary studies to refine chromatographic methods.

Plant tissue part	Radioactivity (d.p.m) Leaf	Rhizome	Root
Radioactivity in original ethanol extract of tissue	173037	100750	163774
Radioactivity in methanol concentrate used for t.l.c	145300	40000	32500
Radioactivity applied to preparative t.l.c *	247200	38800	39760
Radioactivity recovered by elution as			
(1) Phenol (R_F 0.91) (% applied)	52150 (21.1)	0	2100 (5.3)
(2) Catechol (R_F 0.72) (% applied)	34500 (14.0)	14300 (36.9)	8500 (21.4)
(3) Phenyl-β-*D*-GLucoside (% applied) (R_F 0.30)	151300 (61.2)	24500 (63.1)	28000 (70.4)

in all three tissues.

To confirm the identity of the metabolites, portions of the eluate from preparative t.l.c. of the tissue extracts were co-chromatographed with the corresponding authentic materials (10µg). In leaf tissue extracts, suspected catechol and phenyl-β-*D*-glucoside corresponded exactly with authentic materials, and were radioactive Phenyl-β-*D*-glucoside was similarly found in root and rhizome tissues. The suspected catechol gave a barely detectable colour reaction, but the zones corresponding with authentic catechol on chromatograms of this material were weakly radioactive (Fig.4.2).

Authentic catechol was added to portions of the eluate from each of the three tissues, and three recrystallizations were made from hot benzene. The results are given in Table 4.4, and show that [^{14}C]catechol was indeed present in tissue extracts of root, rhizome, and leaf of *P.communis*. A high level of contamination with another compound, also soluble in hot benzene, might account for the variability of the results, especially the large fall in specific radioactivity on the first recrystallization. [U-^{14}C]phenol was a probable contaminant of the early stages of this purification, but second and third recrystallizations yielded results showing clearly that catechol was indeed present.

In leaf tissue, which was chosen for further examination as it contained much radioactive material, phenyl-β-*D*-glucoside was confirmed as the major metabolite of phenol. The results of recrystallization of putative phenyl-β-*D*-glucoside isolated by chromatography are given in Table 4.5, which shows that the compound isolated was very highly purified with comparative ease.

Although the phenyl-β-*D*-glucoside from root and rhizome tissues was not purified to such a degree, the good correspondence of the

Figure 4.2 Chromatography of and detection of radioactivity in a putative metabolite of [U-^{14}C] phenol from tissues of *Phragmites communis*.

Samples (100µl) of putative catechol eluted from preparative chromatograms as described in the text were co-chromatographed with authentic material on cellulose and silica-gel t.l.c. plates in solvents (1,2,3,7, and 9) (Section 2.5), developed with Gibbs' reagent, and scanned for radioactivity (2min/cm or 5min/cm scanning speed, maximum scale deflection 100 or 300cpm, on the instrument described in Section 2.9). Tracings of typical chromatograms obtained in solvent (7) on silica-gel from root extract (Figure A) and in solvent (9) (Figure B) are presented, accompanied by the appropriate radioactivity scan. The R_F values of the isolated, co-chromatographed catechol for these chromatograms are shown on the figures, and in other solvents were, for root, rhizome, and leaf tissues, in the same order throughout, in solvent (1) 0.75, 0.75, 0.76; (2) 0.81, 0.80, 0.80; (3) 0.44, 0.42, 0.42; (7) 0.36, 0.34, 0.36; and (9) 0.26, 0.31, and 0.28.

Sf in the figures denotes the position of the solvent front, and hatched shading the position of the co-chromatographed authentic and isolated catechol (brown colour reaction). Insufficient catechol was present in extracts to yield a colour reaction alone. Unshaded spots indicate the position of authentic and co-chromatographed phenol (blue colour reaction).

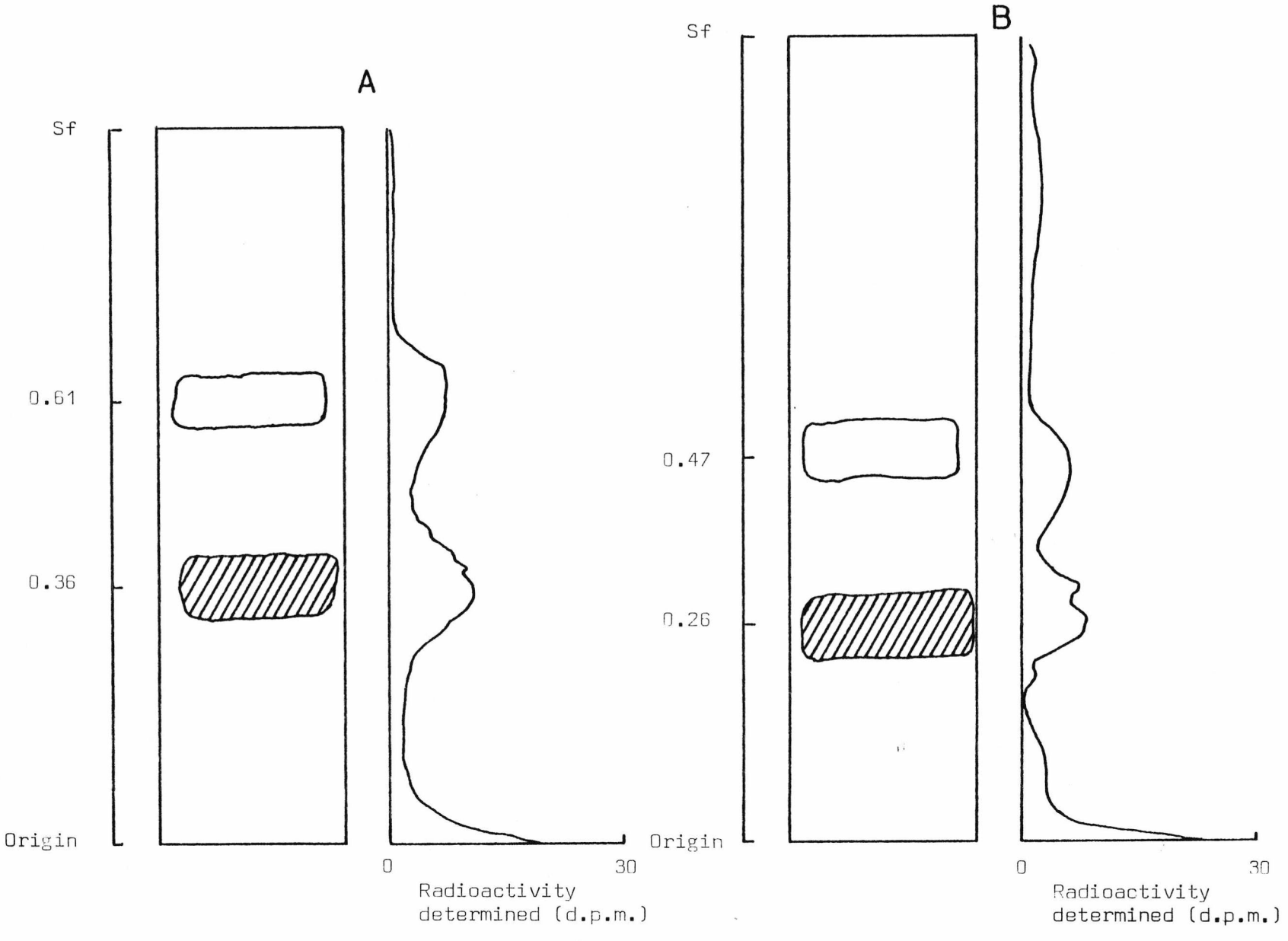

A
Sf
0.61
0.36
Origin
0
30
Radioactivity
determined (d.p.m.)
B
Sf
0.47
0.26
Origin
0
30
Radioactivity
determined (d.p.m.)

Table 4.4. Identification of catechol as a metabolite of phenol by recrystallization to constant specific radioactivity with authentic material.

A portion of the methanol eluate of preparative t.l.c. of each tissue extract (prepared as shown in the text) was reduced to dryness, and authentic catechol (100mg) added with 10ml of hot benzene. After cooling under O_2-freeN_2, the recrystallized material was collected on a sinter, and dried *in vacuo* overnight, after which no further weight loss occurred. A weighed portion of each recrystallized material was sacrificed for determination of radioactivity, and the remainder recrystallized twice more. The weight of the sample taken at each stage is shown. The results are shown as the specific radioactivity (d.p.m./mg), and, in parentheses, the total radioactivity determined at each stage (d.p.m.). Some 10^3 counts were accumulated for each determination. The initial radioactivity of the sample of eluate initially taken was, for leaf tissue 17250 d.p.m, for rhizome 2550 d.p.m, and for root 7150 d.p.m.

	Specific and (Total) radioactivity		
Tissue part	Leaf	Rhizome	Root
Wt of sample (mg)	4.38	4.18	4.06
1st Recrystallization	96 (420)	50 (209)	99 (402)
Wt of sample (mg)	5.61	5.45	5.28
2nd Recrystallization	18 (101)	20 (109)	39 (206)
Wt of sample (mg)	4.34	5.50	3.29
3rd Recrystallization	29 (126)	24 (132)	48 (158)

Table 4.5. Identification of phenyl-β-*D*-glucoside as a metabolite of phenol by recrystallization to constant specific radioactivity with authentic material

To a portion of methanol eluate from preparative t.l.c. of extract of leaf tissue (prepared as shown in the text) (27360 d.p.m) was added 250mg of authentic phenyl-β-*D*-glucoside. The whole was taken up in hot distilled water, and, on cooling, recrystallized material was collected by filtration and dried *in vacuo* to constant weight. A weighed sample was taken for determination of its radioactivity, and the remainder recrystallized 3 times, sampling and counting being repeated at each stage. Some 10^4 counts were accumulated before specific radioactivity was determined.

Recrystallization	Total radioactivity counted (d.p.m.)	Weight of samples (mg)	Specific radioactivity (d.p.m./mg) mean of 2
1	528	6.4	
	617	7.5	82
2	416	5.1	
	499	6.1	82
3	241	2.9	
	199	2.4	83
4	167	2.0	
	218	2.6	83

putative metabolite with the authentic compound on thin-layer chromatograms (Fig.4.3) shows that it was indeed present, and hence it was concluded that phenyl-β-*D*-glucoside is a significant early metabolite of phenol in *P.communis*.

4.3. ISOLATION AND EXAMINATION OF MICROORGANISMS FROM PLANTS

The tissues of twelve species of plants were screened for the presence of phenol-degrading microorganisms by the methods described in Section 2.3.1. and 2.3.2. Both the surfaces and the interior tissues were examined, the former by "printing" the tissue samples firmly onto agar plates, which technique will recover only epiphytic microorganisms, and the latter by homogenization, after surface sterilization, which will allow the isolation and enumeration of the internal microflora of the tissues. Identification of organisms, other than the predominant phenol-degraders and some fungi closely associated with root tissues, was not attempted.

Samples of root, rhizome, stem, and leaf from all plant species other than *E.crassipes* and *L.minor* were taken with sterile precautions to prevent the introduction of laboratory contaminants which might resist surface sterilization. *E.crassipes* samples were of root, leaf laminae, and enlarged aerenchymatous floats, the latter parts being labelled "leaf" and "float" respectively in Table 4.6. *L.minor* was used immediately after its initial collection, as the plant proved very sensitive to hypochlorite and other sterilants.

No count of organisms shown on print plates was attempted, as colonies rapidly became confluent; print plates were made only on phenol-mineral salts agar.

A "control" plant tissue, consisting of 1g of elder pith, soaked in distilled water and sterilized by autoclaving, was carried through

Figure 4.3 Chromatography of and detection of radioactivity in a putative metabolite of [U-^{14}C] phenol from tissues of *Phragmites communis*.

Samples (100µl) of putative phenyl-β-*D*-glucoside eluted from preparative chromatograms as described in the text were co-chromatographed with authentic material on cellulose t.l.c. plates in solvents (1,2, and 3) (Section 2.5), and, after enzymic hydrolysis on the plate (Section 2.5) phenolic compounds were visualized and scanned for radioactivity as described in the legend to Fig. 4.2.

Tracings of typical chromatograms of material from root (B), rhizome (C), and leaf (A) tissue made with solvent (3) are presented, showing the phenyl-β-*D*-glucoside spot (blue colour), with the accompanying radioactivity scan. The R_F value of the phenyl-β-*D*-glucoside obtained is given on each figure, and R_F values of this compound on chromatograms not shown were, for root, rhizome, and leaf extracts respectively, in solvent (1) 0.20, 0.18, and 0.21, and in solvent (2) 0.97, 0.95, and 0.98. Radioactive material corresponded exactly with these positions.

Hatched shading shows the position of co-chromatographed authentic and isolated phenyl-β-*D*-glucoside, and open spots the position of isolated material.

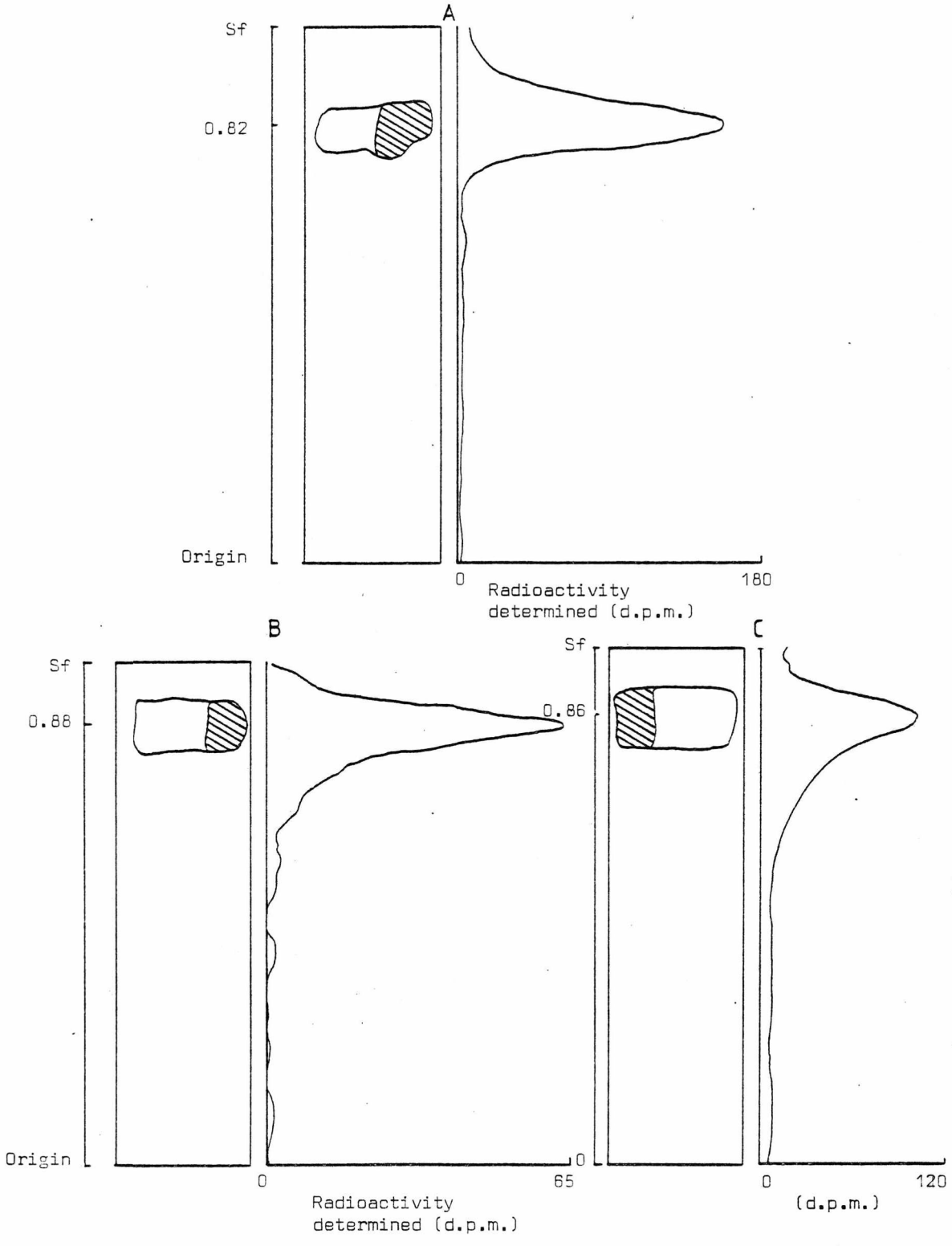

A
Sf
0.82
Origin
0
180
Radioactivity
determined (d.p.m.)
B
Sf
0.88
Origin
0
65
Radioactivity
determined (d.p.m.)
C
Sf
0.86
0
0
120
(d.p.m.)

Table 4.6 Phenol degrading and other bacteria isolated from plant tissues.

Organisms were detected on nutrient agar, nutrient agar plus 1mM phenol, and 1mM phenol - mineral salts agar. Light (+) and heavy (++) growth on 1mM phenol agar print plates are shown in parentheses. Experimental details are given in the text.

Plant species	Tissue part	Viable count/g x 10^{-2}		
		Nutrient agar	Nutrient agar plus 1mM phenol	1mM phenol - mineral salts agar
Phragmites communis	Root	4	1	3 (+)
	Rhizome	4000	50	4 (+)
	Leaf	5	3	3 (+)
Typha latifolia	Root	4000	300	200 (+)
	Rhizome	3000	300	10 (++)
	Leaf	300	10	10 (+)
Typha minima	Root	50000	20000	9000 (++)
	Rhizome	30000	3000	300 (+)
	Leaf	400	100	20 (+)
Eriophorum vaginatum	Root	30000	500	40 (+)
	Rhizome	8000	3000	2000 (+)
	Leaf	20	10	0 (+)
Iris pseudacorus	Root	0	0	0 (+)
	Rhizome	30000	20000	20000 (++)
	Leaf	90000	5000	3000 (++)

Iris foetidissima	Root	0	0	0 (+)
	Stem	40000	40000	30000 (++)
	Leaf	40000	20000	20000 (++)
Mentha aquatica	Root	0.1	0	0 (++)
	Stem	80	0	0 (++)
	Leaf	400	100	30 (++)
Butomus umbellatus	Root	30000	80000	100000 (++)
	Rhizome	2000	1000	2000 (+)
	Leaf	6000	7000	20000 (++)
Alisma plantago-aquatica	Root	200	300	1000 (+)
	Leaf	3000	100	300 (+)
Schoenoplectus tabernaemontani var. *albescens*	Root	10	0	0 (+)
	Rhizome	500	20	300 (+)
	Leaf	2	1	0 (+)
Eichhornia crassipes (see text)	Root	10000	2000	500 (+)
	"Float"	230000	200000	30000 (+)
	"Leaf"	60000	40000	1000 (+)
Lemna minor (see text)	Whole plant	60000	50000	50 (++)

the homogenization procedure to show that the maintenance of sterility throughout this process was successful. One replicate from five of this material showed contamination of 100 organisms per ml of homogenate, but no bacteria capable of growth on phenol agar plates were found. Native plant samples yielding no microorganisms after surface sterilization served as additional internal controls.

At least one of the major tissue parts from all the plant specimens bore a population of microorganisms capable of growth on 1mM phenol-mineral salts agar (Table 4.6). In only six tissue samples of thirty-three was a population of phenol-degrading microorganisms resistant to surface sterilization absent, and only two surface sterilized tissues - roots of *Iris* species - yielded no microorganisms whatever. The counts of organisms on roots of *M.aquatica* were low (10 organisms per g fresh weight) and *P.communis* too yielded consistently small numbers of organisms, confirming earlier results of Seidel (1964, 1969, and 1973b) which suggested that *P.communis*, *M.aquatica*, *Schoenoplectus lacustris*, *Alisma plantago aquatica* and *Iris pseudacorus* reduced the population of microorganisms in water surrounding them. Hydroponic culture media of *M.aquatica* became markedly odorous in some experiments, suggesting leaching or exudation of volatile materials from the plants. Such compounds, especially if of terpenoid character, such as menthol and thymol found in this genus, are toxic or antibiotic.

E.crassipes samples contained the largest total numbers of microorganisms, although other specimens (*B.umbellatus* and *Iris* species) also contained numbers of phenol-degrading organisms of similar order.

No fungal growth was observed on count plates, probably because

the media used were adjusted to a pH more suitable for bacterial growth. It was thought unlikely that plants would be entirely free of fungi, so tissues of *P. communis* were examined microscopically for evidence of fungal contamination. Fifty samples each of root and rhizome pieces (short sections of root and tangential longitudinal sections of rhizome, cut to include epidermis) were taken from each type of culture shown in Table 4.7, and scored positive or negative for the presence of fungal hyphae. Scores are shown in Table 4.7. Greenhouse-grown plant samples gave the highest score, and collection site samples were much less heavily contaminated. Hydroponic laboratory plant cultures had heavily contaminated roots, but rhizomes showed less fungal material than other samples. Agitation of the media may have prevented fungal growth on the smooth rhizome surface, while permitting good growth of fungi in the more obstructed root masses.

Examination of *P. communis* tissues showed no evidence of penetration of the surface by fungi, nor were mycorrhizal structures evident in *P. communis* roots. Only in obviously necrotic areas of root tissues was fungal growth seen within the plant material, and such specimens were routinely avoided when selecting plants for any experiment.

As all plant materials examined for fungi were prepared by washing with distilled water in the same manner as those intended for phenol exposure experiments, or for homogenization, the possibility of transfer of fungal cells to phenol-containing media existed. Root and rhizome tissues of *P. communis* were therefore tested for contamination by phenol-metabolizing fungi. Samples of each tissue from the culture types showing the presence of fungi were blotted, with aseptic precautions, onto the surface of Bavendamm (1928) agar plates (0.5%

Table 4.7. Presence of fungi on samples of root and rhizome tissues of *Phragmites communis* from four sources.

Experimental details are given in the text.

Tissue Sample	Percentage (of 50) samples showing the presence of fungi			
	Source of tissue			
	Greenhouse soil pots	Collection site at Stodmarsh	Laboratory hydroponic culture	Sterile seedlings
Root	92	84	100	0
Rhizome	84	64	42	0

(w/v) yeast extract agar containing 0.17% tannic acid, pH about 6.0) and the plates incubated for 72h at 25°C. Positive results (blackening of the medium caused by extracellular polyphenol oxidase) were obtained from all inocula, and both mycelial and yeast-like fungi showing this activity were detected. Yeast-like forms isolated from such plates failed to grow on 1mM phenol-mineral salts agar, but weak growth of a mycelial fungus was obtained on transfer to this medium. The phenol-degrading fungus did not sporulate when grown on phenol-mineral salts agar, and showed some extracellular polyphenol oxidase activity (darkening of the phenol agar medium). It could not be induced to spore on yeast-extract agar, yeast-extract-potato-dextrose agar, corn-meal agar, malt-extract agar, Czapek-Dox agar, or a Saboureaud-type medium (yeast-extract-peptone-dextrose agar), and therefore remained unidentified. As growth of the organism on phenol was apparent, though, the presence of fungi capable of phenol degradation as well as bacteria in association with *P. communis* specimens was confirmed.

In order that some identification of the fungi associated with *P. communis* roots and rhizomes might be attempted, small (1-2mm) sections of these tissues were taken from washed plant specimens grown in the greenhouse in soil pots and placed directly on the surfaces of plates of each of the fungal media already described. After 24, 72 and 120h incubation at 25°C, specimens of some of the fungi growing from the plant tissue inoculum were examined. Species of *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Cephalosporium*, *Trichoderma*, and an unidentified Basidiomycete were found. The last named was identified by the presence in its hyphae of typical clamp connections, and the others by reference to Smith (1969). *Aspergillus niger* was recognized by its large black sporangia,

growing on all the media used. An organism giving rise to yeast-like, glossy, small colonies, when incubated further on yeast-extract agar, malt extract agar, and yeast-extract potato-dextrose agar (3-5 days, 25°C) produced leathery dark brown colonies, in which thick dark hyphae bore conidia as lateral buds. This was tentatively identified as *Pullularia (Aureobasidium) pullulans*. Several red to pink yeasts grew on malt-extract and yeast extract media, and were probably of the genera *Rhodotorula* or *Sporobolomyces*. Many representatives of these fungal genera, discovered on *P.communis*, have been shown to degrade aromatic compounds, and all are common saprophytes of soil or plant materials.

The bacterium predominating as a contaminant in experiments in which phenol was supplied to plants was tentatively identified as a fluorescent species of *Pseudomonas*. Microscopic examination of samples retained from experiments, cultured on 1mM phenol agar slopes, showed it to be a Gram-negative, possibly flagellate, rod, and colonies of the organism, which were glossy and translucent to creamy-white on phenol agar, fluoresced vividly under ultraviolet light.

Scanning electron microscopy of tissue specimens of *P.communis* failed to show evidence of the internal colonization of any part of this species by microorganisms, which, in view of the ready detection of bacteria in macerates of surface-sterilized tissues, was unexpected. Surfaces of root and rhizome tissue of *P.communis* did, however, bear visible microbial cells (Plates 4.1 and 4.2). These specimen photomicrographs clearly show rod-shaped bacterial cells on the strongly ridged external surface of the rhizome, and fungal hyphae ramifying among root hairs. The specimen of root tissue which yielded this photomicrograph also showed growth of the Basidiomycete fungus reported above.

R

H

R

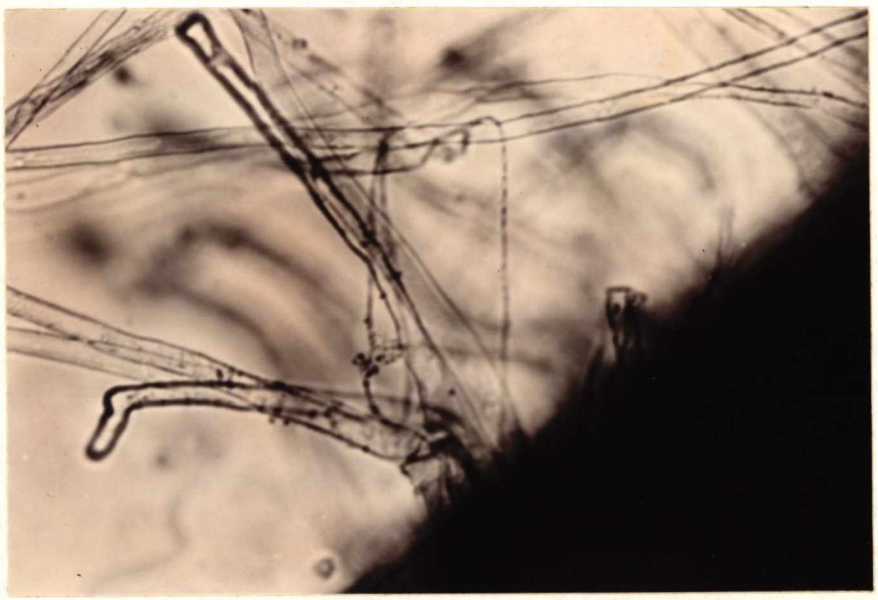


Plate 4.1 Fungal hyphae associated with roots of *Phragmites communis*.

The light photomicrograph shown (magnification, x 90) was of a cottonblue stained preparation (Section 2.3.4) of root tissue not previously exposed to phenol. Fungal hyphae (H) are clearly visible in association with root hairs (R).

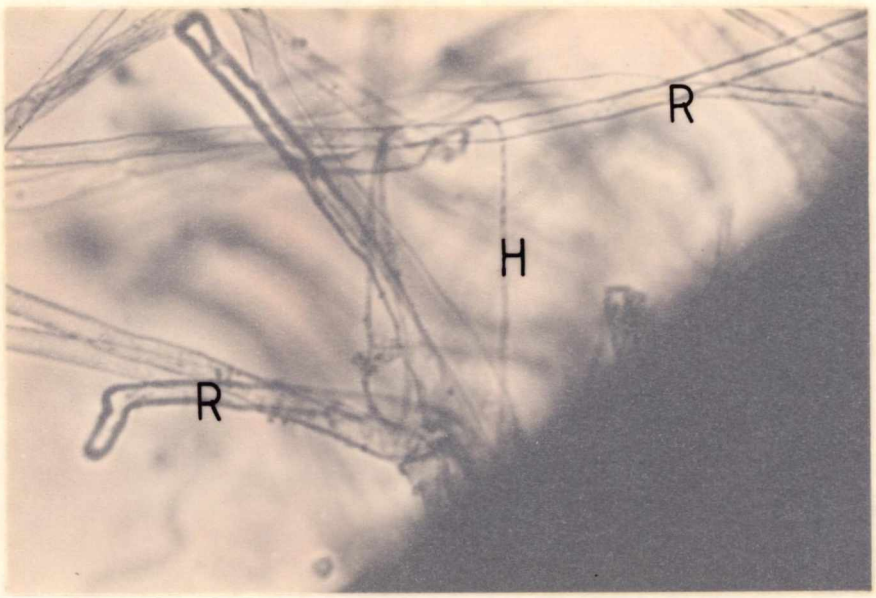


Plate 4.1 Fungal hyphae associated with roots of *Phragmites communis*.

The light photomicrograph shown (magnification, x 90) was of a cottonblue stained preparation (Section 2.3.4) of root tissue not previously exposed to phenol. Fungal hyphae (H) are clearly visible in association with root hairs (R).

B B

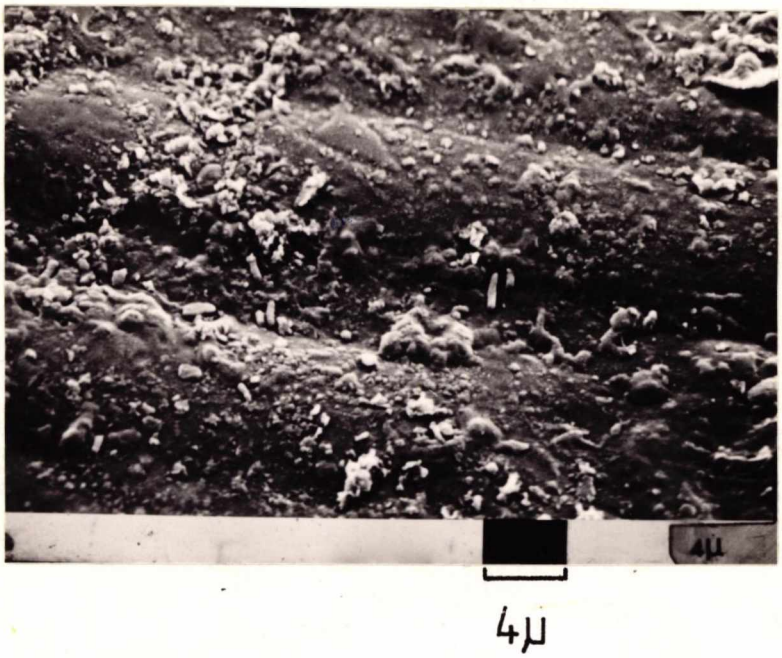


Plate 4.2 Surface bacteria of *Phragmites communis* rhizome.

The photomicrograph shown was of a sample of tissue, prepared for scanning electron microscopy by the methods described in Section 2.3.4, which had not previously been exposed to phenol. Rod -shaped bacterial cells (B) are clearly shown.

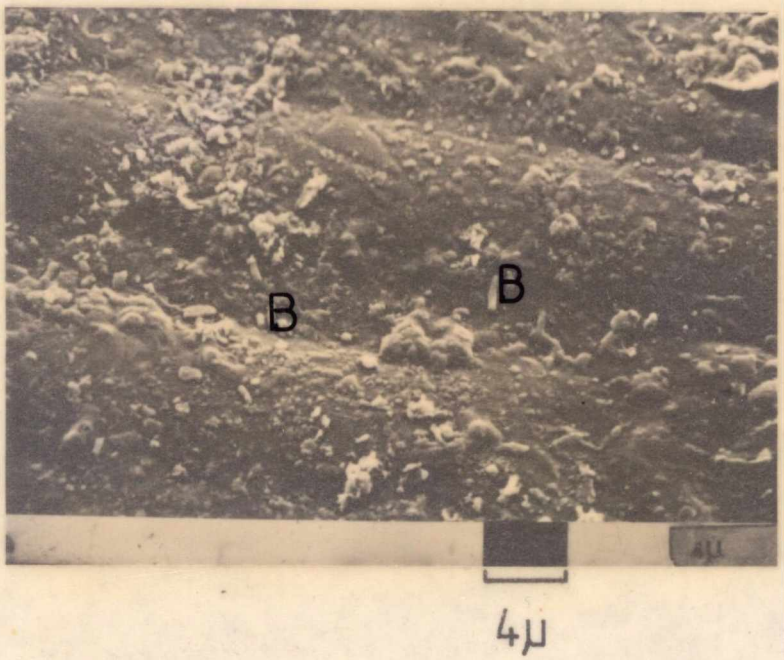


plate 4.2 Surface bacteria of *Phragmites communis* rhizome.

The photomicrograph shown was of a sample of tissue, prepared for scanning electron microscopy by the methods described in Section 2.3.4, which had not previously been exposed to phenol. Rod-shaped bacterial cells (B) are clearly shown.

These experiments thus gave evidence that many microorganisms are intimately associated with the tissues of experimental plants, and that these may provide a phenol-degrading inoculum in plant culture experiments.

SECTION 5 STUDIES ON CELL-FREE PLANT TISSUE PREPARATIONS

5.1 EXPERIMENTS TO DETECT GLUCOSYLATING AND GLUCOSIDASE ACTIVITY IN CELL-FREE PREPARATIONS OF PLANT TISSUES.

5.1.1 Glucosylating activity

Substantial production of phenyl-β-*D*-glucoside was shown in excised tissues of *P.communis*, (Section 4.1), and the enzymes responsible for its formation were therefore sought. Yamaha and Cardini (1960a; 1960b) and Conchie *et al.* (1961) demonstrated the formation of mono-, di-, and triglucosides of certain diphenols in extracts of commercial wheatgerm; broadbean (*Vicia faba*) preparations also possess this activity (Pridham and Saltmarsh, 1963). It was anticipated that germinating barley seeds, shoots, and roots would also possess glucosylating activity, in materials from which extraction of the enzymes might be simpler.

Cell-free extracts of all tissues (root, rhizome, and leaf) of *P.communis* failed to show phenol-glucosylating activity in any of the assay systems described in Section 2.8.4.

Preparations of wheatgerm (from two sources) and broadbeans (commercial seed of two varieties), in which activity with quinol and other substituted phenols has been shown by other workers, were tested exhaustively for activity with phenol, in order to show that the assay systems functioned correctly. Appropriate stoicheiometric results were obtained in assays when commercial UDP was included in model assays, and UDPG could be determined accurately with UDPG-dehydrogenase. Model assays containing extracts of wheatgerm, broad beans, or *P.communis* tissues consumed both UDP and UDPG at a low rate, whereas the phenol concentrations of extracts, estimated by the 4-aminophenazone method, was not altered.

Additions to the extracts, at the homogenization stage or subsequently, of reagents useful for the stabilization of enzymes (EDTA, cysteine, bovine serum albumen, and others), or alteration of the concentrations of these substances when already present in assay methods from the literature, were without effect. Alteration of the buffers described in original references likewise had no beneficial effect.

Paper chromatography of deproteinized extracts from plant material, supplied with phenol as a glucosylation substrate, yielded only phenol; it was therefore concluded that under the test conditions the compound was not a suitable substrate for glucosylating enzymes in any of the plant preparations examined.

In wheatgerm extract (Sigma), prepared as described by Yamaha and Cardini (1960a; 1960b) to the crude extract stage, quinol (1 and 10mM) promoted the formation of UDP from UDPG at a rate in excess of the endogenous UDPG consumption. Using 10mM substrates, the maximum rate of UDPG consumption (measured by use of UDPG dehydrogenase) and pyruvate formation (measured by pyruvate 2,4-dinitrophenylhydrazone formation and in the other assays of Section 2.8.4) was 1.3nmol/min/mg protein. This result suggests that quinol was a substrate of a glucosylating enzyme, and partially confirms the activity of wheatgerm extract, but quinol was not a substrate in extracts from *P.communis*.

5.1.2 Activity of Glucosidase in cell-free preparations of *Phragmites communis*.

Extracts of tissues of root, rhizome, and leaves of *P.communis* were screened for possession of a glucosidase, in an attempt to find out whether the activity of such an enzyme might be responsible for the lack of phenyl-β-*D*-glucoside in extracts supplied with the appropriate substrates (Section 5.1.1).

All tissues possessed some glucosidase activity when *p*-nitrophenyl-β-*D*-glucoside was used as the substrate. The pH optima of the enzymes from the three tissue sources in 0.2M phosphate-0.1M citrate buffers were the same, pH5.5 (Fig. 5.1). When the assays were repeated in 0.1M potassium phosphate buffer, pH7.0, used for tissue extractions, the change of buffer at this pH had no effect on activity. The activity of the preparations was directly proportional to protein concentration in all three cases (Fig. 5.1), but as the extracts of the different tissues contained markedly different amounts of protein, specific activities with respect to both protein and fresh weight of tissue were also variable (Table 5.1). Leaf tissue yielded more protein in extracts than both root and rhizome materials but its specific activity was much lower, whereas the large total yield of protein from this tissue caused the specific activity per gram of tissue to be high.

The Michaelis constant (K_m) of the crude glucosidase extracted from *P.communis* tissues was not rigorously determined, but was estimated to be within the range 1-5mM by comparing the specific activity of extracts from all three tissues at three substrate concentrations.

The specific activity of glucosidase, measured in the presence of 1,5, and 10mM *p*-nitrophenyl-β-*D*-glucoside, and presented in order of ascending substrate concentration, was 13.4, 44.0, and 45.1 nmol/min/mg protein; 12.4, 38.0, and 41.4 nmol/min/mg; and 6.9, 18.3, and 20.2 nmol/min/mg for root, rhizome, and leaf extracts respectively.

Control assays, using heat inactivated (10min at 100°C) extracts showed no activity against 10mM or 1mM *p*-nitrophenyl-β-*D*-glucoside.

The activity of the glucosidases of *P.communis* tissues was also determined with phenyl-β-*D*-glucoside, the expected product of glucosylation of phenol, by measurement of the concentration of phenol formed in incubation mixtures initially supplied with 10mM substrate.

Figure 5.1 pH Optima of glucosidase preparations from tissues of *Phragmites communis*, and effect of concentration of protein on activity.

Assays of total volume 1ml, contained 10µmol of *p*-nitrophenyl-β-*D*-glucoside and 50-250µl of crude cell-free plant extracts (Section 2.7) in 0.2M potassium phosphate – 0.1M citrate buffers, made to the pH values shown on Fig. A, and were performed at 25°C. The reactions were initiated by the addition of the phenolic substrate, and the concentration of product estimated by the methods described in Section 2.8.3, after withdrawal of samples (100µl) after 15,30,45, and 60mins incubation. Assays to determine the linearity of response of activity to protein concentration, (Figs B and C) were performed in the same manner, using 10µmol of substrate and 50-250µl of extracts in 0.2M potassium phosphate–0.1M citrate buffer, pH5.5.

In Figs. A,B, and C, the results for leaf (o), rhizome (●), and root (□) tissue extracts are shown.

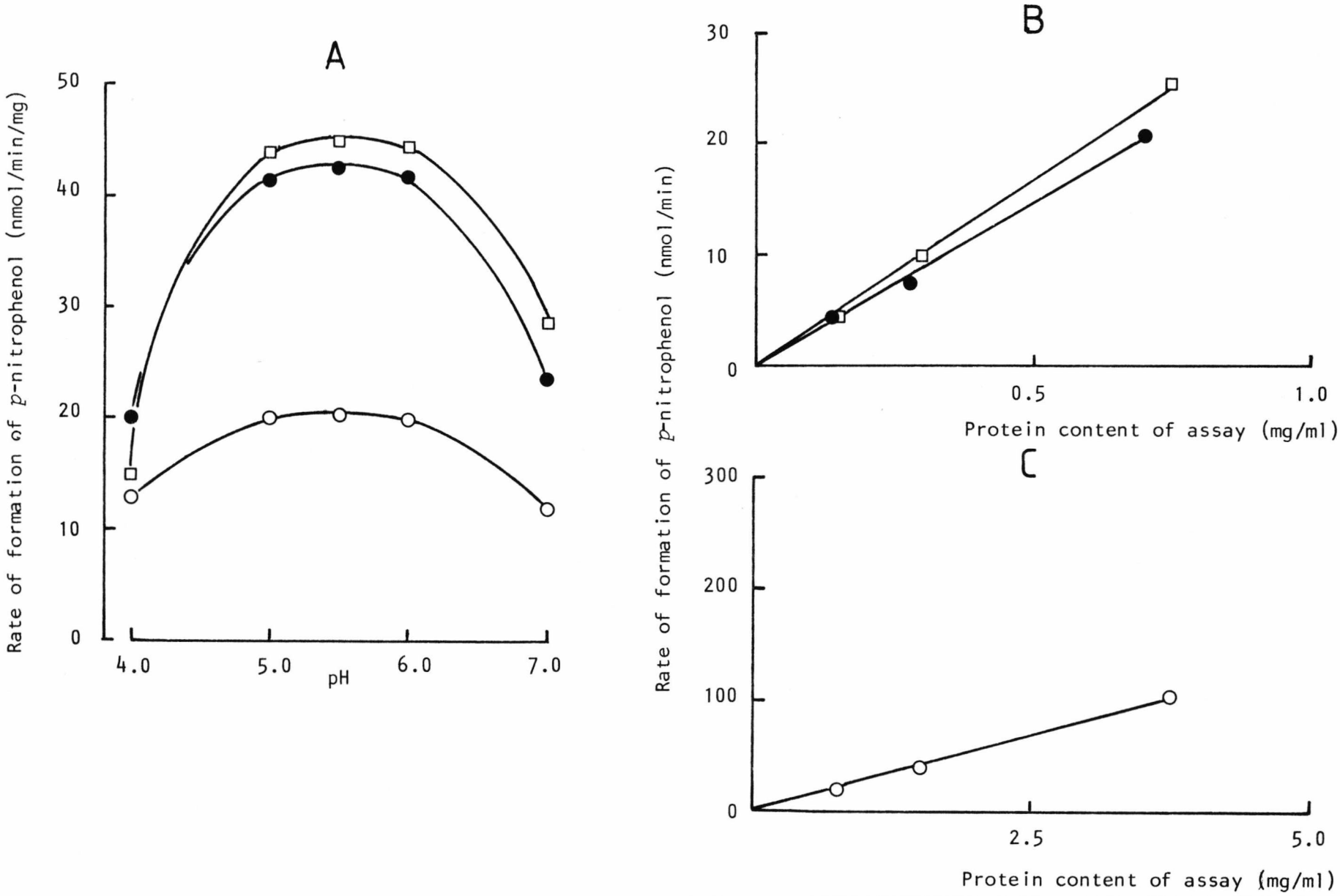

A
Rate of formation of p-nitrophenol (nmol/min/mg)
50
40
30
20
10
0
4.0
5.0
6.0
7.0
pH
B
Rate of formation of p-nitrophenol (nmol/min)
30
20
10
0
0.5
1.0
Protein content of assay (mg/ml)
C
300
200
100
0
2.5
5.0
Protein content of assay (mg/ml)

Table 5.1 Specific activity of glucosidase in extracts of tissues of *Phragmites communis*.

Assays, of 1ml, contained 10µmol of *p*-nitrophenyl-β-*D*-glucoside and 50-250µl of crude cell-free plant extract (Section 2.7) in 0.2M potassium phosphate - 0.1M citric acid buffer, pH5.5, and were performed at 25°C. The reaction was initiated by the addition of the phenolic substrate. The concentrations of the product, *p*-nitrophenol, were estimated by the method shown in Section 2.8.3, after sampling (100µl) at 0,15,30,45, and 60min incubation.

	Plant tissue from which extract was made		
	Leaf	Rhizome	Root
Protein content of tissue extract (mg/ml)	7.50	1.40	1.51
Specific activity (nmol/min/mg protein)	20.2	41.4	45.1
Specific activity (µmol/min/g fresh weight plant tissue)	1.21	0.35	0.27

Assays using each tissue preparation were carried out at the pH optimum already determined with *p*-nitrophenyl-β-*D*-glucoside, and the volume of the assay mixture withdrawn for analysis was increased to 0.5ml, as activity of the enzymes was weaker when supplied with phenyl-β-*D*-glucoside as the substrate, and the larger volume was required to ensure that sufficient phenol was present to be detected colorimetrically. The results, given in Table 5.2, show that phenyl-β-*D*-glucoside was hydrolyzed, yielding phenol, by tissue extracts of *P.communis*, though apparently at a lower rate than *p*-nitrophenyl-β-*D* glucoside.

The results suggest that sufficient β-glucosidase activity was present in *disrupted* tissues to hydrolyse glucosides formed following the addition of phenol to plant extracts. Radioactive phenyl-β-*D*-glucoside was found in *whole tissue* pieces supplied with [U-^{14}C]phenol, where the enzymes were still compartmentalised and rapidly inactivated by extraction with hot ethanol, but glucoside could not be found after prolonged incubations with tissue *extract* supplied with phenol and UDPG (Section 5.1.1). These results do not, however, show why phenol failed to stimulate UDPG-consuming enzymes.

5.2 OXIDATION OF PHENOLIC SUBSTRATES BY EXTRACTS OF PLANT TISSUES: RESUMÉ OF PHENOLIC OXIDATION IN PLANTS: AIMS OF THE SUBSEQUENT STUDIES.

Extraction of tissues of *P.communis* supplied with [U-^{14}C]phenol yielded substantial amounts of [^{14}C]catechol (Section 4.2), which showed clearly that *o*-hydroxylation of phenol had taken place. Further catabolism of phenol (to $^{14}CO_2$) has not so far been shown, but much radioactive material is diverted to ethanol-insoluble material in plant tissues (Sections 3.3.5 and 4.1) suggesting that catechol was

Table 5.2 Hydrolysis of phenyl-β-*D*-glucosides by extracts of *Phragmites communis* tissues.

Assays, of 1ml total volume, contained 10μmol of phenyl-β-*D*-glucoside or *p*-nitrophenyl-β-*D*-glucoside and 50-250μl of crude cell-free extract (Section 2.8.3) of root, rhizome or leaf of *P. communis* in 0.2M potassium phosphate - 0.1M citric acid buffer, pH5.5, and were performed at 25°C. The reaction was initiated by addition of the phenolic substrate, and samples of 100μl (*p*-nitrophenyl-β-*D*-glucoside assays) or 500μl (phenyl-β-*D*-glucoside assays) were removed after 1h and assayed for *p*-nitrophenol and phenol respectively. The results shown are the quantities (μmol) of product detected after 1h incubation, corrected for the amount of protein used.

	Plant tissue from which extract was made		
	Leaf	Rhizome	Root
p-Nitrophenol formed (μmol/mg protein)	1.20	2.51	2.72
Phenol formed (μmol/mg protein)	0.12	0.28	0.27

further oxidised, and was hence bound to cell constituents or polymerised to insoluble "melanins". Further work was intended to show whether (i) the enzymes of aromatic ring fission, leading to CO_2 formation, were present in plant tissues:

(ii) the enzyme(s) of aromatic hydroxylation were present:

and (iii) products of catechol oxidation and the enzyme(s) responsible for this were present.

The enzymes of aromatic ring fission may be detected readily, as their aliphatic products have pronounced ultraviolet absorbances (Section 5.2.1).

Both aromatic *o*-hydroxylation and *o*-diphenol oxidation are reactions of polyphenol oxidase enzymes (Section 1.3) and assays were attempted firstly by simple spectrophotometric methods (Section 5.2.1) to find the characteristic products of catechol oxidase, *o*-benzoquinone or its melanin polymers, and, subsequently, by use of the oxygen electrode.

The latter assay method was used initially to survey extracts of several plant species for activity against monophenols and diphenols, alone or together with two typical cofactors of monophenol hydroxylation by polyphenol oxidase, NADH and ascorbate (Section 5.2.3). Several naturally occurring and other substituted mono- and diphenols were included in the survey assays, to confirm that polyphenol oxidase activities were present, and to show the typically broad substrate specificity of these enzymes.

When catechol oxidase and a putative monophenolase activity were found, the former was more fully investigated in the plant species (*Mentha aquatica*) in which it was most active - this enzyme

was of insufficient activity in crude extracts of *P.communis* to give worthwhile results (Section 5.2.4).

The monophenol and NADH-stimulated activity of *P.communis* extracts was extensively investigated, as it was expected to be of importance in the production of catechol (Section 5.2.4). Careful analysis for hydroxylation products of phenol failed to show that the enzyme was in fact the anticipated hydroxylase, and further studies were made to determine the nature of the interfering activity.

5.2.1 Spectrophotometric detection of reaction products of phenolic oxidation by tissue extracts of *Phragmites communis*.

Spectrophotometric methods may be used to show the formation of coloured oxidation products and strongly u.v.-absorbing ring-fission products from phenolic substrates by appropriate enzymes, so in an initial investigation, the spectral changes catalysed by *P.communis* extracts with a range of mono- and diphenolic substrates were followed.

Extracts of root, rhizome, and leaf tissues of *P.communis* catalyzed no spectral changes with phenol, *p*-cresol, or tyrosine when these substrates were supplied at concentrations of 0.1-10mM.

All three extracts showed activity against catechol, and leaf and rhizome tissue extracts were active in the presence of quinol (Figs 5.2-5.6).

The major absorbance peaks of catechol incubations were at 480-495nm, 502nm, and 465-490nm in incubations of root, rhizome, and leaf extracts respectively. An additional maximum at 342nm arose in the spectrum of leaf incubation mixtures (Figs 5.2-5.4).

Unlike catechol, quinol was observed to autooxidise in reaction mixtures containing no plant extracts or denatured extracts (100°C for 10min), yielding products of high absorbance in the range

Figures 5.2-5.4 Spectra of reaction products of catechol in incubations of tissue extracts of root, rhizome, and leaf of *Phragmites communis*.

Reaction mixtures of 1ml contained 100µmol of potassium phosphate buffer, pH7.0, up to 250µl of tissue extract (Section 2.7) and 10µmol of catechol, and were incubated for up to 4h at 25°C in the constant-temperature cell-holder of a scanning spectrophotometer. At the times shown after initiation of the reaction by addition of the substrate, the absorbance spectra of the reaction mixtures from 250-610nm was determined, scanning rate of the instrument being 5nm/min.

The figures show (5.2) the spectra of a root extract reaction mixture after (A) addition of the substrate at zero time; (B) after 1h incubation; (C) after 2hr; (5.3) the spectra of a leaf extract reaction mixture after (A) zero time; (B) 2h incubation, and (C) 4h incubation; and (5.4) the spectra of a rhizome extract reaction mixture after (A) zero time, (B) 1h incubation, and (C) 2h incubation.

Figure 5.2

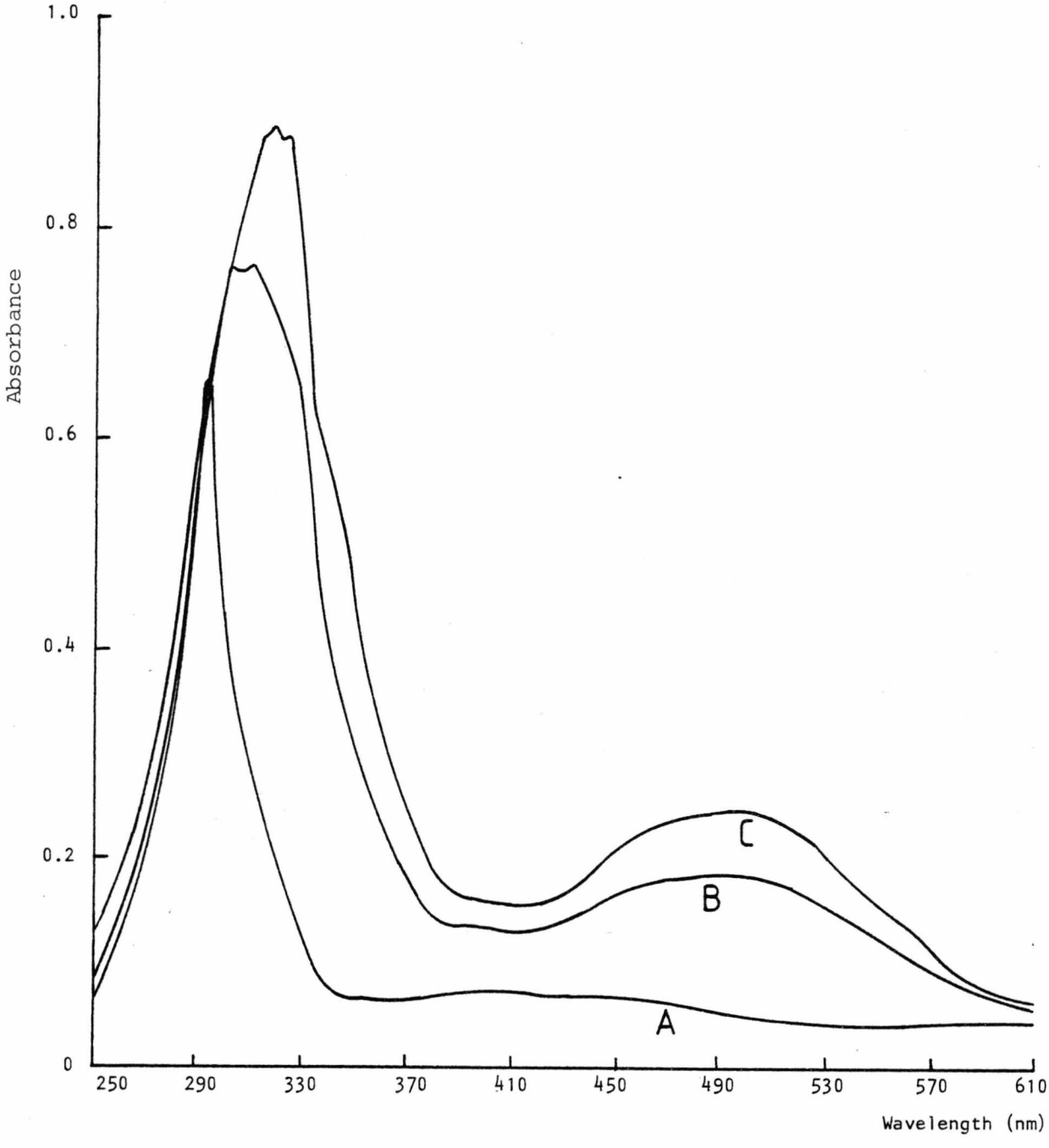

1.0
0.8
0.6
0.4
0.2
0
Absorbance
250
290
330
370
410
450
490
530
570
610
Wavelength (nm)
A
B
C

Figure 5.3

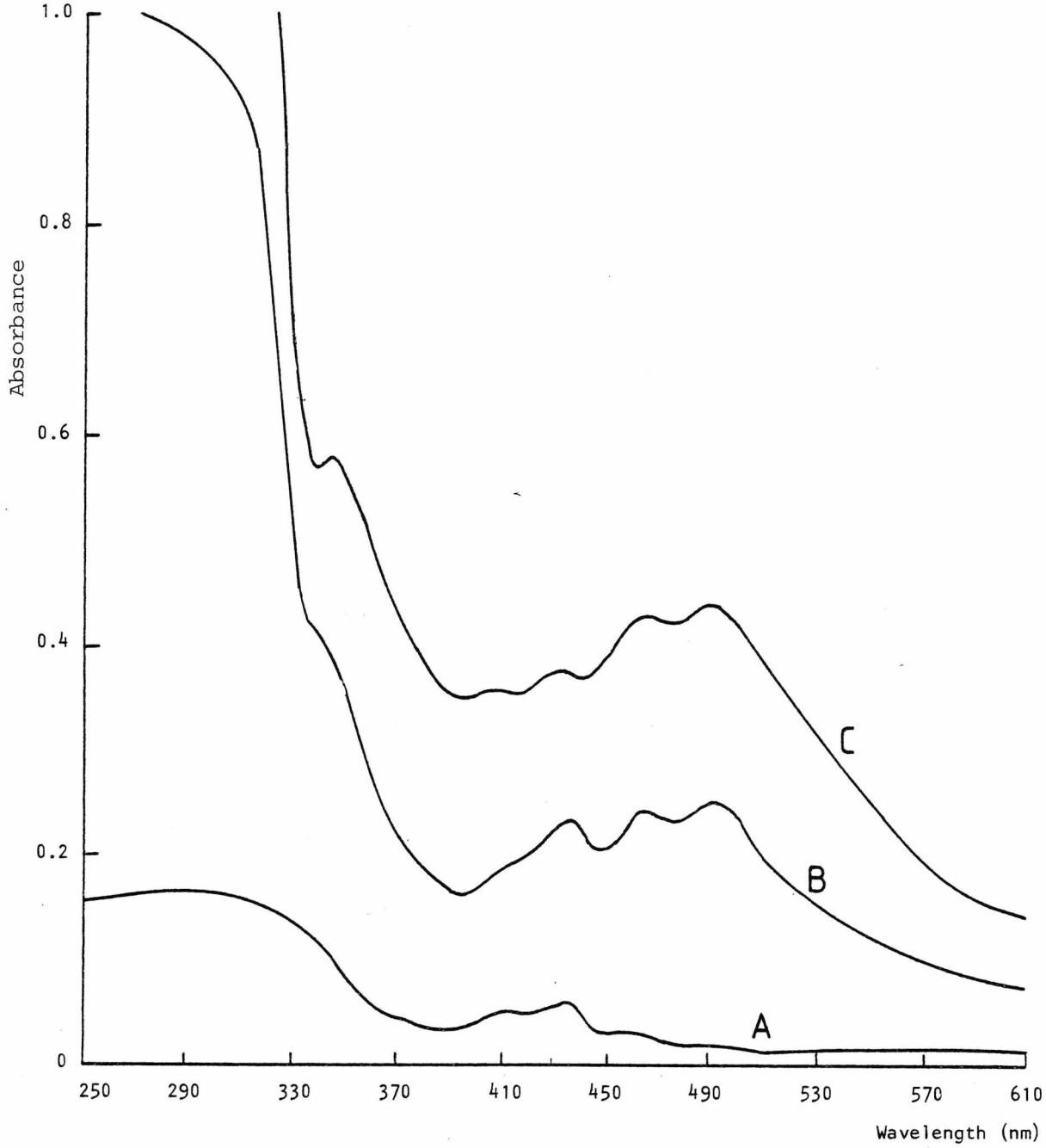
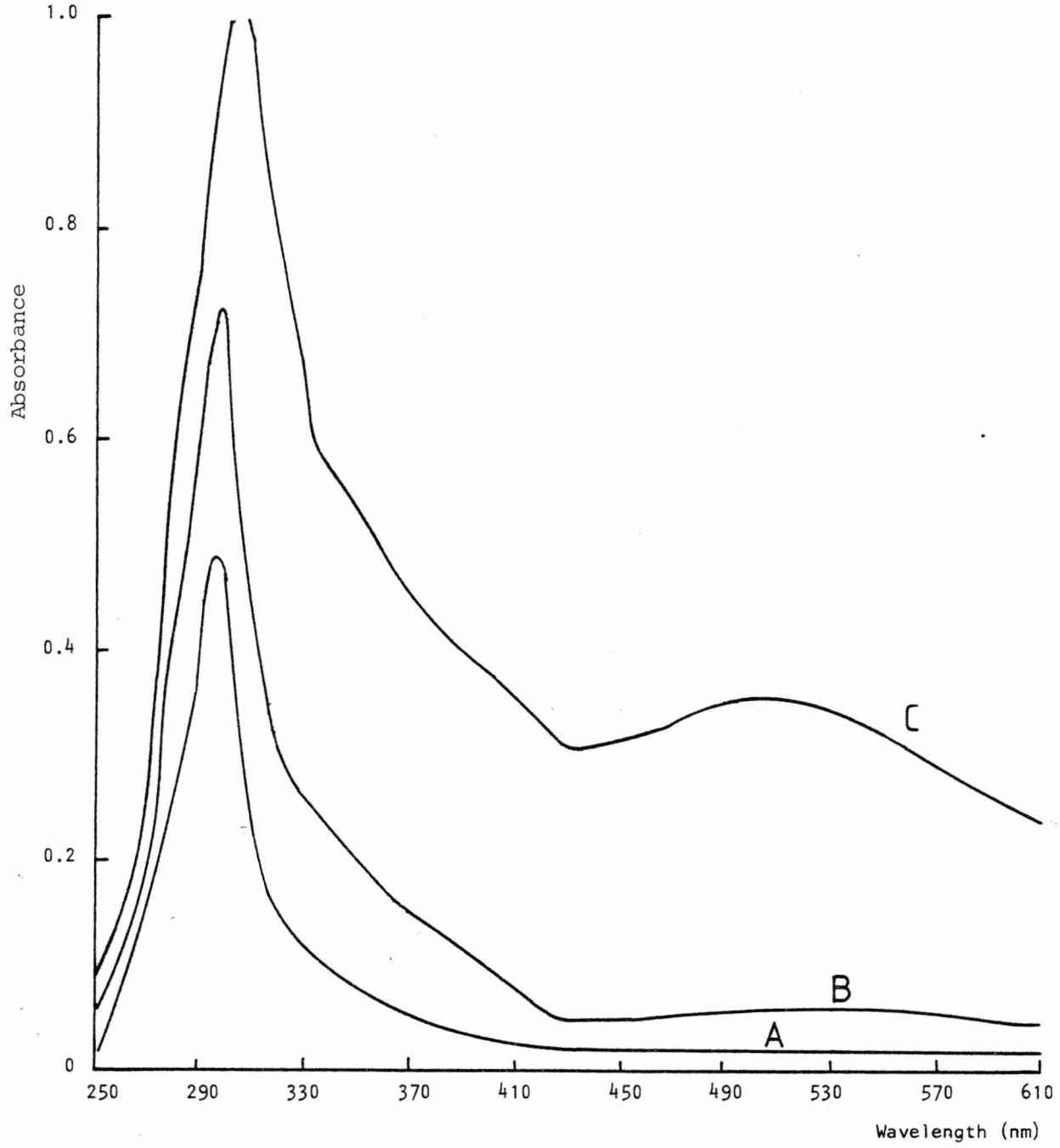

1.0
0.8
0.6
0.4
0.2
0
Absorbance
250
290
330
370
410
450
490
530
570
610
Wavelength (nm)
A
B
C

Figure 5.4


1.0
0.8
0.6
0.4
0.2
0
Absorbance
250
290
330
370
410
450
490
530
570
610
Wavelength (nm)
A
B
C

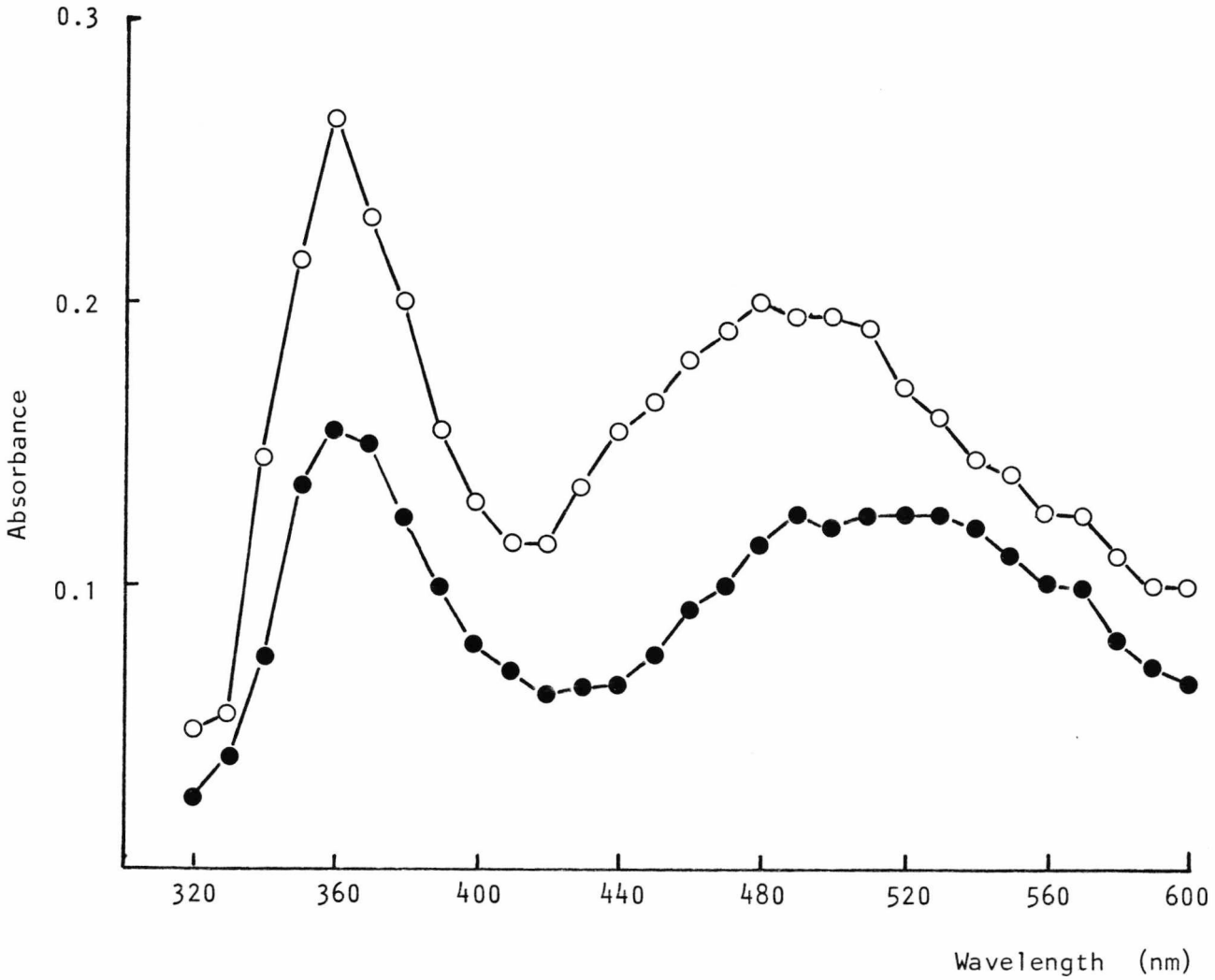


Figure 5.5 Difference spectrum of products of quinol oxidation by extracts of *Phragmites communis* rhizome tissue.

Using experimental methods described in the legends to Figs 5.2-5.4, with the exception that 10mM quinol was the phenolic substrate, the spectrum of the reaction products was determined after 2h (●) and 3h (o) incubation, and to correct for the autooxidation of quinol, similar spectra were obtained from incubation mixtures containing quinol (10mM) and heat inactivated (10min at 100°C) tissue extract. At the points shown in the figure the absorbance of the autooxidation products was subtracted from that of the plant extract-stimulated reaction mixture, and data so obtained are shown.

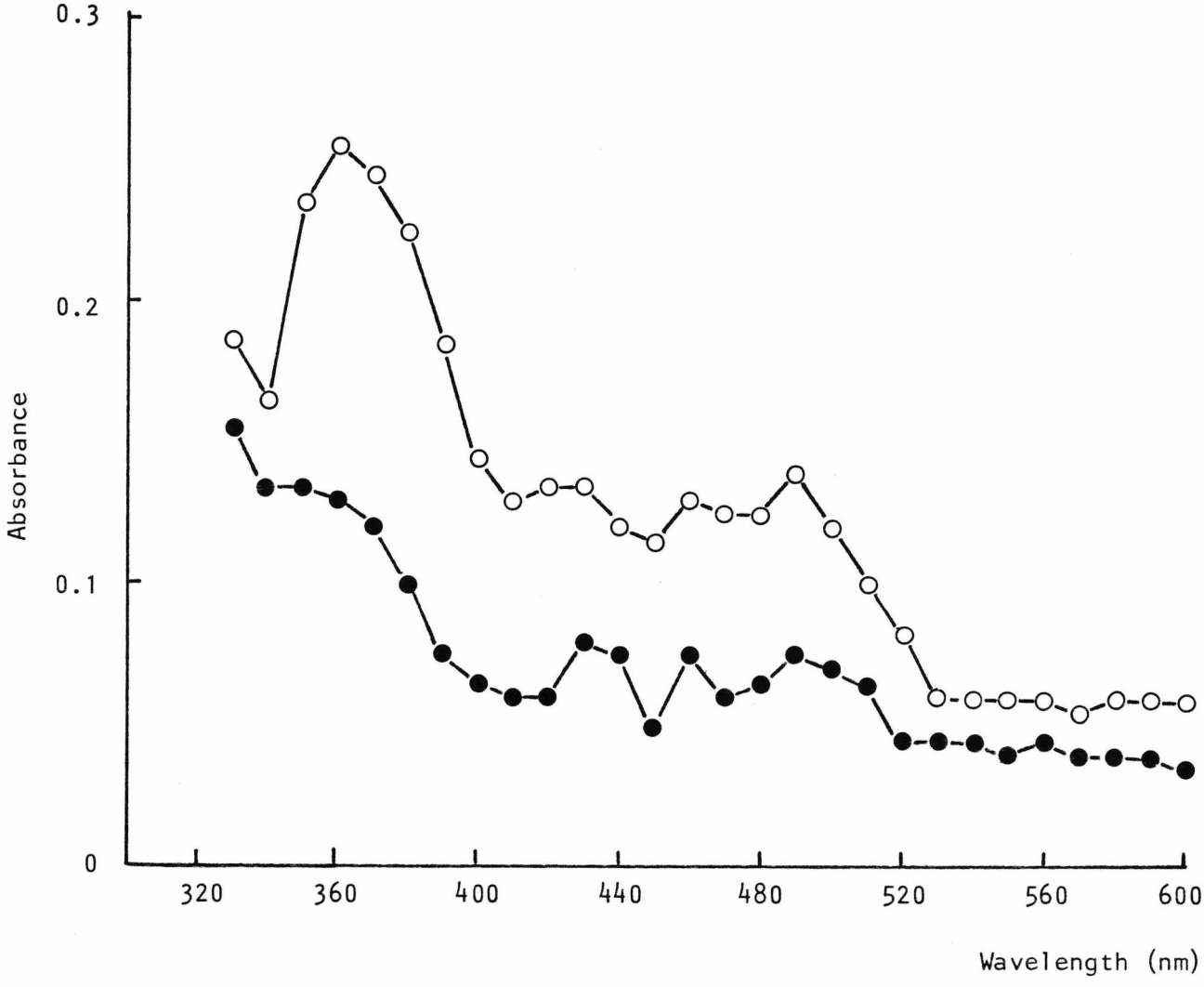


Figure 5.6 Difference spectrum of products of quinol oxidation by extracts of *Phragmites communis* leaf tissue.

Using experimental methods described in the legend to Figs 5.2-5.4, with the exception that 10mM quinol was the phenolic substrate, the spectrum of the reaction products was determined after 2h (●) and 4h (o) incubations, and to correct for autooxidation of quinol, similar spectra were obtained from incubation mixtures containing quinol (10mm) and heat inactivated (10min at 100°C) tissue extract. At the points shown, the absorbance of the autooxidation products was subtracted from that of the plant extract-stimulated reaction mixture, and data so obtained are shown.

420-600nm, which appeared to coincide with the absorbance maxima of products generated in plant extract incubation. Absorbance of denatured extract-quinol incubations at wavelengths of less than 350nm was also very high when 10mM substrate was used, but as lower concentrations of quinol gave very weak activity with all plant extracts tested, this could not be corrected for. Difference spectra which correct for the autooxidation of quinol are given in Figs 5.5 and 5.6, and show that rhizome extract stimulated the formation of products having absorbance maxima of 360nm and 480-530nm. Leaf tissue extracts likewise stimulated the formation of products in excess of autooxidative changes, these having absorption maxima of 360 and 490nm.

There was no evidence in any incubation mixture of the formation of materials having absorption spectra corresponding with those of ring-fission products of catechol.

These experiments gave a useful indication of the presence in plant extracts of enzymes catalysing the formation of characteristic oxidation products of diphenols, but no trace of monophenol activity was found. The apparent lack of ring-fission activity confirms the earlier experiments which showed that excised plant tissues did not cause the production of $^{14}CO_2$ from [^{14}C]phenol.

Further experiments of this kind were used to quantify some of the polyphenol oxidase activities detected.

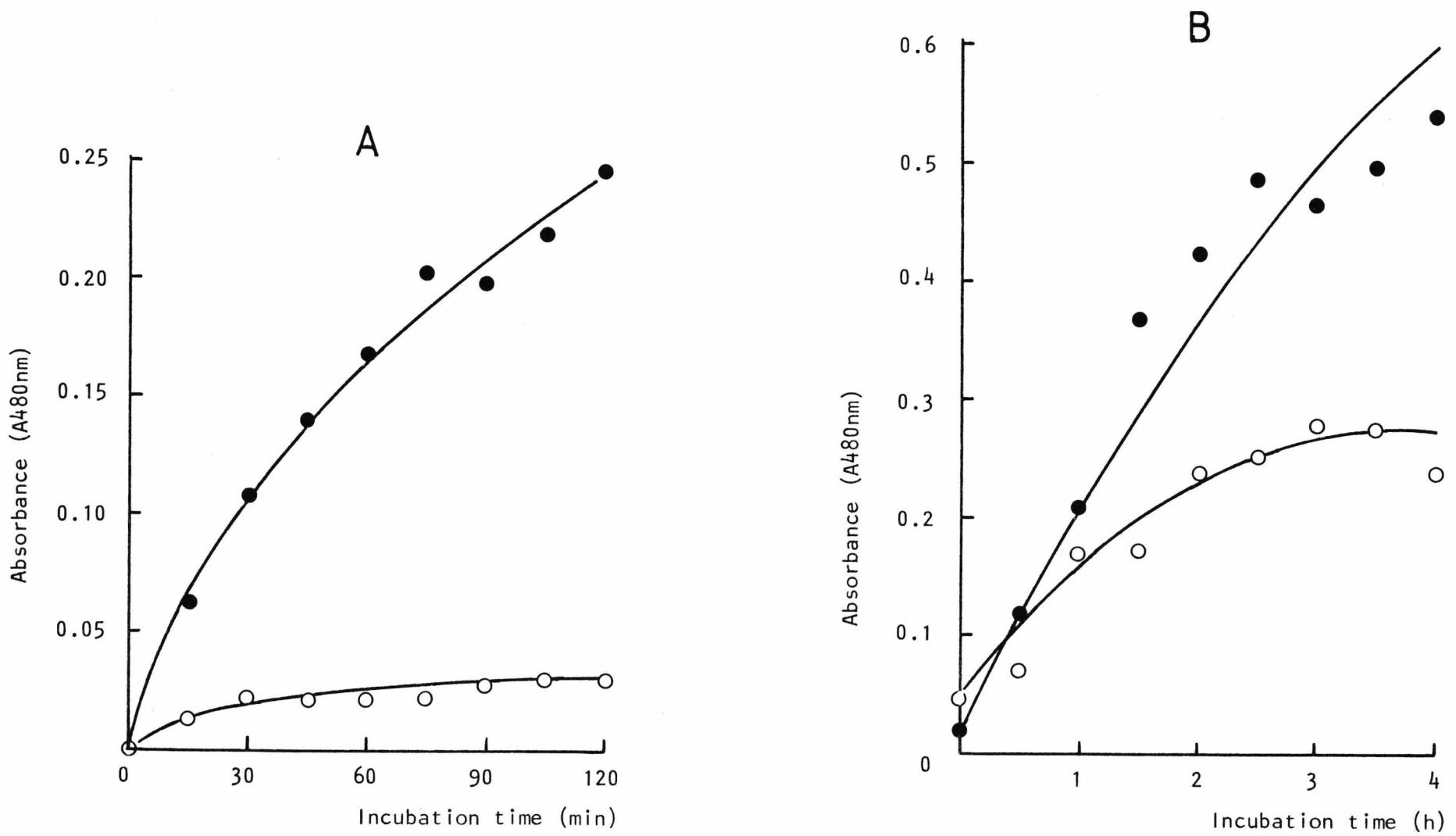
Fig 5.7 shows a typical result from simple kinetic experiments in which the absorbance of reaction mixtures of plant extracts with diphenols was monitored.

All preparations showed low rates of activity and lost activity as the reaction progressed. Approximate maximum reaction rates, determined graphically by construction of tangents to the time-course curves occurred invariably in the presence of 10mM substrates;

Figure 5.7 Oxidation of catechol and quinol by extracts of root tissue of *Phragmites communis*.

Assays, of total volume 1ml, contained (●) 10µmol, or (o) 1µmol, of catechol (Fig. A) or quinol (Fig. B), in catechol assays 0.35mg protein and in quinol assays 0.425mg protein, and 100µmol of potassium phosphate buffer, pH7.0, and were incubated at 25°C. The reaction was initiated by addition of the phenolic substrate, and the change in absorbance at 480nm, for both substrates, was monitored discontinuously. The results for oxidation of quinol (Fig. B) were corrected for autooxidation of this substrate by subtraction from the experimental results of the results from quinol assays containing inactivated (10min at 100°C) tissue extract.

The protein content of root tissue extract was 1.7mg/ml, and the extractable protein content of root tissue was 5.84mg/g fresh weight.


A
0.25
0.20
0.15
0.10
0.05
Absorbance (A480nm)
0
30
60
90
120
Incubation time (min)
B
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance (A480nm)
1
2
3
4
Incubation time (h)

with catechol, for leaf, rhizome, and root extracts respectively the rates were 0.30, 0.19, and 1.36 ΔA/h/mg protein, and with quinol in the same order of presentation, they were 0.18, 0.85 and 0.47. The activity with respect to protein content and the ratios of catechol/quinol activity of these extracts differed markedly, catechol activity being greatest in root extract, and quinol activity greatest in rhizome extract. The higher protein content of leaf extract meant, however, that fresh leaf tissue contained more of both activities than the other tissue.

As a demonstration of polyphenol oxidase activities, the spectrophotometric assay method is useful, but as it is non-quantitative with respect to both product formation and substrate consumption, further kinetic investigations of phenol oxidases were confined to polarographic assays.

5.2.2 Isolation and identification of oxidation products of monophenolic substrates formed in the presence of plant tissue extracts.

In previous experiments (Section 5.2.1), cell-free extracts of *P.communis* tissues promoted the formation of coloured products from diphenolic substrates. The cut edges of tissue samples of *M.aquatica* were observed in other experiments to become blackened when exposed to phenol, suggesting the presence of active polyphenoloxidases.

Cell-free extracts of *I.pseudacorus* and *T.latifolia* also yielded spectra suggesting the presence of diphenoloxidation products. A series of experiments designed to allow the isolation of some of these oxidation reaction products was therefore performed.

Incubation mixtures containing phenolic substrates (100μmol),

crude plant extracts (1000*g* supernatant of Section 2.7, 2ml), and potassium phosphate buffer, pH7.0 (1mmol), in a total volume of 10ml, were incubated with stirring at 25°C for 1h, after which they were inactivated and acidified with 1M HCl to pH 1-2. Precipitated protein was removed by centrifugation, and the supernatant was extracted with 3 x 20ml of diethyl ether. The extracts were combined, dried over anhydrous sodium sulphate for 2h, filtered, and reduced under O_2-free dry N_2 to 1-2ml. Samples of concentrated extracts were chromatographed on 10 x 10cm cellulose and silica-gel t.l.c. plates in solvents 1,2,3,7,8, and 9 (Section 2.6) together with samples of authentic materials. Where the spot formed by a suspected reaction product was too diffuse to allow its unequivocal identification, or was apparently contaminated with an endogenous phenolic or other material, larger volumes of concentrated ether extracts (up to 200µl instead of 50-100µl) were chromatographed separately in solvents known to give good separation of monophenols and diphenols (solvents 3 and 7 for example); after running, the chromatograms (on 20 x 20cm plates) were partly masked, and only those parts bearing authentic markers were visualised with a spray of Gibbs' reagent. The corresponding,undeveloped, product zones were scraped from the chromatograms, and the coating powder eluted with diethyl ether. The supernatant eluate was decanted, concentrated under O_2-free dry N_2, and rechromatographed for final identification.

The monophenolic substrates examined were phenol, *p*-cresol, *p*-hydroxybenzoic acid, and *p*-coumaric acid, and their anticipated products were, respectively, catechol, 4-methylcatechol, protocatechuic acid, and caffeic acid.

After rechromatography on silica-gel, in solvent 7, incubation

mixtures containing extracts of both leaf and root tissues of *M.aquatica* yielded catechol and 4-methylcatechol when supplied with, respectively, phenol and *p*-cresol (Table 5.3). The expected product of *p*-coumaric acid, caffeic acid, was detected in the two solvents in which it was mobile on chromatograms of extracts from both leaf and root incubations. Authentic caffeic acid was immobile in solvents 8 and 9, and results obtained from chromatograms made with these solvents were therefore discounted.

All incubation mixtures, whether supplied with *p*-hydroxybenzoic acid or not, yielded a spot corresponding in all solvents on both chromatography media with protocatechuic acid, so the oxidation of *p*-hydroxybenzoic acid could not be demonstrated by this method.

After detection of the putative products by chromatography, the remainder of the ether extracts from phenol and *p*-cresol incubations with both leaf and root tissue preparations were twice chromatographed by applying them first as bands on 20x20cm silica-gel t.l.c. plates, developed with solvent (9), in which authentic phenol and *p*-cresol (R_F of both, 0.62) could be well separated from catechol and 4-methylcatechol (R_F of both, 0.36). After location, the corresponding product bands were scraped from the backing sheets and eluted with 1-2ml of ethanol. After centrifugation, the ultraviolet (u.v) spectra of the putative products were determined, and compared with those of authentic materials (0.05-0.1mM in ethanol).

Leaf extracts did not yield satisfactory spectra, but ethanolic eluates of the product materials from incubations of root tissue preparations supplied with phenol had a u.v. spectrum corresponding with that of authentic catechol (λmax 218 and 279nm, shoulder 285nm), and the product from incubation with *p*-cresol had a spectrum

Table 5.3 Oxidation products of monophenols identified in extracts from incubation mixtures of *Mentha aquatica* tissue extracts.

Ether extracts from incubation mixtures containing monophenolic substrates of leaf and root tissues were chromatographed on cellulose and silica-gel t.l.c. plates in solvents 1,2,3,7,8, and 9 (Section 2). The table shows the R_F values of the authentic substrate and product markers and their colour reactions with Gibbs' reagent and alkaline overspray (Section 2.6). The identity of isolated products is given with the solvents in which they were successfully detected. The methods of incubation and extraction are given in the text.

Monophenolic Substrate	Monophenolic Substrate							
	R_F in solvent						Colour reaction with Gibbs' reagent and alkaline over-spray	Expected diphenolic product
	1	2	3	7	8	9		
Phenol	0.89	0.89	0.87	0.72	0.60	/	Sky blue	Catechol
p-Cresol	0.88	0.79	0.88	0.71	0.61	/	Slate grey	4-Methyl-catechol
p-Coumaric acid	0.48	0.86	0.58	0.46	0	0.04	Yellow	Caffeic acid

Authentic diphenolic product marker						Colour reaction with Gibbs' reagent and alkaline over-spray	Identified Product (and solvents in which detected)
R_F in solvent							
1	2	3	7	8	9		
0.63	/	0.37	0.38	0.37	/	Purple-brown	Catechol (1,3,7,8)
0.73	0.83	0.50	0.45	0.38	/	Brown	4-Methyl-catechol (1,2,3,7,8)
0.34	/	/	0.13	0	0	Brown	Caffeic acid (1,7)

corresponding to that of authentic 4-methylcatechol (authentic 4-methylcatechol λmax 222 and 284nm, shoulder 218; Fig. 5.8). The ethanol eluate of the suspected caffeic acid did not contain sufficient material for the determination of u.v. spectra.

Incubations of cell-free extracts of *P.communis* leaf, rhizome, and root tissues gave similar results, but insufficient product was obtained from any monophenol to allow its unequivocal identification by spectroscopy. Phenol incubations, however, yielded traces of a product corresponding with catechol in solvent 9 and *p*-cresol incubations yielded a diphenol corresponding with 4-methylcatechol in solvents 7, 8, and 9. Catechol and 4-methylcatechol were eventually isolated from incubations of all three tissue extracts.

Root extract incubations supplied with *p*-coumaric acid yielded a compound corresponding with caffeic acid in solvent 7 alone. No caffeic acid was obtained when *p*-coumarate was supplied to rhizome and leaf tissue preparations.

Cell-free extracts of *I.pseudacorus* and *T.latifolia* tissues, when incubated with monophenolic substrates, produced traces of diphenolic products, which were not readily detected, but all reaction mixtures showed some evidence of the conversion of phenol and *p*-cresol to the corresponding catechols.

Addition of ascorbate (100μmol sodium ascorbate, freshly prepared) to incubation mixtures of extracts of *P. communis* and *T.latifolia* tissues had no qualitative effect on the results obtained by chromatography of ether extracts, but diphenolic products were more readily detected in chromatograms of preparations from *P.communis* incubations, suggesting that their production was increased in the

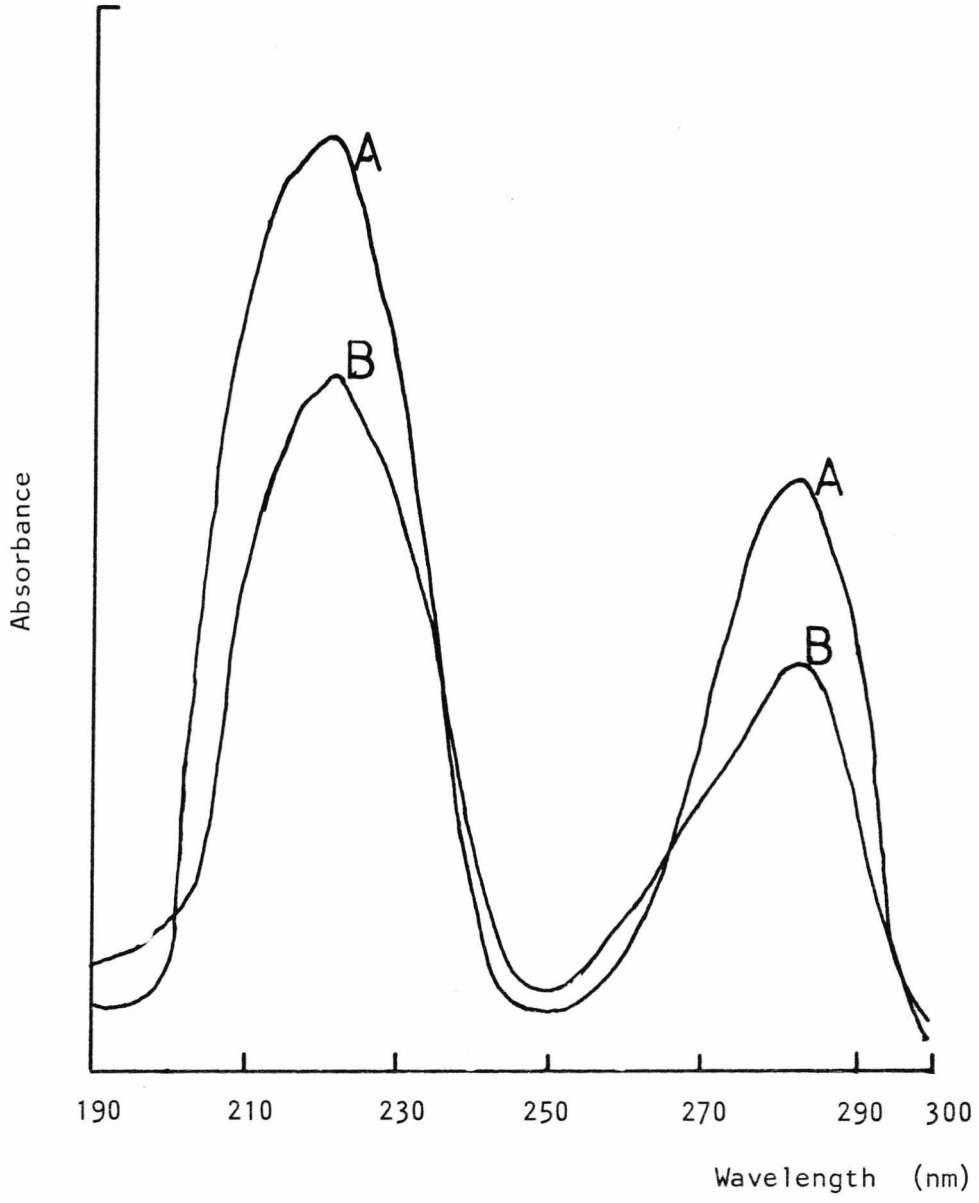


Figure 5.8 Absorbance spectra of authentic and isolated 4-methylcatechol

The ultra-violet spectrum of putative 4-methylcatechol (B), purified by chromatography as described in the text, and dissolved in ethanol, was compared with that of authentic material (0.1mM) (A).

presence of a reducing agent, or that subsequent oxidation of the diphenol by phenol oxidases was prevented. The presence of NADH (10μmol) had a similar effect.

It was concluded that oxidation of monophenols to diphenols in plant extracts took place, although this activity was not detectable by the colorimetric enzyme assay methods of Section 5.2.1. The results confirmed the formation of catechol from phenol in experiments using excised tissue pieces (Section 4.2), and thus suggest an important means of metabolism of phenol in plant tissues.

5.2.3 Polarographic assays of the oxidation of phenolic substrates by cell-free extracts of plant tissues.

In view of the detection of diphenolic products, formed by oxidation of monophenols, and the demonstration by spectroscopic methods of diphenol oxidase activity, extracts from tissues of *P.communis*, *T.latifolia*, *I.pseudacorus*, and *M.aquatica* were examined polarographically for phenol oxidase activities, using a Clark-type oxygen electrode. This method was chosen for its speed and sensitivity, and because it yields directly a quantitative result in terms of O_2 consumption, for the activity of phenol oxidases.

Phenolic substrates were chosen to give information in a wide range of phenolase activities, in the presence and absence of cofactors. The results are given in Tables 5.4-5.9. All tissues examined showed an O_2 uptake stimulated by some of the phenolic substrates, but heated (100°C for 10min) extracts were, without exception, inactive.

Phragmites communis

The various phenolic substrates stimulating O_2 uptake in *P.communis* extracts were fewer in number than in extracts of the other three

Tables 5.4-5.9 Stimulation of O_2 consumption in cell free plant tissue extracts by diphenols and monophenols, with and without cofactors.

The methods used in these assays were essentially as described in Section 2, with the exception, which is shown where necessary, that the total assay volume was either 1 or 2ml. The chamber of the O_2 electrode contained, in these total volumes, 100µmol or 200µmol of potassium phosphate buffer, pH7.0, 10-500µl of plant tissue extracts, 1µmol (2µmol) or 10µmol (20µmol) of phenolic substrate, and when tested, 1µmol (2µmol) of either freshly prepared sodium ascorbate or NADH; the larger quantities of each reagent shown were used in 2ml assays. The buffer was maintained at 25°C, and all assays were made at this temperature. After stabilization of the recorder baseline, plant extracts were added, and the endogenous rate of O_2 consumption was recorded. When used, the reductants were added next, and the O_2 consumption stimulated by them was measured, after which the phenolic substrates were supplied. The rates of O_2 consumption given (nmolO_2/min/mg protein) were determined at once, after addition of the phenols, or, when a lag period was evident, when the rate became linear. All results were corrected for endogenous rates of O_2 consumption before presentation. Where cofactors and phenols were used, the order of addition of these substances was reversed, in a replicate set of analyses for each plant tissue extract.

The data underlined in the tables are those from experiments using 10mM phenolic substrate concentrations; all others were obtained with 1mM substrate, but where a zero result is shown, this was obtained with both 1 and 10mM concentrations. Data given in parentheses are the final linear rates of O_2 consumption obtained in assays where there was an initial lag on addition of the substrates.

The full range of diphenols used was catechol, 4-methylcatechol, chlorogenic acid, caffeic acid, DOPA, resorcinol, quinol, and protocatechuic acid. The monophenols and other aromatic compounds used were phenol, *p*-cresol, *p*-coumarate, tyrosine, phenyl-β-*D*-glucoside, and cinnamic acid. All plant extracts were tested with all these compounds: where no result was obtained with any extract from a plant species, the negative results are omitted.

The suppliers of the phenols and other reagents used in these experiments were: chlorogenic acid, DOPA, cinnamic acid, Koch-Light Ltd., Colnbrook, Bucks; *p*-cresol, resorcinol, Fisons Ltd, Loughborough; protocatechuic acid, quinol, B.D.H. Ltd, Poole, Dorset; 4-methylcatechol, Coal and Coalite Products Ltd, Chesterfield, Derbyshire; tyrosine, *p*-coumarate, phenyl-β-*D*-glucoside, caffeic acid, NADH, ascorbate (ascorbic acid, sodium salt), Sigma.

Table 5.4 Stimulation of O_2 consumption by tissue extracts of *Phragmites communis* by phenols and cofactors.

The total volume of all assays was 2ml.

Diphenolic substrate	Rate of O_2 consumption								
	Leaf extract			Rhizome extract			Root extract		
	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH
Catechol	4	10		33	33		9	9	
4-Methylcatechol	4	14		39	43		10	6	
Chlorogenic acid	2	3		26	26		18	17	
Resorcinol	0	0	2	0	0	186	0	0	206

Phenolic substrate	Rate of O_2 consumption with 1mM NADH		
	Leaf extract	Rhizome extract	Root extract
Phenol	13	201	204
p-Cresol	10	174	469
Tyrosine	(7)	(19)	0
p-Coumaric acid	(151)	(545)	(1329)

Table 5.5 Stimulation of O_2 consumption of tissue extracts of *Typha latifolia* by diphenols.

The total volume of assays of leaf and rhizome extracts was 2ml, and of root extracts 1ml.

Diphenolic substrate	Rate of O_2 consumption								
	Leaf extract			Rhizome extract			Root extract		
	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH
Catechol	3	0		24	14		0	0	
4-Methylcatechol	7	41		57	112		16	0	
Chlorogenic acid	5	0		63	59		20	0	
Caffeic acid	11	9		29	25		59	0	
DOPA	0	34		12	24		9	133	
Quinol	12	13		12	6		125	0	
Resorcinol	0	0	171	0	0	50	0	0	68

Table 5.6 Stimulation of O_2 consumption of tissue extracts of *Typha latifolia* by phenols and cofactors.

The volume of assays of leaf and rhizome extracts was 2ml, and of root extracts 1ml.

Aromatic Substrate	Rate of O_2 consumption								
	Leaf extract			Rhizome extract			Root extract		
	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH
Phenol	0	0	27	0	0	64	0	0	37
p-Cresol	0	4	57	0	0	40	0	0	21
Tyrosine	8	26	9	21	19	10	21	58	0
Cinnamic acid	11	17	(3)	0	0	12	0	0	0
p-Coumaric acid	12	27	(113)	14	0	(60)	14	0	(106)
Phenyl-β-*D*-glucoside	0	0	26	0	0	0	0	0	0

Table 5.7 Stimulation of O_2 consumption of tissue extracts of *Iris pseudacorus* by diphenols.

The volume of all assays was 2ml.

Diphenolic substrate	Rate of O_2 consumption								
	Leaf extract			Rhizome extract			Root extract		
	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH
Catechol	0	0		0	5		5	0	
4-Methylcatechol	14	16		0	20		24	10	
Chlorogenic acid	2	0		0	0		6	0	
Caffeic acid	6	48		4	20		10	4	
DOPA	5	0		114	44		0	0	
Protocatechuic acid	2	0		0	0		0	0	
Quinol	7	0		52	11		46	0	
Resorcinol	0	0	19	0	0	0	0	0	92

Table 5.8 Stimulation of O_2 consumption of tissue extracts of *Iris pseudacorus* by phenols and cofactors.

The volume of all assays was 2ml.

Substrate	Rate of O_2 consumption					
	Leaf extract		Rhizome extract			Root extract
	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	Plus 1mM NADH
Phenol	0	11	0	0	0	81
p-Cresol	0	14	0	0	0	617
Tyrosine	4	0	90	26	54	0
Cinnamic acid	0	0	0	11	13	0
p-Coumaric acid	0	(33)	(10)	12	(59)	(1505)

Table 5.9 Stimulation of O_2 consumption of tissue extracts of *Mentha aquatica* by phenols and cofactors.

The total volume of all assays was 2ml.

Diphenolic substrate	Rate of O_2 consumption			
	Leaf extract		Root extract	
	No Addition	Plus 1mM NADH	No Addition	Plus 1mM NADH
Catechol	395		638	
4-Methylcatechol	1114		1644	
Chlorogenic acid	1286		947	
Caffeic acid	28		633	
DOPA	39		42	
Protocatechuic acid	28		69	
Quinol	7		18	
Resorcinol	0	1	0	10

Phenolic substrate	Rate of O_2 consumption					
	Leaf extract			Root extract		
	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH	No Addition	Plus 1mM Ascorbate	Plus 1mM NADH
Phenol	0	0	16	0	0	22
p-Cresol	28	21	40	0	23	38
Tyrosine	0	7	0	0	0	0
p-Coumaric acid	18	16	60	0	31	56

species, activity in the presence of DOPA and caffeic acid, for example, being entirely absent (Table 5.4). Diphenol-stimulated activity was generally higher in rhizome extract of this species than in root and leaf extracts, and monophenol activity in the presence of NADH (Table 5.4) was greater in both root and rhizome extracts than in leaf extract. In all three extracts of *P.communis*, O_2 consumption in the presence of NADH was greatest when *p*-coumarate was supplied as the monophenolic substrate, and in general, the range of substrates (both monophenols and diphenols) which stimulated O_2 uptake was similar. No monophenol would stimulate O_2 consumption in the absence of NADH.

The lack of activity obtained with quinol as the diphenolic substrate suggests that *p*-diphenol oxidase (laccase) activity was probably absent in *P.communis* (Table 5.4). Among the *o*-diphenols, substituted compounds were more effective in stimulating O_2 consumption than catechol itself.

Ascorbate was without effect when supplied to assays with monophenols, indicating a requirement for activity against these substrates for NADH or some other reductant. When assayed with diphenols, no lag in O_2 consumption on addition of the substrate was evident, but when tyrosine and *p*-coumarate were added to extracts of all three tissues in the presence of NADH, a lag occurred before development of the maximal rate of O_2 consumption (Table 5.4). The monophenols, phenol and *p*-cresol, and the *m*-diphenol, resorcinol, did not show this property, which was found with *p*-coumarate in all subsequent assays of other plant tissues except those of *M.aquatica*. The *total* rate of O_2 consumption in such assays showing a lag (i.e. rate in presence of *p*-coumarate *plus* NADH) was unaffected by the order of addition of the reductant and the phenolic. By adding NADH

first, the lag could be shown to be a property related to the phenolic compound - the rate of O_2 consumption stimulated by NADH alone immediately became linear in all cases.

Typha latifolia

The substrate range of *T.latifolia* was generally wider than that of *P.communis* and in some instances, monophenols alone stimulated O_2 consumption (Tables 5.5 and 5.6). Quinol, caffeate, and DOPA were apparent phenolase substrates of this species. Tyrosine, cinnamate, *p*-coumarate, and, in root extracts, resorcinol, all stimulated O_2 consumption in extracts of *T.latifolia*, and in leaf and rhizome extracts, NADH-dependent monophenol activity occurred in the presence of most of the substrates tested. In contrast with the range of diphenol activities, monophenol activities of *T.latifolia* root extracts covered a similar range of substrates to those of *P.communis*.

Ascorbate also served as the cofactor for the expression of activity in the presence of many monophenolic substrates, indicating that monophenol-stimulated activities with a cofactor specificity very different from those of *P.communis* were present in all extracts. The diphenol-stimulated activities of *T.latifolia* were generally higher than those of *P.communis*.

Again, *p*-coumarate, among the monophenols, caused the greatest NADH-dependent O_2 consumption, and substituted catechols were more effective than catechol itself in extracts of *T.latifolia*. Indeed, *catechol* oxidase was absent in root extracts of this species, but the stimulation of O_2 consumption brought about by quinol suggested that laccase activity was present.

Iris pseudacorus

In *I.pseudacorus* tissue extracts, catechol-stimulated activity was weak or absent, but quinol-dependent activity was comparatively

pronounced (Table 5.7) in rhizome and root extracts. In leaf and root tissue extracts of this species, 4-methylcatechol was effective, but the best apparent substrate of *o*-diphenol oxidase in rhizome tissue extract was DOPA. In general, the diphenol activities of rhizome extracts were very different from those of leaf and root extracts. Rhizome extract showed monophenol activity without cofactors (Table 5.8), and showed activity with a markedly different range of monophenol substrates from those found in *P.communis* and *T.latifolia*. Tyrosine, cinnamate, and *p*-coumarate were all effective in rhizome extract, and, unusually, tyrosine and *p*-coumarate stimulated nearly equal rates of O_2 consumption when NADH was present. Tyrosine, in this tissue, was also oxidised rapidly when no cofactor was present. In leaf and root tissue extracts of *I.pseudacorus*, as in most of the other extracts examined, *p*-coumarate was again the best stimulator of O_2 consumption in the presence of NADH. The generally high activity of *I.pseudacorus* rhizome extract in the presence of tyrosine and its *o*-diphenolic hydroxylation product, DOPA (Tables 5.7 and 5.8) suggested that this tissue possessed very different phenol-oxidising enzyme(s) - probably a more specific tyrosinase - from those found in other genera.

Mentha aquatica

The results from experiments using *M.aquatica* extracts stand in marked contrast to all others (Table 5.9) in that very high diphenol oxidase activity occurred in the presence of all the diphenols tested. Quinol-stimulated O_2 consumption was present in both leaf and root extracts, and protocatechuate, which had no significant effect in any other experiment, was also effective. Catechol oxidase was highly active in *M.aquatica* extracts, but as in most other experiments, substituted catechols were yet more effective in

enhancing O_2 consumption.

Among the monophenols (Table 5.9), *p*-coumarate stimulated O_2 consumption in both *M.aquatica* preparations by the greatest amount, and this stimulation also occurred when cofactors were lacking. *p*-Cresol was a very effective stimulator of O_2 consumption in leaf extracts, and was also responsible for some stimulation of activity in the absence of cofactors. *p*-Cresol activity was apparent in root tissue extracts too, but required cofactors in this case.

Of particular interest with reference to the present study were the indications that phenol in the presence of cofactors, and its hydroxylation product catechol, both stimulated O_2 consumption in most of the plant tissues examined, albeit weakly in some. The results suggested that further examination of these phenol oxidases should be made, to determine whether phenol itself was a substrate of such enzymes, and could be detoxified by their activity.

5.2.4 Characterization of phenol oxidase activities of plant tissues

Tissues of *M.aquatica* and *P.communis*, examined in other experiments, were shown to possess oxygen-consuming activities stimulated by phenols. Extracts of both species, supplied with monophenols, produced substances suspected to be hydroxylation products, and both species, when supplied with [U-^{14}C]phenol, immobilized a substantial amount of applied radioactivity in ethanol-insoluble material. The catechol and monophenol oxidase activities of tissues of the two plants were therefore partially characterized.

Mentha aquatica

Root and leaf tissue homogenates of this species, when screened for activity, showed substantial enhancement of O_2 consumption when supplied with catechol, 4-methylcatechol, and chlorogenic acid. Caffeic acid greatly stimulated O_2 consumption by root tissue

homogenate, but was less effective in leaf preparations. Both tissue extracts showed typical Michaelis-Menten kinetics when oxidising all four substrates, but the saturating velocity (V_{max}) of the reaction, obtained by calculation from Lineweaver-Burke plots was not observed in any instance, using 10mM substrate concentrations. Table 5.10 shows the observed maximum velocity and V_{max} and the Michaelis constant (K_m) of the diphenol oxidase of *M.aquatica* tissue extracts supplied with four substrates, and Figs 5.9 and 5.10 show typical plots of the data obtained with leaf and root preparations respectively, using chlorogenic acid and 4-methylcatechol as substrates.

Fig. 5.11 shows that for both root and leaf extract preparations, the observed rate of reaction was proportional to the volume of extract, and hence the quantity of protein, used in the assays. These results, using two substrates, were typical of all four substrates used.

Phragmites communis

P.communis leaf and root extracts were assayed for catechol oxidase in the same manner as *M.aquatica*, but as the activity towards diphenolic substrates was so low (Table 5.4), further quantitative estimates of the characteristics of catechol oxidase from this species were not attempted.

Monophenol-stimulated activity of *Phragmites communis* extracts.

Extracts of tissues of *P.communis* showed substantial enhancement of O_2 consumption when supplied with monophenols and a reductant, NADH (Table 5.4). This activity was (i) dependent on the presence of plant extract, (ii) was related to the amount of plant extract assayed (Fig. 5.12), and (iii) was not observed when heat-inactivated

Table 5.10 Apparent kinetic parameters of *Mentha aquatica* diphenol oxidase activities and observed maximum velocities of the reactions with four substrates.

Assays, of 2ml total volume, were performed as shown in Section 2.8.2 and contained up to 100µl of cell-free extract (Section 2.7). Velocities are expressed as oxygen consumption ($\mu mol O_2$/min/mg protein) under these conditions. The Michaelis constant (K_m) and saturating velocity (V_{max}) were calculated from Lineweaver-Burke plots of the data, fitted by eye.

Tissue extract	Substrate	Michaelis constant K_m (mM)	Calculated V_{max} ($\mu mol O_2$/min/mg protein)	Observed maximum velocity V at 10mM substrate; relative to chlorogenic acid = 100	
Leaf	Catechol	4.8	1.10	0.70	(40)
	4-Methylcatechol	2.0	1.80	1.35	(77)
	Chlorogenic acid	2.7	2.35	1.75	(100)
	Caffeic acid	2.0	1.15	0.95	(54)
Root	Catechol	14.4	5.00	2.15	(59)
	4-Methylcatechol	3.6	7.15	5.55	(152)
	Chlorogenic acid	5.6	6.45	3.65	(100)
	Caffeic acid	3.0	0.35	0.26	(7)

Figure 5.9 Kinetics of *Mentha aquatica* leaf tissue diphenol oxidase.

Assays, of total volume 2ml, were performed using the methods of Section 2.8.2, using up to 100µl of cell-free extract (Section 2.7 1000*g* supernatant). The main figure shows the double reciprocal ($\frac{1}{V}$ *versus* $\frac{1}{S}$) plot of the relationship between substrate concentration (S) and the velocity of the reaction (V) using two substrates; chlorogenic acid (o) and 4-methylcatechol (●); and the inset shows this relationship (V, µmolO_2/min/mg protein, *versus* S, mM) presented conventionally. Values of K_m and V_{max} derived from these figures are given in Table 5.16.

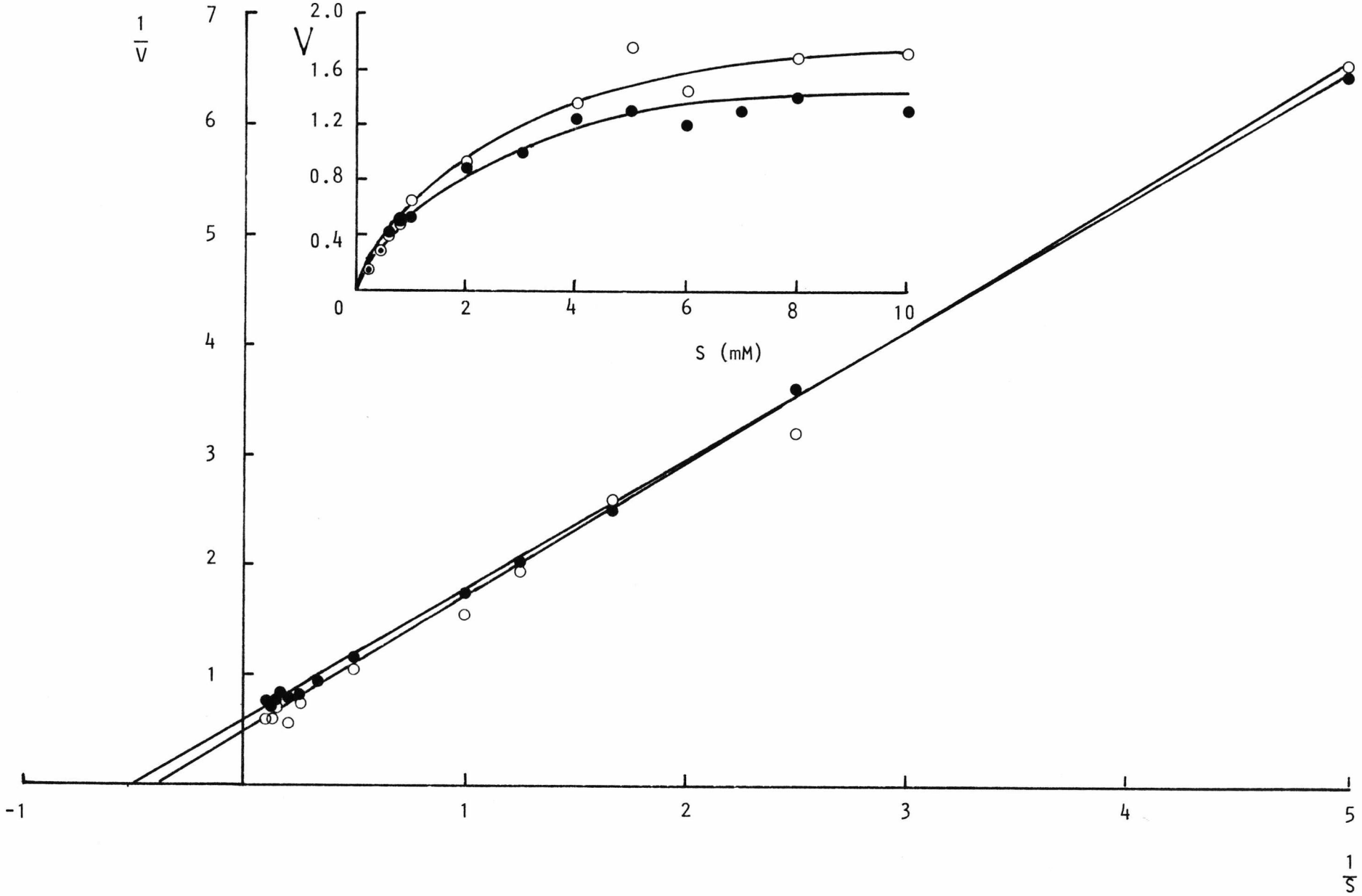

1/V
7
6
5
4
3
2
1
-1
1
2
3
4
5
1/S
V
2.0
1.6
1.2
0.8
0.4
0
2
4
6
8
10
S (mM)

Figure 5.10 Kinetics of *Mentha aquatica* root tissue diphenol oxidase.

Assays, of total volume 2ml, were performed using the methods of Section 2.8.2, using up to 50µl of cell-free extract (Section 2.7 , 1000g supernatant). The main figure shows the double-reciprocal ($\frac{1}{V}$ *versus* $\frac{1}{S}$) plot of the relationship between substrate concentration (S) and the velocity of the reaction (V) using two substrates; chlorogenic acid (o) and 4-methylcatechol (●); and the inset shows this relationship ($\frac{V}{S}$) presented conventionally. Values of K_m and V_{max} derived from these figures are given in Table 5.16.

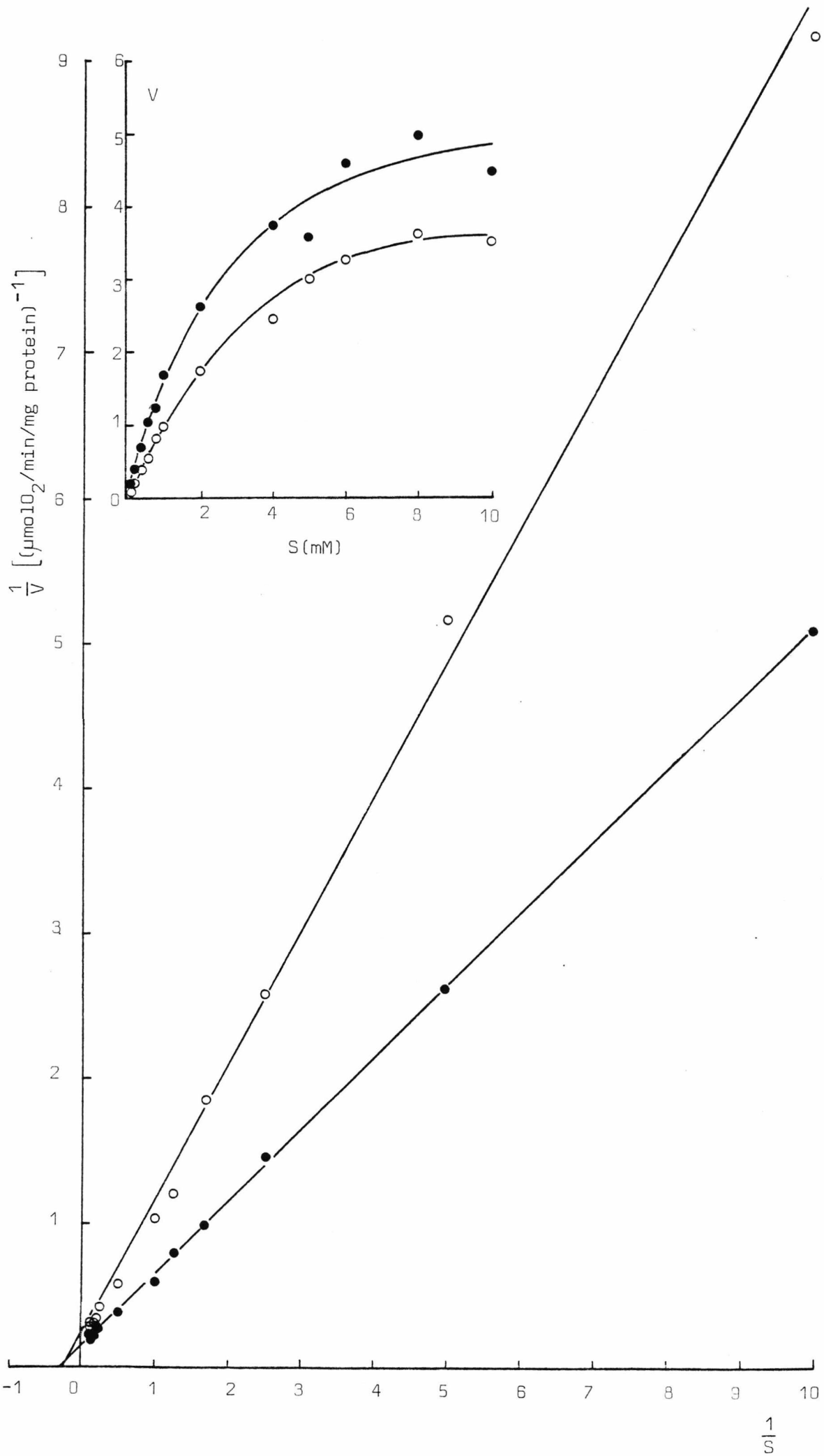
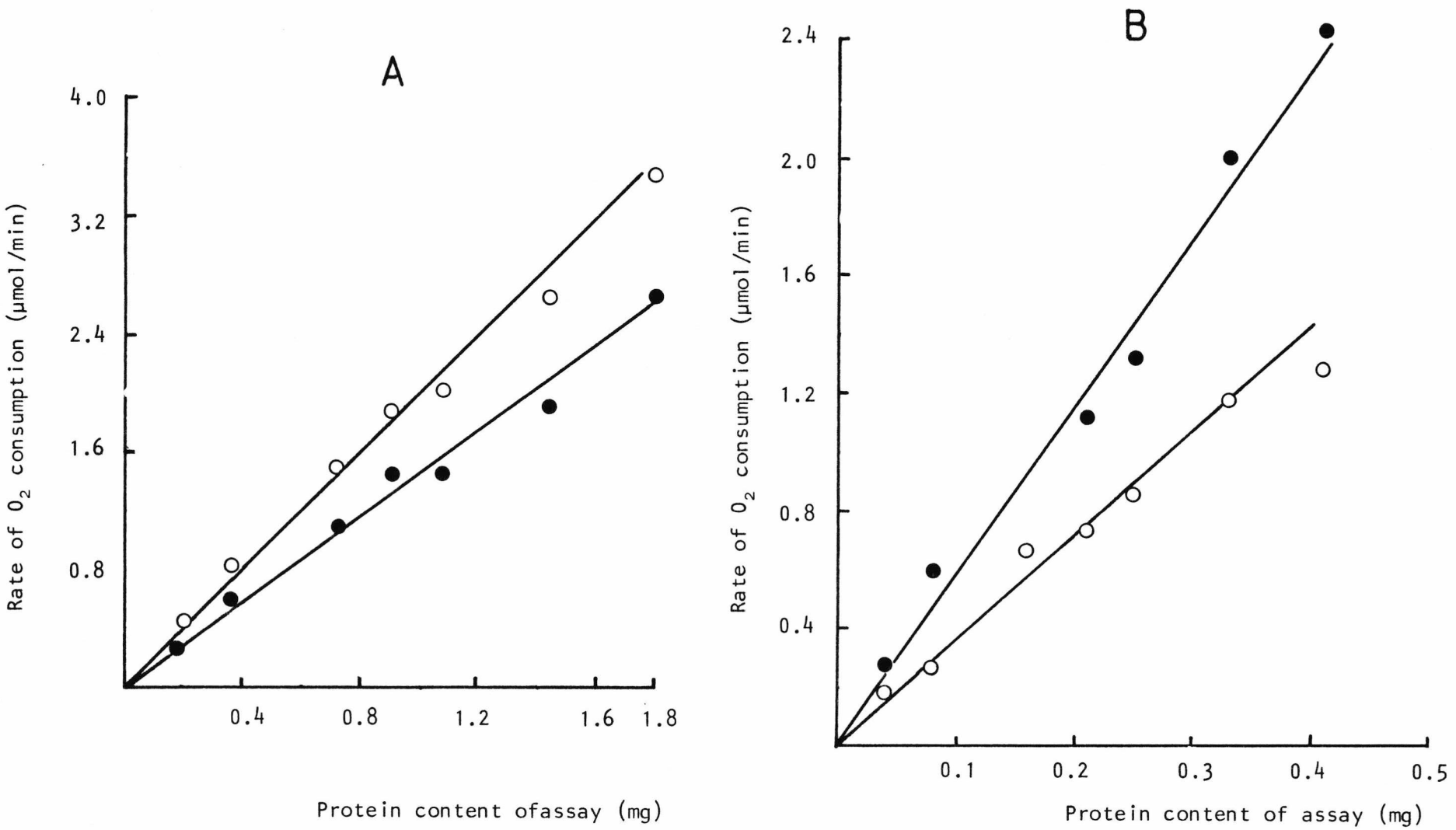

V
S(mM)
$\frac{1}{V}$ [(μmolO_2/min/mg protein)$^{-1}$]
$\frac{1}{S}$

Figure 5.11 Properties of *Mentha aquatica* diphenol oxidase: relationship between the rate of reaction and the amount of extract used in assays.

Assays (2ml total volume) were performed by the methods of Section 2.8.2, and contained 10mM chlorogenic acid (o) or 10mM 4-methylcatechol (●). Leaf extract experiments (Figure A) contained up to 200µl of extract containing about 9mg/ml protein, and root extract experiments (Figure B) used up to 100µl of extract containing about 4mg/ml of protein.

Inactivated extracts (10min at 100°C) or assays containing no plant extract showed no activity.


A
4.0
3.2
2.4
1.6
0.8
Rate of O_2 consumption (μmol/min)
0.4
0.8
1.2
1.6
1.8
Protein content ofassay (mg)
B
2.4
2.0
1.6
1.2
0.8
0.4
Rate of O_2 consumption (μmol/min)
0.1
0.2
0.3
0.4
0.5
Protein content of assay (mg)

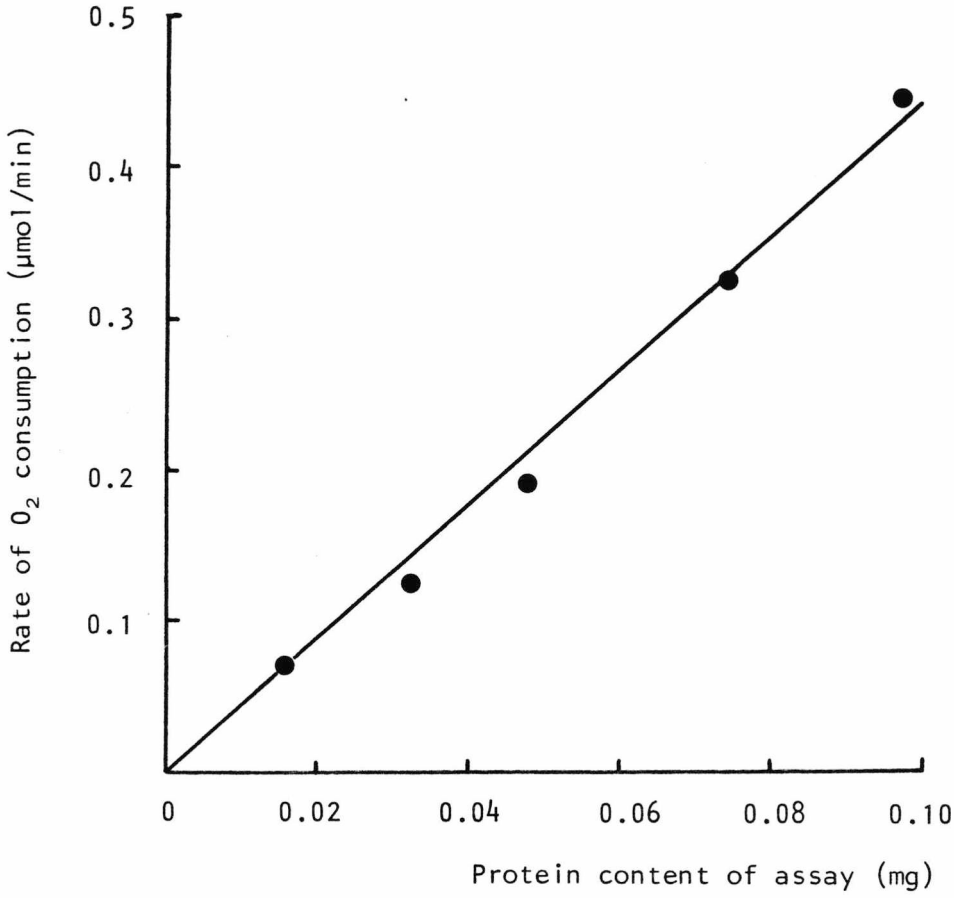


Figure 5.12 Proportionality of monophenol-stimulated O_2-consuming activity of *Phragmites communis* leaf extract with respect to the quantity of extract assayed.

Assays, of 2ml total volume, contained 200µmol of potassium phosphate buffer, pH7.0, 20µmol each of NADH and *p*-coumarate, and volumes of leaf tissue HSS yielding the amounts of protein shown. Reactions, at 25°C, were initiated by the addition of phenolic substrate. The plant tissue extract contained 3.225mg/ml protein, and the specific activity of the O_2-consuming activity was 4.59µmolO_2/min/mg protein under these conditions.

(10min at 100°C) extract was used. Table 5.11 shows some kinetic parameters of this activity, determined in assays of leaf and root tissue extracts. Some combinations of stimulating substrates failed to show Michaelis-Menten kinetics, and are noted in the Table. This phenomenon was observed most clearly when attempting to determine the K_m with respect to *p*-cresol and phenol in the presence of 10mM NADH with leaf extract, and is illustrated by Fig. 5.13. The sigmoid relationship between substrate concentration and reaction velocity is characteristic of "cooperativity" in allosteric enzymes (Segel, 1976). Application of the Hill equation to the data of Fig. 5.13 (C) shows that when supplied with phenol and *p*-cresol respectively, the enzyme system has n_{app} for the two substrates of 1 and 2, and hence that it possesses these minimum numbers of binding sites exhibiting cooperativity effects. Further study of such complex characters in crude extracts is of little value; that *P.communis* preparations possess such properties is nevertheless clear.

The affinity of the enzymes for *p*-coumarate, and the rate of O_2 consumption stimulated by this substrate, was greater than for both phenol and *p*-cresol, while *p*-cresol showed both lower K_m and greater stimulation than phenol. The K_m of the enzymes for O_2 (Table 5.11) was very much lower than for phenolics and NADH, and invariably exhibited typical Michaelis-Menten kinetics. NADH in the presence of any phenolic substrate, and *p*-coumarate in the presence of NADH also showed these typical characters, illustrated in Figs. 5.14 and 5.15.

Table 5.11 Kinetic parameters of NADH-requiring monophenol-stimulated O_2-consuming activity of *Phragmites communis* tissue extracts.

Assays, at 25°C, of 1ml (2ml) total volume contained 100 (200) µmol of potassium phosphate buffer, pH7.0, up to 100µl of plant extract (HSS of Table 5.12) and up to 10 (20) µmol each of NADH and the monophenol. For determination of velocity of reaction at varying concentrations of O_2, the contents of the oxygen electrode chamber were purged with N_2 (Section 2.8.2). The protein content of leaf extracts was 3-5mg/ml, and of root extracts 0.5-1.5mg/ml.

ND - Not Done

Tissue	Substrate	K_m with respect to Phenol (mM)	NADH (mM)	O_2 (µM)	Observed maximum velocity at 10mM phenolic and NADH ($\mu mol O_2$/min/mg protein)
Leaf	*p*-Coumarate	2.5	3.16	1.5	4.2
	p-Cresol	2.7*	ND	ND	3.6
	Phenol	15.4*	3.52	8.5	1.05
Root	*p*-Coumarate	2.6	3.84	1.45	9.0
	p-Cresol	ND	ND	ND	5.95
	Phenol	12.0	ND	ND	1.15

* These data failed to show Michaelis-Menten kinetics, and typical results are presented in Fig.5.13.

Figure 5.13 Effect of substrate concentration on the (NADH + phenols) - stimulated O_2-consuming activity of *Phragmites communis* leaf extract.

Assays, of 1ml total volume, contained 100µmol of potassium phosphate buffer, pH7.0 , up to 100µl of plant extract (HSS of Table 5.12), 10µmol of NADH, and the concentrations shown of (o) phenol and (●) *p*-cresol.

The results are shown -

A, in conventional form,

B, in double-reciprocal form,

and C, in the form of the Hill plot (Segel, 1976)

For the presentation of C, V_{max} for phenol and *p*-cresol were estimated from double-reciprocal plots as 4.44 and 3.08µmolO_2/min/mg protein respectively. The gradient of plots C shows n_{app} representing the minimum number of substrate binding sites per molecule of enzyme, from the logarithmic form of the Hill equation

$$\log \frac{v}{V_{max}-v} = n\log [S] - \log K'$$

where v is the velocity of the reaction at substrate concentration [S], and K' is a constant comprising substrate binding site interaction factors and the intrinsic dissociation constant K_s.

For phenol, $n_{app} = 1.00$ and for *p*-cresol $n_{app} = 1.83$.

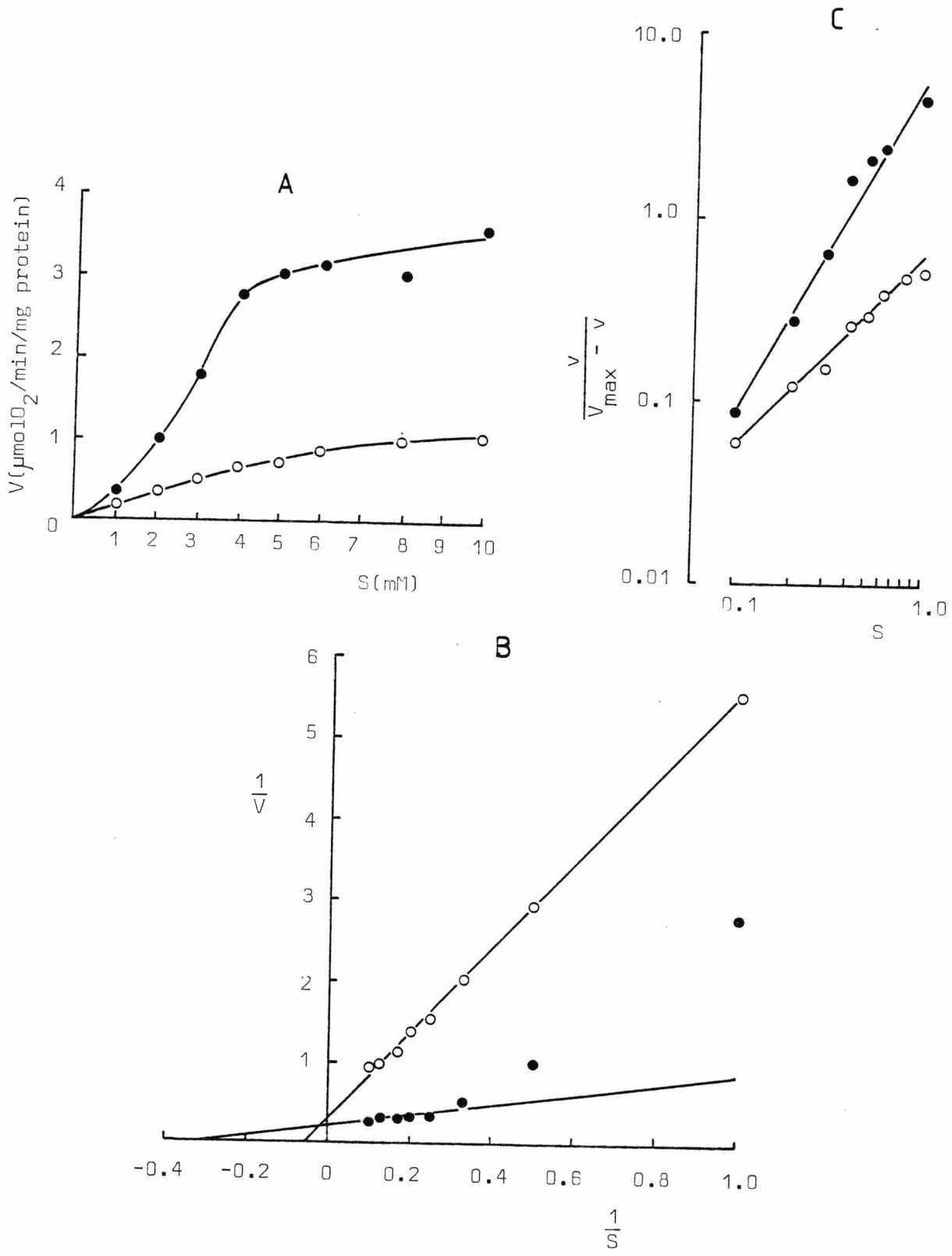
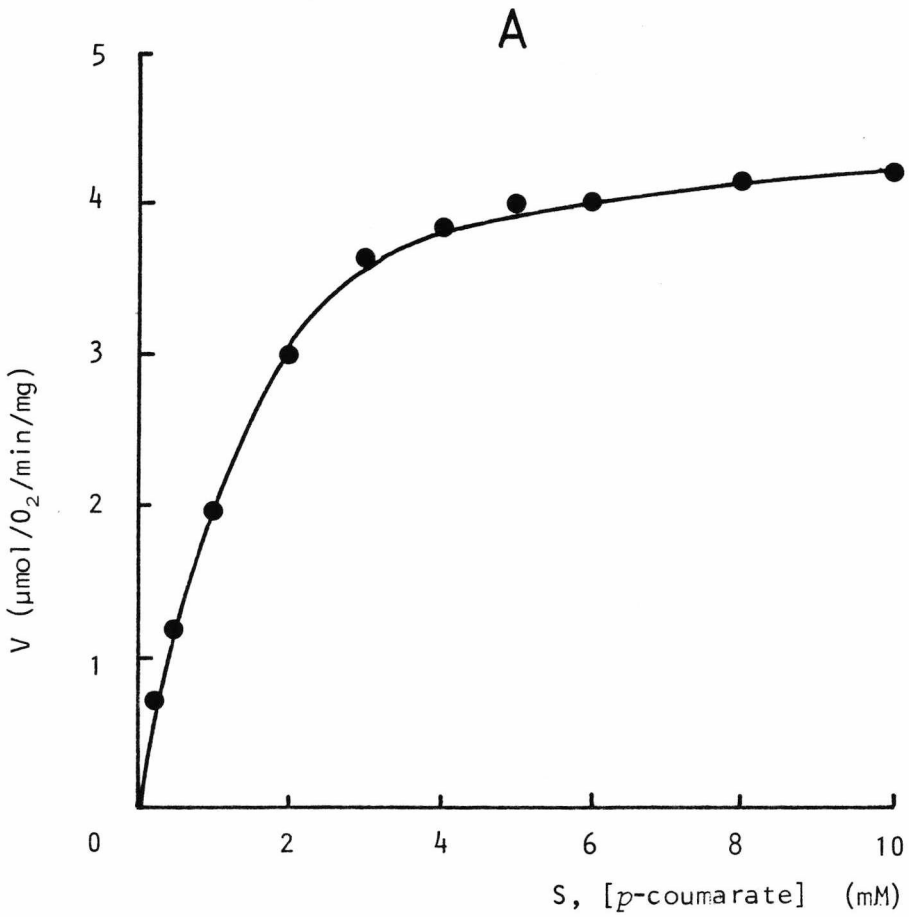

A
V(µmol O_2/min/mg protein)
S(mM)
B
$\frac{1}{V}$
$\frac{1}{S}$
C
$\frac{v}{V_{max} - v}$
S

Figure 5.14 Effect of substrate concentration on the NADH and *p*-coumarate-stimulated O_2-consuming activity of *Phragmites communis* leaf extract.

Assays, of total volume 1ml, contained 100µmol of potassium phosphate buffer, pH7.0, up to 100µl of plant extract (HSS of Table 5.12), and 10µmol of NADH, and the concentrations shown of *p*-coumarate. Assays were performed at 25°C, and the results are shown (A) conventionally, and (B) in double-reciprocal form.


A
5
4
3
2
1
0
2
4
6
8
10
V (µmol/O2/min/mg)
S, [p-coumarate] (mM)

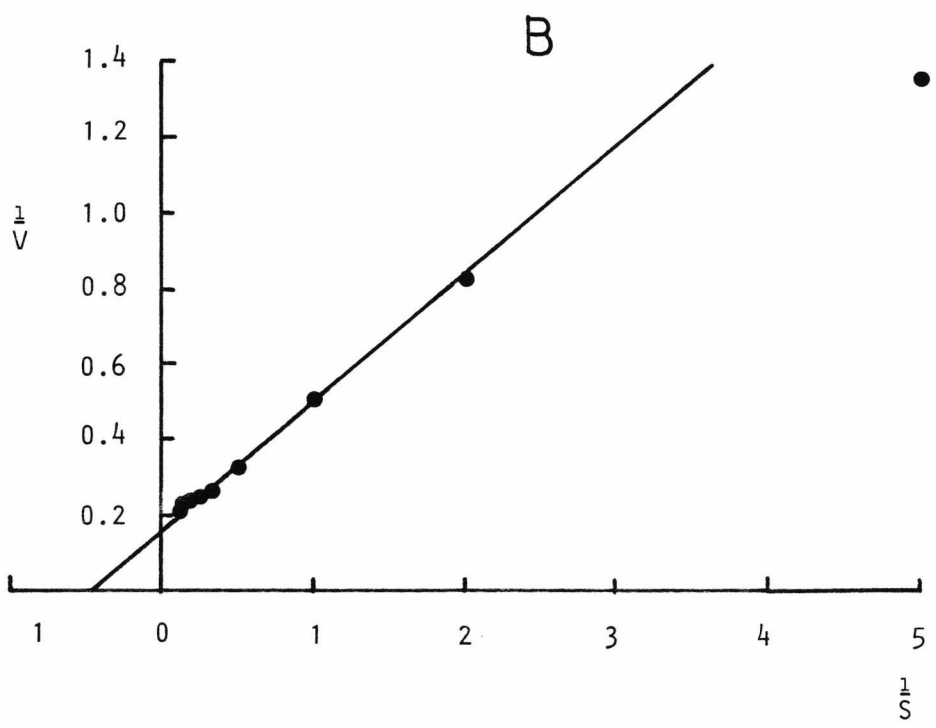
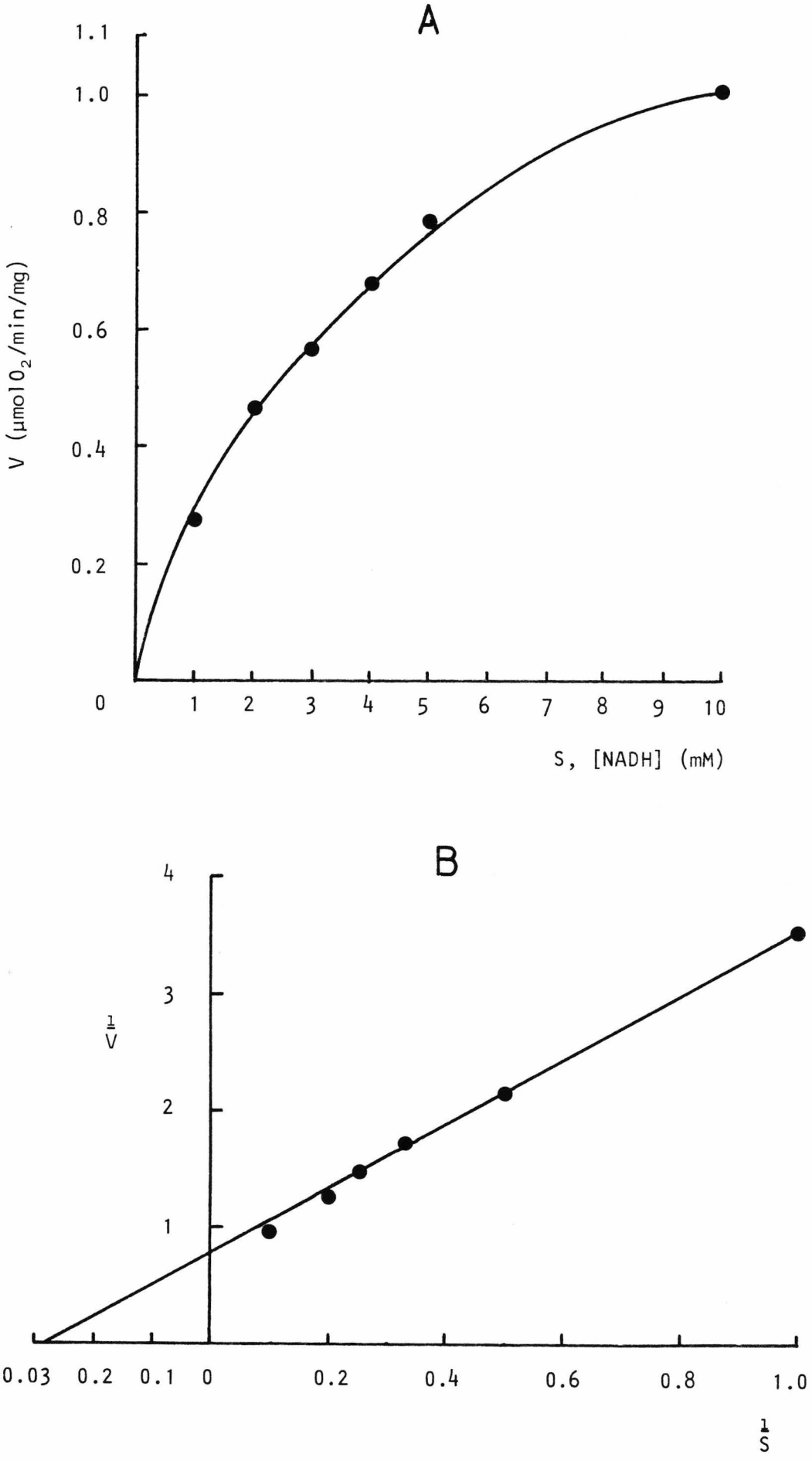

B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
0
1
2
3
4
5
1/V
1/S

Figure 5.15 Effect of substrate concentration on NADH and phenol-stimulated O_2 consuming activity of *Phragmites communis* leaf extracts.

Assays, of total volume 1ml, contained 100µmol of potassium phosphate buffer, pH7.0, up to 100µl of plant extract (HSS of Table 5.12), 10µmol of phenol, and the concentrations shown of NADH. Assays were performed at 25°C, and the results are shown (A) conventionally, and (B) in double-reciprocal form.


A
1.1
1.0
0.8
0.6
0.4
0.2
0
V (μmol O_2/min/mg)
1
2
3
4
5
6
7
8
9
10
S, [NADH] (mM)
B
4
3
2
1
$\frac{1}{V}$
-0.03
0.2
0.1
0
0.2
0.4
0.6
0.8
1.0
$\frac{1}{S}$

Fractionation of *Phragmites communis* monophenol-stimulated activity.

Fractionation of crude extracts (Section 2. 7) of *P.communis* tissues by centrifugation showed that 89% of the activity originally present in root homogenate was cytoplasmic, and remained in the high speed supernatant (HSS) resulting from centrifugation at 200,000*g* for 4h. Some 85% of the initial activity of leaf homogenate also remained in the HSS.

Table 5.12 gives the results of fractionation of a typical root homogenate, showing that the small amount of activity lost was present in the 100,000*g* pellet (11.4%). Leaf extract, which was not fractionated in such detail, lost 8.5% of its original activity in the 10,000*g* pellet and a further 5.5% in the 200,000*g* pellet. Crude extracts of increased protein content could be made by extensive regrinding of the residue from extraction (Section 2.7) but this served only to decrease specific activity, probably by the release of nonfunctional protein, and little or no further phenol-stimulated activity was released.

Table 5.13 shows that the ratio of specific activity in the presence of three phenolic substrates, *p*-coumarate, *p*-cresol, and phenol showed only minor variations in the different fractions. After 1000*g* centrifugation, the ratio of activities of the supernatant fraction altered little from 1:0.68(± 0.06) : 0.14(± 0.04), indicating that all 3 activities remained in the soluble fractions. Only the pellets varied at all from this ratio, but by amounts of insufficient significance to ascribe the activity to a particular sub-cellular fraction.

Stability of the monophenol-stimulated activity of *Phragmites communis* extracts

An extract of leaf tissue, prepared to HSS, and showing a specific

Table 5.12 Fractionation of *p*-coumarate-stimulated O_2-consuming activity of *Phragmites communis* root tissue.

The fractionation régime was essentially derived from Bonner (1976). The homogenate, strained, was taken from the first stage of the method of Section 2.7 , without centrifugation, and the 1000*g* (30min) supernatant was the usual crude extract of this method. The 10,000*g* fractions were generated by centrifugation for 2h and 100,000 and 200,000*g* fractions by centrifugation for 4h. All operations were carried out on ice or at 4°C, and pellet activities were determined after washing by resuspension and recentrifugation. The activity of the washings was ignored in the account of activity as their protein contents and activities were very small. Assays were performed in the presence of 10mM NADH and *p*-coumarate, and activities are expressed as µmolO_2 consumed/min/mg protein. As the protein content of different homogenate preparations varied the results shown are from a typical fractionation, containing initially 0.625mg/ml protein. The protein content of each *pellet* is shown corrected to the volume of extract from which it was obtained.

Fraction	Protein content (mg/ml)	Specific activity ($\mu mol O_2$/min/mg)	Total activity present (%)
Homogenate, strained	0.625	6.31	100
1000*g* Pellet	0.055	0.23	1.99
1000*g* Supernatant	0.570	6.44	93.10
10 000*g* Pellet	0.040	2.05	0.89
10 000*g* Supernatant	0.530	6.97	93.60
100 000*g* Pellet	0.070	6.43	11.40
100 000*g* Supernatant	0.460	7.60	88.60
200 000*g* Supernatant (HSS)	0.460	7.60	88.60

Table 5.13 Ratios of activities from fractions of *Phragmites communis* leaf tissue using three monophenols.

Specific activities with respect to each monophenol are expressed as the ratio with respect to that for *p*-coumarate; all were derived in the presence of 10mM NADH.

Fraction	Ratio of specific activity of fraction *p*-coumarate : *p*-cresol : phenol (*p*-coumarate = 1)
Homogenized extract	1 : 0.74 : 0.18
1000*g* Pellet	1 : 0.40 : 0.19
1000*g* Supernatant	1 : 0.66 : 0.13
10000*g* Pellet	1 : 0.41 : 0.15
10 000*g* Supernatant	1 : 0.70 : 0.15
100 000*g* Pellet	1 : 0.43 : 0.10
100 000*g* Supernatant	1 : 0.66 : 0.13
200 000*g* Supernatant	1 : 0.66 : 0.13

activity of 4.1μmolO_2/min/mg with 10mM *p*-coumarate and NADH, was stored for 48h at 4°C, during which time no change in the ratios of activity (*p*-coumarate: *p*-cresol: phenol, Table 5.13) or of the specific activity took place.

Monophenol-stimulated activity in the presence of mixed substrates.

Table 5.14 shows the results of assays of leaf HSS monophenol-stimulated activity performed with mixtures of the effective monophenolic substrates. Both phenol and *p*-cresol inhibited *p*-coumarate-stimulated activity, *p*-cresol being more inhibitory. Addition to phenol reaction mixtures of *p*-coumarate caused an increase to a rate similar to that obtained when phenol was supplied to *p*-coumarate reaction mixtures, but *p*-cresol and phenol as mixed substrates had different effects, depending on the order in which they were added. Phenol-saturated enzyme (assays in which phenol was added first) did not recover full activity against cresol, but when supplied as a mixture, the two compounds caused stimulation equal to that of *p*-cresol alone, suggesting that once saturated with phenol, the enzymes could not bind *p*-cresol, but when added first or in a mixture, the higher affinity of the enzyme for *p*-cresol prevented this. Phenol, supplied later to *p*-cresol incubations, had no effect, when saturation with *p*-cresol was complete. Addition of *p*-coumarate to assays containing either phenol or *p*-cresol resulted in a stimulation of O_2 consumption to a rate very similar to that achieved by the addition of both phenol and cresol to *p*-coumarate-saturated assays. Hence, *p*-coumarate, which had a higher affinity for the enzyme than either of the other substrates, was preferentially oxidised.

The results obtained show that in no case were additive rates apparent. Were two or more enzymes responsible for the O_2 consumption,

Table 5.14 Effect of monophenol mixtures on the activity of *Phragmites communis* leaf extracts.

Assays, of 1ml total volume, contained 100µmol of potassium phosphate buffer, pH7.0, 5µl of leaf tissue extract HSS (Table 5.12) containing about 0.015mg protein, and 10µmol of NADH. To the assay mixture was added 10µmol of the appropriate monophenol and the linear rate of O_2 consumption was allowed to develop.

The second monophenol (10µmol) was then added, and the modified rate recorded. When the effects of substrate mixtures were to be examined, aliquots of mixed monophenol stock solutions containing 10µmol of each were added to the cuvette after the NADH. The rates recorded in the table are of O_2 consumption (µmol/min/mg protein) in the presence of single, sequentially added, and simultaneously added combinations.

Substrate or substrate combination added to reaction mixtures.	Rate of reaction
p-Coumarate alone	4.83
p-Coumarate, then phenol	3.85
p-Coumarate plus phenol, mixed	3.92
p-Coumarate, then *p*-cresol	3.51
p-Coumarate plus *p*-cresol, mixed	3.41
Phenol alone	0.64
Phenol, then *p*-cresol	1.19
Phenol, then *p*-coumarate	3.83
Phenol plus *p*-cresol, mixed	2.20
p-Cresol alone	2.20
p-Cresol, then phenol	2.20
p-Cresol, then *p*-coumarate	3.51

the addition of, say, phenol to *p*-coumarate assays should have resulted in an arithmetic increase in activity, and addition of *p*-coumarate to phenol or *p*-cresol assays would also give a rate approximating to the sum of their separate activities. This was clearly not the case. It was therefore concluded that one enzyme, binding all three monophenols with varying degrees of efficiency, was probably responsible for the O_2 consuming activity, as previously inferred from the consistent ratios of activity with three monophenols (Table 5.13).

Alternative reductants for the monophenol-stimulated activity of *Phragmites communis* extracts.

NADPH was tested as a reductant for the activity, with *P.communis* leaf tissue HSS. In the presence of 1mM *p*-coumarate, 0.5µmol NADPH replaced NADH, but gave rise to a rate of O_2 consumption of only 33% of that observed with 0.5µmol of NADH. Ascorbate (1 or 10mM) was ineffective (Table 5.4).

Stoicheiometry of the NADH and monophenol-requiring activity.

The stoicheiometry of the reaction catalyzed by *P.communis* tissue extracts was examined.

Phenol and *p*-coumaric acid, proposed substrates of the reaction, and catechol and caffeic acid, their respective *o*-hydroxylated products, were assayed discontinuously by colorimetric methods, and consumption of NADH was monitored at 340nm. Consumption of oxygen could be determined by use of smaller quantities of the substrates in oxygen-electrode assays run to organic substrate (not O_2) exhaustion. Thus the stoicheiometry with respect to monophenolic substrate and NADH, monophenolic substrate and diphenolic product, NADH and O_2, and monophenolic substrate and O_2 could be determined separately.

Results presented in Fig. 5.16 show the time course of reaction of phenol and *p*-coumarate and NADH in the presence of leaf tissue HSS. Although NADH was oxidised virtually to completion, there was no evidence that either monophenolic substrate was consumed, or that any diphenolic product appeared. The rate of NADH consumption bore little relationship to rates of O_2 consumption obtained in polarographic assays previously reported, the probable reason for this being that O_2 diffusion into the reaction mixture, although it was stirred, was too slow to sustain the maximum rates of reaction observed elsewhere.

As previous experiments had indicated that catechol was formed from phenol in leaf tissues, this experiment was repeated with crude extract, and monophenol, NADH, and putative diphenol product were assayed only at the beginning and end of the incubation periods; no conversion of phenolic substrates took place. In a further crude extract assay *p*-cresol was also not metabolised. Lest high (10mM) phenolic substrate concentrations might cause substrate inhibition of diphenol production, phenol and *p*-coumarate assays were repeated with 1mM concentration of these substrates, although NADH was maintained at the initial concentrations of 10mM. On assay at a single end point, no conversion of the phenolic substrates was observed.

Table 5.15 shows the results of determinations of the stoicheiometry of the reaction with respect to NADH and O_2. The molar ratio of NADH supplied/O_2 consumed, for both substrates, was about 2, indicating that the consumption of 1mol of NADH required 0.5mol of O_2 in the presence of 10mM phenolic substrate, i.e. the stoicheiometry foran NADH oxidase -

$$NADH + H^+ + \tfrac{1}{2}O_2 \longrightarrow NAD + H_2O$$

Figure 5.16 Stoicheiometry with respect to NADH, *p*-coumarate, caffeate, phenol, and catechol of the monophenol-stimulated activity of *Phragmites communis* leaf tissue.

Assay mixtures, of total volume 3ml, contained 300µmol of potassium phosphate buffer, pH7.0, 150µl of leaf tissue HSS, and 30µmol each of NADH, and (A) *p*-coumarate, or, (B) phenol and were incubated in small flasks at 25°C with constant stirring. Samples were removed at the times shown for assay of NADH (●), phenol (△), catechol (▲), *p*-coumarate (□) and caffeate (■). NADH was assayed after rapid dilution of the sample by its absorbance at 340_{nm} and the phenolic substrates and products were assayed colourimetrically (Section 2.6). The results shown are the averages of duplicate assays performed on duplicate experiments.

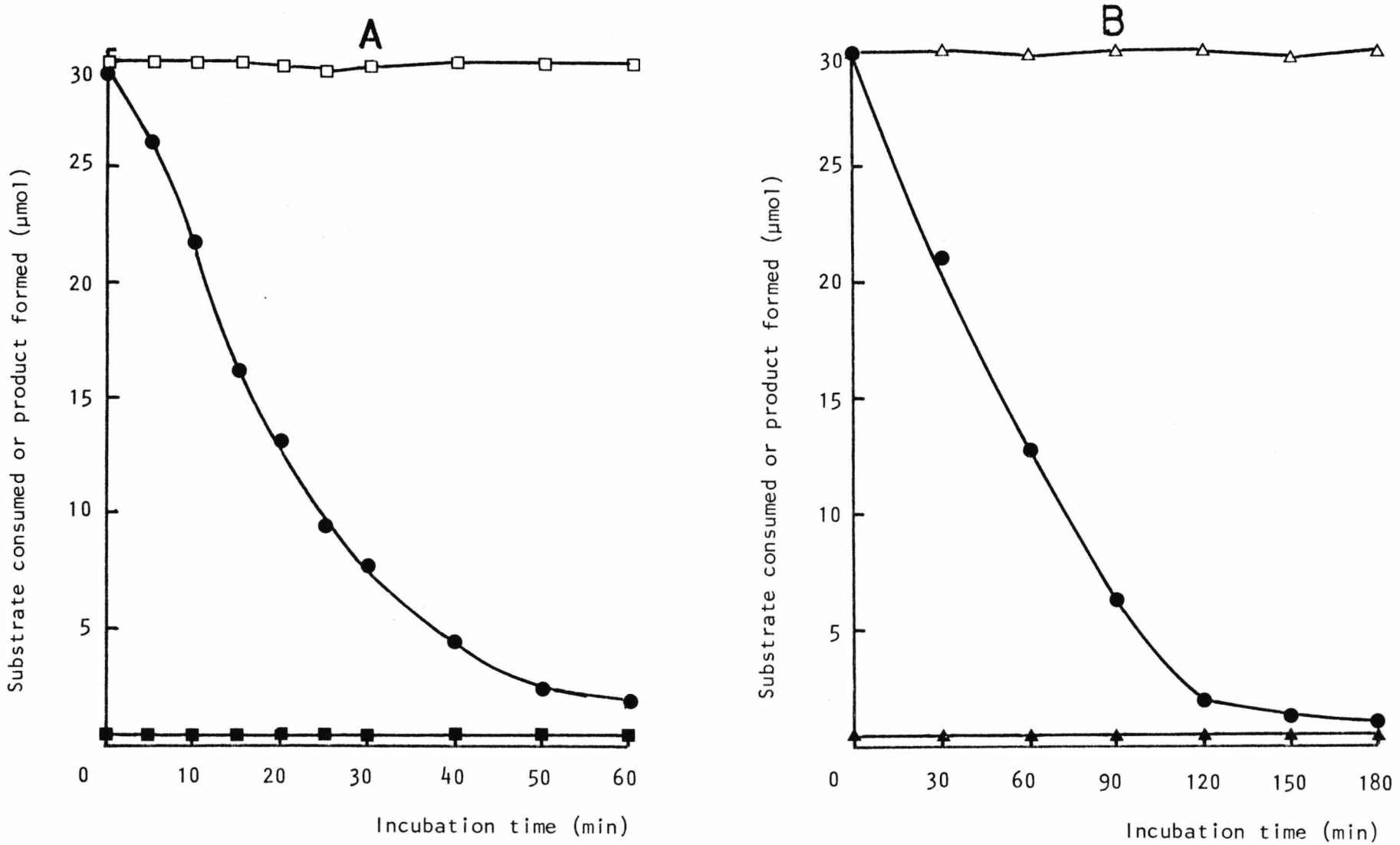

A
B
Substrate consumed or product formed (μmol)
Incubation time (min)
0
5
10
15
20
25
30
40
50
60
90
120
150
180

Table 5.15 Stoicheiometry of the monophenol-stimulated activity of *Phragmites communis* leaf tissue with respect to NADH and O_2 in the presence of phenol and *p*-coumarate.

Assays of 2ml total volume contained 200µmol of potassium phosphate buffer, pH7.0, 20µmol of either *p*-coumarate or phenol, and 0.016mg of protein in 5µl of leaf tissue HSS, and were maintained at 25°C. To the assay mixture, showing a stable endogenous O_2 consumption rate, was added the quantity of NADH shown. The assay was run until the accelerated rate of O_2 consumption returned to the endogenous rate, and the perpendicular displacement of the chart trace, representing the decrease in O_2 concentration within the cell caused by the addition of NADH was found. The results given are the averages of duplicate determinations.

Phenolic Substrate (10mM)	NADH Supplied (µmol)	O_2 Consumed (µmol)	Molar Ratio $\frac{NADH}{O_2}$
p-Coumarate	0.4019	0.205	1.96
	0.1608	0.064	2.51
Phenol	0.4849	0.247	1.96
	0.1961	0.110	1.78

The determination of stoicheiometry with respect to monophenols and O_2 could not be carried out, as the addition to assay mixtures of small quantities of phenolic substrates, in the presence of 1 or 10mM NADH, resulted in the development of the appropriate rate of O_2 consumption proceeding to O_2 exhaustion. This result, obtained with *p*-cresol, phenol, and *p*-coumarate, suggested that the action of the monophenols was catalytic, rather than as substrates, and facilitated an NADH oxidase activity. Such results explain the data of Fig. 5.16. The NADH-requiring O_2 consumption is clearly stimulated by monophenols, but these latter remain unaffected in a reaction, which is probably that of an uncoupled monophenol oxygenase or similar phenol-stimulated system.

The stoicheiometry of the reaction observed fits only one of two alternatives for NADH oxidation. Uncoupled aromatic hydroxylases usually divert O_2 to H_2O_2 (Spector and Massey, 1972; White-Stevens and Kamin, 1972; Neujahr and Kjellen, 1978), which is clearly inappropriate. The phenol-stimulated aerobic NADH oxidase activity of peroxidase, however, has the stoicheiometry observed here (Akazawa and Conn, 1958). This enzyme, essential in plants for lignin production (Harkin and Obst, 1973), and hence found widely in plant tissues, therefore seemed the most likely source of the phenol-stimulated activity against NADH.

Studies of peroxidase and inhibitors of monophenol-stimulated activity of *Phragmites communis* extracts.

As it was suspected that peroxidase might be responsible for some of the phenol-stimulated NADH and O_2-consuming activities of plant extracts, assays with commercial horseradish peroxidase (Sigma, Type 1) were made in the presence of phenols and NADH in the same manner as with *P.communis* preparations, and plant extracts were

examined specifically for peroxidase and H_2O_2. The responses of commercial horseradish peroxidase (HRP) and plant extracts to a number of inhibitory substances were compared.

The results of Table 5.16 and Fig. 5.17 show that peroxidase was indeed present in both freshly prepared and aged (48h at 4°C) *P.communis* leaf extracts. Aged extract required exogenous H_2O_2 to exhibit any activity, but freshly prepared extract yielded a positive spot test for peroxidase and H_2O_2 in the presence of 10mM guaiacol indicating the presence of H_2O_2 in the tissues. The H_2O_2 present in such extracts was very short-lived, as the plant material also contained catalase (Table 5.16). No positive reaction was obtained when aged plant extract (100µl of crude strained homogenate of Table 5.12) was added to 1ml of 10mM guaiacol solution only 30min after its initial homogenization. When assayed as shown in Table 5.16, both fresh and aged extract gave identical results.

Pure HRP catalysed the oxidation of NADH at a low rate at both pH5.0 and pH7.0 (Fig.5.18), and this activity was markedly stimulated by the addition of Mn^{2+}, alone or in conjunction with phenols (Table 5.17). Phenol alone, however, did not stimulate this activity.

The total inhibition of NADH oxidase by catalase (Sigma, from bovine liver, type C-40) and catechol confirmed that this activity was similar to that described by Halliwell (1978) and Akazawa and Conn (1958) with commercial horseradish enzyme. The activity was further confirmed at higher concentrations of NADH by measuring O_2 consumption at pH7.0 (Fig. 5.19). NADH was not oxidised by heat inactivated (10min at 100°C) HRP, and did not autooxidise at a significant rate alone or in the presence of Mn^{2+} or phenols, separately or together.

When assayed at high concentrations of substrate the oxidation of NADH by HRP showed no evidence of saturation by 10mM NADH (Fig. 5.20),

Table 5.16 Assays of commercial peroxidase and catalase and of these activities in *Phragmites communis* leaf extract.

i) Assays of peroxidase were carried out with guaiacol as the donor substrate, under the conditions of (A), Bergmeyer *et al.* (1974); (B), with both guaiacol and H_2O_2 supplied in 10mM concentration.

Assays for (A) contained, in 1ml total volume, potassium phosphate buffer, pH7.0, 100µmol; guaiacol, 0.3µmol; and H_2O_2, 0.12µmol, and were performed at 25°C. The reaction was initiated by the addition of 10-100µg of commercial horseradish peroxidase or 10-100µl of *P.communis* extract.

Assays for (B) were identical, except that both guaiacol and H_2O_2 were present at 10µmol.

Both methods were monitored continuously at 430nm, and rates of reaction are reported in $\Delta A430nm$.

ii) Assays of catalase were carried out (C) by monitoring the concentration of H_2O_2 at 240nm (A_{240nm} of H_2O_2= 72) or (D) by following the evolution of O_2 in the oxygen electrode, both at 25°C.

Assays for (C) contained, in a total volume of 1ml, 10µmol of H_2O_2 and 1-100µg of commercial catalase or 10-100µl of *P.communis* extract. The reaction was initiated by addition of the enzyme, after determination of the spontaneous rate of H_2O_2 decomposition.

For assays by method (D), 1ml of 100mM potassium phosphate buffer, pH7.0, was deoxygenated in the cell of the O_2 electrode by bubbling with dry O_2-free N_2 until the recorder indicated zero concentration of O_2. H_2O_2 solution (100mM) was added to yield a final concentration of 1mM, and its spontaneous decomposition rate was determined. Addition of 1-20µg of commercial enzyme or 10-100µl of plant extract caused a linear evolution of O_2, which was recorded in the usual way.

ND - Not Done

Table 5.16

Assay	Specific Activity of Plant extract	Specific Activity of commercial enzyme
Peroxidase		
A (ΔA/min/mg)	ND*	41.5
B (ΔA/min/mg)	8.09	2593
Catalase		
C (μmolH_2O_2/min/mg)	ND†	140
D (μmolO_2/min/mg)	8.11	ND

* Plant extract failed to show peroxidase under the low substrate concentration of this assay method.

† Plant crude extract could not be assayed by this method as ultraviolet absorbance of the material was excessive.

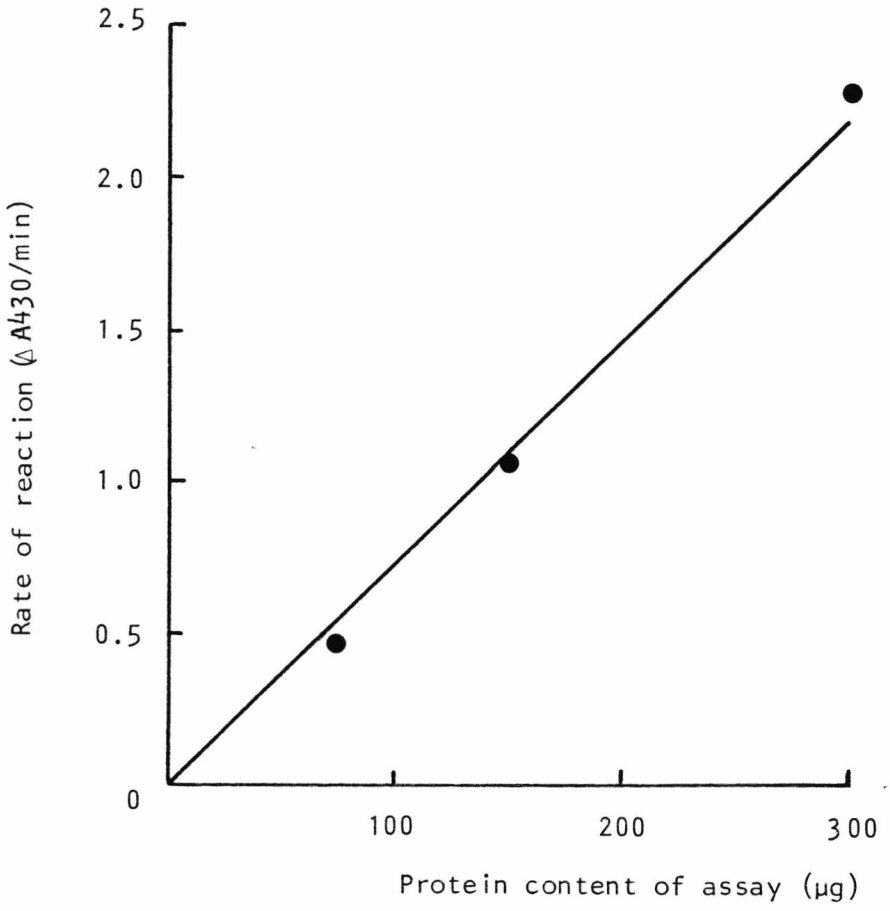


Figure 5.17 Presence of peroxidase in extracts of *Phragmites communis* leaf tissue.

Assays, performed under the conditions described in the legend to Table 5.16B, contained the amounts of plant extract protein shown in the figure.

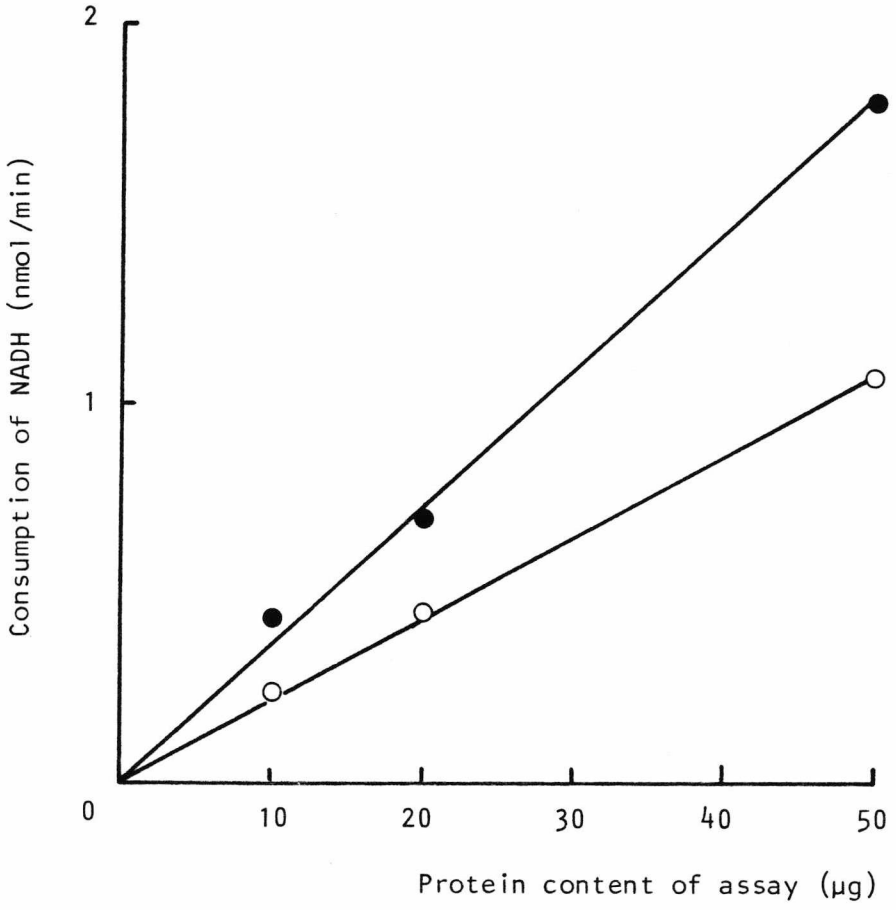


Figure 5.18 Oxidation of NADH by commercial horseradish peroxidase.

Assays, at 25°C, in a total volume of 1ml, contained potassium phosphate buffer, pH5.0 (●) or pH7.0 (o) 100µmol, NADH, 0.1µmol, and the amounts of HRP shown. Consumption of NADH was monitored continuously, after addition of the enzyme, at 340nm. Blank assays contained no HRP.

Table 5.17 Oxidation of NADH by commercial horseradish peroxidase.

Assays, at 25°C in a total volume of 1ml, contained potassium phosphate buffer, pH7.0 or pH5.0, 100µmol; HRP, 1-100µg; NADH, 0.1µmol, and the stated amounts of stimulating or inhibitory substances. Consumption of NADH was monitored continuously at 340nm and blank assays contained no HRP. The initial rate of reaction was recorded.

Additions to assay	Rate of NADH oxidation (nmol/min/mg protein)	
	pH5.0	pH7.0
None	35.5	21.5
1µmol phenol	35.5	21.5
1µmol $MnCl_2$	3500	520
1µmol phenol + 1µmol $MnCl_2$	26700	
1µmol *p*-cresol + 1µmol $MnCl_2$	55400	
1µmol *p*-coumarate + 1µmol $MnCl_2$	69500	
1µmol H_2O_2	750	
1µmol H_2O_2 + 1µmol $MnCl_2$	≧100 000	≧100 000
1µmol H_2O_2 + 1µmol $MnCl_2$ + 1µmol phenol	≧100 000	≧100 000
Catalase 100µg	0	0
1µmol catechol	0	0

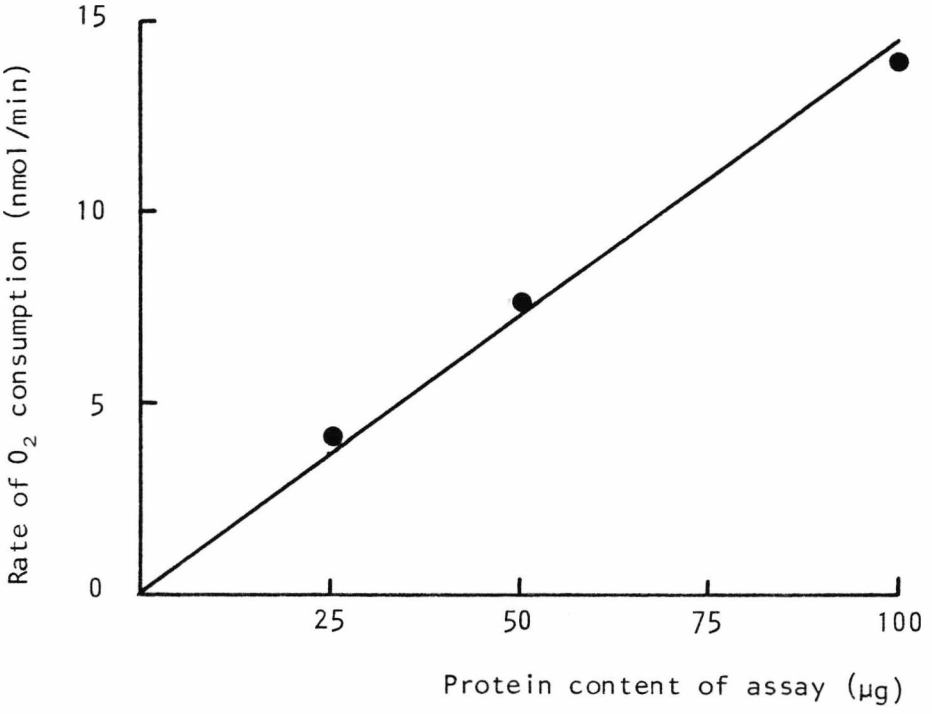


Figure 5.19 Oxidation of NADH by commercial peroxidase.

Assays, of 1ml total volume in the chamber of the oxygen electrode, contained 100μmol potassium phosphate buffer, pH7.0, 1μmol NADH, and the amounts shown of HRP. Assays, performed at 25°C, were initiated by the addition of enzyme.

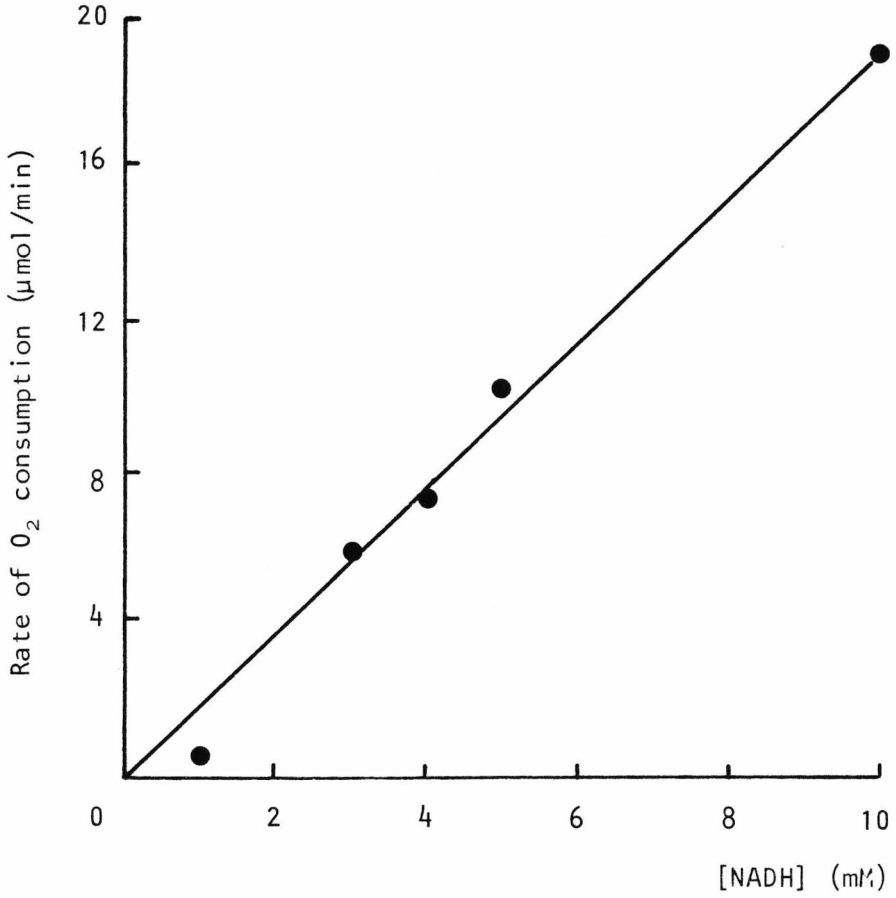


Figure 5.20 Effect of substrate concentration on the oxidation of NADH by commercial peroxidase.

Assays, of 1ml total volume in the chamber of the oxygen electrode, contained 100µmol potassium phosphate buffer, pH7.0, NADH as shown, and were initiated by the addition of 1-100ug of HRP.

but when stimulated by phenols alone showed some evidence of saturating kinetics with respect to NADH (Fig. 5.21) in the presence of 10mM *p*-coumarate (the optimal phenolic stimulator), but this was not described by a simple double-reciprocal plot of the data. The results of Table 5.18 further demonstrate the activity of HRP against NADH in the presence of phenols and Mn^{2+}. As with *P.communis* extract, (Table 5.11) *p*-coumarate was the most effective phenolic stimulant of O_2 consumption, but phenol and *p*-cresol gave differing results. Comparison with the results of assays of a typical *P.communis* leaf extract, included in Table 5.18, show that most features of phenol and Mn^{2+}-stimulated NADH oxidation of HRP were similar to the plant extract activity, with the exceptions that the specific activity of the commercial enzyme was very much greater, and that plant extract activity was not affected by Mn^{2+}. The crude plant extract might have contained sufficient Mn^{2+} to saturate the smaller quantities of enzyme present.

Low concentrations of NADH (0.1mM) were oxidised at a low rate in the presence of plant extract, but the addition of phenols or Mn^{2+} failed to stimulate this activity, perhaps because the catalase present in the extract (Table 5.16) interfered with the reaction. The basal rate of NADH oxidase of plant extract, measured spectrophotometrically, was only 3.13nmol/min/mg protein, but the results of Fig. 5.16 confirmed that the loss of NADH in the presence of phenols did occur when NADH was present at higher concentration.

When treated with inhibitors, further similarities between plant extract activity and HRP-catalysed NADH oxidation became apparent (Table 5.19). For these experiments, inhibition of HRP-NADH oxidase in the presence of both 1mM and 10mM concentrations of NADH and phenolics was studied, for comparison with inhibition of plant extract activity under 10mM conditions; activity of plant extracts with 1mM

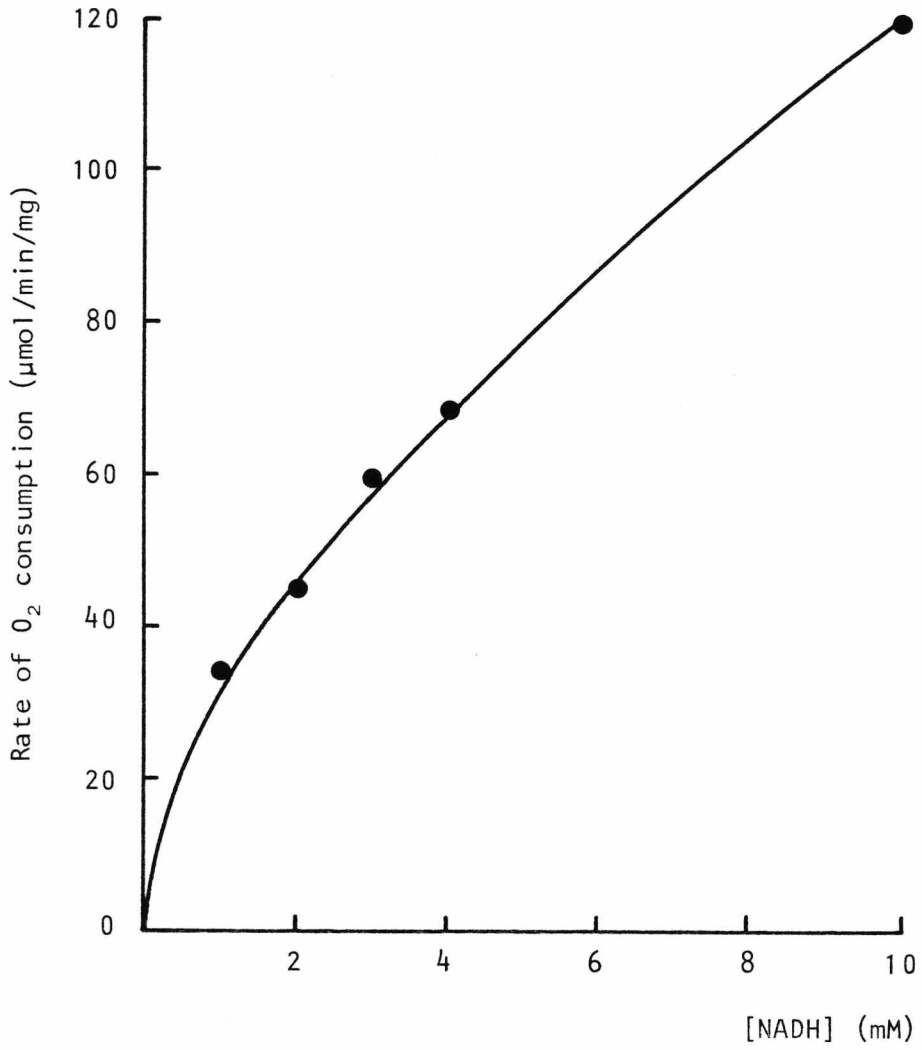


Figure 5.21 Effect of reagent concentration on the phenol-stimulated oxidation of NADH by commercial peroxidase.

Assays, performed as shown in the legend to Fig. 5.20, contained 100µmol potassium phosphate buffer, 10µmol *p*-coumarate, and NADH as shown, and were initiated by the addition of 5-100µg HRP.

Table 5.18 Stimulation by phenols and Mn^{2+} of NADH oxidation by commercial peroxidase and *Phragmites communis* leaf tissue extract.

Assays, of 1ml total volume at 25°C, contained 100µmol of potassium phosphate buffer, pH7.0, 1-100µg of HRP or 10-100µl of plant extract and the stated amounts of additional reagents. The assay was initiated by the addition to the O_2 electrode cell of the reagent shown last in each case.

Additions to assay	Specific activity of commercial peroxidase ($\mu mol O_2/min/mg$)	Specific activity of plant extract ($\mu mol O_2/min/mg$)
1µmol NADH	0.505	0.028
10µmol NADH	19.1	0.098
1µmol NADH + 10nmol $MnCl_2$	0.644	0.028
1µmol NADH + 1µmol $MnCl_2$	2.029	0.028
1µmol NADH + 1µmol Phenol	7.558	0.060
1µmol NADH + 1µmol *p*-Cresol	6.835	0.090
1µmol NADH + 1µmol *p*-Coumarate	9.161	0.180
1µmol NADH + 1µmol *p*-Coumarate + 1µmol $MnCl_2$	25.250	0.180
10µmol NADH + 10µmol *p*-Coumarate	119.5	4.2

Table 5.19 Inhibition of NADH oxidising activity of commercial peroxidase and *Phragmites communis* leaf extract.

Assays, of 1ml total volume in the cell of the O_2 electrode at 25°C, contained 100µmol of potassium phosphate buffer, 1-10µg of commercial horseradish peroxidase or 10-100µl of plant extract, and either 1µmol each of NADH and *p*-coumarate or 10µmol each of these reagents. The reaction was initiated by the addition of the phenolic, and after the linear rate of O_2 consumption had developed, the stated amount of inhibitor was added. Parentheses indicate the percentage inhibition brought about by each substance (uninhibited = 0). Abbreviations used for the inhibitors are as follows: NaN_3, 10mM sodium azide solution; KCN, 10mM potassium cyanide solution, freshly prepared; DIECA, 10mM sodium diethyldithiocarbamate solution; EDTA, 10mM solution of ethylene diamine tetraacetic acid, sodium salt; 8HQ, 8-hydroxyquinoline, 10mM solution; $CuSO_4$, 10mM solution of cupric sulphate.

Concentration of substrates	Specific activity of Commercial peroxidase ($\mu mol O_2/min/mg$) 1mM		Specific activity of Commercial peroxidase ($\mu mol O_2/min/mg$) 10mM		Specific activity of Plant extract ($\mu mol O_2/min/mg$) 10mM	
Inhibitor						
None	9.161		119.5		4.06	
NaN_3 (1µmol)	4.122	(55)	119.5	(0)	4.06	(0)
KCN (1µmol)	0	(100)	0	(100)	0.307	(92)
DIECA (1µmol)	0	(100)	78.6	(34)	2.177	(46)
EDTA (1µmol)	0	(100)	0	(100)	2.079	(49)
8HQ (1µmol)	2.11	(77)	107.1	(10)	1.065	(74)
Catechol (1µmol)	0	(100)	0	(100)	0.149	(96)
Caffeate (1µmol)	0	(100)	2.251	(98)	0.149	(96)
Catalase (100µg)	0	(100)	0	(100)	0.830	(80)
$CuSO_4$ (1µmol)	0	(100)	5.97	(95)	0.033	(99)

substrates was too low to permit accurate estimation of the effects of inhibitors.

HRP was strongly inhibited by potassium cyanide (KCN), sodium diethyldithiocarbamate (DIECA), ethylene diaminetetraacetic acid (EDTA), 8-hydroxyquinoline (8HQ), catechol, caffeate, catalase, and Cu^{2+} but high concentrations of substrates appeared to protect the enzyme against inhibition by some of these, notably DIECA and 8HQ. Inhibition by sodium azide (NaN_3) and 8HQ was less effective under both low and high substrate regimes, and in all cases, the order of addition of the inhibitor (before or after the substrates) was immaterial. The inhibition of HRP activity by catalase was observed by Halliwell (1978), and Akazawa and Conn (1958) reported inhibition by catechol, KCN, Cu^{2+} and catalase, while finding that 0.17mM NaN_3 and 33μM DIECA were not inhibitors.

P.communis leaf extract activity was likewise inhibited by all the substances tested except NaN_3; strong inhibition was shown by KCN, catechol, caffeate, catalase, and Cu^{2+}, which were also very effective against HRP. EDTA was moderately effective against plant extract activity, while showing complete inhibition of HRP, and 8HQ and DIECA were more effective against plant extract activity than against HRP. However, no inhibitor completely abolished *P.communis* activity, suggesting either that other components of the crude extract protected the activity or that some other enzymic activity remained.

Three of the most potent inhibitors of the NADH oxidase activity of HRP, catechol, catalase, and Cu^{2+}, which were found in a separate series of assays to inhibit the "endogenous" NADH oxidase activity of HRP (i.e. activity in the absence of phenols), completely, were entirely without effect on the endogenous NADH oxidase of plant extracts. This result partially confirms the suggestion that a small proportion of *P.communis* activity could survive inhibition, and might

not be due to peroxidase.

Nevertheless, the present studies have shown clearly that peroxidase, even in the absence of endogenous H_2O_2, was responsible for the consumption of NADH and O_2 stimulated by phenols in plant tissues.

SECTION 6 DISCUSSION

Pollution by Phenolic Compounds

Phenolic pollution is characteristic of manufacturing and raw materials conversion processes, many of which, listed by the Water Research Centre (1980), are "heavy" industries, economically significant, and producing large volumes of contaminated wastes. While the effluent-treatment capabilities of some major industries are generally efficient (Section 1.4), low-level contamination with phenolic materials is very wisespread. Analyses of rivers, effluent outfalls, and landfill runoff waters from the United States and many Northern European countries, which are readily available (Water Research Centre, 1980), show that phenol itself is among the compounds most frequently sought and found, probably because it is so characteristic of heavy chemical industries.

Seidel (1976) observed that early workers regarded clean water as a prerequisite for healthy growth of aquatic plants; hence the discovery (Seidel, 1963) that *Schoenoplectus lacustris* specimens absorbed phenol from waters containing a comparatively high concentration of the pollutant, suggested that not only were some aquatic plants likely to tolerate low-level phenolic pollution, but that they might also serve to reduce such contamination. This is further confirmed by the results of the present study.

Toxicity of phenol to *Lemna minor* and *Phragmites communis*.

The toxicity of phenols and (naturally occurring) phenolic acids to plants and eucaryotic microorganisms is expressed by several effects, which include:-

(i) inhibiting the germination of seeds (Van Sumere *et al.*, 1972; Reynolds, 1978);

(ii) reduction of growth (dry weight yields) of seedlings (Guenzi and McCalla, 1966a; Glass, 1976);

(iii) interfering with the integrity and function of the cell membrane; uptake of phosphate (Glass, 1973; 1975; McClure *et al.*, 1978) and potassium (Glass, 1974) ions is reduced by 50% by (0.1-1mM) phenolic acids. When depolarization of the trans-membrane potential difference was used as a measure of membrane integrity, the severe effects of cinnamic and salicylic acids were evident at a concentration of 0.25mM (Glass and Dunlop, 1974);

(iv) uncoupling oxidative phosphorylation (Van Sumere *et al*, 1972; Marinos and Hemberg, 1960; Demos *et al.*, 1975), which can be shown by an increase in O_2 consumption in the presence of the phenols;

(v) inhibition of enzymes *in vivo* or *in vitro*, at phenol concentrations in the range 0.01-10mM (Rothe, 1976). Among major metabolic systems which are inhibited are protein synthesis, RNA metabolism, and sugar transport (Van Sumere *et al.*, 1975).

When examined in the present study, phenol in the culture medium of *L.minor* (Section 3.2.1) reduced the growth rate of the plant, by increasing the doubling time of exponentially growing cultures from 2.2 to 3.5 days at 0.5mM and to 10.5 days at 1mM. Lower concentrations of phenol (0.1mM), however, were not toxic to *P.communis* seedlings when the fresh weight of the plants was used as an index of toxicity, nor did this concentration affect the growth of mature plants (Section 3.2.2), but germination of *P.communis* seed

was inhibited by 50% by 1mM phenol solution (Section 3.2.2). Reynolds (1978) also found 50% inhibition of the germination of seeds of *Lactuca sativa* by a phenol concentration of similar order (1.4mM). The concentrations of phenol (10-100μM) subsequently used in experiments were non-toxic to *P.communis*, but were generally in considerable excess of the environmental pollution levels of phenol surveyed by the Water Research Centre (1980). In general, phenol toxicity decreases with increasing hydroxylation of the aromatic nucleus (Reynolds, 1978). Thus protocatechuic and caffeic acids are markedly less toxic than *p*-hydroxybenzoic or *p*-coumaric acids; similarly, quinol is less toxic than phenol. Reynolds (1978) noted, however, that the *o*-diphenol, catechol, was more toxic than expected in lettuce-seed germination tests. This unexpected toxicity of the *o*-hydroxy substitution in some compounds was also noted by Glass and Dunlop (1974) and Glass (1974; 1975) in root cell permeability studies with salicylic (*o*-hydroxybenzoic) acid.

The toxicity of phenolic compounds is also affected by pH; phenol is a weak acid (pK_a 10, Dawson *et al.*, 1969) and dissociates only to a limited degree at physiological pH values. Correspondingly, significant dissociation of phenol takes place only at pH values where pH itself rather than the remaining concentration of undissociated phenol might influence plant growth. This was clearly the case for cultures of *L.minor*, where growth at pH10 was markedly restricted, even in the absence of phenol (Section 3.2.1). Experiments to assess the influences of pH on phenol toxicity are therefore hampered by the need to use media of pH values at which phenol is always predominantly undissociated. Investigations of the effects of low ratios of undissociated: dissociated phenol must thus be interpreted carefully.

Nevertheless, the present investigation showed that below its pK_a, decreasing pH raised the toxicity of phenol to *L.minor* (Section 3.2.1),

implying that phenol exerted a major part of its toxic effects on cell membranes, or gained more ready access to the tissues in undissociated form. Without investigation of some membrane-dependent process (ion influx or efflux, or trans-membrane potential difference, for example), toxicity of phenol cannot be ascribed unequivocally to membrane effects, but many examples (Glass, 1973; 1974; 1975; 1976; Glass and Dunlop, 1974) make it clear that such influences of aromatic substances can be expected. Glass and Bohm (1971) confirmed in barley roots that quinol was more readily taken up at lower pH. In general, the toxicity and permeability of organic solutes are related to pH and hence the degree of dissociation (Simon and Beevers, 1952; Lee, 1977), and to the lipid solubility of the molecule, (Lee 1977; Reynolds,1978). In the case of phenolic compounds, which are often strongly lipophilic, Reynolds (1978) observed that while inhibition of seed germination was broadly related to lipophilicity, no relationship between pK_a and inhibition could be deduced, and Glass and Dunlop (1974) found that the depolarization of the trans-membrane potential difference in barley roots was similarly related to the lipid solubility of the benzoic acids used. Both lipophilicity and pK_a correlated well with the phosphate uptake inhibition caused by benzoic acids in barley root tissues (Glass, 1975).

When supplied with phenol at pH10 *L.minor* showed a pronounced increase in growth rate. At this pH, phenol is 50% dissociated, and the potential toxic effects of the undissociated molecule might be sufficiently reduced to show a stimulatory influence of the phenol anion. Seidel (1965) has shown that phenol at concentration of 50mg/1 (about 0.5mM) and greater, at unspecified pH in culture media, increased the fresh weight of *Schoenoplectus lacustris* specimens, although the physiological and biochemical reasons for this are far

from clear, and were not explained by the author. Fresh weight data such as these may be equated with *L.minor* culture results, as leaf counts correspond well with weight measurements in this species.

Loss of phenol from plant cultures.

The simple, non-sterile, hydroponic culture experiments of Section 3.3.1 showed that phenol was removed from the media bathing the roots of twelve species of plants, of which only two had been examined before by other workers. *Eichhornia crassipes* was shown by Wolverton (1975) and Wolverton and McKown (1976) to remove phenol from its culture medium, and *Alisma plantago-aquatica* was examined for its response to phenol by Seidel (1963). In common with several other species, the latter was sensitive to phenol (10-20mg/1), but Seidel (1971) subsequently found that *A.plantago-aquatica* grew vigorously in polluted waters and absorbed phenol from them. The present results for both these species failed to confirm their phenol-removing capability. In general, such non-sterile culture experiments served to demonstrate the difficulties encountered in attributing phenol loss to plants.

That plant specimens themselves contributed to the phenol losses observed was, however, confirmed by subsequent experiments using sterile seedlings of *P.communis*. Although some of these cultures (Section 3.3.2) eventually lost sterility, those which remained sterile continued to lose phenol. The rate of phenol loss in cultures of younger plants was more rapid, but whether this was the result of more vigorous vegetative growth (Seidel, 1965), or of the greater surface/volume ratio of younger (smaller) roots was not pursued.

In axenic cultures of *L.minor*, there was no loss of phenol from the medium in excess of that required to equilibrate with the tissues of the plant inoculum (Section 3.3.3). This response was very different

from that of rooted aquatic plants, including sterile seedlings and mature plants (Section 3.3.4) of *P.communis*. When tissues of such plants were supplied with phenol, uptake markedly in excess of tissue equilibrium was almost always observed (Section 4.1).

The apparent lack of phenol-detoxicating activity in *L.minor* has also been shown by Pridham (1964), who found that this species, among several other aquatic plants, was unable to glucosylate phenols - a reaction that certainly was important in *P.communis* (Section 4.2) and many other species in Pridham's (1964) study. As *L.minor* seemed unable to reduce phenol concentrations in its medium, no further work with the species was attempted.

Experiments of Section 3.3.4 showed unequivocally that [^{14}C]phenol was rapidly lost from plant cultures, and that the presence of mature (12-week-old) *P.communis* specimens resulted in more rapid and extensive decreases in phenol concentration than occurred in (plant-free) control cultures. Sampling the plant tissues for radioactivity confirmed that a major part of the radioactivity lost from the media was indeed present in the plants, and that after 120h incubation, such radioactivity was not present as [^{14}C]phenol or any of its putative, simple, metabolic products at concentrations permitting identification.

The lag phase of phenol uptake from *P.communis* cultures was attributed to an induced process which was not evident when the plants were immediately re-exposed to fresh phenol-containing media. As the plants were washed with sterile water before re-exposure, a process which would reduce the microbial population, subsequent re-exposure to phenol should, if the inducible phenomenon was microbial, have shown similar induction kinetics as the residual population grew, at the expense of the phenol, but no such phenomenon was observed. The lag phase in plant-free cultures of 1, 5, 10 and 50µM phenol

concentration was much longer than that in plant cultures of the same phenol concentrations, suggesting that an inducible process in the plant was more rapid to respond to phenol than the exclusively microbial population of plant-free cultures. An inducible component in the processes responsible for phenol loss was also observed in experiments with sterile *P.communis* seedlings, where a lag phase in phenol loss was observed in most sterile cultures, under conditions where microbial growth does not complicate interpretation. The rate of loss of [^{14}C]phenol in cultures previously exposed to phenol was markedly greater at 50 and 100μM initial phenol concentrations than in similar, once-exposed cultures, another property expected of an inducible system.

As a consequence of the variable degree of microbial contamination of plant cultures (Table 3.4) it was difficult to correct their phenol losses for microbial influences. It may be more useful to regard such non-sterile systems, which are similar to those in Nature, as a single "mixed" culture, in which the plant was demonstrably a significant phenol-removing component. Plant cultures often contained many fewer microorganisms than (no-plant) control cultures, and in view of the large proportion of radioactive material found within the plant, significant microbial metabolism of phenol, at least over the initial, rapid phase of phenol loss, can largely be discounted.

Whether corrected for losses in control cultures or not, the rate of phenol loss from cultures was greater at higher initial phenol concentration than at low concentration; the rate of loss was maximal at 50μM initial phenol concentration. This compares with the saturation of quinol uptake by barley root tissues above 5-10mM, with an apparent K_m of 1.7mM (Glass and Bohm, 1971). Uptake of this substance by *Salvinia* was not saturated at 50mM but was probably non-

metabolic in both cases, the apparent K_m being that of an internal detoxicating process. The study of the time-course of quinol uptake by Glass and Bohm (1971), which showed characters consistent with rapid free-space permeation followed by slower non-metabolic intracellular distribution, confirmed this. The very much longer time-course of phenol uptake by *P.communis* cannot properly be equated with these tissue permeation studies, and the relationship of loss rate with concentration cannot be defined in such rigorous kinetic terms.

In control (no-plant) cultures however, the rate of loss of phenol was quite strictly related on a linear basis to its initial concentration, corresponding with the effect of limiting nutrient concentration on the growth of microbial populations (Stanier *et al.*, 1970).

The progress of phenol loss from cultures of *P.communis* did show some similarity with the results of Wolverton and McKown (1976) for *E.crassipes*. Whereas in (plant-free) control cultures phenol loss was slow and often showed a prolonged lag, in plant cultures the phenol loss was immediate and very fast, or showed a sigmoid time-course with a poorly defined lag. Specimens of *E.crassipes* of dry weight 2.75g absorbed phenol completely from cultures of initial phenol concentration of 25, 50 and 100ppm (about 0.27-1.1mM) in 48-72h. The maximum amount of phenol absorbed in the experimental period (72h) was 36mg/g dry weight of plant material (380µmol/g), which greatly exceeds the amount absorbed by *P.communis* specimens or lost from their media - about 5.8µmol/g fresh weight at the end of the 120h experimental period at 100µM initial concentration (estimated from the radioactivity losses and recoveries measured in these experiments; Section 3.3.4). The phenol uptake of *S.lacustris* specimens (Seidel, 1963; 1965; 1966) is difficult to calculate in terms similar to these, but plants of 300g fresh weight removed 35g of phenol in 3 years (Seidel, 1966) when the

phenol was regularly replenished; an uptake rate of about 33µg phenol/g fresh weight/h (8.4µmol/g fresh weight/day) can be estimated for this species from other results of Seidel (1963). This rate of phenol removal could be sustained when phenol was replenished. A comparable maximum rate of phenol loss for cultures of *P.communis* specimens initially containing 100µM of phenol (Section 3.3.4) was 16.19µg/g fresh weight/h (4.13µmol/g fresh weight/day). *P.communis* was capable of less rapid phenol removal than *S.lacustris* on this basis, although the rate for the former species was enhanced on its second exposure to phenol. Seidel (1965; 1966) explored some aspects of the speed of phenol loss from *S.lacustris* cultures and plantations, observing that loss was more rapid, in field conditions, in the aerial growth season, but always showed a lag, even when phenol was repeatedly re-supplied; the lag shortened after repeated exposures, and was shorter when lower phenol concentrations were used. The persistence of the lag was attributed to a reinduction process required every time phenol concentration was reduced to zero, but as neither field nor culture experiments with *S.lacustris* accounted for the contribution of the microflora to phenol loss, the attribution to *S.lacustris* of a highly responsive inducible phenol degradation system is impossible to substantiate.

Autoradiography of *P.communis* specimens supplied with [^{14}C]phenol (Section 3.3.7) clearly showed that radioactive material had entered the plant within 4h, during which period extensive microbial metabolism of phenol was unlikely. Similarly, in experiments using excised tissue pieces (Sections 4.1 and 4.2), radioactive material was detected within the tissues after short exposure times, in the presence of demonstrably small microbial populations. Using chromatographic methods after rapid solvent extraction, [^{14}C]phenol was recoverable from excised *P.communis* tissues after short treatment periods,

confirming that phenol entered the plant tissues readily and persisted as such for at least 5h. Of the [^{14}C]phenol applied to *P.communis* tissue pieces (Section 4.2), 0-21% of that absorbed was recovered unchanged. After 120h incubation, phenol could not be reisolated from whole plants (Section 3.3.5), probably as the result of complete conversion of phenol to other substances. The varying absorption of phenol by the three tissue types used in experiments of Section 4.2 probably reflected differing permeability and metabolic activities of the tissues; *leaf tissue* with its readily permeable mesophyll is likely to be perfused readily with medium bearing [^{14}C]phenol, allowing the substantial phenol uptake observed, whereas in *rhizome slices* containing many open vessels, the low recovery of [^{14}C]phenol was unexpected. Structural and morphological differences alone, however, are unlikely to account entirely for the results obtained, as rhizome tissue contained substantial amounts of ^{14}C-labelled products derived from phenol, indicating phenol penetration and rapid conversion to other products.

Distribution of phenol absorbed by plant tissues and whole plants.

When specimens of *P.communis*, supplied with [^{14}C]phenol *via* the roots and rhizome, were analysed (Section 3.3.5), radioactive material was found throughout the plants, and autoradiography of similar specimens (Section 3.3.7) showed that the radioactive materials had penetrated the uppermost leaf tips of the plants within 18h. Phenol or its metabolic products were therefore as mobile within *P.communis* as was observed in *S.lacustris* (Seidel, 1963; 1966). Specimens of the latter secreted phenol at the cut ends of shoots; after 3 days' treatment *via* the roots and rhizomes, 21.7, 58.2, and 18.8% of the phenol recovered was found in the stems, rhizome, and roots respectively (Seidel, 1963). Although [^{14}C]phenol applied to

the basal meristem and rhizome of *S.lacustris* plants, by, respectively, injection or infusion through a tube, was metabolised to an unidentified compound (Seidel *et al.*, 1967), and radioactive materials were mobile within the stem, Seidel and Kickuth (1965) isolated volatilised phenol in the air passing over the aerial parts of specimens fed phenol at their roots and rhizomes, confirming that phenol itself was readily mobile in this species.

Naturally-occurring phenols such as caffeic and ferulic acids, and catechol, quinol, resorcinol and phloroglucinol are mobile basipetally in plants. The rate of movement of the last four down the stem of large (30cm high) *Vicia faba* plants was about 50-60cm/h, and resorcinol applied to the main vein of an apical leaf, was distributed throughout the plant within 30min. Movement of the phenolic acids, however, was less rapid (MacLeod and Pridham, 1966). Arbutin, *m*-hydroxyphenyl-β-*D*-glucoside, aesculin and salicin were very rapidly translocated, showing clearly that conjugated phenols are also very readily mobile in whole plants. Transport of unconjugated phenols in *V.faba*, and of endogenous phenolic compounds in *V.faba* and *Salix daphnoides* was confirmed by aphid-stylet methods and by solvent extraction (MacLeod and Pridham, 1966).

There is clearly an important functional difference between acropetal and basipetal transport of solutes in plants, and free phenolic compounds can be shown to move in both fashions. Basipetal transport of free and conjugated phenols is a clear indication of phloem or symplastic transport, where the solutes move *via* the interconnected protoplasts of parenchyma cells and are transported over greater distances in the continuous channel provided by the phloem. Movement of phenols by this means is in marked contrast with conclusions of Ashton and Crafts (1973), that phenolic herbicides are not symplastically transported, but the less toxic materials

considered by MacLeod and Pridham (1966) may exhibit more typical behaviour.

Both apoplastic and symplastic transport processes may be responsible for acropetal transport of solutes, though materials subject to apoplastic transport were originally thought to be unable to penetrate the plasmalemma of cells. However, more recent investigations have shown that some compounds (notably pesticides), generally regarded as apoplastic, can penetrate plant cell membranes (Peterson and Edgington, 1976). Crisp (1972) suggested that weak acids may be loaded into the symplasm in undissociated form, and transported in dissociated form in the phloem (the "weak acid hypothesis"), and Tyree *et al.* (1979) observed that some chemicals are "ambimobile" in plants, penetrating the symplasm by virtue of an ability to pass the plasmalemma, but able to leave the symplasm and enter the apoplast as a result of the same permeability.

Phenol, at physiological pH, is predominantly undissociated, and was shown to penetrate the cells of *P.communis* tissues by its reisolation from washed samples and by isolation of radioactive products from the tissues (Section 4.2). Results of autoradiography of *P.communis* plants (Section 3.3.7), and of experiments with *S.lacustris* (Seidel, 1963; 1966) showed that phenol, or radioactive products derived from it, was rapidly mobile acropetally. Basipetal movement of free phenols, on the other hand, was shown by MacLeod and Pridham (1966), so it seems likely that both free [^{14}C]phenol and its products were transported in *P.communis*, though the mechanism of transport cannot be deduced.

Distribution of absorbed [^{14}C]phenol between ethanol-soluble and insoluble fractions of plant tissues.

The radioactivity recovered from plant tissues after their exposure to [^{14}C]phenol was distributed between two major fractions. Part of the material was recoverable with boiling aqueous ethanol, and another, generally larger, fraction was ethanol-insoluble, especially in plants and plant tissue sections treated for 24h or longer with phenol (Sections 3.3.5, 3.3.6, and 4.1). This simple initial fractionation may usefully distinguish between two major modes of metabolism of the applied phenol;

(i) it may be converted to soluble products or

(ii) bound to or converted to ethanol-insoluble (polymeric) materials.

The extractant, aqueous ethanol, is a good solvent of phenolic and many other low-molecular weight plant constituents, and leaves most proteins and other polymers in the insoluble residue (Harborne, 1973).

The major tissue parts of *P.communis* specimens treated *via* the roots and rhizomes with [^{14}C]phenol, and most of the excised tissue parts of plants bathed in phenol media showed differing distributions of recovered radioactivity between ethanol-soluble and-insoluble fractions. In whole plant experiments (Section 3.3.5) the roots contained both the greater part of absorbed radioactivity, and a larger proportion of insoluble radioactivity. Tissues more distant from the site of application of phenol retained less total radioactivity, and a lesser fraction of insoluble material. This distribution is essentially similar to that found in terrestrial plant specimens and tissue samples when treated with radioactive aromatic compounds (Taylor, 1968; Barnes and Friend, 1975). In intact wheat (*Triticum aestivum*) plants fed [^{14}C]phenylalanine, the exposed roots retained

most (81%) of the recovered radioactivity, and parts of plants progressively more distant from the roots contained lesser amounts; cut plants responded in a similar manner (Barnes and Friend, 1975). Chlorogenic acid was strongly bound in *Xanthium pensylvanicum* leaves, restricting its mobility in these tissue samples (Taylor, 1968).

In whole *P.communis* plants, progressive metabolism of applied [^{14}C]phenol may occur in three fashions:

(i) If phenol was subject to reactions producing ethanol-insoluble material in the organs exposed or was bound to cell constituents, less would be available for transport to other tissues:

(ii) Phenol may be converted at, or near, the site of entry to the plant into soluble derivatives less likely to be converted to immobile materials:

(iii) Both processes may operate.

In view of the results for these and subsequent experiments, in which substantial conversion of phenol to its glucoside and, to a lesser extent, to catechol, was observed, the third mechanism seems likely.

Experiments of Sections 4.1 and 4.2, with tissue pieces of several species, showed that tissues and plant species differed widely in their response to exogenous [^{14}C]phenol. Differences in the relative fractions of soluble and insoluble radioactive material were indeed the result of biochemical and permeability properties of the tissues. Leaf tissue of *P.communis*, for example, when treated with [^{14}C]phenol under conditions where the response could not be modified by the passage of phenol through other tissues still rendered a lesser proportion of the applied label immobile than did root or rhizome tissues. This property of normally subterranean or submerged plant parts was also shown clearly in *T.latifolia* and *M.aquatica* tissues.

Most of these tissues, and particularly those of *P.communis*, contrast markedly with *S.lacustris*, examined by Kickuth (1970). This species retained comparatively little label when supplied with [^{14}C]phenol for 16h; of that absorbed by the roots, 90% of applied radioactivity was entirely lost, and was unaccounted for, and of the remainder, 8.1% was found in phenol and its metabolites. This species rendered little phenol insoluble, but phenol-exposed plants gained fresh weight (Seidel, 1965), though whether the weight gain was the result of a growth-stimulating property of phenol, or whether phenol was assimilated into polymeric material *via* the products isolated or proposed by Kickuth (1970) is not clear. In the only other species tested with [^{14}C]phenol in similar, whole plant, experiments, Wolverton (1975), and Wolverton and McKown (1976) found no phenol or any other identified product in *E.crassipes*, and concluded that phenol applied to this species was rapidly metabolised and lost. Little assessment of microbiological contributions to either *S.lacustris* or *E.crassipes* phenol removal processes were made, so these radioactivity accounts and degradation proposals should be treated with reserve.

The tissues of all plant species in the present study formed ethanol-insoluble material from a large proportion of the phenol supplied to them, and in the absence (Section 4.1) of evidence for the large losses of phenol to CO_2 claimed for *S.lacustris* and other species, the immobilising reactions are of importance.

Reactions immobilizing phenol in plant tissues.

Reactions leading to the immobilization of phenol, in the absence of demonstrable ring-fission activity, must be those of the whole molecule, and can be either physical or chemical.

Phenol and the cresols bind to proteins (Van Sumere *et al.*, 1975),

the methyl-substituted compounds more strongly. This binding activity has suggested a mode of action for phenolic disinfectants (Van Sumere *et al.*, 1975). Strong binding of complex and (often) naturally-occurring phenols results in protein "tanning", for which various explanations are possible. The weakest bond between phenols and proteins, and probably that binding phenol itself, is the hydrogen bond. Weak ionic bonds between phenols and cationic groups of protein molecules probably account for a proportion of tanning activity. Hydrogen bonding and ionic bonding are both reversible, but covalent binding, after oxidation of phenols, is not, and results in irreversible tanning (Swain, 1965; Loomis and Battaile, 1966; Shuttleworth *et al.*, 1968; Loomis, 1969). Shuttleworth *et al.* (1968) concluded that tanning was a solvent-reversible cross-linking process in which hydrogen bonding was the major mechanism. Classical tannins are usually considered to be of high molecular weight (Swain, 1965; 1979), hence phenol, which cannot cross-link protein, but which may bind it, cannot be considered as such. Nevertheless, the ability of phenol to bind proteins may provide temporary immobilization of the compound in plant tissues.

After oxidation to catechol (Sections 4.2 and 5.2.2) phenol may be immobilized more readily. The immediate product of catechol oxidation by catechol oxidase is the corresponding *o*-quinone, a highly reactive molecule (Pugh and Raper, 1927; Mason, 1949; 1955; Waite, 1976). The *o*-quinones of catechol and substituted catechols can interact strongly with proteins (Mason, 1955; Pierpoint, 1970: Van Sumere *et al.*, 1975). Pierpoint (1970) suggested likely reactions between *o*-quinones and proteins, and further suggested ways in which low molecular-weight phenols might be polymerized to form a polyphenol complex with tanning properties. Some specific products of quinone interactions with aminoacids - the red-pigmented aminoquinones, were found by Jackson and

Kendal (1949).

In addition to their complex reactionswith protein, quinones undergo nonenzymic polymerization reactions to form melanins. Both carbon-carbon and carbon-oxygen linkages are produced when melanins form, yielding complex and often indeterminate polymers (Nicolaus *et al.*, 1964; Nicolaus and Piattelli, 1965; Pridham and Woodhead, 1977), which are very resistant to degradation, solution, and analysis. The simplest forms, catechol melanins, are probably formed by successive condensations of *o*-benzoquinone monomers (Mason, 1949; 1955; 1959). Forsyth and Quesnel (1957) and Forsyth *et al.* (1960) found that dimeric products were formed from catechol in the presence of mushroom polyphenol oxidase, and one of these, diphenylene dioxide-2,3-quinone, was produced by enzymes from spinach leaves and *Tecoma stans* (Nair and Vining, 1964; Kandaswami and Vaidyanathan, 1973a; 1973b). The *T.stans* enzyme was not a typical polyphenol oxidase, forming only this product, nor was another enzyme from this species, which catalyzed the formation of another dimer, 3,4,3'4'-tetrahydroxydiphenyl (Kandaswami *et al.*, 1969; Kandaswami and Vaidyanathan, 1972), but the formation of such compounds usefully illustrates the formation of covalently linked materials which may be models for the formation of more complex polymers.

The reactions rendering insoluble the large proportions of [^{14}C]phenol applied to plant tissues therefore probably proceeded from the formation of catechol, which was detected in some of the plant extracts supplied with phenol, and accounted for the radioactivity recovered from *P.communis* tissues so treated (Section 4.2).

That plant tissues possessed an activity against catechol was initially shown in simple spectrophotometric experiments (Section 5.2.1). When supplied with catechol, extracts of *P.communis* tissues catalyzed

the formation of coloured substances with absorption maxima in the red-brown part of the visible spectrum, that is in the ranges 480-495 and 465-490nm, and at 340 and 500nm. These results do not compare with those of Waite (1976) or Mason (1949), who found that the absorption maximum of *o*-benzoquinone was at 390nm at pH5.4-6.8. Shorter incubations of reaction mixtures like those of Section 5.2.1, monitored at 390nm, also failed to show that *o*-benzoquinone was produced transiently in detectable concentrations. Spectrophotometric assays of polyphenol oxidases with catechol as the substrate have been performed at 390nm at pH7.0 (Duckworth and Coleman, 1970), and at 480nm at pH5.98 (Baruah and Swain, 1953), and often at intermediate wavelengths of 400-430nm (Wong *et al.*, 1971; Lanzarini *et al.*, 1972; Benjamin and Montgomery, 1973; Taneja and Sachar, 1974; Kahn, 1976). Such a variation suggests that the immediate product of catechol oxidation cannot always be found, but the absorbance increases found at 465-495nm in *P.communis* extracts are suggestive of those at 480nm with potato polyphenol oxidase found by Baruah and Swain (1953). Reddish-brown colours and appropriate absorbances are characteristic of melanins (Pridham and Woodhead, 1977), and the coloured products of quinones and cell constituents (Jackson and Kendal, 1949)

The action of *P.communis* extracts against quinol likewise produced reddish-brown materials, which did not have clear absorption maxima characteristic of *o*-benzoquinone. This substance has absorption maxima (in water) of 250, 303, and 424nm (International Critical Tables, 1929) The similarity of the maxima obtained with *P.communis* extracts with catechol and quinol suggests that complex products were formed which could not be analysed spectroscopically, perhaps by cooxidation with endogenous tissue components (Mayer and Harel, 1979).

Having shown that plant extracts catalyzed changes in diphenolic substrates, some characteristics of phenol oxidases were apparent in the simple spectrophotometric assays and the subsequent screening and kinetic experiments (Sections 5.2.1, 5.2.3, 5.2.4).

Phenolase activities in plant tissue extracts

The use of the oxygen electrode for assays of phenol-oxidising enzymes is rapid and convenient, and avoids the confusion arising in spectrophotometric assays from further reactions of phenolic oxidation products. For kinetic investigations Mayer and Harel, (1979) and Bull and Carter (1973) favoured this method, but it should be emphasised that when assaying activity against monophenols, the O_2 consumption observed is the result of both monophenol hydroxylation and diphenol oxidation. As a screening assay though, and when used to investigate the diphenol oxidase activity of *M.aquatica*, the method served to show several properties of plant extracts which were worthy of further investigation.

Extracts of *P.communis* showed a restricted range of activity with both monophenols and diphenols, contrasting with the other species examined. The low specific activity of diphenol-stimulated O_2 consumption in extracts of this species showed that further investigation of diphenol oxidase would be of little value, but helped to confirm the tentative results of Section 5.2.1, where some activity against catechol was observed spectrophotometrically. *P.communis* lacked activity against quinol in these experiments (Section 5.2.3), conflicting with earlier results (Section 5.2.1), which had indicated that laccase might be present. Genuine laccase activity is rare in higher plants, though common in fungi; such apparent laccase activity might result from the coupled oxidation by *o*-diphenol oxidase of *p*-diphenols in the presence of endogenous *o*-diphenols (Mayer and Harel, 1979). As the rapid oxidation of *p*-diphenols is generally regarded as diagnostic of

the presence of laccase (Mayer and Harel, 1979) it may be concluded that this enzyme was lacking in *P.communis*. Other species showed considerably greater *p*-diphenol-stimulated activity than *P.communis*, indicating either that laccase might be present, or that coupled oxidation was more apparent.

M.aquatica extracts showed substantial activity in the presence of *o*-diphenols (Section 5.2.3), and the properties of this activity were characteristic of *o*-diphenol oxidases (Section 5.2.4). The substrate specificity of the extracts was broad, a feature of reports of polyphenol oxidases in plants and fungi (Mayer and Harel, 1979). Catechol itself is comparatively rare in the Plant Kingdom, though it has been isolated from species of *Allium* (Link and Walker, 1933), *Gaultheria* (Towers *et al.*, 1966), *Salix* (Vazquez *et al.*, 1968), and *Populus* (Hubbes, 1962; Pearl and Darling, 1965a; 1965b), and may be thought an unlikely natural substrate for polyphenol oxidase for this reason. Several attempts to find "preferred" substrates for the enzyme have shown that the source of the enzyme itself accounts for some aspects of substrate specificity (Sisler and Evans, 1958; Lavollay *et al.*, 1963), and thus enzymes may be adapted in some way to endogenous substrates.

The electronic configuration of the substrate molecule is generally suggested as the property responsible for differences in activity. For both plant and fungal polyphenol oxidases, the rate of oxidation of a range of substrates generally corresponds with the electron-withdrawing or -donating character of the *p*-substituent (Mayer, 1962; Duckworth and Coleman, 1970; Lanzarini *et al.*, 1972).

With crude lettuce polyphenol oxidase, (Mayer, 1962) the order of rate of reaction was 4-methylcatechol = DOPA = caffeate>catechol> protocatechuate; Lanzarini *et al.* (1972) gave the order chlorogenic acid> 4-methylcatechol>caffeate>catechol>DOPA>protocatechuate for *Prunus avium*

enzyme. Electron-donating *p*-substituents caused greater reactivity, and electron-withdrawing *p*-substituents decreased the rate of oxidation, though steric effects and obscure substrate "preferences" may interfere with the rankings (Mayer, 1962; Lanzarini *et al.*, 1972).

M.aquatica extracts showed the property, shared with *P.communis* in survey assays (Section 5.2.3), that *p*-substituted diphenols stimulated greater activity than catechol itself. Extract of leaf of *P.communis* showed activity in the order 4-methylcatechol>chlorogenic acid>catechol, rhizome extract activities were in the order 4-methylcatechol>catechol>chlorogenic acid, and in root extract the order was chlorogenic acid>4-methylcatechol>catechol. Activities in the two tissue parts of *M.aquatica* in the survey assays showed that the same three substrates gave most activity, though the order again varied, and, generally, activity with catechol exceeded that with acidic diphenols such as protocatechuate and caffeate, but was less than that caused by other diphenols. The results of Table 5.10 confirmed the ranking of activity in *M.aquatica* extracts.

The assays of extracts of *M.aquatica* failed to show the maximum velocity of reaction expected (Table 5.10), a property shared with *P.communis* extract tested with catechol (Section 5.2.1). Inhibition by catechol has been observed in polyphenol oxidase from several sources; in preparations from mushrooms (Smith and Krueger, 1963), in *Neurospora crassa* (Fling *et al.*, 1963), lettuce seeds (Mayer, 1962), banana (*Musa cavendishii*) (Padron *et al.*, 1975), and potato tubers (Alberghina, 1964). This effect, often more prominent with catechol as the substrate, is due to interaction of the product with the enzyme protein (Ingraham *et al.*, 1952; Ingraham, 1955; Fling *et al.*, 1963; Wood and Ingraham, 1965; Kahn and Pomerantz, 1980).

The Michaelis constants calculated for *M.aquatica* (Table 5.10) corresponded with those for olive fruit preparations (Ben-Shalom *et al.*,

1977) where K_m values for 4-methylcatechol, chlorogenic acid, and catechol were ranked in the same order, having values of, respectively, 3.3, 5.0 and 8.3mM. The K_m of many plant catechol oxidases is generally of the order of 1mM (Mayer and Harel, 1979), and the results for *M.aquatica* are in broad agreement with this. Very high K_m values for catechol (46-53mM) were obtained by Benjamin and Montgomery (1973) in cherry fruit preparations. Microbial enzymes, in contrast, have generally lower values (Mayer and Harel, 1979), although the tyrosinase of *Streptomyces glaucescens* seems to be an exception, with K_m for catechol, chlorogenic acid, and caffeic acid of 4.51, 0.75, and 0.37mM respectively (Lerch and Ettlinger, 1972).

Hence the assays for phenolase in plant tissues showed that such activity was generally present, and in some instances showed the expected characters. These activities, if indeed they were responsible for the eventual immobilization of phenol, would however require catechol as the substrate, so the production of this compound was investigated in preference to further examination of catechol oxidation products and catechol-oxidising enzymes.

Reactions producing mobile metabolites of phenol

Three mobile products of phenol were recovered from aquatic plants. Catechol, phenyl-β-*D*-glucoside, and CO_2 were products of the metabolism of phenol in incubations of tissues or cultures of *P.communis* and incubations of preparations of some other species yielded diphenols when supplied with monophenols.

Production of $^{14}CO_2$

The results which showed production of $^{14}CO_2$ from [^{14}C]phenol in whole plant cultures of *P.communis* (Section 3.3.6) were equivocal. The medium bathing the roots was contaminated with a population

of microorganisms, but only a small fraction of the applied radioactivity (0.82%) was recovered on a 0.22μm filter. Although the material so recovered was not weighed or scored for microorganisms this result suggests that phenol was oxidised to CO_2 rather than cell mass, or that the microorganisms responsible remained attached to the plant roots and rhizome. The plant specimen absorbed and immobilised a substantial fraction of the applied phenol, but diverted none of this to respired $^{14}CO_2$ in the leaves and stem. Were the plant itself to biodegrade phenol, such respiratory loss was regarded as inevitable, and its absence was a good indication that the aerial parts of *P.communis* were incapable of the ring-fission of phenol. This was confirmed by the absence of product spectra of catechol ring-fission products in spectrophotometric experiments using root, rhizome, and leaf tissue extracts (Section 5.2.1), and the absence of significant amounts of $^{14}CO_2$ in tissue-piece incubation experiments with this or any other plant species (Section 4.1).

Had the microorganisms isolated from plant tissues (Section 4.3) or observed attached to them (Section 4.3) resisted washing in sterile water or antibiotic treatment, and been responsible for phenol degradation, their activity was expected in tissue incubations (Section 4.2) and extract experiments (Section 5.2.1). As certain of the fungi isolated from plant roots and rhizome were capable of growth on phenol media, and were among those shown by Cain *et al.* (1968) to degrade aromatic compounds, these were clearly insignificant influences in such experiments. The predominant phenol-degrading bacterial contaminant isolated from plants, plant cultures and die-away experiments (Sections 3.1, 3.3.1, 3.3.2, 3.3.4, 4.1 and 4.3) did not interfere in tissue or extract experiments either; had significant numbers of any organism been present, much [^{14}C]phenol should have been converted to $^{14}CO_2$ *via* ring-fission reactions.

While insignificant in tissue-piece and extract experiments, microbial contaminants of the plant specimens were present in large numbers in culture media, and were isolated readily from tissues and tissue surfaces. The major bacterial contaminant, a putative *Pseudomonas* species, and the fungal isolates, (species of *Aspergillus*, *Mucor*, *Penicillium*, *Rhizopus*, *Cladosporium*, *Cephalosporium*, *Trichoderma*, *Pullularia* (*Aureobasidium*), *Rhodotorula* or *Sporobolomyces* and a Basidiomycete) were representative of organisms associated with plant surfaces and plant litter (Burges, 1965; Katznelson, 1965; Apinis *et al.*, 1972; Willoughby, 1974; Blakeman and Brodie, 1976). Of these the Pseudomonad was shown to grow on phenol medium, and of the fungi, species of *Aspergillus*, *Mucor*, *Penicillium*, *Cephalosporium*, *Aureobasidium*, *Rhodotorula*, and *Sporobolomyces* all showed evidence of aromatic catabolism in the studies of Cain *et al.* (1968).

The presence of any of these organisms, or a mixed culture of them, can account for degradation of phenol in the root zone of plant specimens (Section 3.3.6).

Formation of phenyl-β-D-glucoside.

The experiments of Section 4.2 yielded sufficient phenyl-β-*D*-glucoside for conclusive identification. All three major tissue parts of *P.communis* were capable of glucosylation. Although the enzymes responsible for the production of this compound could not be detected (Section 5.1.1), the demonstration of activity in intact tissues confirmed that *P.communis*, in common with the plant species examined by other workers (Nystrom *et al.*, 1959; Yamaha and Cardini, 1960a; Pridham and Saltmarsh, 1963; Pridham, 1964; and reviews by Hopkinson, 1969; Miller, 1973) was capable of the glucosylation of a toxic, exogenous phenolic compound. Phenol itself was not a substrate of the comparatively well-defined wheat-germ glucosylating enzymes (Yamaha and

Cardini, 1960a), nor was it glucosylated in broad beans (Pridham, 1958), suggesting that the compound was an unsuitable substrate for enzymes glucosylating naturally occuring phenols, or that it was sufficiently toxic to damage plant materials before detoxication processes could operate. The report by Nystrom *et al.* (1959) that phenol itself was glucosylated in wheat and barley leaves is an exception to this otherwise negative picture.

The plant species which degraded phenol to CO_2 or other aliphatic compounds [*E.crassipes* (Wolverton and McKown, 1976), tea and grapevine (Durmishidze *et al.*, 1969), the algae examined by Ellis (1977), and *S.lacustris* (Kickuth, 1970)] or those which are presumed to degrade benzene *via* phenol and catechol (Durmishidze and Ugrekhelidze, 1969; Jansen and Olson, 1969), were not exhaustively examined for *conjugated* products of exogenous phenolic or aromatic compounds so the coexistence of these two complementary pathways of aromatic detoxication in whole plants is not well established. In plant cell cultures, however, Ellis (1971) demonstrated both the ring-fission and glucosylation of catechol; hence detoxication by degradation and conjugation may not be mutually exclusive.

Production of catechol.

A small amount of a substance corresponding on chromatography with catechol appeared in the root medium of *P.communis* specimens (Section 3.3.6) supplied with phenol. Although its further identification was not pursued in this experiment, the formation of catechol was considered one of the most likely initial reactions of phenol detoxication, in plants or in microorganisms (Section 1.).

Further and more substantial evidence for the formation of catechol was obtained when plant tissue extracts were incubated with phenol (Section 5.2.2). Sufficient quantities of catechol for

chromatographic and spectroscopic identification were isolated from *M.aquatica* extract incubations, and *P.communis* extracts also yielded catechol, identified by chromatography. Excised tissues of *P.communis* all yielded sufficient [^{14}C]catechol after incubation with phenol and subsequent ethanol extraction to allow its recrystallization to constant specific radioactivity (Section 4.2). The formation of catechol, and the presence in most tissues of phenolases, suggests that phenol could be immobilised by conversion to catechol and subsequent polymerization or binding with other cell constituents. It is of interest in this respect that root and rhizome tissues, which contained greater fractions of immobilized radioactive material when fed [^{14}C] phenol than leaves (Sections 3.3.5, 4.2), also converted more of the absorbed phenol to catechol (Section 4.2).

The response of *S.lacustris*, which contained very little phenol-derived carbon in insoluble form (Kickuth, 1970) seems atypical in this respect. All the species examined in the present study formed insoluble phenol-derived materials (Section 4.1), and immobilization of phenol therefore seems to be a more general detoxication response than the proposed (Kickuth, 1970) ring-fission systems of *S.lacustris*

The demonstration of phenol hydroxylation in higher plant tissues appears to be novel. *p*-Cresol but not phenol was a substrate of polyphenol oxidase from apple (Harel *et al.*, 1964), but phenol is rarely even tested as a potential substrate of plant enzymes. Reports of the hydroxylation of substituted monophenols are however very common (Mayer and Harel, 1979). Microorganisms in contrast certainly are capable of phenol hydroxylation (Evans *et al.*, 1951; Seidman *et al.*, 1969; Ribbons, 1970; Chapman, 1972; Bayly and Wigmore, 1973; Neujahr and Gaal, 1973; Buswell, 1975; Gaal and Neujahr, 1979) and sometimes polyphenol oxidases rather than flavoprotein hydroxylases are responsible (Nakagawa and Takeda, 1962; Jayasankar and Bhat, 1966; Nambudiri *et al.*, 1972)

In order to find the enzymes responsible for catechol formation in *P.communis*, the screening assays of plant extracts (Section 5.2.3) were first considered. These showed clearly that extracts of *P.communis* tissues lacked O_2-consuming activity in the presence of phenol, alone or with ascorbate, and therefore that these extracts did not contain classical cresolase. Although some plant polyphenol oxidase preparations lack monophenol activity altogether (Mayer and Harel, 1979), even when they are prepared with precautions to protect the more labile hydroxylase (Kahn and Pomerantz, 1980; Vaughan and Butt, 1969b), ascorbate is a cofactor for several well-defined hydroxylating enzymes (Vaughan and Butt, 1969a; 1969b; 1972; Stafford and Dresler, 1972; Nambudiri *et al.*, 1972; and review by Mayer and Harel, 1979). Screening assays showed in fact that only extracts of *M.aquatica* leaf tissue had an O_2-consuming activity stimulated by phenol alone. Nevertheless, the experiments of Section 5.2.2 where diphenolic products of phenol, *p*-cresol, and *p*-coumarate were found, and of Section 4.2 where [^{14}C]catechol was isolated from tissues fed [^{14}C] phenol showed that phenol hydroxylation occurred, so the influence of another cofactor of phenol hydroxylation was considered.

When NADH was added with monophenols, marked stimulation of O_2 consumption was observed in most plant tissue extracts tested (Section 5.2.3). The stimulation in the presence of *p*-coumarate was particularly marked in most cases; this result, obtained using a naturally occurring phenol of central importance in plant metabolism (Neish, 1964; Pridham, 1965; Stafford, 1974; Hahlbrock and Grisebach, 1975; 1979; Gross, 1977; 1979) suggested the presence of hydroxylating enzymes similar to those of Vaughan and colleagues (Vaughan and Butt, 1967; 1968; 1969a; 1969b; 1970; 1972; Vaughan *et al.*, 1975; McIntyre and Vaughan, 1975; Butt,1979). The spinach beet (*Beta vulgaris*) leaf enzyme used by these workers also possessed diphenolase activity against caffeate, and had monophenolase

activity in the presence of ascorbate as well as NADH. Both cofactors were effective with *p*-coumarate and *p*-cresol in extracts of *M.aquatica*, and with *p*-coumarate in some *Iris pseudacorus* and *Typha latifolia* extracts.

The cell-free extracts of *P.communis* showed some further properties which were consistent with the presence of such hydroxylating phenolases (Section 5.2.4). NADPH was a less effective electron donor than NADH in the spinach beet enzyme-catalyzed hydroxylation of *p*-coumarate (Vaughan and Butt, 1969b), and was also less effective in stimulating O_2 consumption in incubations of *P.communis* preparations with monophenols (Section 5.2.4). Caffeate did not replace ascorbate or NADH as the reductant required for hydroxylation (Vaughan and Butt, 1969b) although this compound was oxidised by, and was necessary for the function of the enzyme, abolishing, even in catalytic amounts, the lag period observed before *p*-coumarate hydroxylation in the presence of cofactors occurred (Vaughan and Butt, 1969a; 1969b; 1970; 1972; Butt, 1979). Diphenols supplied in 1µM-1mM concentration to assays of *P.communis* extract monophenol activity failed to stimulate O_2 consumption when any monophenol was present alone; when supplied at higher concentration (1-10mM), they stimulated only the rate of O_2 consumption appropriate for that diphenol. Extracts of *M.aquatica* tissues responded in the same way. The lag period observed when *p*-coumarate and NADH were supplied to assays of *P.communis* extracts was not, however, abolished by addition of diphenols.

Characterization of the monophenol-stimulated activity of *Phragmites communis* preparations.

Although resembling the spinach-beet enzyme in some respects the O_2-consuming activity of *P.communis* failed to catalyse the formation of detectable amounts of diphenols from *p*-coumarate, *p*-cresol, or

phenol (Section 5.2.4); NADH was nevertheless rapidly oxidised. The failure in subsequent experiments to show that O_2 consumption was concurrent in any way with monophenol consumption suggested that the action of the monophenols was catalytic, possibly promoting NADH oxidase activity.

The spinach beet hydroxylating enzyme, in the presence of NADH, consumes more O_2 than is needed to oxidise its phenolic substrate whether the molar ratio of substrate: reductant is 1 or 2, and yields the caffeate product from *p*-coumarate (Vaughan and Butt, 1972). Substitution of tetrahydropteridine as the reductant, however, gave the stoicheiometric O_2 uptake (Equation 1; AH_2 represents the reductant)

$$AH_2 + p\text{-coumaric acid} + O_2 \longrightarrow \text{caffeic acid} + A + H_2O \quad ....(1)$$

A similar reaction did not occur in experiments with *P.communis* preparations; the stoicheiometry of the reaction with respect to NADH and O_2 in the presence of monophenols corresponded with the formation of water and oxidation of NADH, not with monophenol hydroxylation (Equation 2)

$$2NADH + 2H^+ + O_2 \longrightarrow 2NAD^+ + 2H_2O \quad .....(2)$$

Two possibilities may account for this:

(i) certain hydroxylases are known to be uncoupled by phenols, oxidising NADH without corresponding generation of the hydroxylated product;

(ii) peroxidase has a well-established NADH oxidase activity.

Uncoupling of the oxidase and oxygenase functions is a characteristic of flavoprotein hydroxylases, and is brought about by the binding of effector molecules to the enzyme protein. Such compounds may be either *substrate effectors*, which are hydroxylated by the enzyme but which bring about some uncoupled oxidase activity or which uncouple the activity of the enzyme under certain conditions (Neujahr and Kjellen, 1978), or *nonsubstrate effectors*, which bind

with the enzyme and cause uncoupled oxidase activity without themselves being altered. Examples of the latter are the benzoate-stimulated NADH oxidase activity of salicylate hydroxylase (White-Stevens and Kamin, 1972) and 3,4-dihydroxybenzoate- and 6-hydroxynicotinate-stimulated NADH oxidase activity of *p*-hydroxybenzoate hydroxylase (Spector and Massey, 1972).

The product of O_2 reduction by the reduced nicotinamide nucleotides in uncoupled oxidation by flavoprotein hydroxylases is H_2O_2 (Spector and Massey, 1972; White-Stevens and Kamin, 1972; Neujahr and Kjellen, 1978, and further examples in the reviews of Hayaishi, 1974; Gunsalus *et al.*, 1975), the stoicheiometry of which can be strictly determined.

The phenol hydroxylase of yeasts (Neujahr and Kjellen, 1978) for which phenol itself was a substrate effector, diverted O_2 to H_2O_2 in the presence or absence of the effector.

While the stoicheiometry of the NADH oxidase activity of *P.communis* extracts was affected by both the nature of the putative phenolic effector and the concentration in which NADH was supplied, the molar ratio of NADH:O_2 did not deviate sufficiently from 2 to account for the formation of H_2O_2 rather than water; the reaction cannot therefore be equated with a classically uncoupled hydroxylase.

Peroxidase is capable of the aerobic oxidation of NADH (Akazawa and Conn, 1958; Halliwell, 1978). Low pH conditions were originally thought necessary for peroxidase-catalysed NADH oxidation (Yokota and Yamazaki, 1965; Yamazaki and Yokota, 1973), but Halliwell (1978) obtained activity at pH7 with horseradish peroxidase (HRP). Peroxidase-catalyzed activity is strongly stimulated by Mn^{2+} and phenols, alone or together, though Halliwell (1978) measured NADH oxidation in the absence of these additives.

Using purified HRP as well as impure preparations, Akazawa and Conn (1958) determined the stoicheiometry of the aerobic peroxidase-

catalysed NADH oxidase in the presence of phenols (resorcinol in this instance) and Mn^{2+}, and found that 0.5mol of O_2 was consumed per mol of NADH; this result equates with that obtained for *P.communis* extracts.

Peroxidase activity, using the characteristic guaiacol assay, was subsequently detected in extracts of *P.communis* (Section 5.2.4), suggesting that this enzyme was the probable source of NADH and O_2 consumption in assays of Section 5.2.3 and 5.2.4. Further examination of the characteristics of monophenol-stimulated NADH oxidation in *P.communis* extracts, summarised and compared with results from other experiments with commercial peroxidase and with data of other authors in Table 6.1, gave conclusive evidence that peroxidase activity had interfered with all attempts to discover the mechanisms responsible for phenol hydroxylation in aquatic plants.

As only traces of O_2-consuming activity in the presence of monophenols survived the inhibition of peroxidase-catalyzed NADH oxidation in *P.communis* tissue extracts (Section 5.2.4), further characterization of the catechol-forming activity in crude extracts is not possible.

Summary of results and conclusions.

I have shown in this study that *Phragmites communis* plants possess responses to phenol which are of some biochemical and environmental significance.

Over short growth periods, plants specimens *resisted* the toxic effects of phenol, supplied at concentrations (up to 100μM) in considerable excess of some reported levels of contamination, suggesting that this species is sufficiently robust to withstand moderate amounts of phenolic pollutants.

Table 6.1 Comparison of some characters of Peroxidase-catalyzed NADH oxidase and plant extract monophenol-stimulated activity.

The data summarised below were taken from Section 5.2.4 for *Phragmites communis* (plant extract) activity, and from (I) Akazawa and Conn (1958) and (II) Halliwell (1978). The commercial peroxidase used in the present studies and in all literature reports was from horseradish.

N.D. - Not Done

Characters of peroxidase activity	Found in: Plant extracts	Commercial Peroxidase	Literature
1. NADH oxidase activity in absence of additives	Yes, pH7.0	Yes pH7.0, and pH5.0	No, pH7.4(I) Yes, pH5.0 and pH7.0(II)
2. Stimulated by: phenols alone	Yes	No	No(I) Yes (II)
Mn^{2+} alone	No	Yes	No(I) Yes (II)
phenols plus Mn^{2+}	Yes	Yes	Yes (I)
H_2O_2 (with other additives)	Yes, Strongly	Yes, Strongly	Yes, Strongly(I;II)
3. Relative rate stimulation by phenols	*p*-coumarate > *p*-cresol > phenol	*p*-coumarate > *p*-cresol > phenol	*p*-cresol > phenol(I) *p*-coumarate > *p*-cresol(II)
4. Salicylate ineffective as phenolic stimulator	Yes (Section 5.2.3)	N.D.	Yes (I;II)
5. Stoicheiometry; NADH:O_2	2	N.D.	2 (I)

6. Saturating kinetics for phenolic stimulators	Yes (Section 5.2.4)	N.D.	Yes; concentration of: resorcinol, K_m 38μM(I), *p*-coumarate, optimal 83μM(II), *p*-cresol, optimal 17μM(II)
7. Saturating kinetics for NADH	Yes (Section 5.2.4)	Yes (Section 5.2.4)	
8. Inhibitors	(Section 5.2.4)	(Section 5.2.4)	
	Catalase	Catalase	Catalase (I;II)
	KCN	KCN	KCN (I)
	NaN_3	NaN_3	Ineffective(I)
	DIECA	DIECA	Ineffective(I)
	EDTA	EDTA	
	8HQ	8HQ	
	Catechol	Catechol	Catechol (I)
	Caffeate	Caffeate	
	$CuSO_4$	$CuSO_4$	$CuSO_4$ (I)
9. Addition of more than one phenol gave non-additive stimulation	Yes (Section 5.2.4)	N.D.	Yes (II)

Whole *P. communis* plants, and, in common with other species, excised tissue pieces, *removed* phenol from surrounding media. Absorbed phenol was converted to two unequivocally identified metabolites, catechol and phenyl-β-*D*-glucoside, and a large fraction was rendered inaccessible to routine ethanol extraction. Conversion to the glucoside is an effective detoxication reaction itself, and oxidation to catechol is the first stage of a putative pathway leading to the immobilization of the toxic pollutant in inert bound or polymeric form. The immobilization response was important in all the species examined, suggesting a more common response to exogenous phenolic compounds than the proposed ring-fission pathways of higher plants, for which the present studies found no evidence.

Absorption of phenol, followed by conjugation or immobilization, nevertheless brought about reductions in the phenol content of the media of experimental *P.communis* plants, showing that this species, in common with *Schoenoplectus lacustris*, might usefully withstand and ameliorate contaminated water.

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