ENZYMOLOGICAL ASPECTS OF PHYTOALEXIN ACCUMULATION IN *PHASEOLUS VULGARIS*

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ABSTRACT

In wounded cotyledons of *Phaseolus vulgaris* the accumulation of the 5-hydroxy isoflavonoids 2'-hydroxygenistein and kievitone preceded the major increases in the levels of the 5-deoxy compounds coumestrol and phaseollin. Increased phytoalexin (kievitone and phaseollin) levels were preceded by transient increases in the extractable activities of L-phenylalanine ammonia-lyase (EC 4.3.1.5), chalcone synthase and chalcone isomerase (EC 5.5.1.6). ^{13}C-NMR analysis of kievitone and phaseollin produced after feeding [1,2-$^{13}C_2$] acetate to wounded cotyledons demonstrated the incorporation of intact acetate units into the aromatic A-rings. Phaseollin showed a specific folding of the polyketide chain whereas kievitone exhibited a randomisation of the label in keeping with the intermediacy of a 2',4',6'-trihydroxychalcone during its formation. The biosynthetic routes to these 5-hydroxy and 5-deoxy isoflavonoids thus diverge prior to chalcone formation. The results suggest the involvement of a 6'-deoxy chalcone synthase in the biosynthesis of 5-deoxy isoflavonoid compounds. However, efforts to locate the activity of such an enzyme were unsuccessful.

6'-Hydroxy chalcone synthase and chalcone isomerase were purified from cell suspension cultures of *P. vulgaris*. Chalcone synthase showed many similarities to the enzyme purified from *Petroselinum hortense* but was much less stable, had significantly different apparent K_m values for the two substrates and produced different *in vitro* release products following purification. With malonyl CoA as variable substrate, double reciprocal plots for naringenin formation were sigmoidal suggesting positive cooperativity which may favour the formation of chalcones *in vivo*. Only a single form of chalcone isomerase was found; however, the enzyme catalysed the isomerisation of both 6'-hydroxy and 6'-deoxy chalcones to the corresponding flavanones. Kievitone strongly inhibited the activity of both enzymes whilst coumestrol was a potent inhibitor of chalcone isomerase. A range of other 5-hydroxy and 5-deoxy isoflavonoid compounds tested had little or no effect on the activity of the two enzymes. The possible relevance of the inhibitory effects of such compounds is discussed in relation to the role of both enzymes in the induced accumulation of isoflavonoid phytoalexins in *P. vulgaris*.

CONTENTS

ABBREVIATIONS

A_{259}	Absorbance at 259 nm
ACP	Acyl carrier protein
AMP	Adenosine monophosphate
Ar	Aromatic ring
ARC	Agricultural Research Council
ATP	Adenosine triphosphate
b.p.	Boiling point
BSA	Bovine serum albumin
CA4H	Cinnamic acid 4-hydroxylase
CHI	Chalcone isomerase
CHS	Chalcone synthase
C_i	Curie
4CL	4-Coumarate:CoA ligase
CMI	Commonwealth Mycological Institute
CoA	Coenzyme A
cpm	Counts per minute
2,4-D	2,4-Dichlorophenoxyacetic acid
DEAE	Diethylaminoethyl
DMAPP	Dimethylallylpyrophosphate
DNase	Deoxyribonuclease
D4NA	Diazotised 4-nitroaniline
dpm	Disintegrations per minute
DTT	Dithiothreitol
δ_c	Chemical shift (relative to TMS)
ε	Molar extinction coefficient
EDTA	Ethylenediaminetetraacetic acid
FAS	Fatty acid synthase
$g_{av.}$	Gravity (average)
h	Hour
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
Hz	Hertz
J_{cc}	Carbon-carbon coupling constant
kat	Katal = enzyme activity effecting conversion of 1 mole substrate/sec.
K_m	Michaelis-Menten constant
K_i	Inhibitor constant
$\lambda_{max/min}$	Wavelength of maximum/minimum UV absorbance

m.p.	Melting point
M_r	Relative molecular mass
min.	Minute
mRNA	Messenger RNA
NAD(H)	β-Nicotinamide adenine dinucleotide (reduced)
NADP(H)	β-Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
PAL	Phenylalanine ammonia-lyase
pH	$-\log [H^+]$
pI	pH of isoelectric point
PP_i	Inorganic pyrophosphate
ppm	Parts per million
ppt.	Precipitate
PPO	2,5-Diphenyloxazole
psi	Pounds per square inch
PVP	Polyvinylpolypyrrolidone
R_f	Mobility relative to solvent front
rev.	Revolution
rpm	Revolutions per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
[S]	Substrate concentration
sh	Shoulder (in UV spectra)
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNBS	Trinitrobenzoylsulphate
Tris	Tris(hydroxymethyl)methylamine
[U-]	Universally labelled
v	Initial velocity
V_e	Elution volume
V_o	Void volume
V_{max}	Maximum velocity
W	Watt

Organics

HOAc (acetic acid); Me_2CO (acetone); $CHCl_3$ (chloroform); $(CD_3)_2CO$ (deuterated acetone; $CDCl_3$ (deuterated chloroform); Et_2O (diethylether); DMF (dimethylformamide; EtOH (ethanol); EtOAc (ethyl acetate); HCO_2H (formic acid) ; MeOH (methanol); NaOAc (sodium acetate); EtOMe (methoxyethanol).

CHAPTER 1

Introduction

1.1 Biology and Physiology of Plant-Parasite Interactions

1.1a Resistance and Susceptibility

Biochemical plant pathology is a field which encompasses a vast array of complex molecular events that take place during interactions between potentially pathogenic microbial races and host plant cultivars. Whether a particular interaction results in a susceptible or resistant reaction depends on the genotype of both host and pathogen.

The initial response of the host to the pathogen may be determined primarily by the presence or absence of surface recognition phenomena which then affect the nucleic acid metabolism of the host. In a resistant response this leads to the induction of active defence mechanisms including increased de novo synthesis of enzymes which catalyse the formation and subsequent accumulation of low molecular weight, broad-spectrum, antibiotic substances termed phytoalexins, from distant pathway intermediates. (Greek: "phyton", meaning plant and "alexin", meaning to ward off). Although phytoalexin accumulation is not necessarily the primary determinant of disease resistance it has been shown to play a major role in the expression of a resistance response in some cases (Bailey, 1982).

In many plants, notably members of the Leguminosae and Solanacae, phytoalexin accumulation alone provides a satisfactory explanation for the restriction of microbial growth during some resistant interactions, whilst in other plants the relationship between phytoalexin accumulation and microbial inhibition is not so clear (Mansfield, 1982).

Plant defence mechanisms can be conveniently divided into two groups: pre-exisiting mechanisms that are passive and comprise properties of the plant which either prevent pathogen entry, or which prevent its further development if it does enter, and post-infectional defence mechanisms which are active and dynamic and are generally considered to be of greater significance in protecting plants against pathogens.

General, non-host resistance in plants seems to result from several mechanisms which may be effective at different stages of infection. They include physical barriers such as cuticularised epidermal cells and bark, and metabolic barriers such as preformed toxins and nutritional factors. The best example of resistance based on compounds present and active in uninfected tissue is found in relation to *Colletotrichum circinans*, the causal agent of smudge disease in onion bulbs. Both protocatechuic acid (Link et al., 1929) and catechol (Link and Walker, 1933) occur constitutively in the outer scales of resistant bulbs. Furthermore, the presence of the two compounds only in resistant progeny of crosses between resistant and susceptible cultivars of onion supported the idea that they were the resistance factors. Another example concerns *Botrytis cinerea* which, in spite of its wide host range, is not normally a parasite of tulip, which is attacked by the specialised *B. tulipae*. Tulip resistance toward *B. cinerea* seems to be stictly related to the presence of the toxin tuliposide, which is a preformed unsaturated lactone (Schönbeck, 1976). Although general resistance does not confer complete protection it can retard the rate of spread of a pathogen and has the advantage of not being rapidly overcome by new races of fungi or bacteria.

Specific (induced) resistance mechanisms also fall into two groups, i.e. physical or metabolic barriers. Resistance to fungi is frequently expressed by the failure of infection hyphae to penetrate into or through plant cell walls (Heath, 1980). Various types of deposit

(papillae) have been found to accumulate within living cells beneath sites of attempted penetration. It has been suggested that papilla formation and other localized changes in cell wall structure including lignification (Vance et al, 1980; Henderson and Friend, 1979), silicification (Heath, 1980) and suberisation (Kolattukudy, 1975) may provide purely physical barriers to the continued progress of the invading hyphae. However, the formation of these physical barriers is as frequently associated with successful as with non-successful invasion and thus their role in the resistance response is ambiguous. The important factor in the efficiency of such structures appears to be the rate at which they are formed. Metabolic barriers are often more effective than physical barriers at restricting pathogen development and perhaps the most important of these is the induced accumulation of phytoalexins, although agglutinins (Kuć, 1979) and protease inhibitors (Kuć, 1979) have also been implicated in some host-pathogen interactions. Phytoalexins are not normally present in healthy uninfected tissues but can accumulate rapidly in some interactions and can thus provide an effective defence mechanism against some pathogenic microorganisms.

1.1b Race Specific Resistance

The Dwarf French bean *Phaseolus vulgaris* L. provides an ideal system for investigations into the molecular aspects of phytoalexin accumulation due to its host-specific relationship with *Colletotrichum lindemuthianum*, a facultative pathogen and the causal agent of anthracnose in beans. *C. lindemuthianum* is a seed borne pathogen which invades the lower regions of seedling hypocotyls. It is able to grow biotrophically in susceptible cultivars of *P. vulgaris* while in resistant cultivars the inhibition of pathogen growth is associated with premature death of the infected cell (Bailey, 1982).

Table 1.1

Differential Reaction of Cultivars of *P. vulgaris* to races of *C. lindemuthianum*

Cultivar	Physiological race		
	β	γ	δ
Immuna	HR	HR	S
Kievit	HR	S	HR
Red Kidney	S	S	HR

HR = Hypersensitive flecking only; S = Spreading brown water-soaked lesions (Bailey and Deverall, 1971).

Spores of *C. lindemuthianum* behave similiarly when placed on young hypocotyls of resistant and susceptible cultivars of *P. vulgaris*. Germination takes place within 24h to 48h and short germ tubes with accompanying appresoria are formed. Differences between resistant and susceptible hypocotyls only become evident when infection hyphae from the appresoria penetrate the epidermal cells. On excised hypocotyls of susceptible cultivars, infection by a virulent race causes no observable host response and hyphae continue to grow between the cell wall and the plasma membrane of epidermal cells without deleterious effects to either themselves or the infected cells. After a period of biotrophy the infected cells die, the tissue collapses and a brown limited lesion is produced.

On resistant hypocotyls the initially infected cell and several adjacent cells die soon after infection and appear as scattered flecks on the hypocotyl surface (Table 1.1). This process however, known as "hypersensitivity", is not sufficient on its own to account for the

observed restriction of growth of the intracellular hyphae of this facultative pathogen and is accompanied by the production of one or more fungitoxic isoflavonoid phytoalexins which accumulate to high concentrations and inhibit further growth of the pathogen (Figure 1.1). Where phytoalexin accumulation makes a major contribution to resistance, virulence is associated with failure of the inhibitors to reach or maintain antimicrobial concentrations around the invading micro-organisms. Phytoalexin accumulation almost invariably follows necrosis of isolated challenged cells during infection development. The phytoalexins are synthesized primarily by adjacent living tissue before diffusing into the dead cell(s), which themselves appear to be the trigger of phytoalexin synthesis (Bailey, 1982).

1.1c Molecular Mechanisms of Disease Resistance

Study of interactions between flax and flax rust (*Melampsora lini*) have led to the formulation of a gene-for-gene hypothesis which states that for each gene determining resistance in the host there is a specific and related gene determining virulence in the pathogen. That is, for each gene in the host capable of mutating to give resistance there exists a gene in the pathogen capable of mutating to overcome that particular resistance (Flor, 1946, 1947; reviewed by Ellingboe, 1981).

The mechanisms which give rise to and control host-pathogen specificity have been explained by considering the fundamental macromolecular events which must be involved, and several models have been proposed to explain the various observed interactions. It should be stressed that any molecular models should be consistent with the observed genetics. Day (1974) proposed one such model for the interaction between an avirulent parasite and a host possessing the corresponding resistance gene. Firstly, an active elicitor (the product of the

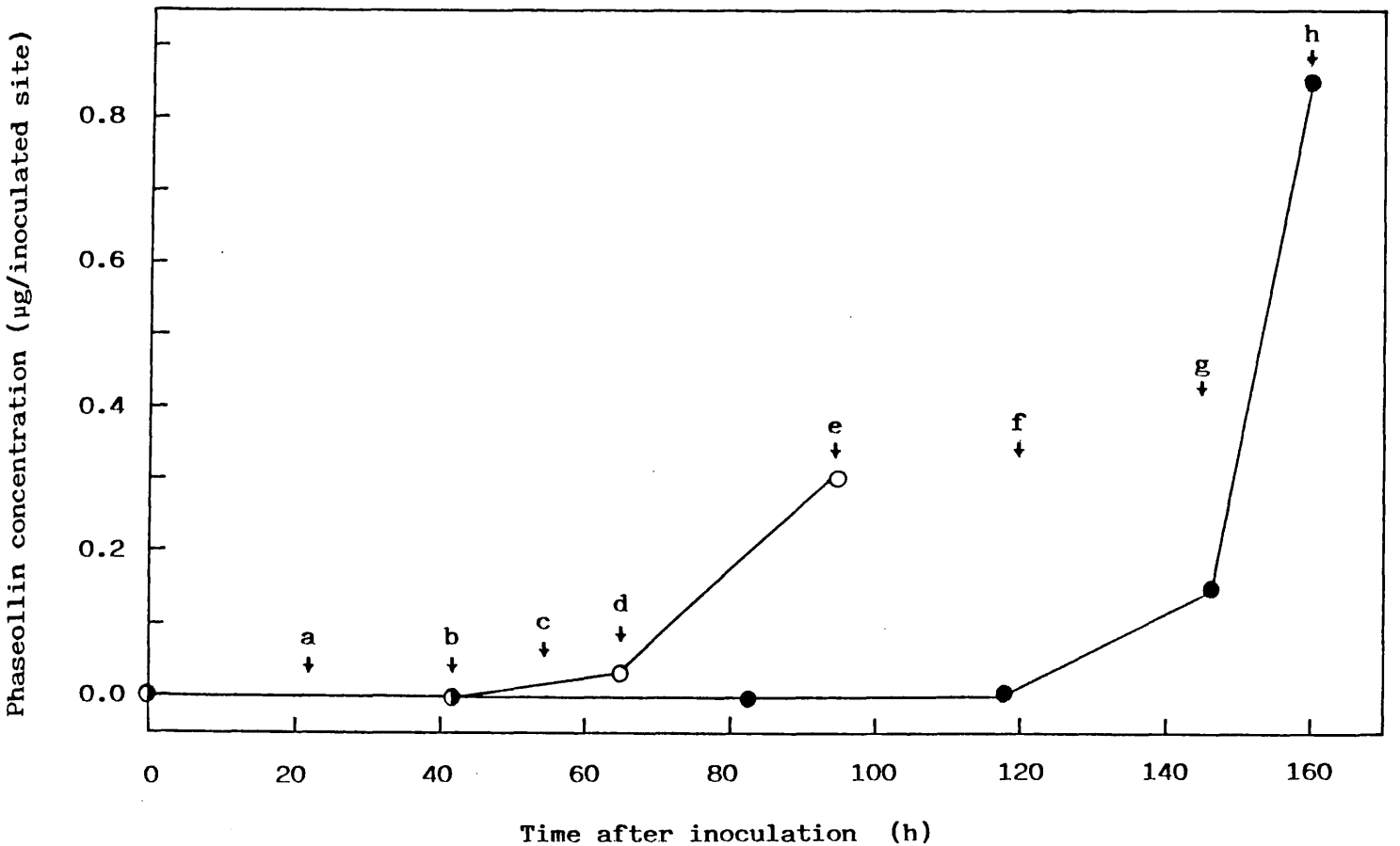


Figure 1.1 Phaseollin concentration during the interaction of *Phaseolus vulgaris* cv. Kievit with races γ (●) and δ (○). a) Appressorium formation by race δ and γ ; b) cellular responses to race δ visible; c) penetration by race γ : no visible cellular response; d) partial browning of cells infected with race δ - hyphae of race γ present in epidermal cells: no visible cellular response; e) hypersensitive response to race δ complete; f) 1% lesions by race γ; g) 80% lesions by race γ ; h) 100% lesions by race γ. (Bailey and Deverall, 1971).

pathogen's avirulence gene) interacts with a receptor (the product of the host's resistance gene) on the host cell surface. In an incompatible interaction, elicitor-receptor recognition triggers the transmission of a message to the host cell nucleus. Such intracellular substance(s) then interact with resistance genes and "switch-on" an activator RNA molecule. The binding of such activator RNA's to structual gene cistrons leads to their de-repression resulting in de novo protein synthesis, phytoalexin induction and limitation of the pathogen's growth. Mutations which prevent the recognition by producing either inactive elicitor or inactive receptor prevent the triggering of phytoalexin accumulation and result in a compatible interaction.

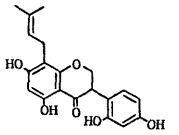
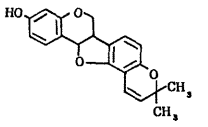
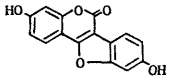
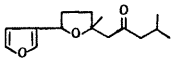
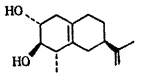
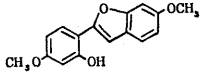
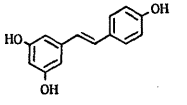
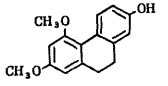
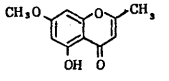
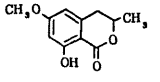
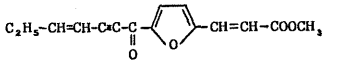
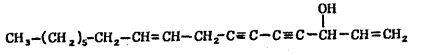
1.2 The Phytoalexin Response

1.2a Structure, Occurence and Biological Activity

Müller and Börger first proposed the phytoalexin concept in 1940. During the past four decades the isoflavonoid-pterocarpan phytoalexins of the Leguminosae and the terpene derived furano- and bicyclic sesquiterpene phytoalexins of the Solanacae, Convolvulacae and the Malvacae have been the subject of the majority of phytoalexin biosynthetic studies. However, a wide variety of other compounds are also now accepted as phytoalexins and many of these fall into clearly defined chemical classes (Table 1.1). In addition to the isoflavonoids other shikimate-polymalonate derived compounds act as phytoalexins; these include arylbenzofurans from *Vigna unguiculata* (Leguminosae), stilbenes from *Vitis vinifera* (Vitacae) and the dihydrophenanthrene derivatives from *Orchis* and *Loroglossum* (Orchidacae).

Several classes of phytoalexin are acetate-polymalonate derived and these include chromones, isocoumarins, furanoacetylenes and polyacetylenes. Some furano- and polyacetylenic phytoalexins which are characteristic

Table 1.1 Structual Diversity of Phytoalexins from a Number of Sources

Chemical class	Trivial name	Structure	Species and Family	Reference
Isoflavanone	Kievitone		*Phaseolus vulgaris* Leguminosae	Smith et al., 1973.
Pterocarpan	Phaseollin		*Phaseolus vulgaris* Leguminosae	Cruickshank and Perrin, 1971.
Coumestan	Coumestrol		*Phaseolus vulgaris* Leguminosae	Lyon and Wood, 1975.
Furanosesquiterpene	Ipomeamarone		*Ipomea batatas* Convolvulaceae	Kubota and Matsura, 1953.
Bicyclicsesquiterpene	Rishitin		*Solanum Tuberosum* Solanaceae	Sato et al., 1968.
2-Arylbenzofuran	Vignafuran		*Vigna unguiculata* Leguminosae	Preston et al., 1975.
Stilbene	Resveratrol		*Vitis vinifera* Vitaceae	Langcake and Pryce, 1977.
Dihydrophenanthrene	Orchinol		*Orchis militaris* Orchidaceae	Fisch et al., 1973.
Chromone	Eugenin		*Daucus carota* Umbelliferae	Stoessl and Stothers, 1978.
Isocoumarin	6-Methoxymellein		*Daucus carota* Umbelliferae	Goodliffe and Heale, 1978.
Furanoacetylene	Wyerone		*Vicia faba* Leguminosae	Fawcett et al., 1968.
Polyacetylene	Falcarinol		*Lycopersicon esculentum* Solanaceae	de Wit and Kodde, 1981.

of the Compositae, occur in both the Leguminosae eg. wyerone in *Vicia faba* (Fawcett et al., 1968) and the Solanaceae eg. falcarinol in *Lycopersicon esculentum* (de Wit and Kodde, 1981). These compounds occur together with the isoflavonoid and terpene derivatives usually associated with these plant species and represent some of the few cases where the taxonomic distribution of the chemical classes of phytoalexin deviates from the expected pattern. In general, the chemistry of phytoalexins is uniform within a plant family but diverse within the plant kingdom.

The diversity of phytoalexin structure implies a complex integration of synthetic organisation. This statement is reinforced by the fact that many plant species produce a variety of phytoalexins and related induced compounds in response to infection. For example, *Solanum tuberosum* produces rishitin, phytuberin, isolubimin and at least eight other related compounds in response to *Phytophthora infestans* (Katsui et al., 1968; Kuć et al., 1976) and the Dwarf French bean (*Phaseolus vulgaris*) produces the isoflavonoids phaseollin, phaseollidin, phaseollinisoflavan, 2'-hydroxygenistein, dalbergioidin, kievitone, coumestrol and others in response to *Colletotrichum lindemuthianum* (Lyon and Wood, 1975; Biggs, 1975; VanEtten and Pueppke, 1976; Woodward, 1979a, 1979b, 1980a, 1980b).

The production of several phytoalexins by a plant might be advantageous if the spectrum of antibiotic action was widened to afford protection against a greater number of potential pathogens. Furthermore, the probability of a specific microorganism overcoming a resistance response might be reduced if several inhibitors were present. An example of this is provided by the interaction of carrot (*Dacus carota*) phytoalexins with several non-pathogens of the plant. Thus, chlorogenic acid inhibited *Helminthosporium carbonum* and *Venturia inequalis* but did not inhibit *Ceratocytis fimbriata* which was, however, inhibited by the isocoumarin 6-methoxymellin, which is also produced by carrot (Condon and Kuć, 1960).

The amounts and relative ratios of the accumulating phytoaleins can vary and have been shown to be dependent not only on the fungal species used to induce (Bailey and Burden, 1973; Smith et al., 1975; VanEtten and Pueppke, 1976) but also on fungal elicitor concentration (Dixon et al., 1981), the plant tissue used (Bailey and Burden, 1973; Theodorou and Smith, 1979), temperature (Murch and Paxton, 1980) and light (Rowell and Bailey, 1982).

Some isoflavonoids which act as phytoalexins in some systems are found as constitutive components of healthy tissues. For example, biochanin A and formononetin occur in *Cicer arietinum* (Saya-gener et al., 1969; Wengenmayer et al., 1974) and luteone occurs in the leaves of *Lupinus albus* (Harborne et al., 1976). Maackian occurs as a phytoalexin in *Pisum sativum* and *Trifolium pratense* (Stoessl, 1972; Dewick, 1975) and is also present in a modified form, mainly as the β-D-glucoside trifolirhizin, in heartwood, callus or root tissue of several species including *Trifolium* and *Sophora* (VanEtten and Pueppke, 1976). Other isoflavonoids such as daidzein and coumestrol are present in healthy tissues of *Glycine max*, but their concentration increases during infection (Keen and Kennedy, 1974). These pre-existing isoflavonoids would constitute a passive defence mechanism if they acted as a determinant factor in disease resistance.

Despite the considerable information on the antimicrobial activity of phytoalexins, little is known about the mechanism of toxicity. Unlike some microbial antibiotics and synthetic fungicides, phytoalexins do not appear to have discreet "biochemical targets" but instead appear to function as non-specific membrane antagonists (VanEtten and Bateman, 1971). For example, phaseollin caused ion-leakage from treated fungal cells and glyceollin, phaseollin and medicarpin caused bursting of red blood cells (VanEtten and Bateman, 1971). Similarly, phytuberin and

other sesquiterpene phytoalexins from *Solanum tuberosum* caused rapid lysis of *Phytophthora infestans* zoospores (Harris and Dennis, 1976). Phaseollin and other phytoalexins can also affect the permeability of the plant cells in which they accumulate (Shiraishi et al., 1975; El-naghy & Heitfuss 1976) and thus membrane dysfunction, particularly that of the plasmalemma, appears to be instrumental in phytoalexin toxicity. Early investigations into the structure-activity relationships suggested that the aplanar configuration of the pterocarpan phytoalexins may be essential for their fungitoxicity (Perrin and Cruickshank, 1969), but it was subsequently shown that several 6a-hydroxypterocarp-6a-enes which do not show the same configuration had essentially unchanged activity (VanEtten , 1976).

At least two isoflavonoids from *Phaseolus vulgaris*, namely genistein and 2'-hydroxygenistein, accumulate along with other compounds but have only weak antimicrobial properties (Biggs, 1975). Isopentenyl substitution makes both these compounds considerably more active (Fukui et al., 1973; Ingham et al., 1977). Wighteone (6-isopentenylgenistein) and luteone (6-isopentenyl-2'-hydroxygenistein) both exhibit high anti-fungal activity against *Cladosporium cucumerinum* (Ingham et al., 1977). Similarly both kievitone and phaseollin are more antifungal than their unprenylated precursors. This has led to the proposal that lipophilicity is an important contributor to antifungal activity and may reflect a compound's ability to interact with and disrupt fungal plamsa membranes. Thus, in *P. vulgaris* the inducibility and specificity of prenyl trans-ferases may be important factors in the plant's defence response.

Coumestrol is not a true isoflavonoid phytoalexin although it accumulates with these compounds and several related coumestans in *P. vulgaris* and other plants (Olah and Sherwood, 1971; Dixon and Bendall, 1978b). The biological activity of coumestrol is antibacterial rather

than antifungal (Lyon and Wood, 1975).

Phytoalexins are natural compounds and it is therefore resonable to expect that they can be further metabolised by either the host or the pathogen. In the host it has been shown that the half-lifes of some phytoalexins such as glyceollin are as long as 100h (Moesta and Grisebach, 1981) and thus increases in phytoalexin concentration are due to increased de novo synthesis as opposed to decreased turnover of exisiting pools of phytoalexin. In the pathogen, detoxification of phytoalexins has been widely reported over the last decade. This ability is generally, though not exclusively, limited to fungi which are pathogenic to legume species and in certain cases the ability to detoxify phytoalexins may be a major determinant of fungal pathogenicity (VanEtten et al., 1982).
A variety of different biochemical modifications have been observed during the detoxification of isoflavonoid derivatives. These generally tend to decrease the lipophilic nature of the compound and include hydration, demethylation, aromatic hydroxylation, ring opening and oxidation.
Cultures of *Fusarium solani* f. sp. *phaseoli* are able to convert both kievitone and phaseollidin to less toxic compounds by hydration of their isopentenyl groups (Kuhn et al., 1977; Smith et al., 1980). Furthermore, an enzyme catalysing the formation of kievitone hydrate has been isolated from cell-free culture filtrates of the fungus (Kuhn and Smith, 1979) and the observation that pre-treatment of cultures with kievitone led to a more rapid rate of kievitone disappearance suggests that the enzyme system is inducible.

The degradation of phytoalexins to non-toxic derivatives can be regarded as a form of active suppression of resistance and there are a number of diseases where the host range of a particular fungal pathogen appears to be related to its ability to metabolise certain phytoalexins (Ward and Stoessl, 1972).

1.2b Elicitors of the Phytoalexin Response

The mechanisms responsible for initiating the biosynthesis and subsequent accumulation of phytoalexins in plant tissues have been the subject of a considerable amount of research. Phytoalexins are induced in plant cells by phytopathogenic fungi, bateria and nematodes. However, the original conclusion that phytoalexins were of host origin was based on the demonstration that phytoalexins were produced in tissues after various chemical treatments. Since then a wide variety of structually unrelated compounds have been shown to act as unnatural (abiotic) elicitors. These include heavy metal salts, polyamines, organic acids, chloroform, fungicides, autoclaved RNase and DNase, the action of localised freezing and thawing, detergents and short-wave ultraviolet irradiation (Van Etten and Pueppke, 1976). The induction of the phytoalexin response by fungi is believed to be due to fungal metabolites which act as natural (biotic) elicitors. Such molecules have been isolated from fungal culture filtrates and mycelial cell walls and appear to be either polysaccharides (Anderson-Prouty and Albersheim, 1975; Ayers et al., 1976) or glycoproteins (Daniels and Hadwiger, 1976; Stekoll and West, 1978; Dow and Callow, 1979; de Wit and Roseboom, 1980).

Detailed physiological studies have led to the proposal that induction of phytoalexin accumulation by the various abiotic agents involves host cell damage or death which causes the release of plant metabolites and these then stimulate the synthesis of phytoalexins in surrounding healthy cells (Bailey, 1982). Hargreaves and Bailey (1978) demonstrated the existence of such metabolites by freeze-thaw treating one half of split hypocotyls and then placing the two halves back together. They found that phytoalexins accumulated in the dead section and interpreted the results as showing that a constitutive elicitor had been released from the dead cells and had elicited phytoalexin synthesis in the living

tissue. Similiar intercellular transmission of elicitation, across a dialysis membrane, was observed in tissue cultured cells of *P. vulgaris* in response to the macromolecular abiotic elicitor, RNase (Dixon et al., 1983).

These results indicate that a low molecular weight (diffusible) substance(s) is produced or released from elicited/damaged cells which then diffuses into and acts as an elicitor in unelicited cells. Bailey (1982) suggests that in undamaged cells the constitutive (endogenous) elicitor is inactive, perhaps bound to or compartmentalised within the cells and that all types of elicitor cause cell injury which releases the constitutive elicitor(s) and this then leads to phytoalexin accumulation. These suggestions are supported by the fact that extracts of autoclaved bean hypocotyls act as elicitors (Hargreaves and Selby, 1978). However, Dixon et al., (1983) have demonstrated that no intercellular transmission occurs in response to biotic elicitor preparations from *Colletotrichum lindemuthianum* which suggests that different mechanisms may operate for the overall transmission of the phytoalexin response to biotic and abiotic elicitors.

1.2c Phytoalexin Induction in *Phaseolus vulgaris*: Biosynthesis and Enzymology

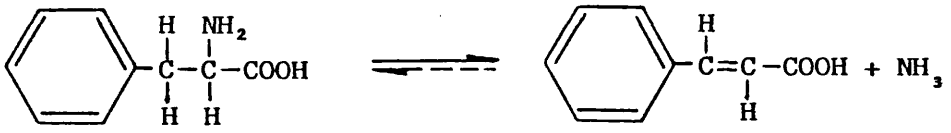
1.2c-1 The Central Phenylpropanoid Pathway

Isoflavonoid phytoalexins are based on a C_{15} molecular skeleton which results from the convergence of two unrelated metabolic routes i.e. the acetate-malonate and shikimic acid pathways (Grisebach, 1967) both of which are ubiquitous in higher plants. One function of the shikimic acid pathway in primary metabolism is the synthesis of phenylpropanoid units such as phenylalanine and tyrosine. These two compounds can enter the central or "core" phenylpropanoid pathway by reductive deamination to the

corresponding acids which are then activated by conversion into hydroxycinnamoyl coenzyme A (CoA) thiol esters. The esters of 4-coumaric and caffeic acid are precursors in the biosynthesis of flavonoids (including anthocyanins and condensed tannins), isoflavonoids, chlorogenic acid, coumestans and pterocarpans, whereas the esters of ferulic and sinapic acids, after oxidation to the corresponding alcohols, may undergo oxidative polymerisation to yield lignin.

The enzyme L-phenylalanine ammonia-lyase (PAL, EC. 4.3.1.5) represents the primary control point in overall phenylpropanoid biosynthesis (Lamb and Rubery, 1976; Hahlbrock and Grisebach, 1979) and catalyses the first committed step in the biosynthesis of flavonoid and other phenylpropanoid secondary products; the elimination of ammonia from L-phenylalanine to yield *trans*-cinnamic acid (Reaction 1).

Reaction 1

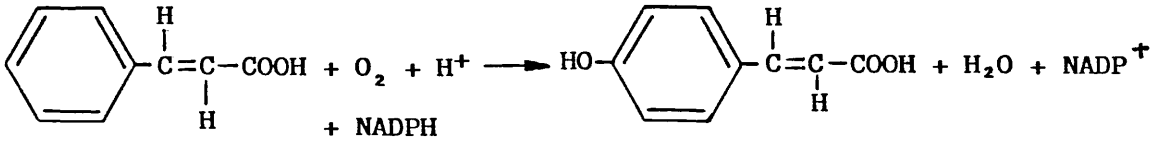


Treatment of both *Glycine max* and *Phaseolus vulgaris* cell suspension cultures with biotic elicitor preparations from *Phytophthora megasperma* var. *sojae* and *Colletotrichum lindemuthianum* respectively, (Ebel et al., 1976; Dixon and Bendall, 1978b) caused rapid but transient induction of PAL followed by the accumulation of isoflavonoid phytoalexins. The induced accumulation of such compounds occurred in the absence of changes in the levels of other phenylpropanoid derivatives such as leucoantho-

cyanins, flavonol glycosides and hydroxycinnamic acid compounds which occur as constitutive compounds in *P. vulgaris* (Rathmell and Bendall, 1971; Dixon and Bendall, 1978a), and thus reflects a specific change in host metabolism within a group of compounds that are closely related biosynthetically. This evidence clearly indicates that further regulation of the pathway leading to isoflavonoid phytoalexins occurs at points subsequent to the PAL reaction.

Conversion of *trans*-cinnamic acid to 4-coumaric acid is catalysed by the enzyme cinnamic acid 4-hydroxylase (CA4H, EC. 1.14.13.11), a mixed function mono-oxygenase, which catalyses the insertion of an atom of oxygen into cinnamic acid from molecular oxygen with resultant oxidation of NADPH (Reaction 2).

Reaction 2



As with other enzymes catalysing related aromatic hydroxylations in both animals and plants, CA4H is a cytochrome P_{450}-linked microsomal enzyme and *in vitro* requires the presence of a low concentration of a thiol reagent such as 2- mercaptoethanol for maximal activity (Billett and Smith, 1978). In contrast to the rapid transient induction of PAL, CA4H activity increases linearly for 48h after elicitor treatment in *P. vulgaris* cell suspension cultures (Dixon and Bendall, 1978b).

4-coumaric acid and other hydroxycinnamic acid derivatives are

activated by thiol esterification to CoA by hydroxycinnamoyl-CoA ligases (EC. 6.2.1.12) in a two step process involving the formation of an enzyme-bound adenylate derivative with release of inorganic pyrophosphate followed by a CoA exchange reaction (Reaction 3).

Reaction 3

$$\text{Enzyme} + \text{R-CH=CH-COOH} + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{Enzyme[R-CH=CH·CO·AMP]} + PP_i$$

$$\text{Enzyme[R-CH=CH·CO·AMP]} + \text{CoA·SH} \rightleftharpoons \text{R-CH=CH·CO·S·CoA} + \text{AMP} + \text{Enzyme}$$

Two isoenzymic forms of the CoA ligase have been isolated from cell suspension cultures of *Glycine max*. Substrate specificity studies have suggested that one form, which is generally active with ferulic and sinapic acids, may be involved in lignin biosynthesis whilst the other form, which is inactive with sinapic acid but shows highest activity with 4-coumaric and caffeic acids, may be involved in flavonoid/isoflavonoid biosynthesis (Knobloch and Hahlbrock, 1975). The two enzymes were inhibited to different degrees by AMP, the extent being dependent on ATP levels. The possibility of regulation via energy charge has led to the suggestion that the CoA ligase isoenzymes might represent a sensitive control point for the divergence of the lignin and isoflavonoid pathways (Hahlbrock and Grisebach, 1979). Treatment of cell suspension cultures of *P. vulgaris* with the abiotic elicitor RNase induced 4-coumaric acid:CoA ligase activity in a similiar manner to that observed with CA4H, i.e. a gradual increase over 48h (Dixon and Bendall, 1978b).

A detailed knowledge of the substrate specificities of the CoA ligases induced by elicitors may have important implications for isoflavonoid phytoalexin biosynthesis. All isoflavonoid phytoalexins characterised to date are hydroxylated or further substituted at position 4' on the

B-ring (isoflavonoid numbering, see Appendix A); this corresponds to position 4 of the hydroxycinnamic acid precursor. Further hydroxylation of the B-ring may occur at the isoflavonoid stage, i.e. at the 2' position; however, phytoalexins such as pisatin and maackian possess a 4',5'-methylene dioxy substituent on the B-ring. The initial 4',5'-dihydroxy oxygenation pattern could arise at either the level of the C_6C_3 phenylpropanoid precursor or, on the evidence of labelling studies (Dewick and Ward, 1978; Dewick and Banks, 1980), at the C_{15} stage. If the oxygenation pattern is established at the C_6C_3 stage then the involvment of a CoA ligase with activity towards caffeic acid would be predicted. Such substrate specificity would also extend to other enzymes on the pathway to isoflavonoid phytoalexins including the next enzyme of the pathway, chalcone synthase.

1.2c-2 Enzymic Formation of Chalcones

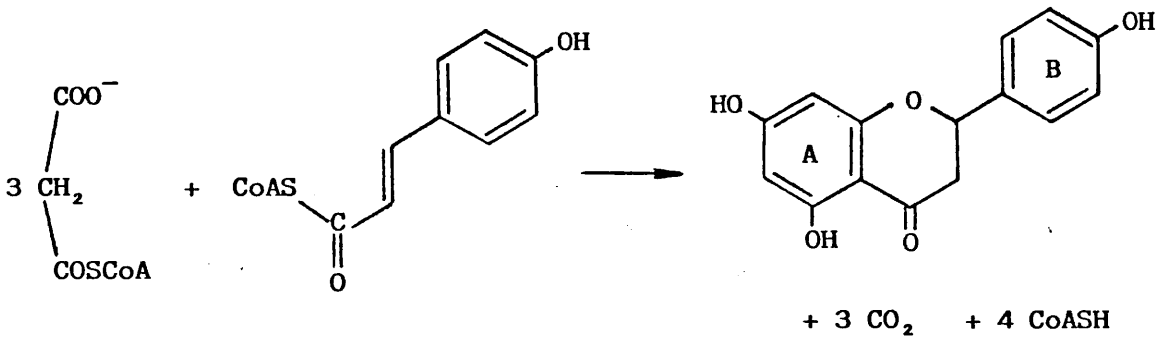
Incorporation experiments with radiolabelled 2',4,4'-trihydroxychalcone have demonstrated the intermediacy of chalcones in the biosynthesis of isoflavonoid phytoalexins in seedlings of *Medicago sativa* and *Trifolium pratense* treated with $CuCl_2$ (Dewick, 1975; Dewick and Martin, 1979b), and also in hypocotyls of *Glycine max* infected with *Phytophthora megasperma* f. sp. *glycinea* (Keen et al., 1972) and during coumestrol biosynthesis in *Phaseolus aureus* (Dewick et al., 1970).

Chalcone synthase (formerly called flavanone synthase) was first detected in cell suspension cultures of *Petroselinum hortense* following irradiation with UV light (Kreuzaler and Hahlbrock, 1975a), a treatment that results in the formation of flavone glycosides. The enzyme activity has since been observed in several plant systems including cotyledons of *Glycine max* (Zähringer et al., 1978), anthers of *Tulipa* c.v. "Apeldoorn" (Sütfeld et al. , 1978), cell suspension cultures

of *Phaseolus vulgaris* (Dixon and Bendall, 1978b) and *Haplopappus gracilis* (Saleh et al., 1978) and in seedlings of *Pisum sativum*, *Brassica oleracea* and *Spinacia oleracea* (Hrazdina and Creasy, 1979; Hrazdina et al., 1980).

Under the original assay conditions employed by Kreuzaler and Hahlbrock (1975a) the enzyme from *Petroselinum hortense* catalysed the formation of the flavanone naringenin from one molecule of 4-coumaroyl CoA and three molecules of malonyl CoA, the C_6C_3 unit forming the B-ring of the flavanone and the A-ring being formed by the head-to-tail condensation of acetate(malonate units) as originally predicted by Birch and Donovan (1953) and confirmed experimentally by labelling studies (Grisebach, 1967). (Reaction 4, see also Figures 1.4, 4.1 and 5.1).

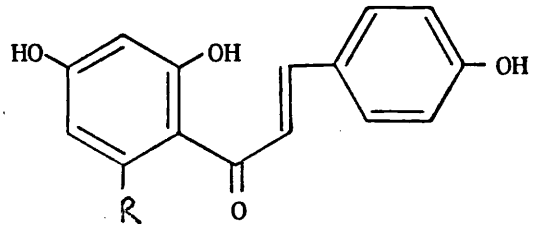
Reaction 4



The formation of the flavanone naringenin instead of the isomeric chalcone (Figure 1.2) was unexpected since both labelling experiments (Wong, 1968) and genetic evidence (mutants of *Callistephus chinensis*, deficient in chalcone isomerase (responsible for the interconversion of chalcones and flavanones) accumulated naringenin chalcone-2'-glycoside (Kuhn et al., 1978)), suggested that the chalcone was the more immediate precursor of flavonoid and isoflavonoid compounds than the corresponding flavanone. Further evidence that the chalcone biosynthetically precedes

the flavanone came from ^{13}C-NMR experiments which indicated the direct intermediacy of the chalcone during the formation of kaempferol from contiguously ^{13}C-labelled acetate in cell suspension cultures of *Petroselinum hortense* (Light and Hahlbrock, 1980). Naringenin is formed in assays of chalcone synthase as the chalcone is unstable at the usual pH of the assay (pH 8.0). However, using a special "trapping" technique it has been possible to demonstrate that naringenin chalcone is the main product of chalcone synthase. Sütfeld and Wiermann (1980) used a biphasic assay with enzyme from *Tulipa* present in a viscous lower phase and substrates present in an upper phase (pH 4.0) where chalcone could accumulate without isomerisation and under these assay conditions was found to be the main product.

Figure 1.2



R = H : Isoliquiritigenin

R = OH : Naringenin chalcone

An interesting feature of CHS is release from the enzyme of short-chain polyketide intermediates when assayed in the presence of high concentrations of thiol reagents such as 2-mercaptoethanol and dithiothreitol. These "release" or "derailment" products (see Figure 5.1), have been obtained from partially purified preparations of the synthase from *P. hortense* but not from the *Tulipa* enzyme. Bis-noryangonin formation is the result of release from the enzyme of a polyketide formed from one molecule of 4-coumaroyl CoA and two molecules of malonyl CoA (Kreuzaler and Hahlbrock, 1975b) whereas the hydroxyphenylpyrone and

4-hydroxybenzalacetone products are released after condensation with only one molecule of malonyl CoA (Hrazdina et al., 1976). Similiar release of a partially completed polyketide (as triaceticacid lactone) occurs with the enzyme 6-methylsalicylic acid synthase from *Penicillium patulum* (Dimroth et al., 1970).

CHS from *P. hortense* differs considerably from 6-methylsalicylic acid synthase (which also catalyses the synthesis of an aromatic ring from malonyl CoA), and type I fatty acid synthase (FAS). The latter two enzymes are both high molecular weight complexes ($M_r > 1 \times 10^6$) and catalyse several related reactions including acetyl and malonyl transfer from CoA to the enzyme, chain elongation and the reduction of a carbonyl group. Both enzymes also contain a pantetheinyl residue which is the functional part of the acyl carrier protein (ACP condensing enzyme) in type I FAS. CHS on the other hand, is a low molecular weight ($M_r \approx 7.7 \times 10^4$) dimer which lacks a pantetheinyl residue (Kreuzaler et al., 1979). However, there are several similarities between CHS from *P. hortense* and FAS: both are strongly inhibited by sulphydryl reagents and the antibiotic cerulenin (from *Cephalosporium caerulens*) which specifically inhibits the ß-keto ACP synthetase of the FAS complex (Kreuzaler and Hahlbrock, 1975a); both catalyse the transfer of malonate from malonyl CoA to pantetheine (Kreuzaler and Hahlbrock, 1975a); and both can decarboxylate malonyl CoA to acetyl CoA (Kreuzaler et al., 1978). Furthermore, CHS catalyses CO_2 exchange between malonyl CoA and $^{14}CO_2$ (Kreuzaler et al., 1978). The occurence of the CO_2 exchange and decarboxylation reactions catalysed by CHS indicates that the acetyl carbanion which was postulated as an intermediate in chalcone synthesis (Kreuzaler et al., 1978) reacts as a CoA ester and not as enzyme-bound malonate. This direct intermediacy of a CoA-thiol ester as substrate for the condensing reaction is the most significant difference between CHS and the related enzymes FAS and 6-methyl-

salicylic acid synthase and explains why a free ACP or enzyme-bound pantetheinyl residue is not required, as malonyl CoA itself is the immediate substrate in chalcone formation. It is possible that the two established catalytic activities of CHS i.e. the formation of the primer acetyl carbanion on the enzyme surface and condensation (chain elongation) (Kreuzaler et al., 1979) may be sufficient to catalyse the formation of chalcones. Other activities e.g. thiolesterase, may not be required if the chalcone is released from the enzyme by spontaneous cyclisation of the final tri-β-ketoacyl derivative. A scheme to account for the observed reactions catalysed by CHS is shown in Chapter 5 (Figure 5.1).

In spite of the differences outlined above, a strong similarity exists between CHS and the β-ketoacyl-ACP synthetase of type II (non-aggregated) FAS and this has led Kreuzaler et al. (1979) to suggest that CHS may have arisen by gene duplication.

CHS from all sources so far investigated catalyses the formation of chalcones with pholoroglucinol-type A-ring hydroxylation patterns, the three hydroxyl groups originating from the malonyl CoA carboxyl groups involved in the CoA-thiol ester linkage (see Figure 4.1) and this enzyme is presumably involved in the biosynthesis of 5-hydroxy isoflavonoid phytoalexins such as kievitone and licoisoflavone A in *Phaseolus vulgaris*, wighteone in *Glycine wightii* and luteone in *Lupinus albus*. Many isoflavonoid phytoalexins including phaseollin, glyceollin, pisatin and the related compound coumestrol lack the A-ring hydroxyl at position 5; however, incorporation studies with radiolabelled naringenin and liquiritigenin demonstrated conclusively that 5-hydroxy isoflavonoids were not metabolically converted to 5-deoxy isoflavonoids (Patschke et al., 1964; Keen et al., 1972; Dewick, 1975) and although the direct dehydroxylation of a phloroglucinol substituted aromatic ring occurs in nature during the urinary conversion of genistein to equol in the fowl (Cayen et al., 1965)

there is no evidence of a similiar step in *P. vulgaris*. It is therefore necessary to postulate the existence of a separate enzyme which catalyses the formation of 6'-deoxy chalcones and hence 5-deoxy isoflavonoid phytoalexins.

Although attempts to detect and characterise a 6'-deoxy CHS in a cell-free system have been made (Dixon and Bendall, 1978b) they proved unsuccessful and, to date, the activity of this enzyme remains to be demonstrated. It has been suggested that the formation of 5-deoxy isoflavonoids may be catalysed by an enzyme system in some ways analogous to the 6-methylsalicylic acid synthase from *Penicillium patulum* (Dimroth et al., 1976). 6-methylsalicylic acid is synthesised on a multienzyme complex from a primer molecule of acetyl CoA and three molecules of malonyl CoA. The complex contains 4-phosphopantetheine and is very similiar to type I (aggregated) FAS from yeast and from *P. patulum* itself. The enzyme catalyses an NADPH-dependent reduction followed by dehydration of the enzyme bound polyketide at the triacetic acid level prior to the final condensation with malonyl CoA (Dimroth et al., 1976). A study of the incorporation pattern of ^{13}C-acetate into the A-ring of the pterocarpan phytoalexin pisatin in *Pisum sativum* indicated that a similiar type of mechanism may be involved. Although reduction and dehydration of the carbonyl moiety at C-5 occurred prior to the cyclisation of the aromatic ring it was not possible to determine whether this event occurs before or after the condensation of the final malonyl unit (Stoessl and Stothers, 1979). Furthermore, the identity of the required reductant is unknown; incubation of cell-free extracts from *P. vulgaris* with CHS substrates and either an NADH or NADPH generating system only resulted in the formation of the 5-hydroxyflavanone naringenin (Dixon and Bendall, 1978b).

Plants synthesise fatty acids via a type II (non-aggregated) FAS

system and it interesting to postulate that the enzyme synthesising 6'-deoxy chalcones employs activities very similiar to the separate β-ketoacyl ACP dehydrogenase and the β-hydroxyacyl ACP dehydratase of type II FAS as well as the 'condensing activity' of 6'-hydroxy CHS (similiar to β-ketoacyl synthase). Such a possibiltiy awaits further investigation.

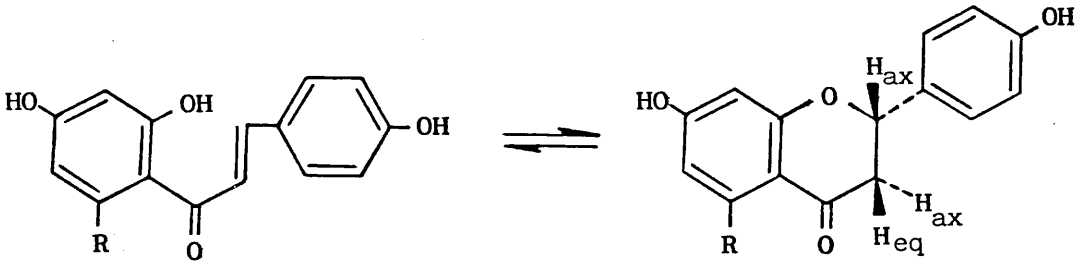
In spite of the important position of CHS as the first committal step in flavonoid/isoflavonoid biosynthesis, little work had been conducted on the regulation of CHS activity in plant cells producing phytoalexins. Rapid, transient increases in CHS activity have been shown to occur after PAL induction and slightly preceding phytoalexin accumulation during the synthesis of both phaseollin in *Phaseolus vulgaris* cell suspension cultures in response to the abiotic elicitor RNase (Dixon and Bendall, 1978b) and glyceollin in cotyledons of *Glycine max* in response to a biotic elicitor from *Phytophthora megasperma* f. sp. *glycinea* (Zähringer et al., 1978).

In *P. vulgaris* cell suspension cultures, levels of wall-bound phenolics increase in addition to increases in the levels of phaseollin (Dixon and Bendall, 1978a) and it is likely that PAL controls the overall flux of the phenylpropanoid pathway whilst CHS behaves as a secondary control element regulating the entry of material into the flavonoid/isoflavonoid branch.

Chalcones produced by CHS can be further metabolised in one of two ways depending upon the plant species. As has been pointed out earlier, substitution, in the form of glycosylation, can occur in mutants of *Callistephus chinensis*. Alternatively, chalcones can be isomerised to flavanones by the enzyme chalcone isomerase (CHI, EC. 5.5.1.6) which catalyses the isomerisation stereospecifically producing (-)(2S) flavanones (Reaction 5, see also Figure 1.4)(Hahlbrock et al., 1970a).

The enzyme has been purified from many sources including *Glycine max* (Moustafa and Wong, 1967; Boland and Wong, 1975), *Petroselinum hortense* (Hahlbrock et al., 1970b), *Cicer arietinum* (Hahlbrock et al., 1970b) and *Phaseolus aureus* (Hahlbrock et al., 1970a). The substrate specificities of the enzyme generally reflect the aromatic substitution patterns of the flavonoids found in the particular plant source (Hahlbrock et al., 1970b). Thus, the enzyme from *Petroselinum hortense* is specific for 6'-hydroxy chalcones (corresponding to 5-hydroxy, flavonoid numbering) and the plant only accumulates 5-hydroxylated flavonoid derivatives.

Reaction 5



R = H : Isoliquiritigenin

R = OH : 2',4,4',6'-Tetrahydroxy-chalcone

R = H : Liquiritigenin

R = OH : Naringenin

Chemical models for the synthesis of isoflavonoids had predicted the direct formation of isoflavones from chalcones (Grisebach, 1967) and this implied that CHI had no direct role in the biosynthesis of isoflavonoid phytoalexins. However, recent work by Hagmann and Grisebach (1984) has detected an 'isoflavone synthase' in microsomal preparations from elicitor-challenged *Glycine max* cell suspension cultures which catalyses the rearrangment of a flavanone to an isoflavone (see Section 1.2c-3). This new evidence has firmly established the role of CHI during isoflavonoid

phytoalexin biosynthesis. CHI activity increases in induced cell suspension cultures of both *Glycine max* (Partridge and Keen, 1977) and *Phaseolus vulgaris* (Dixon and Bendall, 1978b; Dixon and Lamb, 1979) but the slow response and high basal level might suggest the lack of any key regulatory role for this enzyme in isoflavonoid phytoalexin biosynthesis.

1.2c-3 Formation of Isoflavones

Incorporation studies in several plant species have demonstrated conclusively the intermediacy of isoflavones in the formation of isoflavonoid phytoalexins. For example, formononetin was incorporated into several phytoalexins including medicarpin and sativan in *Medicago sativa* (Dewick and Martin, 1979b) and daidzein was a good precursor of coumestrol in *Phaseolus aureus* (Dewick et al., 1970).

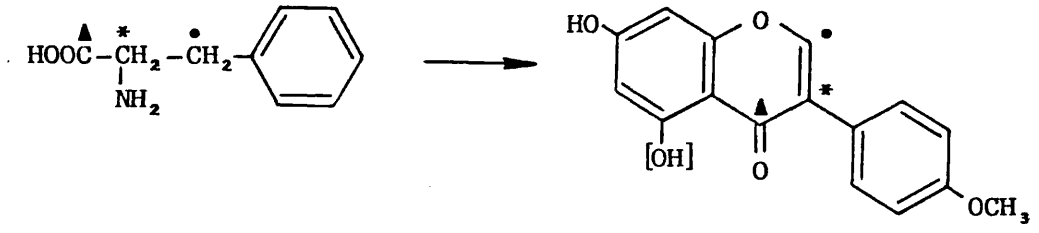
Early evidence predicted the formation of isoflavones from chalcones (Wong, 1968; Keen et al., 1972). Keen isolated radiolabelled daidzein from hypocotyls of *Glycine max* infected with *Phytophora megasperma* f. sp. *glycinea* after feeding [9-^{14}C]-2',4,4'-trihydroxy-chalcone. However, recent evidence has demonstrated for the first time the enzymatic rearrangement of the flavanones naringenin and liquiritigenin to the isoflavones genistein and daidzein respectively in *Glycine max* (Hagmann and Grisebach, 1984). Importantly, the substrates for the 'isoflavone synthase' were the (-)(2S) flavanones, the exact stereospecific conformation produced by the preceding enzyme, CHI (see Section 1.2c-2).

The unique aryl migration step catalysed by the 'isoflavone synthase' was demonstrated originally by labelling experiments in *Cicer arietinum* where DL-phenylalanine labelled in either the 1,2 or 3 position was incorporated into formononetin and biochanin A (Figure 1.3, Grisebach, 1959; Grisebach and Doeur, 1959).

The 'isoflavone synthase' characterised by Hagmann and Grisebach

(1984) required NADPH and O_2 as cofactors for the reaction and a hypothetical pathway from (-)(2S)-flavanone to isoflavone involving keto-enol tautomersim of the flavanone and the formation of an epoxide intermediate was proposed.

Figure 1.3 Labelling Evidence for Aryl Migration During the Formation of Isoflavones



1.2c-4 Further Elaboration of the Isoflavonoid Skeleton: Biosynthesis of Isoflavones, Pterocarpans and Coumestans (Figure 1.4)

In *Phaseolus vulgaris* further elaboration of the isoflavonoid skeleton to phytoalexin compounds proceeds by the two separate 5-hydroxy and 5-deoxy routes. However, the first two steps of both pathways are directly analogous. The isoflavone derivative is first 2'-hydroxylated, presumably by an isoflavone 2'-hydroxylase and then the isoflavone is reduced to an isoflavanone by a reductase. Neither of these two enzymes has been characterised to date although both activities would probably be microsomal. In kievitone formation these reactions represent the sequence genistein → 2'-hydroxygenistein → dalbergioidin; prenylation of dalbergioidin at C-8 produces kievitone (Woodward, 1980a). In phaseollin formation the sequence is represented by daidzein → 2'-hydroxydaidzein → 2',4',7-trihydroxyisoflavanone. Pterocarpan formation is

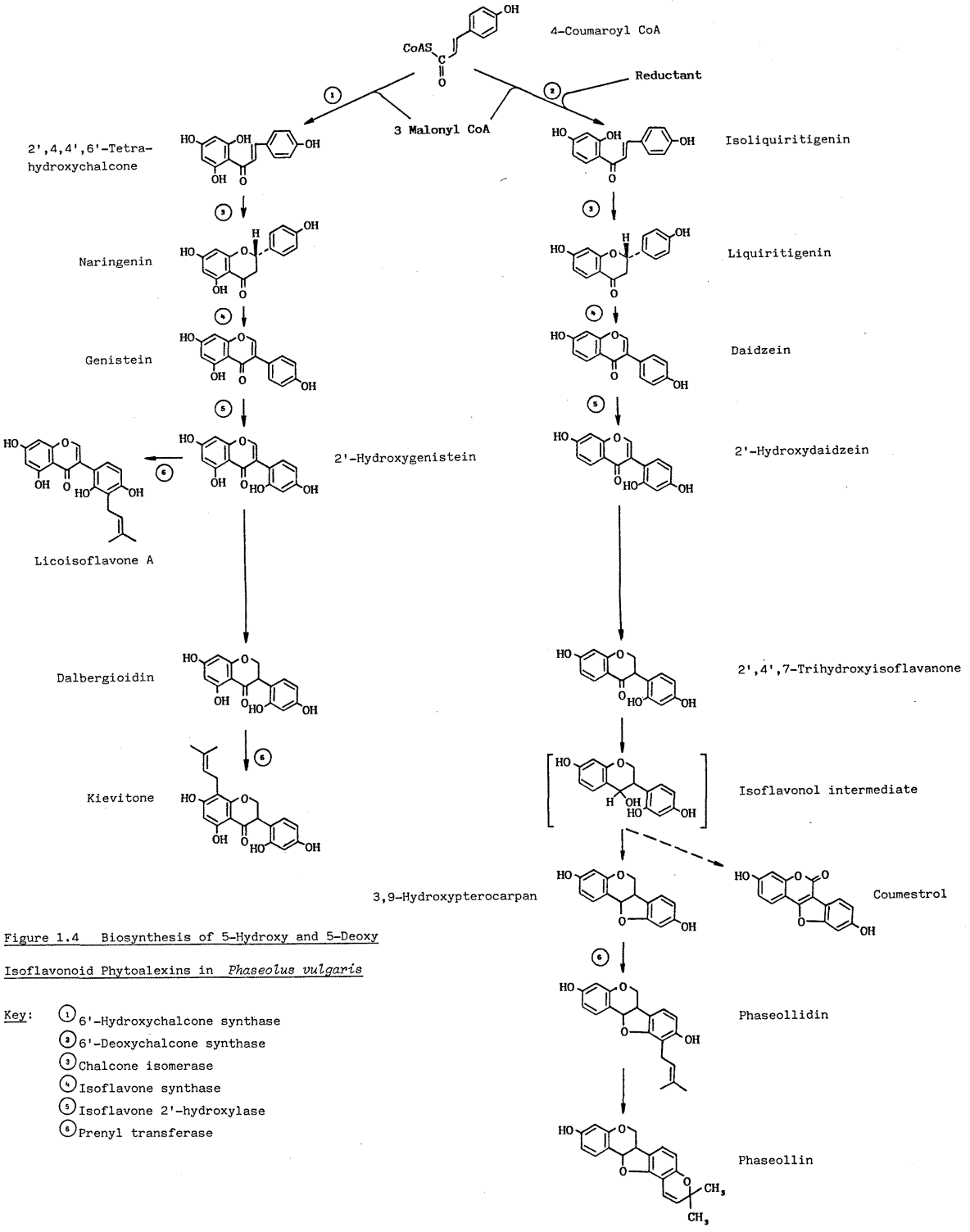


Figure 1.4 Biosynthesis of 5-Hydroxy and 5-Deoxy Isoflavonoid Phytoalexins in *Phaseolus vulgaris*

Key:
1. 6'-Hydroxychalcone synthase
2. 6'-Deoxychalcone synthase
3. Chalcone isomerase
4. Isoflavone synthase
5. Isoflavone 2'-hydroxylase
6. Prenyl transferase

thought to occur via an unstable isoflavonol intermediate formed by reduction of the C-4 carbonyl group of the trihydroxyisoflavanone. This intermediate then loses water and cyclises to form the pterocarpan. Labelling studies have shown that pterocarpans and pterocarp-6a-enes are not incorporated into coumestrol. However, 2'-hydroxyisoflav-3-ene which would result from the dehydration of the isoflavonol intermediate is readily incorporated and it is proposed that it is at this point that the biosynthesis of phaseollin and coumestrol diverge (Dewick, 1977; Martin and Dewick, 1978; Dewick and Martin, 1979a; 1979b). Prenylation of the pterocarpan at C-10 gives rise to phaseollidin, and phaseollin is formed by the cyclisation of the prenyl side chain to form a dimethylpyrano ring (Woodward, 1980a; Dewick and Steele, 1982).

Many isoflavonoid derivatives, both induced and constitutive, have a C_5 prenyl group attached to their aromatic rings. There may be one free prenyl group as in kievitone (Smith et al., 1973), phaseollidin (Burden et al., 1972; Ingham, 1978), luteone (Harborne et al., 1976) or licoisoflavone A (Woodward, 1979a); two free prenyl groups as in the di-prenyl genisteins (Singhal et al., 1980) or prenyl groups cyclised to hydroxy groups resulting in the 2,2-dimethylpyrano ring structure of, for example, phaseollin (Rathmell and Bendall, 1971) and glyceollin (Keen et al., 1972; Keen and Kennedy, 1974). Prenylated coumestans also occur, an example being psoralidin (6-isopentenylcoumestrol) which occurs in *Phaseolus lunatus* (Rich et al., 1977).

During the biosynthesis of both the 5-hydroxy and 5-deoxy classes of phytoalexin in *Phaseolus vulgaris*, prenylation can occur at either the isoflavone (licoisoflavone A), isoflavanone (kievitone) or pterocarpan (phaseollidin) stage. This clearly raises the important question of substrate specificity of the prenyl transferase(s) (Woodward, 1980b). In addition to the above stages of prenyl addition, there is also the

question of whether the enzyme(s) are specific for the two distinct A-ring hydroxylation patterns encountered (5-hydroxy or 5-deoxy), or the position (3'-,6- or 8-, isoflavone numbering) of insertion of the prenyl group.

Plant enzymes transferring prenyl residues to aromatic rings have been described in *Ruta graveolens*, where a dimethylallylpyrophosphate (DMAPP): umbelliferone dimethylallyl transferase is involved in the biosynthesis of furanocoumarins (Ellis and Brown, 1974); in elicitor-treated hypocotyls of *Glycine max*, where a DMAPP:trihydroxypterocarpan dimethylallyl transferase catalyses the penultimate step in the biosynthesis of the glyceollins (Zähringer et al., 1979) and in *Lupinus albus* where a DMAPP: isoflavone dimethylallyl transferase catalysed the prenylation of of both genistein and 2'-hydroxygenistein to produce wighteone and luteone respectively (Schröder et al., 1979). The prenyl moeity arises by the classical mevalonic acid pathway as demonstrated by the incorporation of [2-^{14}C]-mevalonic acid into glyceollin (Zähringer et al., 1979).

The prenyl transferases from both *R. graveolens* and *G. max* appeared to be located in the plastids and the activity of the *G. max* enzyme is induced in response to elicitor treatment (Zähringer et al., 1979). However, treatment of lupin hypocotyls with a fungal elicitor did not increase the dimethylallyl transferase activity; this is in keeping with the observation that both wighteone and luteone occur as constitutive isoflavonoids in this plant (Harborne et al., 1976).

1.3 Scope of the Present Thesis

A major goal of research in molecular plant pathology is to understand the regulation of plant responses at the gene level; in order to achieve this aim, a detailed understanding of the enzymological aspects of responses such as phytoalexin synthesis is required.

In *Phaseolus vulgaris* a variety of isoflavonoid phytoalexins

accumulate in response to elicitation and these can be divided into two classes, namely 5-hydroxy and 5-deoxy. The point at which the biosynthetic pathways leading to the two classes of compound diverge is thought to be at the level of chalcone formation; however, the regulatory and mechanistic details of this event are not clearly understood. It is therefore important to develop an experimental system in which both pathways are expressed in order to enable investigations to be made in this area.

CHS from all sources so far investigated catalyses the synthesis of 6'-hydroxychalcones; these would ultimately form 5-hydroxy substituted isoflavonoid phytoalexins such as kievitone. However, no enzyme system has been isolated to date which is capable of catalysing the formation of 6'-deoxychalcones, as would be required during the biosynthesis of 5-deoxy isoflavonoid phytoalexins such as phaseollin.

A detailed study of the induction, control, physical features and requirements of bean CHS (6'-hydroxy) may produce a clearer understanding of the synthesis of 5-hydroxy isoflavonoid compounds. It is also possible that characterisation of this enzyme may help in attempts to locate 6'-deoxy CHS activity.

It is probable that the synthesis of chalcones represents the most important control point in isoflavonoid phytoalexin biosynthesis. However, enzymes catalysing reactions subsequent to chalcone formation may also be important and detailed investigations into the induction and properties of the next enzyme, CHI, will also provide valuable information about the regulation of isoflavonoid phytoalexin production; chalcone isomerase at present represents the latest enzyme in the phytoalexin pathway which is amenable to analysis *in vitro*.

The present thesis attempts to provide information concerning the above points by considering a) the patterns of phytoalexin induction in bean tissues in relation to the suitability of a system for studying

induction of both 5-hydroxy and 5-deoxy isoflavonoids in the same tissue b) the mechanism of 6'-hydroxy and 6'-deoxy chalcone synthesis as determined by ^{13}C-labelling and nuclear magnetic resonance studies c) the isolation and characterisation of bean CHS and CHI and d) possible procedures for the demonstration of 6'-deoxy CHS activity *in vitro*.

Detailed discussions of each of these related areas is provid ed in the final sections of the individual chapters. A final summary and discussion, with pointers to future approaches, appears in the last chapter of the thesis.

CHAPTER 2

General Materials and Methods

2.1 Growth of Plant Material

Cell suspension cultures of dwarf French bean (*Phaseolus vulgaris* var. Canadian Wonder) were initiated and maintained by regular sub-culture at 14 day intervals in a modified Schenk and Hildebrandt medium (Dixon and Fuller, 1976) (Table 2.1).

Cultures were grown at 25°C in a Gallenkamp model INR-401 orbital incubator at 110 rpm and illuminated by a single 30 W fluorescent tube approximately 0.5 m above the cultures. All cultures used in experiments were in the exponential growth phase (6-7 days after sub-culture).

Hypocotyls and cotyledons of *P. vulgaris* var. The Prince were obtained by germinating seed in boxes of moist vermiculite in the dark for 7 days at 25°C. In each experiment reported all data were derived from material from the same batch of seedlings.

2.2 Growth of Fungal Material

Cultures of *Colletotrichum lindemuthianum* (Commonwealth Mycological Institute (CMI), isolate IMI 112166) were maintained on a semi-solid glucose neo-peptone medium (Mathur et al., 1949) (Table 2.2) in a continuous black light incubator. For liquid cultures, conidia were removed from the mycelial mat by vigorous shaking with sterile distilled water, and transferred to 500ml batches of the Mathur medium (modified by the addition of 15g glucose/l) in 2 l conical

Table 2.1 Modified Schenk and Hildebrandt Medium used for *Phaseolus vulgaris* Callus and Cell Suspension Cultures (Dixon and Fuller, 1976)

Component	Concentration of Stock Solution		Stock/l	Final Molarity ("rounded up")
Major salts:				
KNO_3	1.00M		25.00ml	25.00mM
$MgSO_4.7H_2O$	0.10M		15.00ml	1.50mM
$NH_4H_2PO_4$	0.10M		25.00ml	2.50mM
$CaCl_2.2H_2O$	0.10M		15.00ml	1.50mM
Minor salts:				
$MnSO_4.4H_2O$	1.32g	per liter	10.00ml	60.00μM
H_3BO_3	0.50g			125.00μM
$ZnSO_4.7H_2O$	0.10g			3.50μM
KI	0.10g			6.00μM
$CuSO_4.5H_2O$	0.20g			0.80μM
$Na_2MoO_4.2H_2O$	0.01g			0.40μM
$CoCl_2.6H_2O$	0.01g			0.40μM
$FeSO_4.7H_2O$	1.50g	per liter	10.00ml	55.00μM
Na_2EDTA	2.00g			55.00μM
Organic supplement:				
Thiamine-HCl	50.00mg	per 100 ml	10.00ml	15.00μM
Nicotinic acid	50.00mg			40.00μM
Pyridoxin-HCl	5.00mg			2.50μM
myo-Inositol	Solid		1.00g	5.50μM
Sucrose	Solid		30.00g	88.00μM
Growth regulators:				
2,4-D	1.00mM		2.00ml	2.00μM
4-Chlorophenoxy-acetic acid	1.00mM		10.00ml	10.00μM
Kinetin	1.00mM		0.50ml	0.50μM

pH 5.8-5.9. Agar is added at 6g/l for semi-solid media.

flasks. These were incubated on an orbital incubator at 120 rpm for 14 days at 25°C.

Table 2.2

Medium for Growth of Liquid Cultures of *C. lindemuthianum*

Component	g/l
D-glucose	15.00
$MgSO_4.7H_2O$	1.23
KH_2PO_4	2.72
Bacterial grade peptone	2.00

For semi-solid medium; as above but with 2.8g glucose and 20g agar/l.

2.3 Preparation of Fungal Elicitor

Mycelium of *C. lindemuthianum* from liquid cultures was harvested on four layers of muslin cloth. The collected material was washed repeatedly with distilled water and then homogenised in a Waring blendor for 3 min. with 5ml water/g fungal mycelium. The resulting homogenate was filtered on paper by suction through a Buchner funnel and the collected solid re-homogenised as above. After washing the solid with distilled water until the filtrate was clear it was further washed with $CHCl_3$:MeOH (1:1, 25ml/g solid) and then Me_2CO (10ml/g solid). This procedure resulted in a white, cell wall material which was air dried until fluffy and then stored at -20°C. The elicitor solution was prepared by autoclaving the cell wall material with 100ml water/g walls at 120°C for 30 min. (Anderson-Prouty and Albersheim, 1975). The autoclaved mixture was filtered through sintered glass (Whatman # 3), clarified by centrifugation and dialysed for 24h against three 10 l changes of distilled water. The non-diffusible material was

concentrated approximately 5-fold in vacuo at 50°C and was stored at -20°C until required.

2.4 Methods of Elicitor Application

(a) Samples of phytoalexins were routinely obtained from excised etiolated hypocotyl segments placed in petri dishes containing filter paper discs soaked with a 1mM solution of the abiotic elicitor $HgCl_2$. Accumulated phytoalexins were extracted after 7 days by the methods described in Sections 2.5 and 4.3 .

(b) In experiments investigating elicitor-mediated enzyme induction in cell suspension cultures, or enzyme induction and phytoalexin accumulation in hypocotyls or cotyledons, plant material was treated with the biotic elicitor heat-released from the cell walls of *C. lindemuthianum* as described below.

(1) 40ml batches of *P. vulgaris* cell suspension culture (20ml packed cell volume) were treated for 7h with elicitor at a final concentration of 20µg glucose equivalents/ml culture, under normal culture conditions. Controls were treated with an equal volume of sterile distilled water in place of elicitor solution. Each experiment was performed with cells from the same culture batch, which, after treatment above, were harvested by suction filtration and frozen in liquid N_2 before storage at -20°C.

(2) Excised etiolated hypocotyl or cotyledon tissue of *P. vulgaris* was first prepared by removing the top 2-3mm of cells with a scalpel. At least 5 cotyledons or 2cm hypocotyl segments were used per treatment. After placing the prepared tissue in petri dishes containing moistened filter paper the cut surfaces were treated with elicitor solution (20µg glucose equivalents/ml, 50µl solution/cotyledon or hypocotyl section). The petri dishes were incubated at 25°C in a

dark sealed container lined with moistened Whatman 3mm filter paper to provide a humid enviroment. Tissue samples were harvested by freezing in liquid N_2 before storage at -70°C.

2.5 Extraction, Identification and Estimation of Phytoalexins

Plant material was weighed and then homogenised in a pestle and mortar with 20ml EtOH containing 200nmol of 2',4,4'-trimethoxy-chalcone as internal standard. The extract was filtered through a sintered glass funnel (Whatman # 3) and the residue re-extracted with 20ml pure EtOH. After combining the two extracts the solvent was removed *in vacuo* and the resulting residue was dissolved in 10ml distilled water prior to partitioning two times against 20ml aliquots of Et_2O. The pooled Et_2O fractions were reduced *in vacuo* to approximately 0.5ml. This was then transferred to a small vial and the remaining solvent evaporated under a stream of N_2. The residue was then taken up in a known volume of EtOH (usually 100µl) and 50µl of this solution was spotted as a 1.5cm band onto a TLC plate (Machery Nagel, silica gel G/UV_{254}). The plate was developed in an equilibrated tank with $CHCl_3$:EtOH (100:3) as solvent and after drying, the separated compounds were visualised under short-wave (254nm) ultraviolet light. The separated compounds were scraped off the TLC plates and eluted from the silica with 1.0ml EtOH. An equal area of silica powder was eluted from an undeveloped chromatogram and used as a blank for each compound to compensate for the background absorption of the fluorescent marker.

Spectra were recorded on a Unicam SP 1800 scanning spectrophotometer, and compounds were identified and quantitated on the basis of characteristic shape and λ_{max} of the spectrum using reported extinction coefficients (Bailey and Burden, 1973).

Samples of phaseollin, kievitone and coumestrol were further purified by Sephadex LH-20 column chromatography (Section 4.3c, method A), for use in enzyme inhibition experiments (Sections 5.8l and 6.9e).

Amounts of unidentified compounds are expressed on an absorbance units/g fresh weight basis and all final values are corrected for the % recovery of the internal standard. In a preliminary experiment the % recoveries of the trimethoxychalcone, phaseollin and kievitone were 56.6, 54.8 and 55.1 % respectively.

The properties of unidentified isoflavonoid compounds were investigated in more detail after the compounds had been purified further on silica gel TLC plates developed in either toluene:$CHCl_3$:Me_2CO (40:25:35 by volume) or $CHCl_3$:EtOH (100:5 or 100:7).

A comparison of spectral shifts which occur characteristically when isoflavonoid compounds are treated with certain chemical reagents (see Section 2.6) and co-chromatography with authentic samples led to the identification of two previously unidentified compounds (Chapter 3). Several compounds were only partially characterised by these methods. All results, including additional data based on the use of a chromogenic spray for the detection of phenolic compounds, are presented in tabular form in Chapter 3 (Table 3.1).

2.6 Chemical Reagents Used for the Identification of Isoflavonoid Phytoalexins

2.6a Ethanolic $AlCl_3$

This was prepared by the cautious addition of 0.5g $AlCl_3$ to 5ml EtOH. Undissolved solid was removed by centrifugation (Jobling 320 microcentrifuge) and 50µl of the resulting solution was added to both sample and blank cuvettes during spectrophotometric analysis. $AlCl_3$ is used to detect free 5-hydroxyl groups and the degree of batho-

chromic shift is characteristically different for isoflavones (10-14nm) and isoflavanones (20-24nm)(Bailey and Burden, 1973).

2.6b Anhydrous NaOAc

Solid NaOAc (approx. 1mg) was added directly to sample and blank cuvettes. The NaOAc detects free 7-hydroxyl groups by causing them to ionize, and gave 10-20nm bathochromic shifts in the UV spectra of 7-hydroxy isoflavonoids (Mabry et al., 1970).

2.6c Diazotised 4-Nitroaniline (D4NA)

D4NA reagent was prepared by dissolving 0.7g 4-nitroaniline in 9ml of d 1.19 HCl and then making this solution up to 100ml with distilled water. 4ml of this stock solution was diazotised by adding it drop by drop to 5ml of an ice-cold 1% (w/v) solution of $NaNO_2$. The mixture was diluted to 100ml with ice-cold distilled water and was used immediately as a spray for thin layer chromatograms. Phenolic compounds develop a yellow to orange colour within 5 min. of spraying with a more intense colouring being associated with increasing numbers of phenolic groups (Mabry et al., 1970).

2.7 - 2.9 Source of Chemicals

2.7a Purchased Chemicals

[U-^{14}C] Sodium acetate (58μCi/μmol), [2-^{14}C] malonyl CoA (59mCi/mmol) and sodium [^{14}C] bicarbonate (58mCi/mmol) were obtained from the Radiochemical Centre, Amersham, UK. [1,2-$^{13}C_2$] sodium acetate (90% labelled at each C atom) was obtained from MSD Isotopes, Germany, through Cambrian Gases, Croydon, UK. Naringenin, cerulenin, CoA, unlabelled malonyl CoA, aminohexyl-Sepharose 4B, 4-nitrobenzoylazide, 4-coumaric acid, caffeic acid, ferulic acid, NADH, NADPH, ascorbate, D-glucose-6-phosphate

and glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co., Poole, Dorset, UK. Benzaldehyde and hydroxyacetophenone derivatives used in the synthesis of chalcones and flavanones (Section 2.9, Table 2.4) were obtained from Aldrich Chemical Co., Gillingham, Dorset, UK.

All other reagents used were 'Analar' grade obtained from commercial outlets.

2.7b Gifts

Genistein and 3,9-dihydroxypterocarpan were gifts from Dr. P.M. Dewick, Nottingham, UK; 2'-hydroxygenistein and dalbergioidin were gifts from Dr. S. Antus, Budapest, Hungary; eriodictyol and homoeriodictyol were gifts from Dr. W. Heller, Freiburg, Germany and triacetic acid lactone was a gift from Dr. P. Dimroth, Munich, Germany.

2.8 Preparation of Hydroxycinnamoyl CoA Thiol Esters

The CoA esters of 4-coumaric, caffeic and ferulic acids were prepared via the N-hydroxysuccinimide ester according to the method of Stöckigt and Zenk (1975) and were purified from unreacted CoA by column chromatography on Sephadex G-10 (Lindl et al., 1973). Experimental details for the preparation of 4-coumaroyl CoA are given below and are essentially those used for caffeoyl and feruloyl CoA.

2.8a 4-Coumaroyl N-Hydroxysuccinimide Ester

4-Coumaric acid (2.643g) was dissolved in 60ml of warm (50°C) EtOAc. After cooling to 30°C, N-hydroxysuccinimide (1.725g) and dicyclohexylcarbodiimide (3.508g) were added and the mixture shaken and left to stand for 24h. After this period the dicyclohexylurea precipitate was filtered off and the filtrate partitioned against 1M $NaHCO_3$ (2 x 100ml). The EtOAc phase was dried over anhydrous Na_2SO_4, and then evaporated

in vacuo. The extract was taken up in Me_2CO, applied to 0.75mm silica gel G-HR thin layer plates and developed in equilibrated tanks with $CHCl_3$: MeOH as solvent (20:1,or 4:1 for the caffeoyl and feruloyl derivatives). The ester was visualised as a yellow fluorescent band under short-wave (254nm) UV light (R_f 0.65) and recovered by elution with Me_2CO.

Table 2.3

UV-Absorption Data for Hydroxycinnamoyl-CoA Derivatives (Stöckigt and Zenk, 1975)

CoA Derivative	λ_{max}	λ_{min}	log ε
4-Coumaroyl	254,333	285	4.32
Caffeoyl	254,346	287	4.25
Feruloyl	254,346	287	4.28

2.8b 4-Coumaroyl CoA

N_2 gas was bubbled slowly through 2.5ml distilled water whilst CoA (100mg), 4-coumaroyl-N-hydroxysuccinimide ester (63.3mg) and $NaHCO_3$ (210mg) were added. [Note: excess Me_2CO caused $NaHCO_3$ to precipitate out of solution]. Me_2CO was added dropwise until the reactants had dissolved. The mixture was kept at 4°C for 24h after which time the organic solvent was evaporated under a stream of N_2. The remaining aqueous phase was desalted with Dowex 50W-X8 (3g, 10M equivalents), extracted with EtOAc (3 x 20ml) and freeze-dried. The lyophilized material was dissolved in 3ml of HCO_2H (50mM) and applied to a Sephadex G-10 column (72 x 1.5cm) which was eluted with HCO_2H (50mM) at a flow rate of 150ml/h. 3.8ml fractions were collected and measured for absorbance at both 260nm (λ_{max} CoA) and also at the λ_{max} of the CoA thiol ester (Table 2.3).

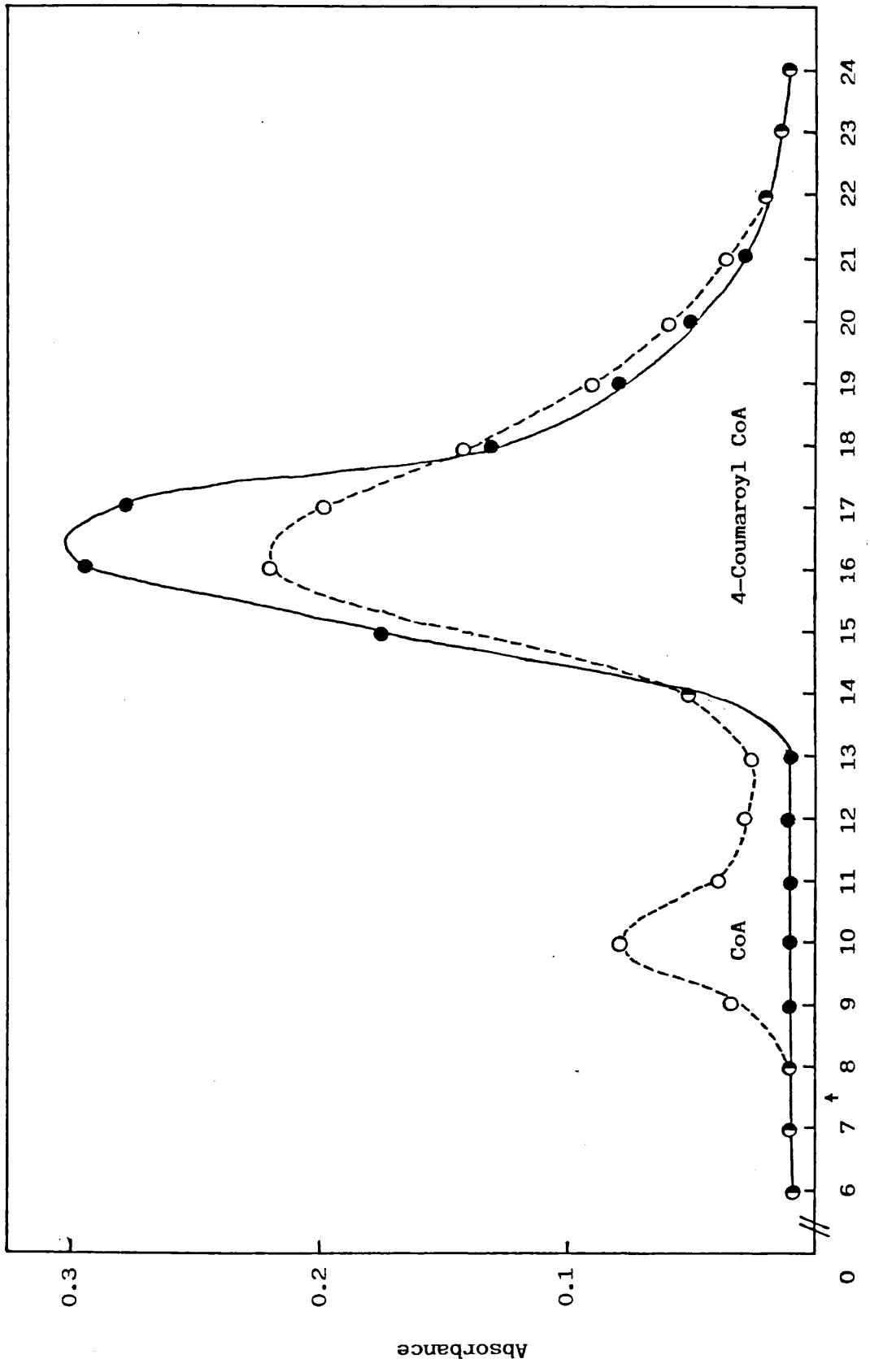


Figure 2.1 Purification of 4-coumaroyl CoA from free CoA by gel-filtration on Sephadex G-10.
(O---O) Absorbance at 260 nm; (●—●) Absorbance at 333 nm; (↑) Void volume.

Those fractions containing the purified thiol ester (Figure 2.1) were combined, freeze-dried and then stored at -20°C. The prepared thiol esters had identical UV spectra to those shown in Table 2.3.

2.9 Synthesis of Chalcones and Flavanones

Most chalcones were prepared by alkaline condensation of the appropriate hydroxyacetophenone with the appropriately substituted benzaldehyde derivative by the method of Geissman and Clinton (1946). Structures of the chalcones and flavanones synthesised for use in the present work are shown in Table 2.4 The purity of synthesised compounds was checked by TLC.

2.9a Basic Method

To a cold (-5°C) suspension of equimolar quantities of the aldehyde and ketone in EtOH (2ml/g ketone) was added, with shaking, a cold (-5°C) 60% solution of KOH (14ml/g ketone). The air in the flask was replaced with N_2 gas and the flask was then securely stoppered and allowed to stand at room temperature for one week with occasional shaking. After this time an equal volume of degassed water was added and the mixture acidified with 6N HCl (temperature not above 3°C). The resulting precipitate of chalcone was filtered off and recrystallised 3 times from aqueous EtOH.

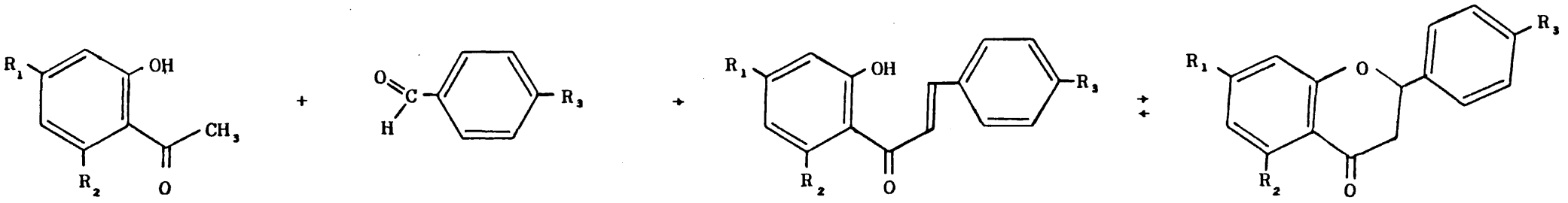
2.9b 2',4,4',6'-Tetrahydroxychalcone

The above compound was prepared by treating naringenin at 100°C for 2 min. with 50% aqueous KOH. The mixture was acidified with 6N HCl and the yellow chalcone recrystallised from aqueous EtOH (Moustafa and Wong, 1967).

2.9c 4',7-Dihydroxyflavanone

The above compound was prepared by treating a solution of

Table 2.4 Structure and Synthesis of Chalcones and Flavanones



	Hydroxyacetophenone	+	Benzaldehyde	→	Chalcone	⇄	Flavanone
(a)	R_1= OH, R_2= H :		R_3= OH :				
	Resacetophenone	+	4-Hydroxybenzaldehyde	→	2',4,4'-Trihydroxychalcone (Isoliquiritigenin)	→	4',7-Dihydroxyflavanone (Liquiritigenin)
(b)			R_3= OMe :				
	Resacetophenone	+	4-Methoxybenzaldehyde	→	2',4'-Dihydroxy-4-methoxy-chalcone		
(c)	R_1= OMe, R_2= H :						
	2-Hydroxy-4-methoxy-acetophenone	+	4-Methoxybenzaldehyde	→	2'-Hydroxy-4,4'-dimethoxy-chalcone		
(d)	R_1= R_2= OMe :						
	2,4-Dimethoxy-acetophenone	+	4-Methoxybenzaldehyde	→	2',4,4'-Trimethoxychalcone		
(e)	R_1= $OCH_2C_6H_5$, R_2= H :						
	2-Hydroxy-4-benzyloxy-acetophenone	+	4-Methoxybenzaldehyde	→	2'-Hydroxy-4'-benzyloxy-4-methoxychalcone		
(f)					2',4,4',6'-Tetrahydroxy-chalcone	→	4',5,7-Trihydroxyflavanone (Naringenin)

isoliquiritigenin (1.3g in 50ml EtOH) with acid (4N HCl), which was added until the solution became turbid. The solution was cleared by the addition of a small amount of EtOH and refluxed over a water bath for 24h. Upon cooling the solution in ice, a light cream precipitate of liquiritigenin formed. A further precipitate was obtained by reducing the solvent in vacuo. The combined precipitates were washed with cold distilled water and recrystallised twice from aqueous EtOH (Nadkarni and Wheeler, 1938).

2.9d 2'-Hydroxy-4'-benzyloxy-4-methoxychalcone

The above compound was prepared by the basic method (Section 2.9a) after first synthesising 4'-benzyloxy-2'-hydroxyacetophenone. This was prepared by the following method.

2,4-Dihydroxyacetophenone (800mg), benzoyl chloride (665mg), dry K_2CO_3 (2g) and dry KI (100mg) were dissolved in 25ml dry DMF. The mixture was heated to 80°C, stirred for 2h and then poured into water (25ml). The precipitate was filtered off, washed and recrystallised from MeOH. Yield 880mg, m.p. 103-105°C lit. 110°C (Dewick and Ward,1978).

2.10 Enzyme Assays

2.10a Extraction and Assay of L-Phenylalanine Ammonia-Lyase (PAL) Activity

Approximately 1g of plant material was weighed out and homogenised with 6ml of 50mM Tris-HCl buffer pH 8.5 containing 42µmol 2-mercaptoethanol, and 0.1g of insoluble polyvinylpolypyrrolidone (PVP) in a chilled pestle and mortar. (The PVP is included to remove plant phenolics which may interfere with the assay). The homogenate was strained through four layers of muslin and then centrifuged at 15,000 x g_{av} for 30 min. at 4°C. Aliquots of supernatant (0.5ml) were incubated at 40°C with 2.5ml Tris-HCl buffer pH 8.5 containing 30µmol L-phenylalanine.

Each assay sample was read against its own blank which contained 0.5ml supernatant and Tris-HCl buffer pH 8.5 containing 30μmol D-phenylalanine. (D-phenylalanine is a substrate inhibitor of PAL and thus prevents any background reaction that may occur with endogenous L-phenyalanine in the supernatant). The absorbance of the reaction mixture was read at 290nm at 5,35 and 65 minutes after the start of the reaction, the increase in absorption being due to cinnamic acid production (Lamb and Rubery, 1976). The change in absorbance per hour was calculated for each sample and converted to a specific activity (μkat/kg protein) after protein determination (Section 2.11) (log ε cinnamic acid at 290nm = 4.0).

2.10b Extraction and Assay of Chalcone Isomerase (CHI) and Peroxidase Activity

The extraction procedure for CHI and peroxidase was essentially the same as that for PAL (Section 2.10a) except that the buffer used was 50mM KH_2PO_4, pH 8.0. This buffer contained 50mM KCN when used for assaying isomerase activity in extracts or fractions containing peroxidase. Apparent peroxidase activity (chalcone oxidation dependent upon endogenous H_2O_2 levels) was estimated by assay in the presence and absence of 50mM KCN; the rate in the presence of KCN (isomerase activity) was subtracted from the rate in the absence of the peroxidase inhibitor.

For determination of chalcone isomerisation or oxidation, 20μl of a 1.0mg/ml solution of 2',4,4'-trihydroxychalcone in EtOH was added to 2.5ml of 50mM KH_2PO_4, pH 8.0 (plus or minus KCN), followed by a suitable aliquot of enzyme (5-50μl). The decrease in absorption at 400nm was monitored at 30°C in a Unicam SP 1800 recording spectrophotometer against a blank containing no chalcone. The initial rate of disappearance of chalcone was used to estimate enzyme activity. When using the unstable 2',4,4',6'-tetrahydroxychalcone as substrate, the rate of non-enzymic

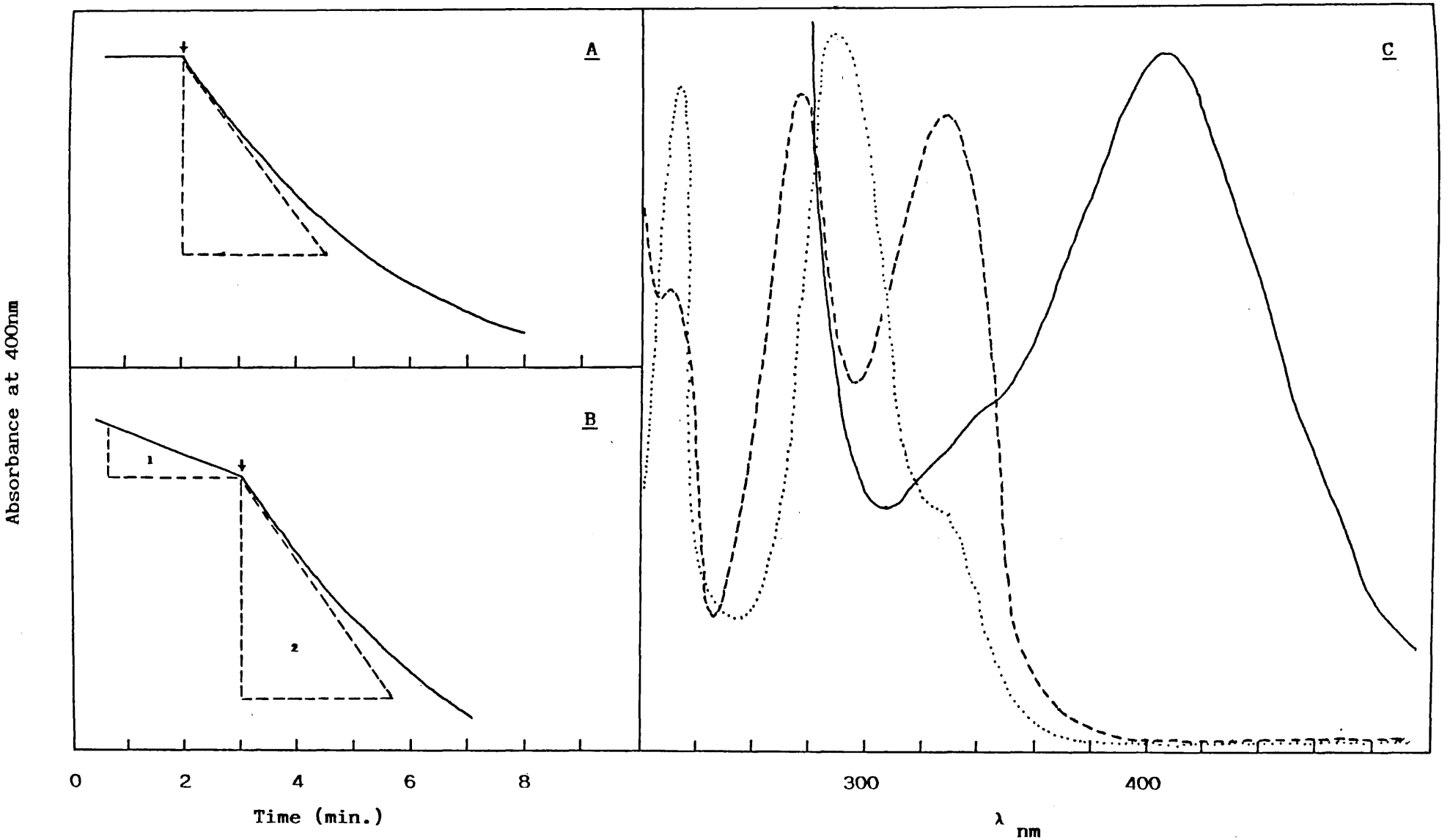


Figure 2.2 Enzymic isomerisation of chalcones. (A) Initial rate of decrease in absorbance at 400nm measured for isoliquiritigenin isomerisation; (B) Isomerisation of 2',4,4',6'-tetrahydroxychalcone; the non-enzymic rate (1) is subtracted from the initial rate obtained with enzyme (2). (↓) = addition of enzyme. (C) UV spectra for isoliquiritigenin (———), liquiritigenin (- - - -) and naringenin (·····) at pH 8.0. [Note: The UV spectrum of the tetrahydroxychalcone is not shown due to the instability of this compound in solution at pH 8.0 (λ_{max} 400nm). At pH 5.0, the spectrum is essentially the same as that of the trihydroxychalcone at pH 8.0 although the λ_{max} is shifted approximately 10nm hipsochromically due to the pH change].

isomerisation was first measured; this was then subtracted from the rate in the presence of enzyme (Figure 2.2). Specific activities (mkat/kg protein) were calculated in a similiar manner to those for PAL (Section 2.10a). (log ε 2',4,4'-trihydroxychalcone = 4.505; log ε 2',4,4',6'-tetrahydroxychalcone = 4.520. Both at 400nm, pH 8.0).

[Note: In the time course experiments (Section 3.3b) both CHS (Section 2.10c) and CHI were assayed from the same material samples extracted with 100mM KH_2PO_4 buffer, pH 8.0].

2.10c Extraction and Assay of Chalcone Synthase (CHS) Activity

The extraction procedure for CHS was essentially the same as that for PAL (Section 2.10a) except that the buffer used was 100mM KH_2PO_4, pH 8.0. In the course of optimising the assay conditions for CHS, the effect of Dowex AG 1-X8 was investigated. Treatment of enzyme supernatants from cotyledon and hypocotyl material with Dowex AG 1-X8 (equilibrated with 100mM KH_2PO_4) led to an increase of between 70% and 130% in CHS activity respectively. In supernatants from extracted cotyledon material, optimal increases in CHS activity were obtained when the weight of Dowex exceeded 0.4g/g fresh weight. However, with supernatants from hypocotyl extracts, optimal increases were obtained with just 0.1g Dowex/g fresh weight; levels exceeding this ratio caused a decrease from the optimal level (Figure 2.3). The activation caused by the Dowex treatment may be due to the removal of inhibitory flavonols (Rhodes, 1977).

In all assays of CHS reported, extracts of hypocotyl and cell suspension culture material were treated with 0.1g/g fresh weight of Dowex AG 1-X8. Cotyledon extracts were treated with 0.5g Dowex/g fresh weight.

(1) Total Chalcone Synthase Activity (Rapid Assay Method)

Reaction vials containing 2.1nmol [2-^{14}C] malonyl CoA (6.6×10^4 dpm) and 9.1nmol 4-coumaroyl CoA in 15µl dilute HCl, pH 3.5,

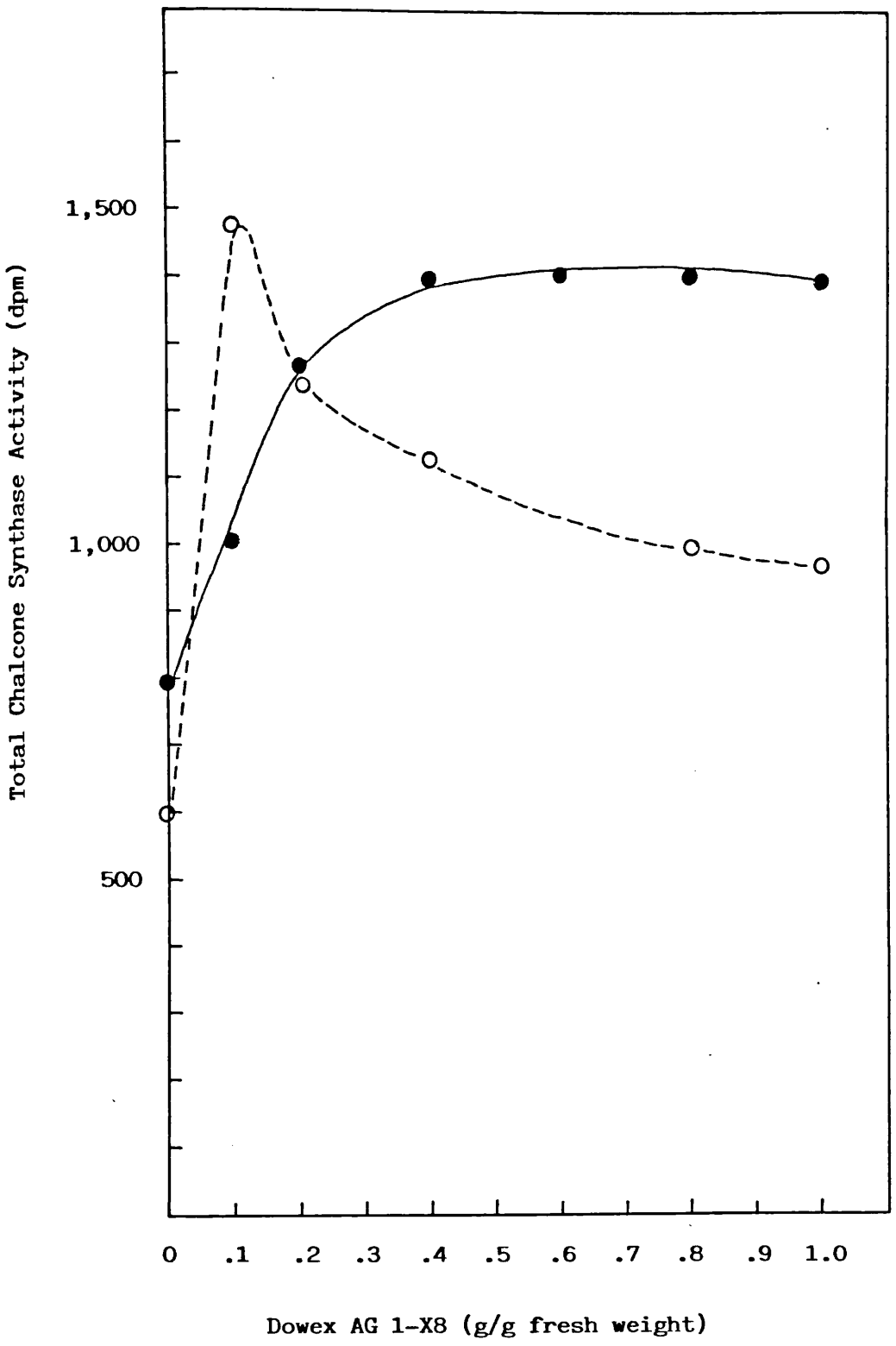


Figure 2.3 Activation of Chalcone Synthase Activity in Cotyledon and Hypocotyl Extracts of *Phaseolus vulgaris* Tissue by Dowex AG 1-X8. Zero weight Dowex represents original activity. (●——●) Cotyledon extract; (O---O) Hypocotyl extract, treated by stirring with increasing weights of Dowex AG 1-X8.

were stored frozen at -20°C. After thawing, 35µl of buffer containing 9.1µmol KH_2PO_4 and 140nmol dithiothreitol (DTT), pH 8.0 was added to each vial, the reaction mixtures were equilibrated to 35°C and enzyme preparation (50µl) was added. For blank controls boiled enzyme preparation was used. Reactions were terminated by the addition of 20µl naringenin in MeOH (1.5mg/ml) (Dixon and Bendall, 1978b). EtOAc (200µl) was then added to each reaction vial and the contents mixed for 1 min. on a vortex mixer. The organic and aqueous layers were separated by centrifugation (Jobling 320 microcentrifuge) for 2 min. An aliquot of the EtOAc phase (75µl) was transferred to a scintillation vial and the solvent removed under a stream of N_2. Radioactivity was determined by liquid scintillation counting using 10ml of a scintillation cocktail consisting of 5g PPO per 1 toluene (Counting efficency was determined by placing a known ammount of ^{14}C in a scintillation vial with 10ml of the scintillation cocktail. The counting effiency was determined to be 95% by this method)(Schroder et al., 1979). References to total activity for CHS therefore represent the conversion of malonyl CoA to EtOAc soluble products.

(2) Naringenin Formation

Aliquots (75µl) of the above EtOAc phase were spotted as 1.5cm bands onto 0.1mm thickness Machery-Nagel cellulose UV_{254} thin layer plates. Chromatograms were developed in an equilibrated tank with 30% HOAc. After drying and visualisation under short-wave UV light (254nm), the naringenin band (R_f 0.44) was cut out and radioactivity determined as above by placing the TLC strip in a scintillation vial with 10ml of the scintillation cocktail (as above)(Counting efficency was determined by spotting a known amount of ^{14}C onto a TLC strip and counting as above. The counting efficency was determined to be 57% by this method). Specific activites (µkat/kg protein) were calculated after protein determination (Section

2.11) taking into account the 3 moles of malonyl CoA required to produce 1 mole of naringenin.

(3) CO_2 Exchange Reaction

Aliquots (50µl) of enzyme were incubated at 35°C with 9.1µmol KH_2PO_4, 140nmol DTT, 30nmol malonyl CoA and 172nmol $NaH^{14}CO_3$ (10µCi) in a total volume of 100µl at pH 8.0. The reaction was terminated by the addition of 50µl of HOAc to the reaction vials (in a fume cupboard as $^{14}CO_2$ is released). An aliquot (50µl) of the reaction mixture was spotted as a 1.5cm band onto a 0.1mm thickness cellulose UV_{254} thin layer plate and the chromatogram developed in isobutyric acid:NH_3:H_2O (66:1:30). The developed chromatogram was cut into 0.5cm strips after drying and radioactivity determined as above.

2.11 Protein Determination

Protein was determined by the method of Lowry et al. (1951) as modified by Leggett-Bailey (1962). A standard curve was established for bovine serum albumin (BSA) using the Folin-Ciocalteau reagent. The blue colour which develops was measured at 750nm on a PYE UNICAM SP 500 spectrophotometer. The standard curve was linear for the range 0-100µg protein (Figure 2.4).

2.12 Carbohydrate Determination

The total carbohydrate content of elicitor preparations was estimated in terms of glucose equivalents by the α-napthol/sulphuric acid colourimetric method of Fuller and Northcote, (1956). A standard curve for glucose by this assay was linear for the range 0-50µg (Figure 2.5).

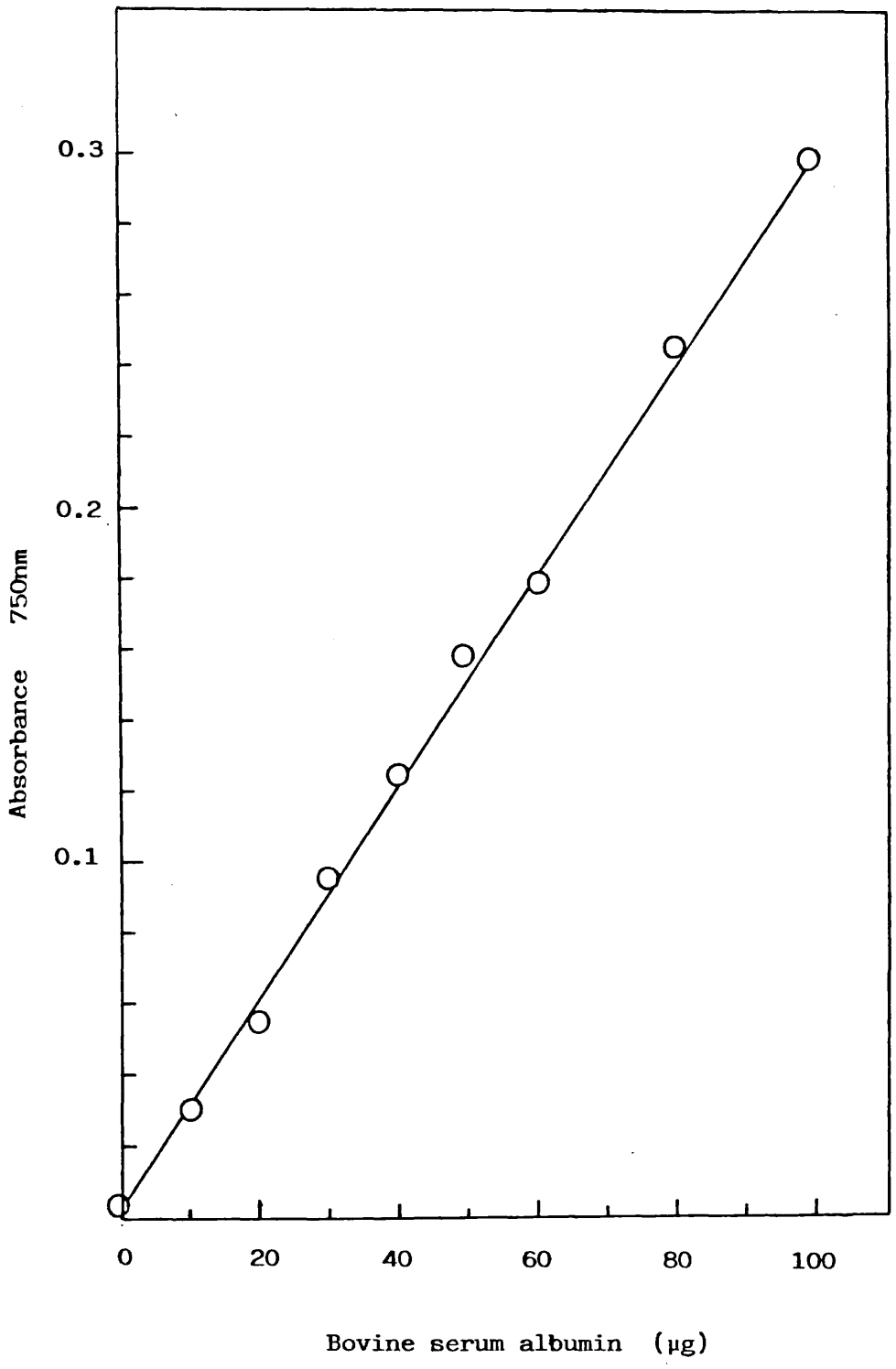


Figure 2.4

Standard curve for bovine serum albumin. After Lowry et al. (1951) as modified by Leggett-Bailey (1962).

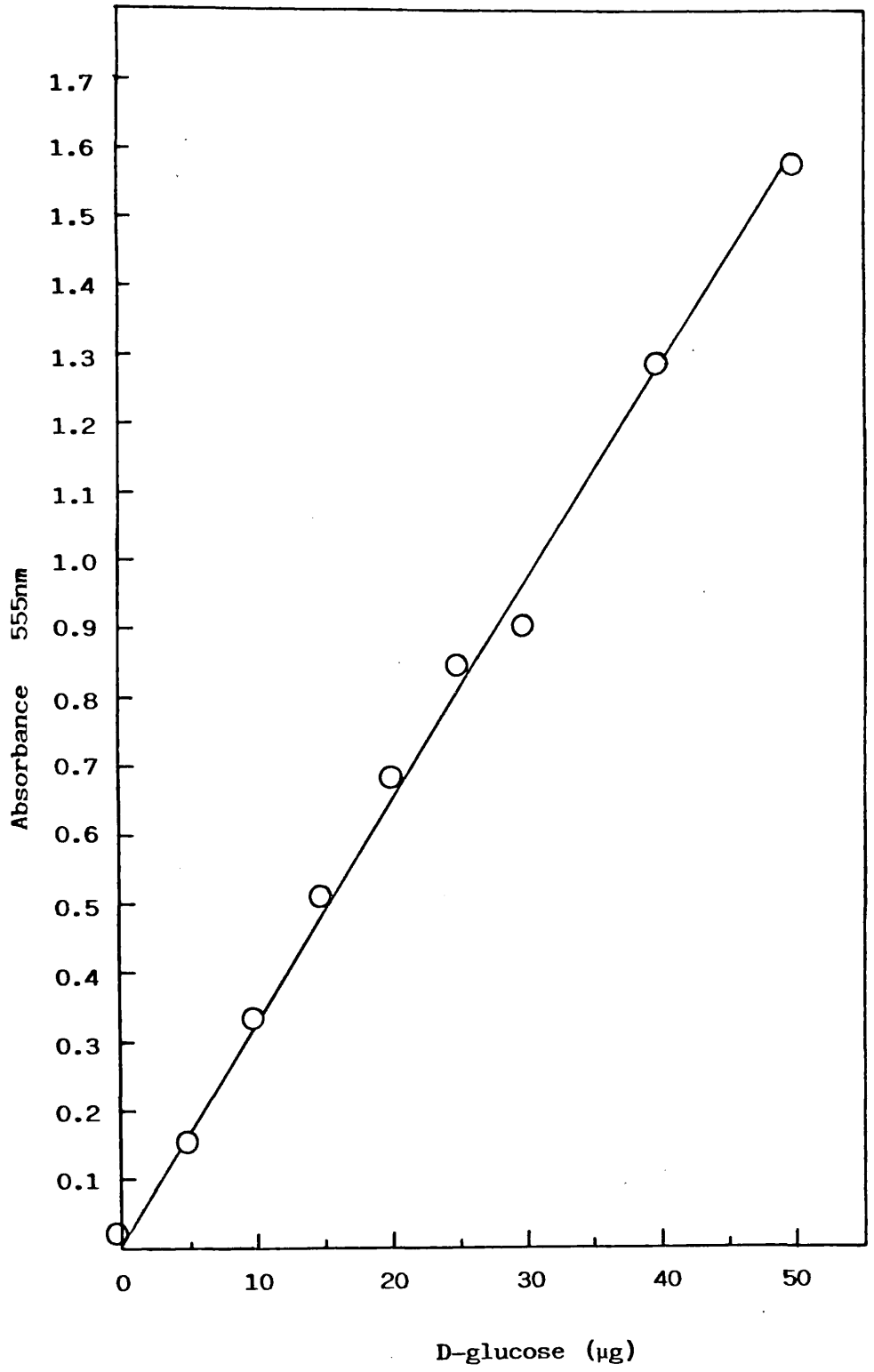


Figure 2.5

Standard curve for D-glucose. By the method of Fuller and Northcote (1956).

2.13 Preparation of Sepharose-Ligand Affinity Column

The ligand 2',4'-dimethoxy-4-hydroxychalcone was immobilized on a Sepharose gel for use in an affinity column for enzyme purification, by the method of Cuatrecasas (1970). This method links the ligand to the Sepharose matrix through a 4-aminobenamidohexyl 'spacer' side arm by the method described below.

2.13a Method

Aminohexyl-Sepharose 4B (1.0g) in 0.2M sodium borate pH 9.3 and 40% DMF (v/v), was treated for 1h at room temperature with 0.07M 4-nitrobenzoylazide. The substitution was complete, as judged by the loss of colour reaction with sodium 2,4,6-trinitrobenzenesulphonate (TNBS) (Section 2.13b). The 4-nitrobenzamidohexyl-Sepharose was washed extensively with 50% DMF and then reduced by reaction for 40 min. at 40°C with 0.1M sodium dithionite in 0.5M $NaHCO_3$, pH 8.5. The effectiveness of this procedure was demonstrated by the orange-red colour produced upon reaction with TNBS. The 4-nitrobenzamidohexyl-Sepharose derivative was washed with distilled water, placed in 0.5N HCl, and was then diazotized by treatment for 7 min. at 4°C with sodium nitrite. Immediately this treatment was completed the chalcone ligand (1g dissolved in saturated sodium borate: DMF, 1:1 v/v, pH 10.0) was added to the diazotised gel. The gel turned red on contact with the ligand and the mixture was stirred for 1h after which excess ligand was removed by washing the gel with 50% DMF. The gel prepared in this way was then washed with water, equilibrated in the appropriate buffer (buffer C, Section 6.3) and stored at 4°C.

2.13b Colour Test with TNBS

The degree of substitution of gel derivatives and of hydrazide

gels can be rapidly and conveniently estimated from the relative colour intensity of washed gels after treatment with TNBS. Saturated sodium borate (0.5ml) was added to a slurry (0.1ml in distilled water) of Sepharose gel. Three drops of a 3% aqueous solution of TNBS was added and the colour of the beads observed after 2h. Unsubstituted Sepharose appears yellow whilst unsubstituted hydrazide derivatives of the Sepharose gel formed a deep orange-red colour.

CHAPTER 3

Phytoalexin Accumulation and Enzyme Induction in Wounded *Phaseolus vulgaris* Cotyledons

3.1 Introduction

A variety of different isoflavonoid phytoalexins and related metabolites accumulate in tissues of *P. vulgaris* in response to biotic and abiotic treatments. In resistant interactions with a parasite, the types of phytoalexin and the speed with which they accumulate may be important criteria for determining the success of the resistant response (Chapter 1). Thus it is important to have a clear understanding of what takes place in stressed *P. vulgaris* tissues both from a chemical and biochemical viewpoint.

The identification of minor isoflavonoid components, coupled with the results of labelling experiments in closely related plants, has led to an understanding of the probable pathways involved in the formation of the phytoalexins kievitone and phaseollin from their initial biochemical precursors in *P. vulgaris* (Chapter 1).

Previously, work with *P. vulgaris* cell suspension cultures demonstrated that the accumulation of phytoalexins was preceeded by a rapid, transient induction of PAL together with associated changes in the activity of four other enzymes related to the synthesis of these compounds (Dixon and Bendall, 1978b).

The present work examines in detail both qualitative and quantitative aspects of the induction of isoflavonoid compounds in *P. vulgaris* cotyledons in response to wounding. The work focuses

on the patterns of accumulation of both 5-hydroxy and 5-deoxy isoflavonoids and the concommitant changes in the levels of enzymes associated with the synthesis of these compounds.

A clear understanding of the events which occur will enable further work to investigate the enzymological basis for the differentiation of the two pathways. In this regard, wounded cotyledons are perfectly suitable for the current investigations which concentrate on the chemical and biochemical rather than the physiological aspects of phytoalexin accumulation in *P. vulgaris*.

3.2 Materials and Methods

The growth and preparation of plant material, experimental conditions, extraction of phytoalexins, assay of enzyme levels and the source of all chemicals has been described in Chapter 2. The time course experiment was performed over a four day period.

3.3 Results

Initial experiments with both hypocotyl and cotyledon material showed that enzyme levels and phytoalexin accumulation could be induced by wounding alone, in the absence of other elicitors. Identical changes in phytoalexin levels were observed regardless of whether or not vigourous maintenance of aseptic conditions was observed.

3.3a Identification of Isoflavonoids

Several isoflavonoid derivatives in addition to phaseollin, kievitone and coumestrol were detected on thin-layer chromatograms of extracts from wounded cotyledons. Table 3.1 tabulates the compounds isolated together with UV data used in their characterisation and identification.

Table 3.1 Isoflavonoid Compounds in Induced *Phaseolus vulgaris* Cotyledon Tissue

#	R_f[1]	UV_{254} appearance	D4NA colour	Ultraviolet Spectral Data λ_{nm}			Comments	log ε	Reference
				EtOH	EtOH/$AlCl_3$	EtOH/NaOAc			
1	0.64	Purple absorbance	-	266min, 345max	-	-	2',4,4'-Trihydroxy-chalcone[2]	4.36	-
2	0.58	Purple absorbance	Yellow	253min, 280max, 285sh, 317.	No effect	-	Phaseollin	4.04	Bailey and Burden (1973)
3	0.39	Purple absorbance	Light orange	250min, 270max, 340sh.	252min, 280max, 360sh.	No effect	A 5-hydroxy isoflavone	-	-
4	0.37	Purple absorbance	Light orange	244min, 266max, 338sh.	245min, 270max, 312, 368sh.	248min, 278max, 338sh.	Free 7-hydroxy group	-	-
5	0.30	Blue fluorescence	Orange	260, 280sh, 347max.	No effect	351max.	A 5-Deoxy coumestan	-	-
6	0.21	Purple absorbance	Orange	242min, 259max, 271sh, 286sh, 336.	252min, 270max, 281sh, 312, 380sh.	242min, 260max, 271sh, 291sh, 340.	A 5-hydroxy isoflavone	-	-
7	0.18	Purple/blue fluorescence	Orange	343max.	No effect	363max.	Coumestrol	4.45	Bailey and Burden (1973)
8	0.15	Purple absorbance	Orange	238min, 262max, 286sh, 341.	270max, 314, 360.	274max, 344.	2'-Hydroxygenistein	4.14	Biggs (1975)
9	0.11	Purple absorbance	Orange	294max, 344sh.	316max, 360sh.	-	Kievitone	4.22	Bailey and Burden (1973)
10	0.08	Purple	Orange	288max.	313max, 364.	270, 310max.	Dalbergioidin	4.31	Farkas et al. (1971)

[1] $CHCl_3$:EtOH (100:3); [2] Internal Standard.

Compound 1 (Figure 3.1)

2',4,4'-Trimethoxychalcone was used as an internal standard to correct for variations in the extraction procedure. The R_f value was higher than that of any of the induced compounds due to its trimethoxy substitution pattern. Another advantage was the stability of the compound. However, one slight disadvantage was the relatively broad band produced on TLC plates.

Compound 2 (Figure 3.2)

This compound was positively identified as phaseollin from its characteristic UV spectrum (Bailey and Burden, 1973).

Compound 3 (Figure 3.3)

This compound was shown to be a 5-hydroxy isoflavone by the characteristic 10nm bathochromic shift observed in the UV spectrum following the addition of ethanolic $AlCl_3$. The relatively high R_f value indicates a low polarity due either to a low number of free hydroxyl groups or possibly a prenyl substituent.

Compound 4 (Figure 3.4)

The addition of ethanolic $AlCl_3$ had no effect on the spectrum of this compound which indicates the lack of a free 5-hydroxyl group. This compound could therefore be a 5-deoxy isoflavone. The 12nm bathochromic shift obtained with NaOAc indicates a free 7-hydroxyl group.

Compound 5 (Figure 3.5)

The blue fluorescence of this compound together with a characteristic UV spectrum indicates that this compound is a coumestan.

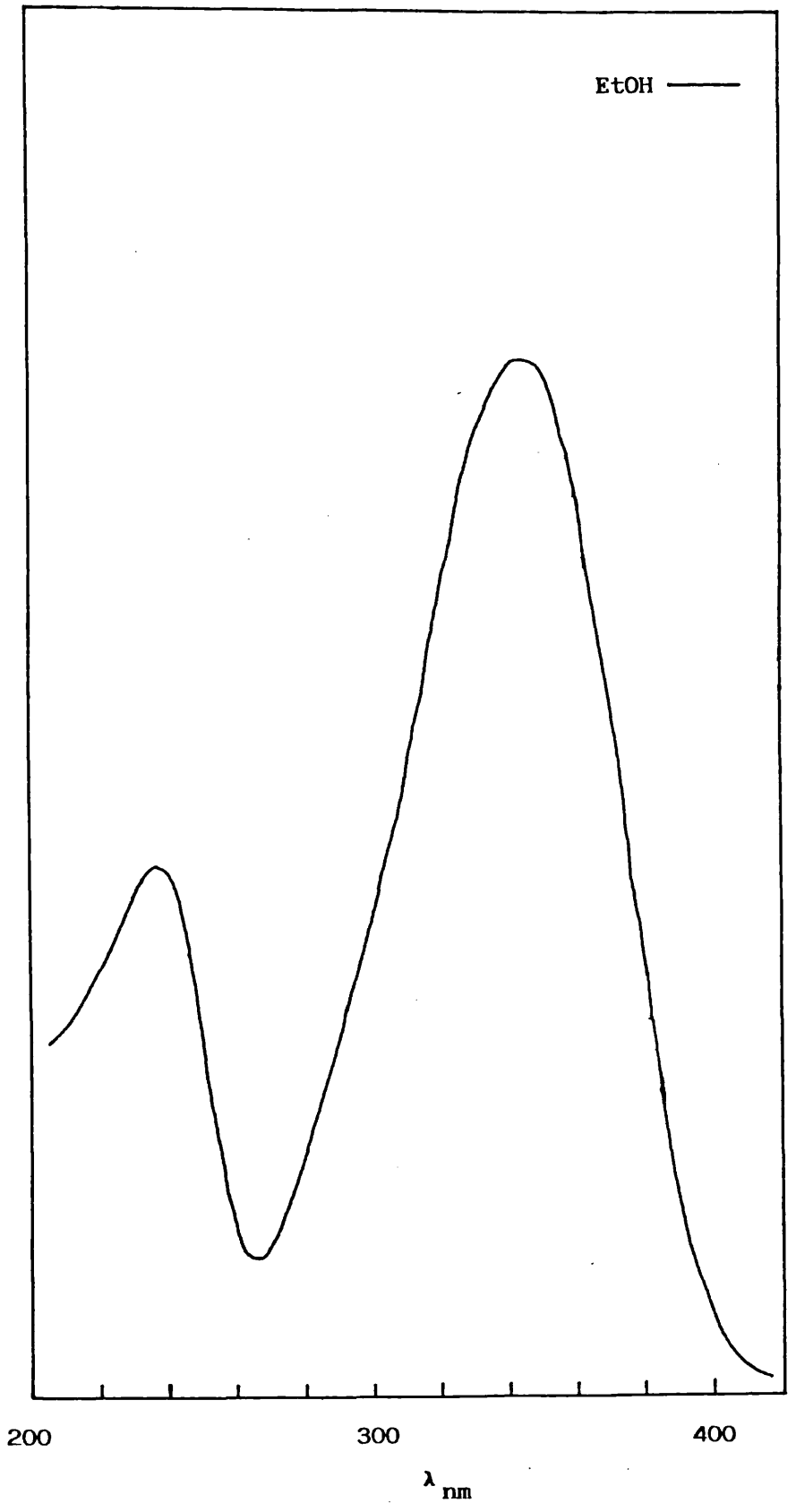
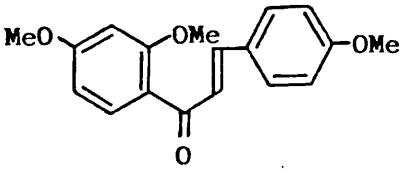


Figure 3.1 UV Spectrum of 2',4,4'-Trimethoxychalcone



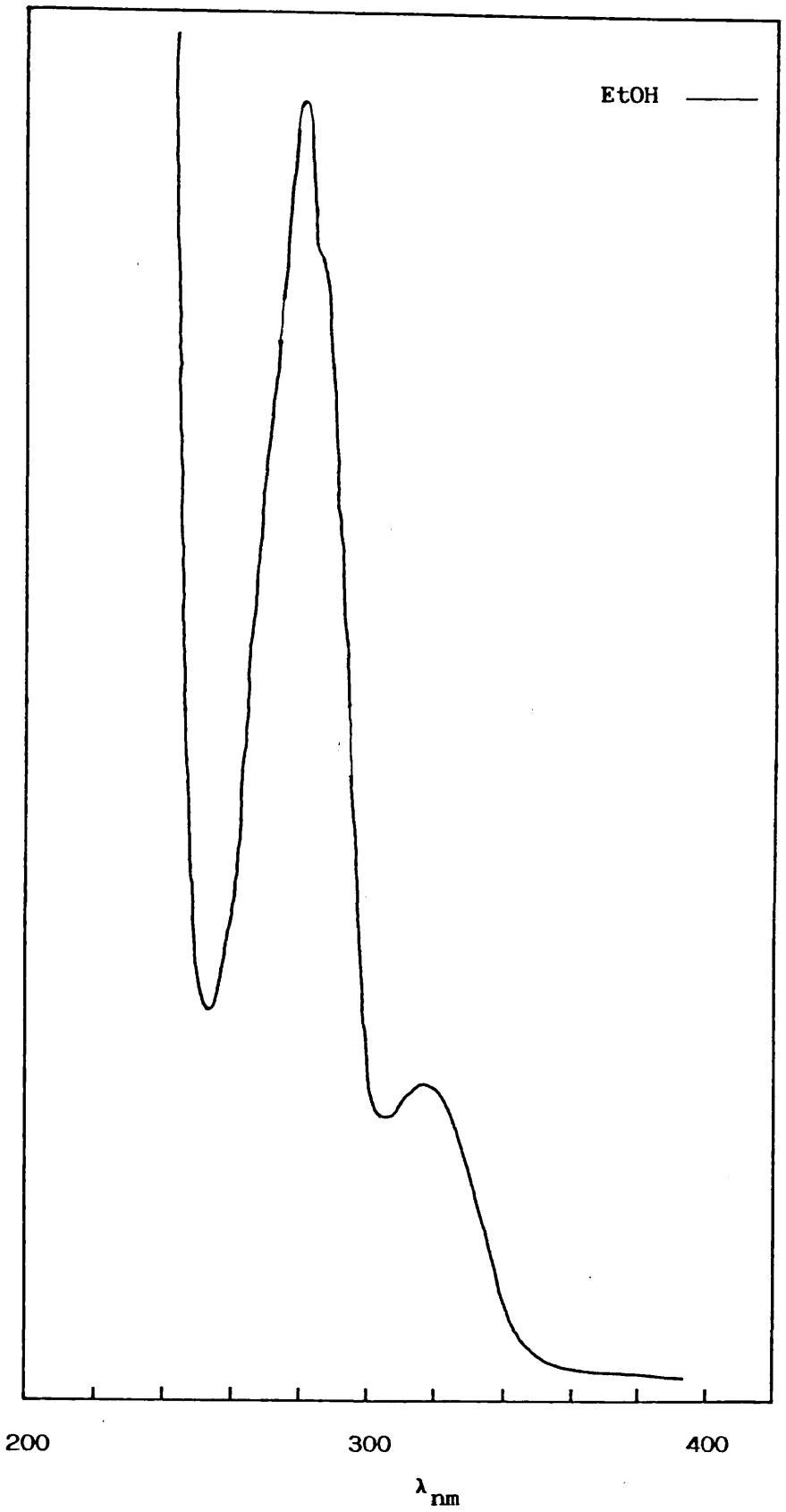
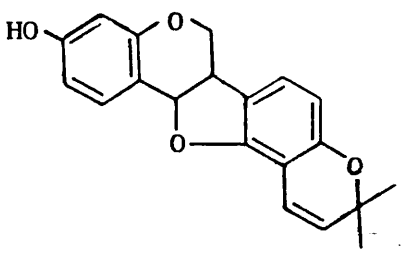


Figure 3.2 UV Spectrum of Phaseollin



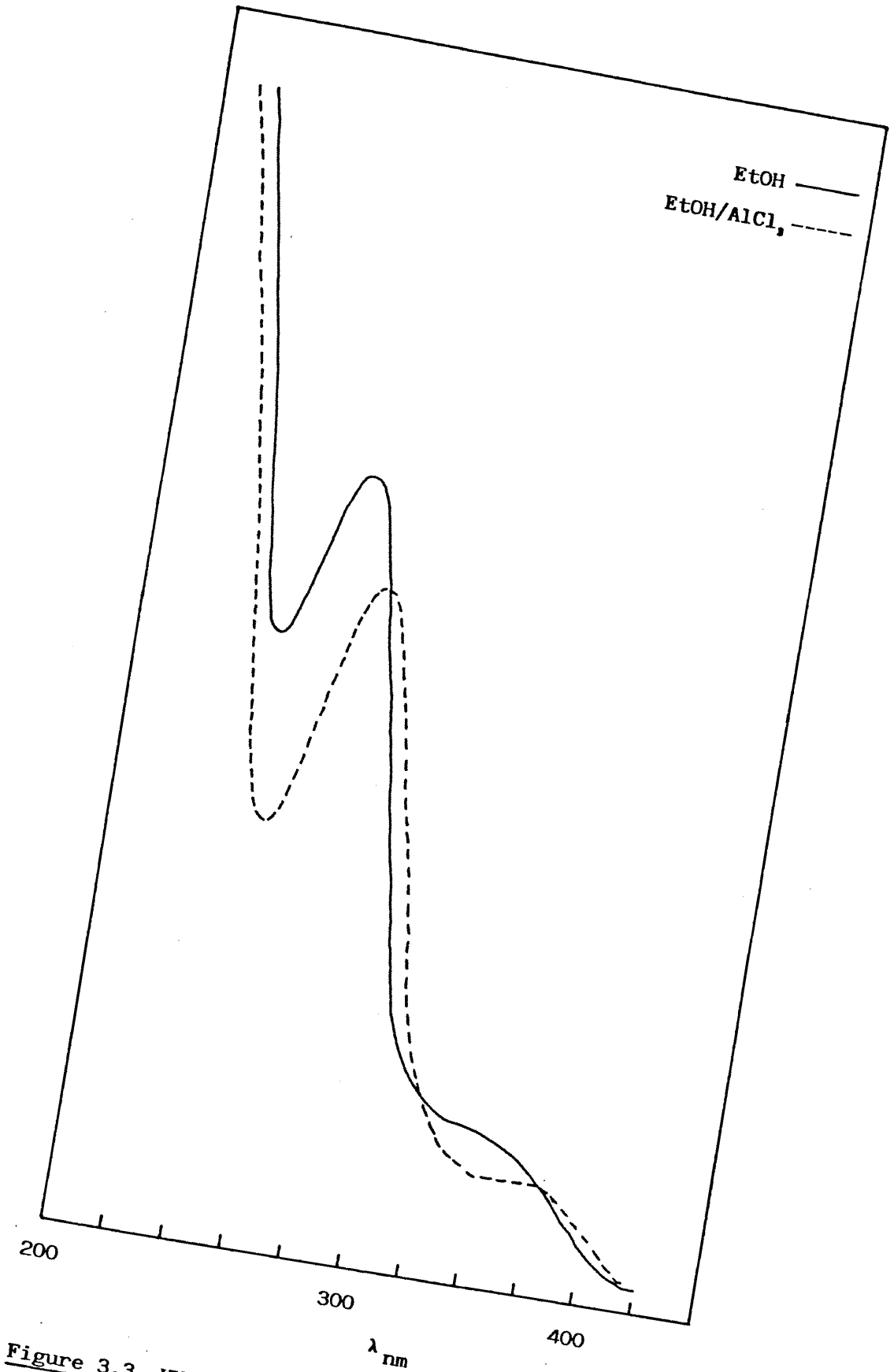


Figure 3.3 UV Spectra of Isoflavone 270

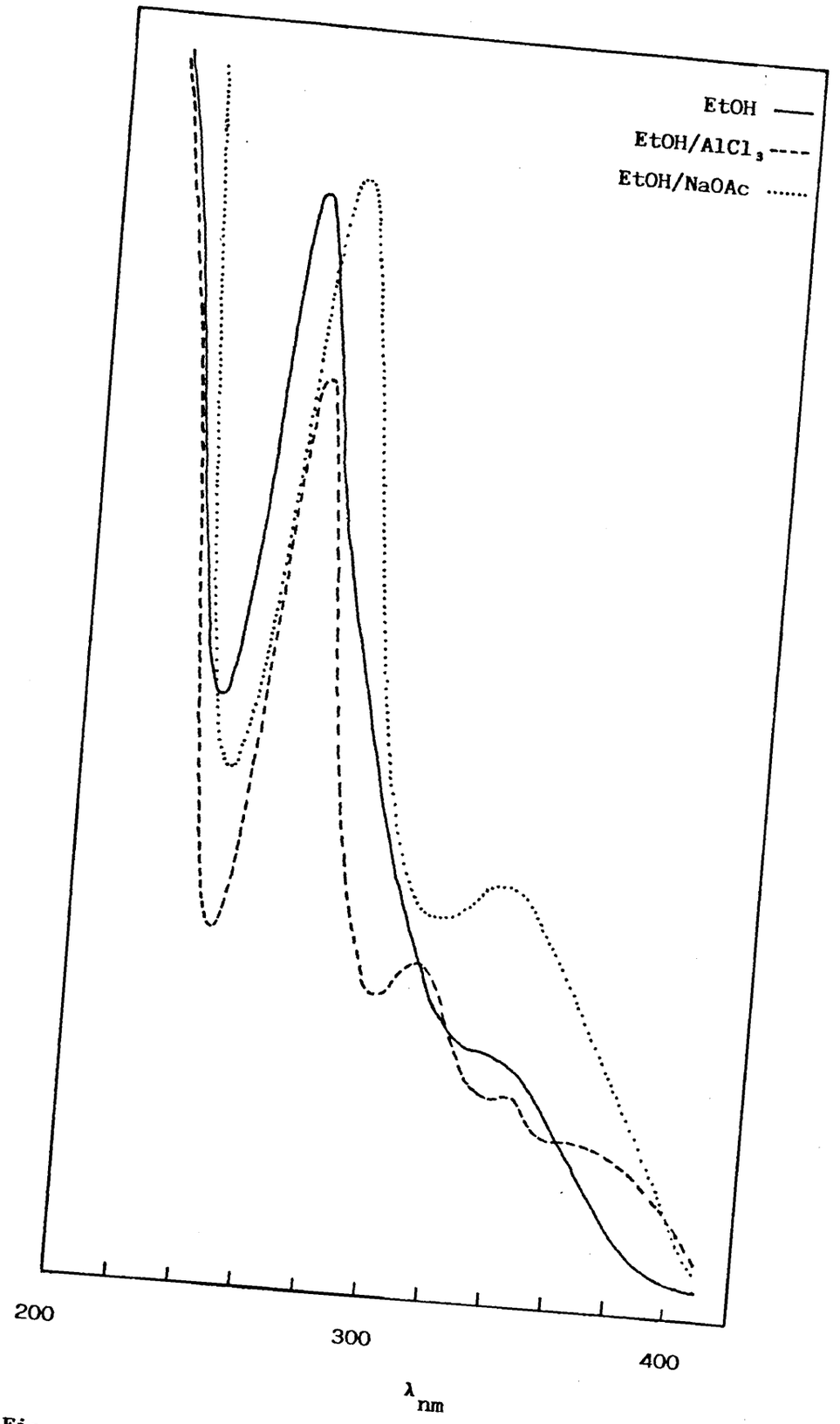


Figure 3.4 UV Spectra of Isoflavone 266

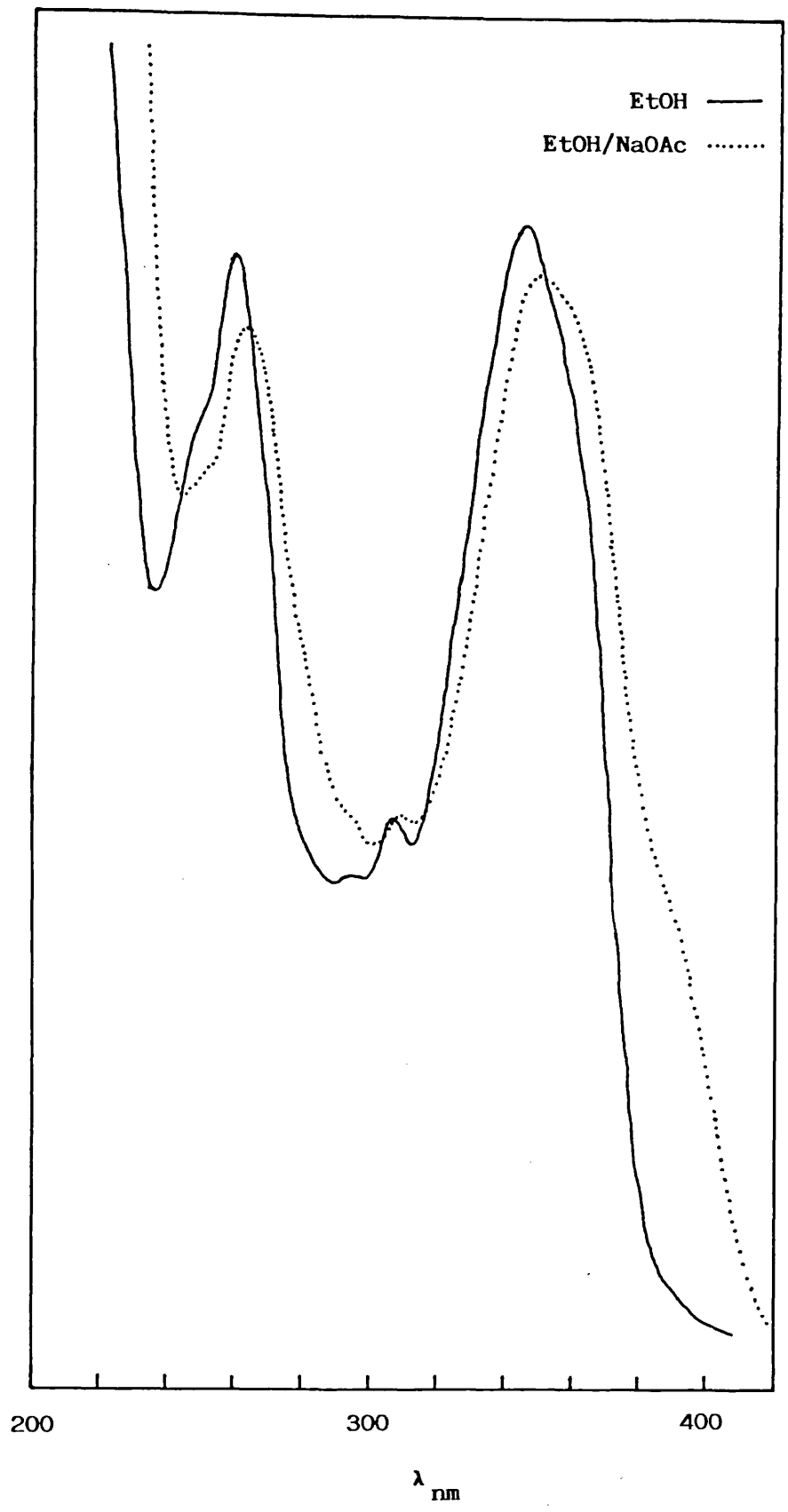


Figure 3.5 UV Spectra of Coumestan 351

Neither $AlCl_3$ nor NaOAc had any effect, which suggests that there are no free hydroxyl groups in either the 5 or 7 positions.

Compound 6 (Figure 3.6)

This compound was shown to be a 5-hydroxy isoflavone by the addition of ethanolic $AlCl_3$. The isoflavone co-chromatographed with an authentic sample of the trihydroxyisoflavone genistein in one system (Cellulose, 30% HOAc). However the UV spectrum, although similiar in shape, had significantly different λ_{max} values (of between 3-15nm). Thus compound 6 is very similiar to, but is not, genistein. [Note: Genistein could be expected to accumulate as it is an intermediate in the formation of kievitone].

Compound 7 (Figure 3.7)

This compound was tentatively identified as coumestrol from its UV spectrum and characteristic 20nm bathochromic shift when treated with NaOAC (Bailey and Burden, 1973).

Compound 8 (Figure 3.8)

This compound was tentatively identified as the isoflavone 2'-hydroxygenistein . The UV spectrum was identical for both compound 8 and an authentic sample of 2'-hydroxygenistein for all the reagents used. The two compounds co-chromatographed in the following TLC systems. Cellulose, 30% HOAc; silica G, $CHCl_3$:EtOH 100:3 and silica G, toluene:$CHCl_3$:Me_2CO 40:25:35.

Compound 9 (Figure 3.9)

This compound was positively identified as kievitone from its characteristic UV spectrum and 22nm bathochromic shift with $AlCl_3$ (Bailey and Burden, 1973).

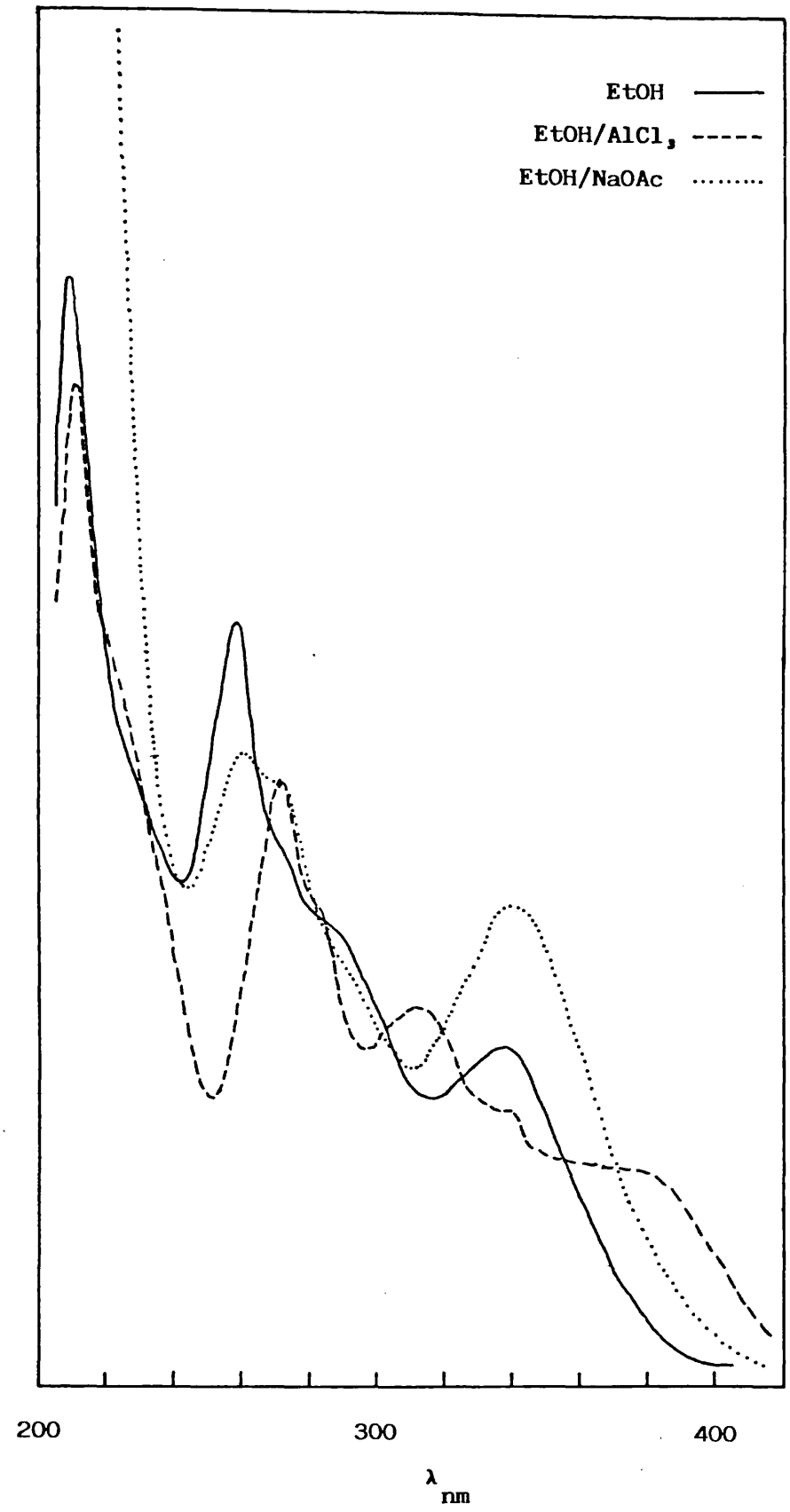


Figure 3.6 UV Spectra of Isoflavone 259

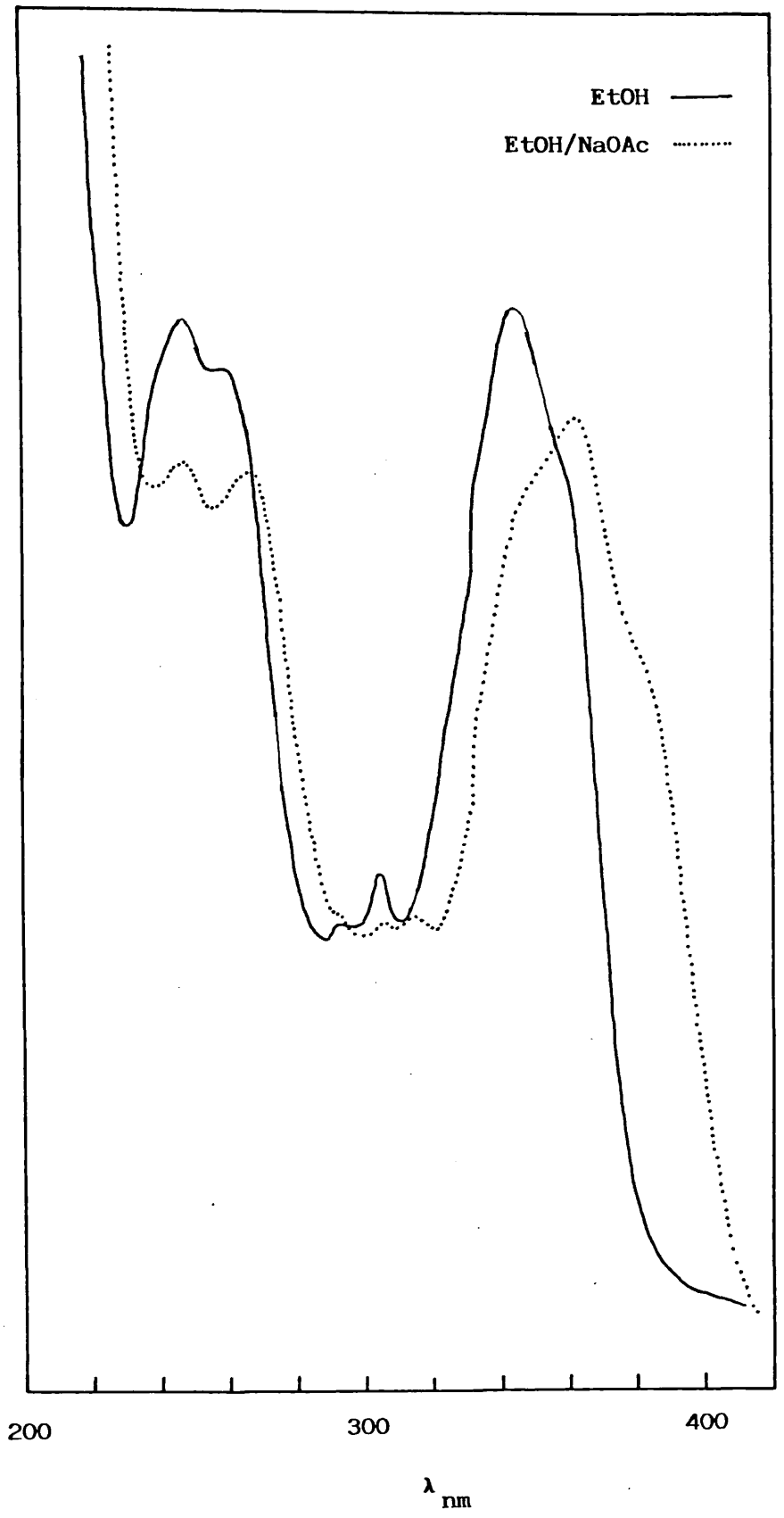
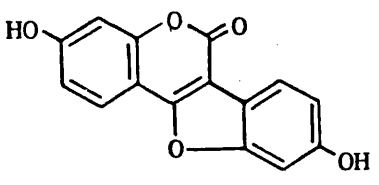


Figure 3.7 UV Spectra of Coumestrol



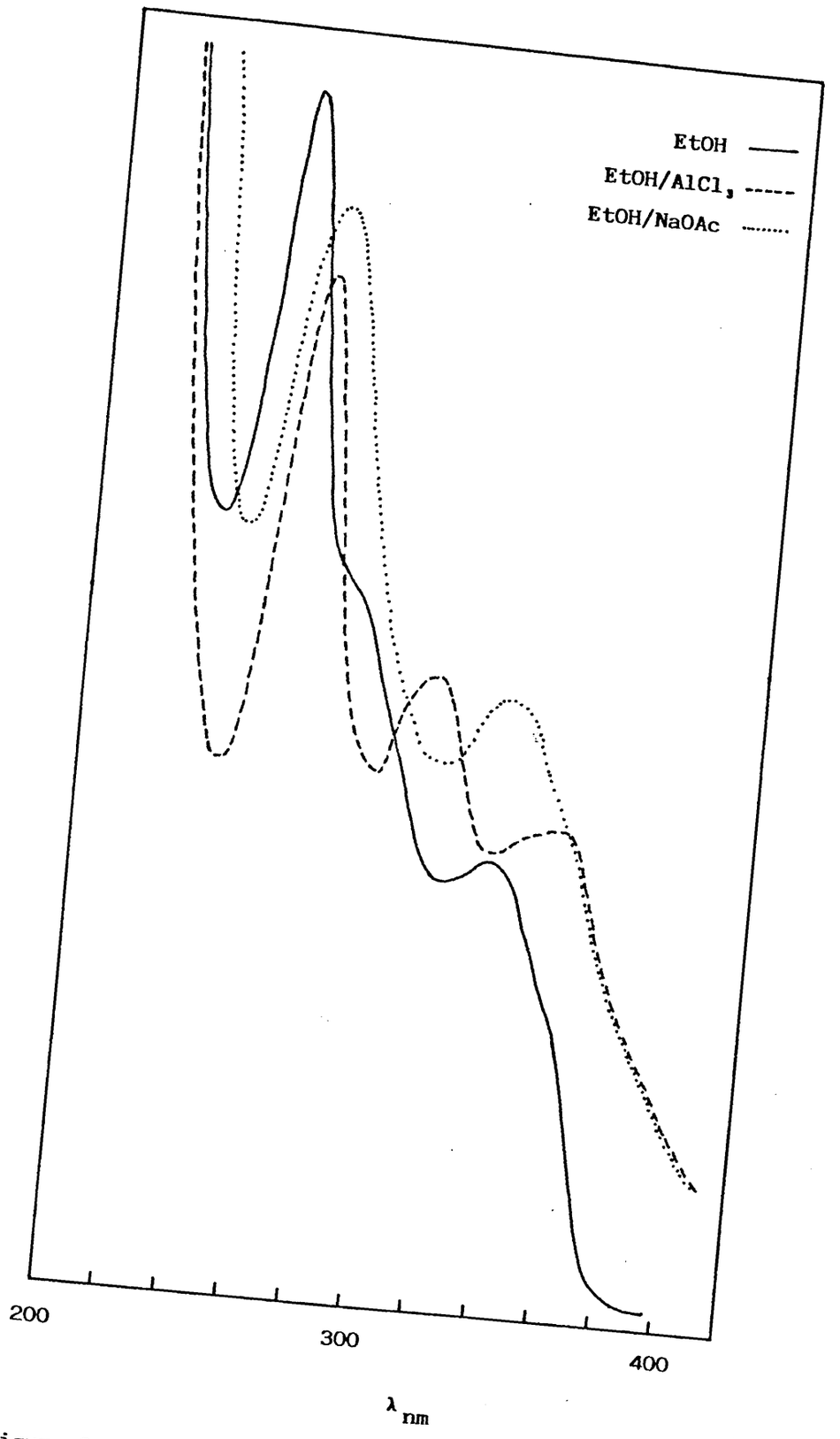
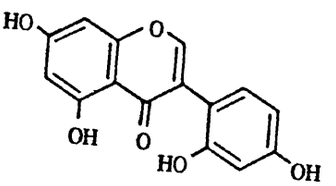


Figure 3.8 UV Spectra of 2'-Hydroxygenistein



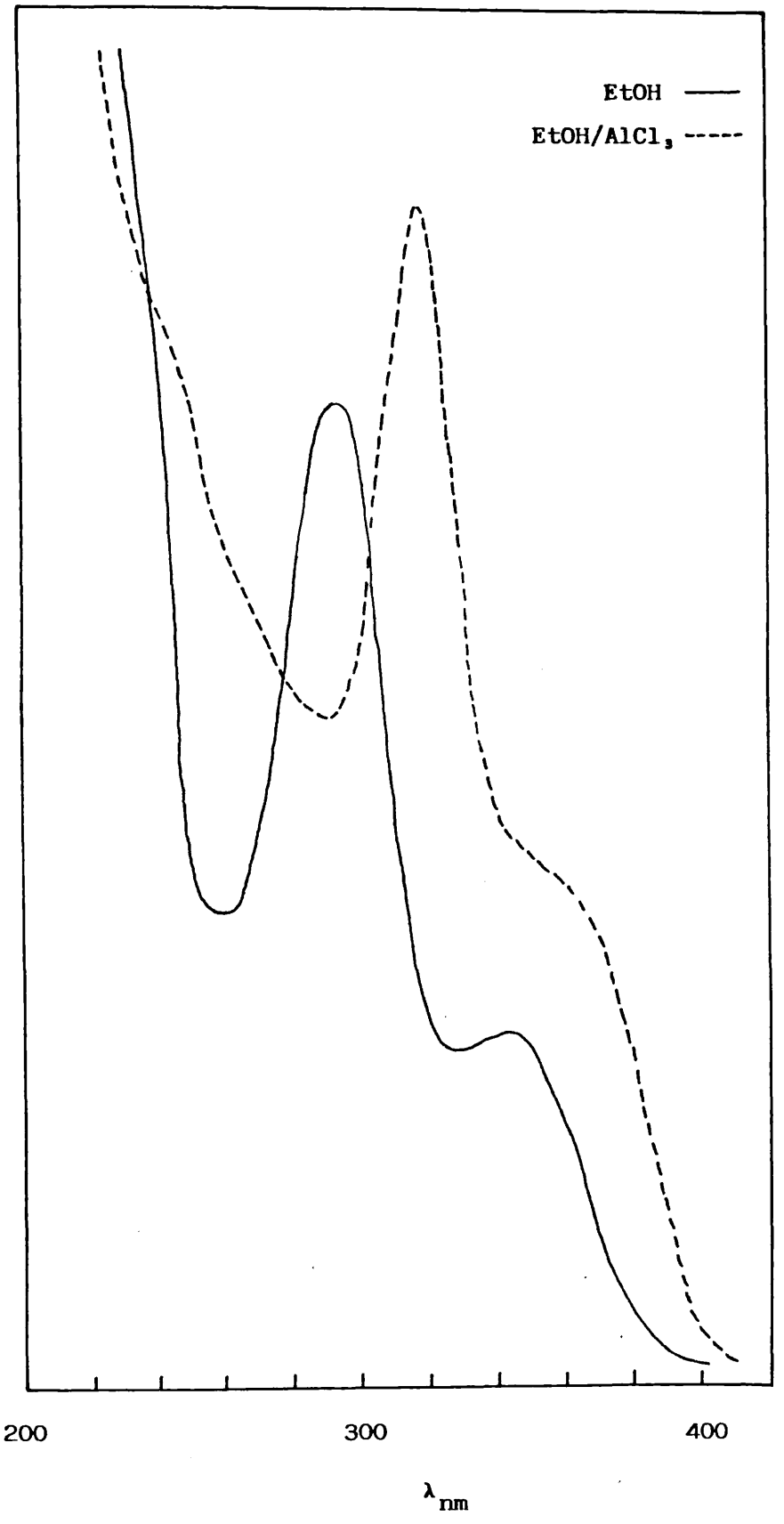
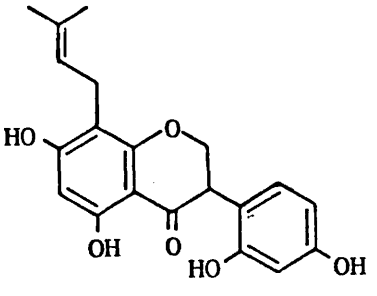


Figure 3.9 UV Spectra of Kievitone



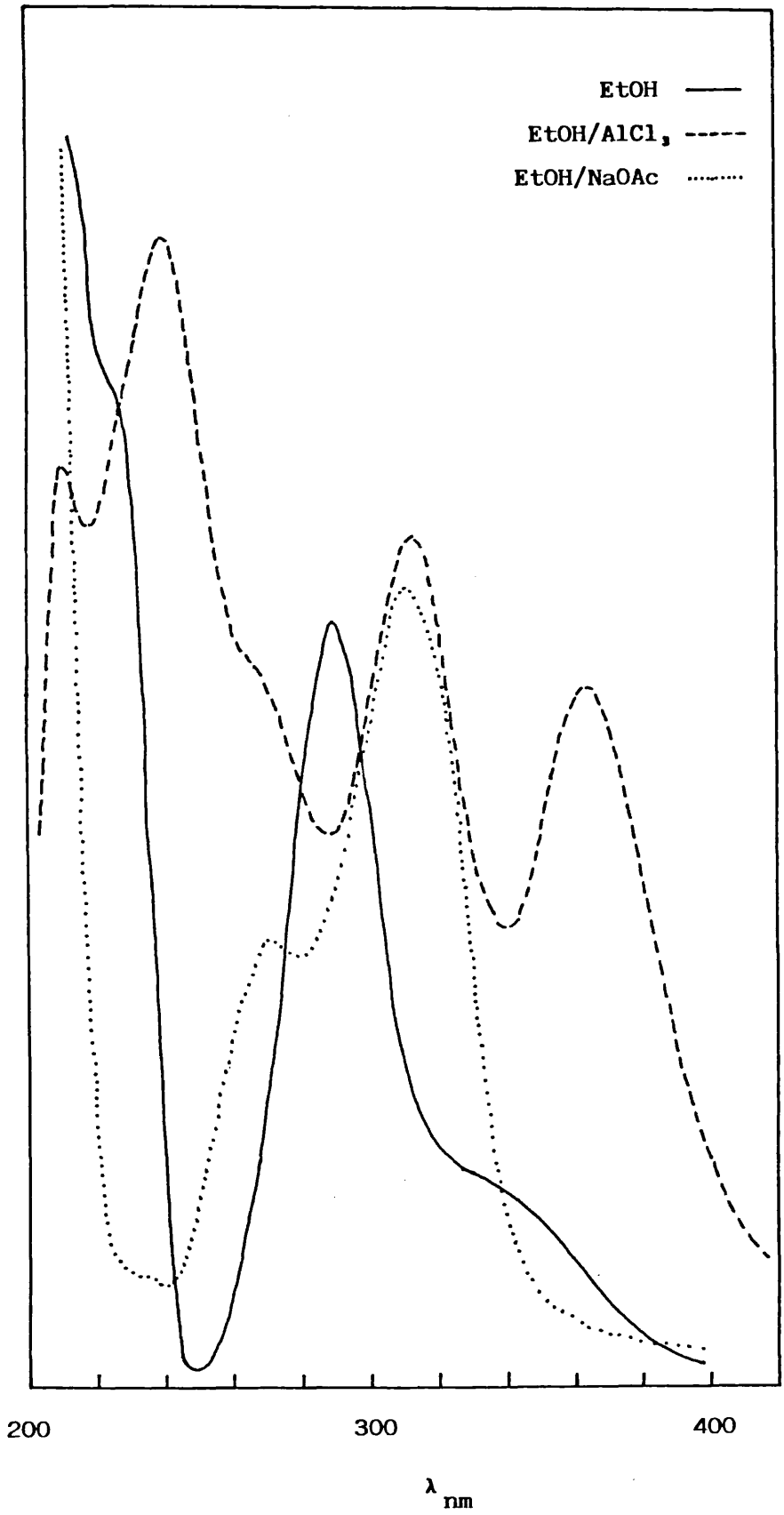
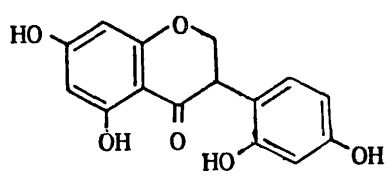


Figure 3.10 UV Spectra of Dalbergioidin



Compound 10 (Figure 3.10)

This compound was tentatively identified as the isoflavanone dalbergioidin. The UV spectrum was identical for both compound 10 and an authentic sample of dalbergioidin for all the reagents used. The two compounds co-chromatographed in the following TLC systems. Cellulose, 30% HOAc; silica G, $CHCl_3$:EtOH 100:3 and $CHCl_3$:$MeCO_2$ 40:25:35.

Compounds with chromatographic or spectroscopic properties corresponding to phaseollidin and phaseollinisoflavan (Bailey and Burden, 1973); licoisoflavone A (phaseoluteone), 5-deoxykievitone and 2,3-dehydrokievitone (Woodward 1979a, 1979b); daidzein and 2'-hydroxydaidzein (Mabry et al., 1970) were not detected. Phaseollinisoflavan was previously detected in material grown in the light but not in the dark as in the experiments reported here.

3.3b Time Course Studies

i) Isoflavonoid Accumulation

A time course study of the accumulation of isoflavonoids in wounded cotyledons (Figure 3.11) indicated that 5-hydroxy substituted isoflavonoids (kievitone, 2'-hydroxygenistein and the isoflavone λ_{max} 259) appeared earlier than the 5-deoxy derivatives (phaseollin, coumestrol and coumestan λ_{max} 347); near maximum levels of kievitone were obtained about 20h after wounding, whereas phaseollin levels did not show their greatest increase until after 60h. Major rates of increase of the 5-hydroxy compounds occured from 8-20h and from 60h onwards, this latter increase corresponding to the increase in the 5-deoxy compounds. The 5-hydroxy isoflavanone, dalbergioidin, was not detected consistently enough during the experiment to allow an accurate time course to be constructed.

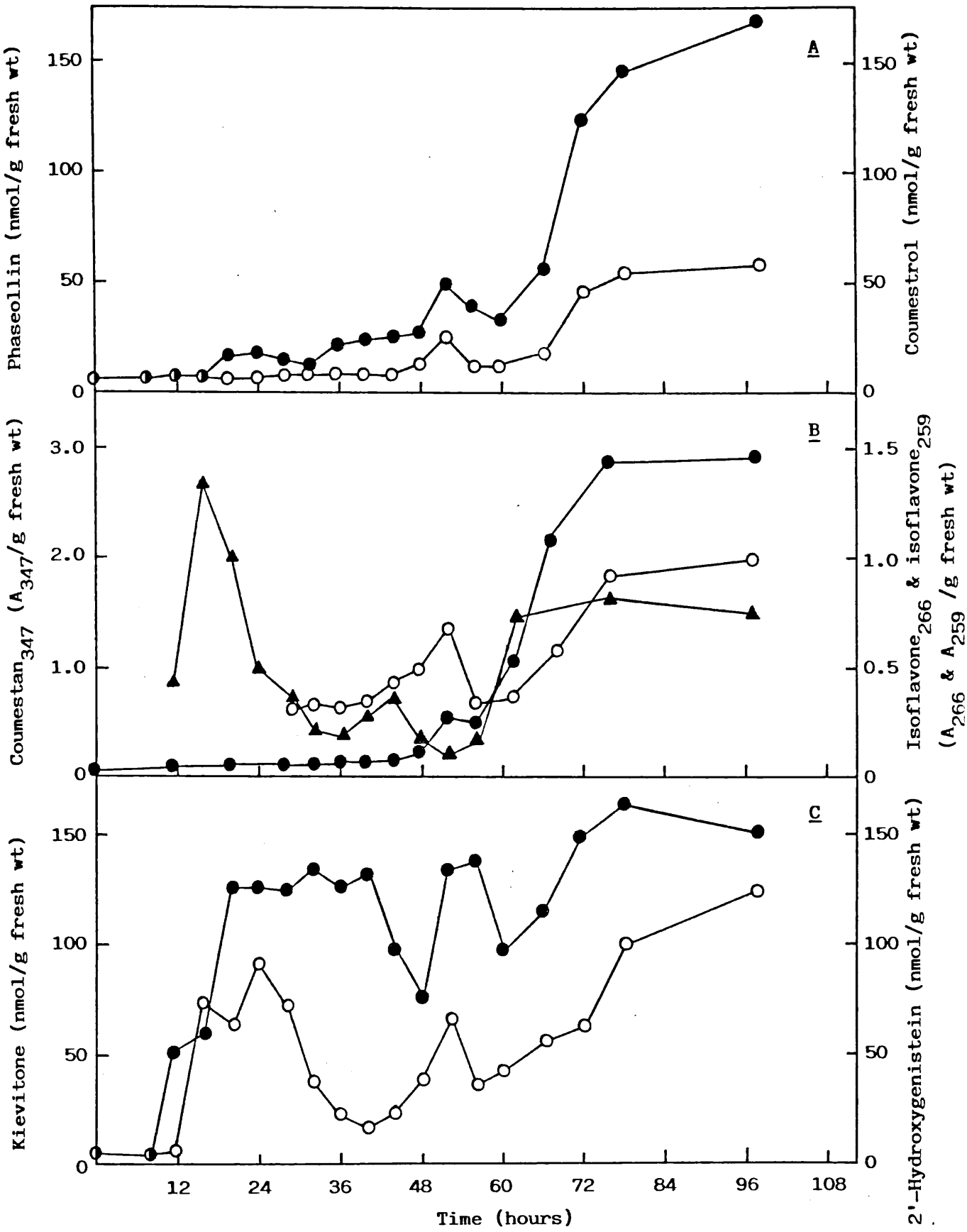


Figure 3.11 A – C

Accumulation of isoflavonoids in wounded cotyledons of *Phaseolus vulgaris*. (A) Phaseollin (●—●), coumestrol (○—○); (B) Coumestan λ_{max} 347nm (●—●), isoflavone λ_{max} 266nm (○—○), isoflavone λ_{max} 259nm (▲—▲); (C) Kievitone (●—●), 2'-hydroxygenistein (○—○).

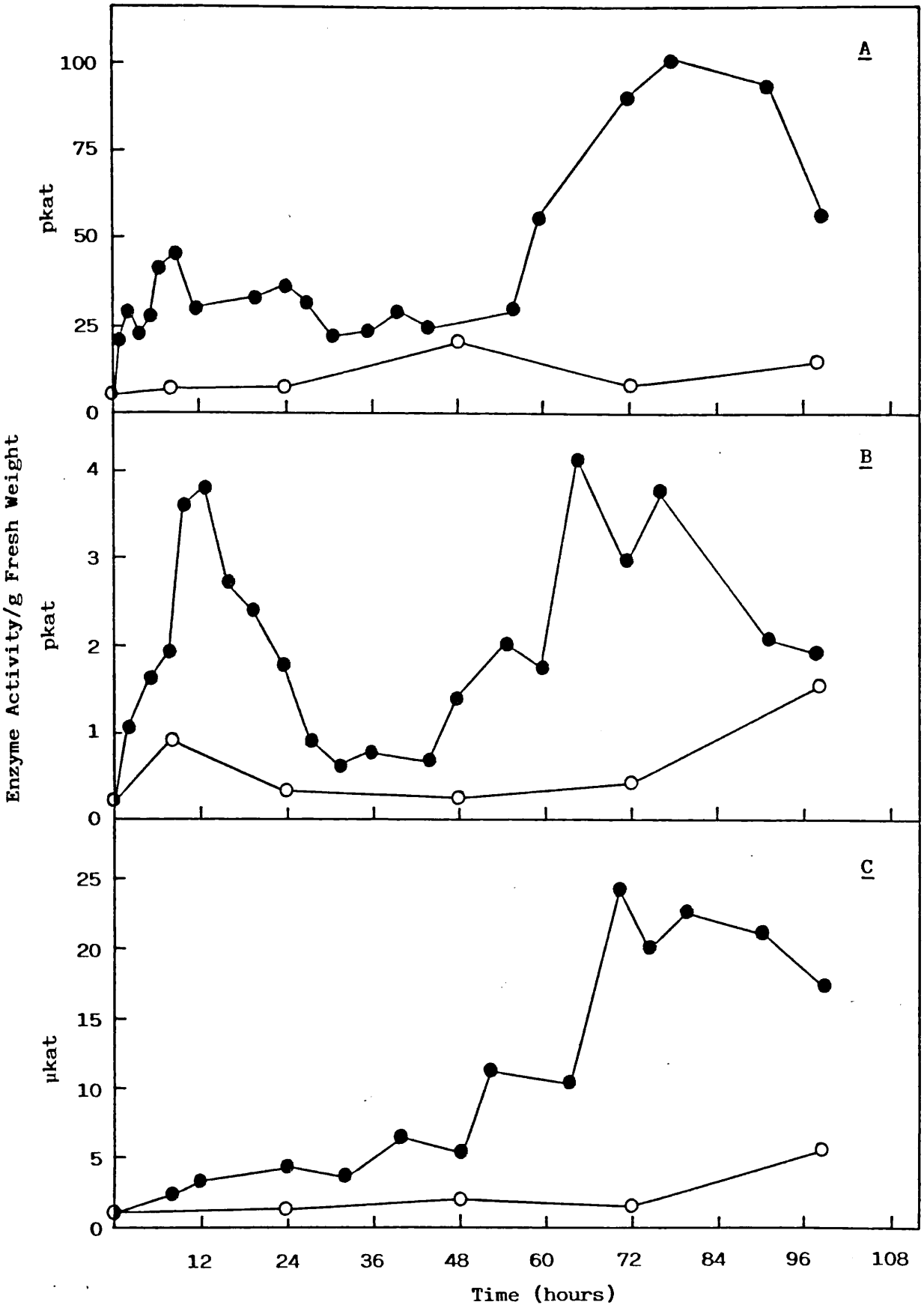


Figure 3.12 A - C

Changes in enzyme levels in wounded cotyledons of *Phaseolus vulgaris* (●—●) and non-wounded controls (○—○). (A) L-Phenylalanine ammonia-lyase; (B) Chalcone synthase; (C) Chalcone isomerase.

No phytoalexin accumulation occured in unwounded control cotyledons during the first 48h; by 96h levels of kievitone and phaseollin had increased to approximately 20 and 30% of the wounded levels respectively.

ii) Enzyme Changes

The major changes in isoflavonoid levels were preceded by transient increases in the biosynthetic enzymes PAL, CHS and CHI (Figure 3.12). CHS and to a lesser extent PAL, both showed two distinct peaks of activity with respect to time.

3.4 Discussion

The accumulation of phytoalexins in non-elicited wounded cotyledons of *P. vulgaris* observed in the present work is in contrast to earlier reports (Anderson-Prouty and Albersheim, 1975; Theodorou and Smith, 1979), where control levels are very low in the absence of fungal preparations. This discrepancy may be explained by several small differences in experimental detail including the variety of bean used, different times of sampling, the harvest of only necrotic tissue instead of whole cotyledon segments and the use of antibiotics (Anderson-Prouty and Albersheim, 1975; Theodorou and Smith, 1979). Although it is possible that contaminating micro-organisms already present on the surface of the cotyledons induced phytoalexin accumulation, surface sterilized cotyledons (soaked in sodium hypochlorite [2% available Cl] for 30 min.) still accumulated the full range of isoflavonoids when wounded under aseptic conditions.

The present results indicate a clear temporal difference between the accumulation patterns of the 5-hydroxy and 5-deoxy isoflavonoid classes (Figure 3.11) and this strongly suggests that the two pathways may be under separate control. The more rapid initial decline in the 2'-hydroxygenistein and isoflavone λ_{max} 259 levels than observed for kievitone is consistent

with the intermediacy of 5-hydroxyisoflavones in the biosynthesis of kievitone where the proposed pathway is believed to be genistein → 2'-hydroxygenistein → dalbergioidin → kievitone (Woodward, 1979a). This implies a high degree of co-ordination in the regulation of the individual steps of the pathway. The maintenance of a constant level of kievitone from 20-40h, followed by a decline, may indicate turnover of the phytoalexin. However, the half-life of the phytoalexin glyceollin in $HgCl_2$-treated soybean cotyledons has been calculated to be around 89h (Moesta and Grisebach, 1980), a value too long to account for the proposed role of turnover in the regulation of phytoalexin levels in this system (Yoshikawa et al., 1979). No information is available as yet on the half-lifes of endogenous phytoalexins in *P. vulgaris*. The changes in enzyme levels (Figure 3.12) exactly preceded the changes in phytoalexin accumulation; the first peaks in PAL and CHS levels occured approximately 10h before the early maximum in 5-hydroxy isoflavonoid levels was attained, with the second main increase in both enzymes and phytoalexins starting at around 60h.

The role of CHI in the induced response was not clear; the increase in isomerase levels is less rapid than those for PAL or CHS and follows the same pattern as 5-deoxy isoflavonoid accumulation. However, the high basal activity of CHI and the similiar K_m values for 6'-hydroxy and 6'-deoxy chalcones (corresponding to 5-hydroxy/5-deoxy flavonoids/isoflavonoids, Chapter 6) suggests that the enzyme is probably not a controlling factor in the phytoalexin response, although new evidence has established the role of the enzyme in the synthesis of these compounds (Hagmann and Grisebach, 1984).

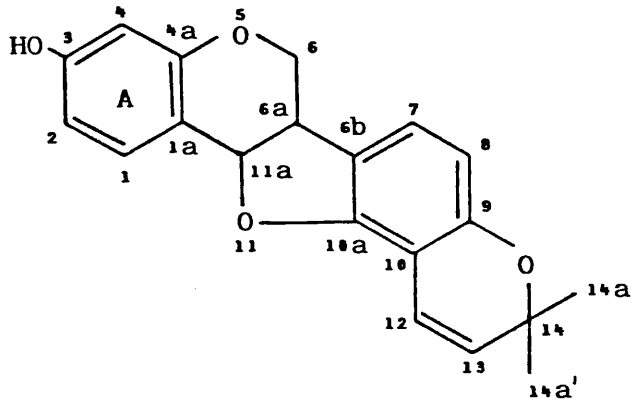
In the next chapter, the enzymological basis for the differentiation of the two pathways is investigated by examining the mechanism of synthesis of both 6'-hydroxy and 6'-deoxy chalcones during the formation of phaseollin and kievitone.

CHAPTER 4

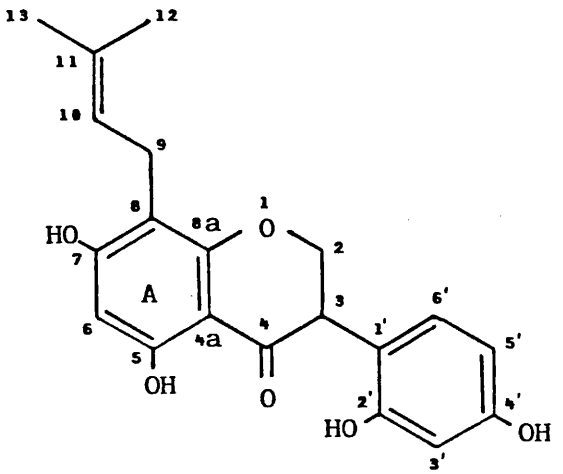
The Use of ^{13}C-NMR Spectroscopy to Investigate the Mechanism of Chalcone Synthesis in *Phaseolus vulgaris* L.

4.1 Introduction

Investigations into phytoalexin biosynthesis were first subjected to the powerful technique of using double labelled ^{13}C-acetate and NMR spectroscopy by Baker et al. (1975) studying the biosynthesis of capsidiol in infected sweet peppers (*Capsicum frutescens*). The method depends on the magnetic interactions (spin-spin coupling) of contiguous ^{13}C atoms revealed as characteristic splitting patterns in the ^{13}C-NMR spectrum. Such patterns will appear in products of biosynthetic studies only if the contiguous atoms are incorporated as an intact unit from the administered precursor.



Phaseollin



Kievitone

Using this method, Baker et al. (1975) were able to demonstrate a predicted methyl migration; a pair of carbon atoms which were contiguously labelled in the precursor, farnesol, gave rise to singlet rather than doublet absorptions in the ^{13}C spectrum of enriched capsidiol, indicating that the two atoms had become detached during the biosynthetic process.

The A-ring of flavonoids and isoflavonoids is derived by the head-to-tail condensation of three acetate-malonate units (Grisebach, 1967. see also Chapter 1, Section 1.2c and Figures 4.1 and 5.1), and two recent studies (Stoessl and Stothers, 1979 and Light and Hahlbrock, 1980) using sodium [1,2$^{13}C_2$] acetate have elegantly displayed significant differences in the biosynthetic pathways leading to the 5-hydroxy and 5-deoxy series of compounds. During the biosynthesis of pisatin in *Pisum sativum*, Stoessl and Stothers were able to show the intact incorporation of double labelled acetate into carbons 1a-1, 2-3 and 4-4a of the A-ring of this 5-deoxyisoflavonoid derivative. This demonstrated that a specific folding of the polyketide chain occured and that reduction of the "missing" oxygen function took place prior to ring closure (Figure 4.1).

The work of Stoessl and Stothers was contrasted by the results of Light and Hahlbrock investigating the biosynthesis of the 5-hydroxyflavonol, kaempferol in *Petroselinum hortense* cell suspension cultures. They found that the A-ring signals in the ^{13}C-NMR spectrum of the enriched material were flanked not by a single pair of satellites (as with pisatin) but by two pairs and further that the coupling constants were sufficiently different to enable the signals to be resolved. These results were interpreted as showing that a mixture of the two possible products was present (Figure 4.1). The randomisation of the label was thought to occur at the chalcone stage by free rotation of the A-ring. Results similiar to these

were obtained with the flavone, apigenin, although less certain since chemical randomisation could have occured during the work up (Light and Hahlbrock, 1980).

Phaseollin and kievitone are representative of the 5-hydroxy and 5-deoxy series of isoflavonoids in *P. vulgaris* and there is a clear temporal difference between the accumulation patterns of the two compounds (Chapter 3, Figure 3.11) which suggests that the two pathways leading to the formation of these and other related compounds are under separate control. Furthermore, it appears that a common, enzyme-bound, open-chain, polyketide precursor (Figures 4.1 and 5.1), may exist during the biosynthesis of both these compounds.

The enzymic formation of 6'-hydroxy chalcones (5-hydroxy by isoflavonoid numbering) by a 6'-hydroxychalcone synthase (CHS), has been demonstrated in *P. vulgaris* and several other plant systems (Chapter 5). However, a 6'-deoxychalcone synthase activity catalysing the formation of 6'-deoxy chalcones has yet to be demonstrated.

A comparison of the ^{13}C A-ring labelling patterns of phaseollin and kievitone should provide information on the mechanism of biosynthesis of these two compounds and allow an insight into the nature of the as yet uncharacterised 6'-deoxy CHS enzyme system.

4.2 Materials and Methods

Seeds (0.5kg) of *P. vulgaris* var. The Prince were germinated as described previously (Section 2.1). Cotyledons were excised, wounded by removing the top 2-3mm of cells from the inner surface with a razor blade and arranged in petri dishes on moist filter paper. Distilled water (50µl) was applied to the cut surface of each cotyledon and the petri dishes were then placed in a dark, humid chamber at 25°C.

Accumulation of both phaseollin and kievitone in cotyledons of *P. vulgaris* is optimal approximately 96h after wounding (Section 3.11).

In the experiment to obtain natural abundance ^{13}C spectra for both compounds the plant material was harvested after this time by transfer to redistilled EtOH (500ml). Phaseollin was purified from material worked up by method A (Section 4.3b) whilst kievitone was obtained from material worked up by method B (Section 4.3d).

Preliminary experiments with [U-^{14}C] acetate (5μCi/mg; 5mg/ml; 50μl/cotyledon) indicated that the optimal incorporation of acetate into both phaseollin and kievitone occured approximately 48h after wounding. Thus, in the experiment to obtain enriched spectra, 50μl of an aqueous solution of [1,2-$^{13}C_2$] sodium acetate (5mg/ml; 90% labelled at each carbon atom; pH 7.6) was applied to the wounded surface of each cotyledon after 48h (total volume 100ml). The incubation of the cotyledons was continued with the labelled acetate for a further 48h period and they were then harvested as above. Phaseollin and kievitone were both purified from material worked up by method B (Section 4.3d).

In the ^{14}C-labelling experiment, under the conditions outlined above, ^{14}C-phaseollin was isolated which, after purification to constant specific activity by TLC, showed an incorporation of label of 5.7% with an isotope dilution of 17.7.

4.3 Extraction and Purification of Phaseollin and Kievitone

4.3a Extraction

The prepared cotyledon material in EtOH (500ml)(Section 4.2) was homogenised in a Waring blendor. The extract was filtered and the residue re-extracted with a further 500ml EtOH. The combined extracts were reduced to dryness *in vacuo*, resuspended in distilled water (100ml) and partitioned extensively against Et_2O (6 x 250ml). The pooled Et_2O fractions were reduced to dryness *in vacuo* and the residue purified by either method A or B below.

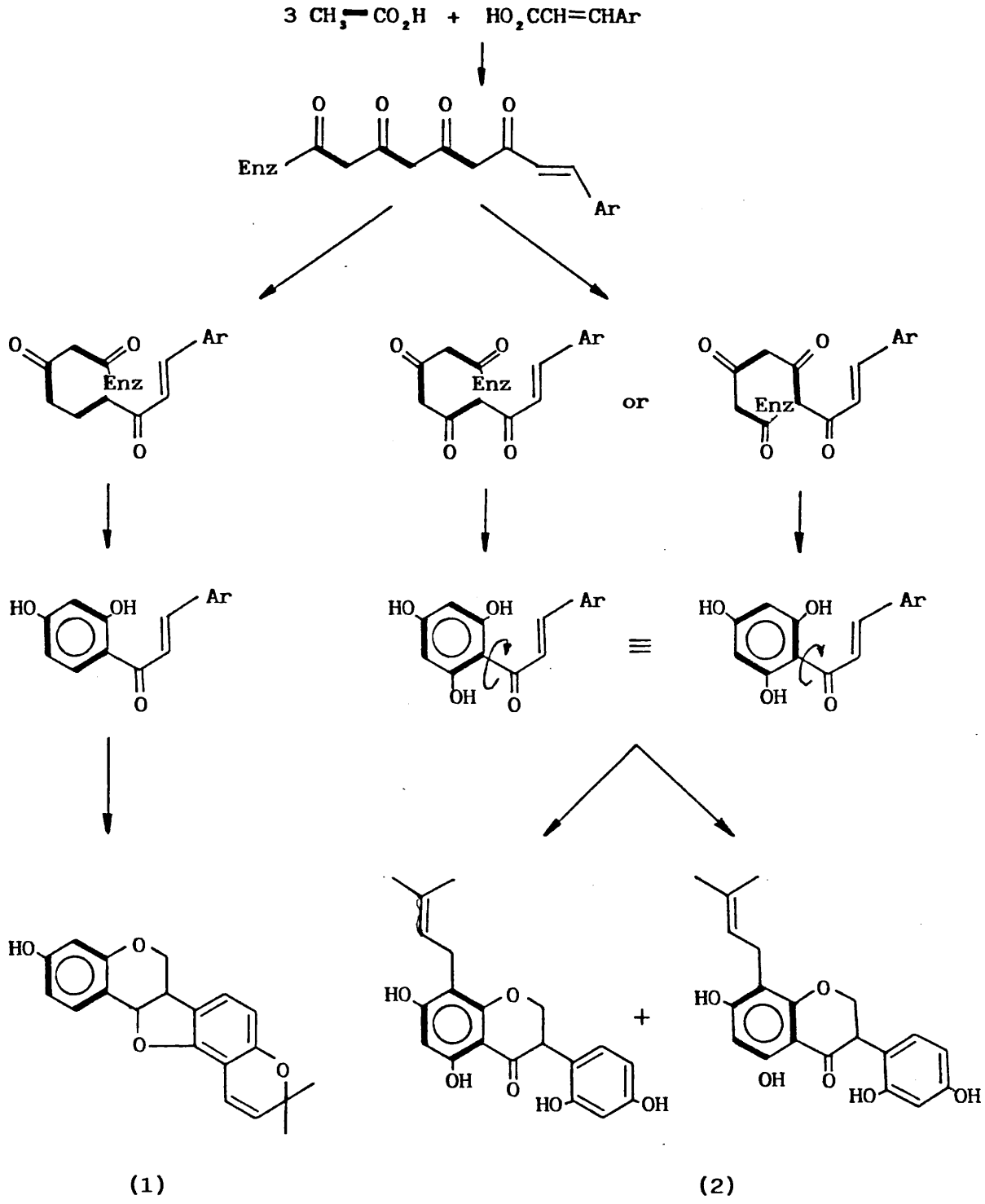


Figure 4.1

Biosynthesis of phaseollin (1) and kievitone (2) from sodium [1,2-$^{13}C_2$] acetate after Stoessl and Stothers (1979) and Light and Hahlbrock (1980).

Enz = Enzyme Ar = Aromatic ring.

4.3b Purification of Phaseollin by Alumina Column Chromatography (Method A)

10g of neutral aluminium oxide (Woelm, Pharma) was covered with petrol (B.P. 40°-60°), deactivated to grade III (6%) by the addition of 0.6ml distilled water and packed into a glass column (11 x 125mm). The extracted residue (Section 4.3a) was dissolved in petrol (5ml) and run onto the column which was then eluted with solvents as shown below in Table 4.2.

Table 4.2 Solvents Used for the Elution of the Alumina Column

Solvent	Volume (ml)	Fraction Eluted
Petrol	100	1 and 2
Petrol:Et_2O (7:3)	150	3 and 4
Petrol:Et_2O (1:1)	200	5
Petrol:Et_2O (3:7)	60	6
Et_2O	100	7
Et_2O:MeOH (95:5)	100	8

The elution profile was recorded using a Uvicord spectrometer at 280nm (Figure 4.2). Eight fractions were separated by this method. From UV spectra and TLC with authentic samples, phaseollin was found to be the major component of fractions 5,7 and 8 (Figure 4.2). Fraction 7 was not consistently observed and could be a column artefact.

4.3c Sephadex LH-20 Column Chromatography

Fractions 5, 7 (when present) and 8 from the alumina column were reduced to dryness, dissolved in EtOH (3ml) and applied separately to a Sephadex LH-20 column (3.4 x 30cm). The column was eluted with EtOH and the elution profile (Figure 4.3) was essentially similiar for all

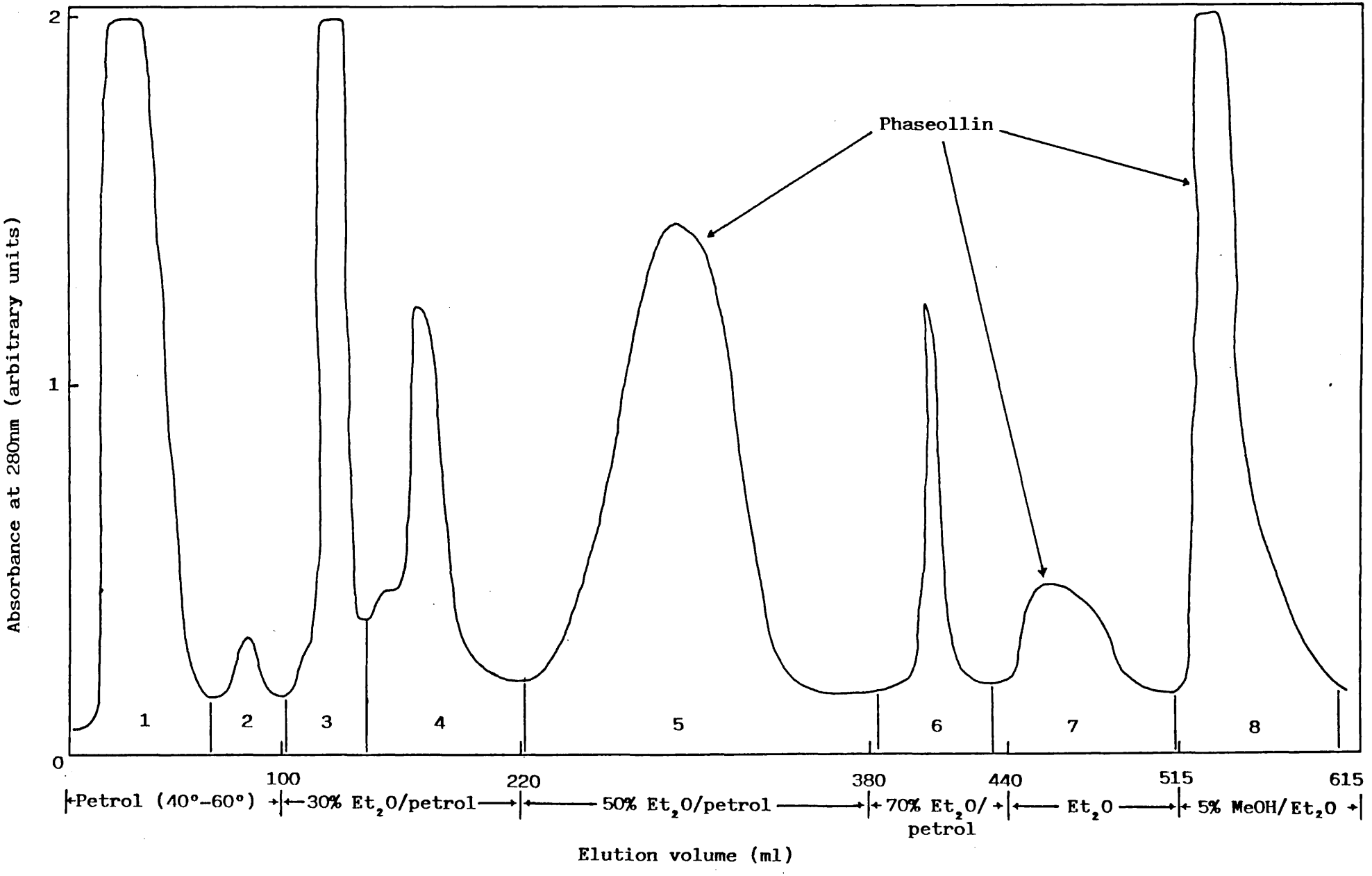


Figure 4.2 Purification of phaseollin by alumina column chromatography. 1-8 = Fractions collected.

three fractions. However, fraction 5 contained a large amount of a yellow pigment which separated on Sephadex LH-20 (Figure 4.3) This compound (λ_{max} 418, 440, 470) was tentatively identified as the carotenoid violaxanthin (Davies, 1976); it is possible that this compound caused phaseollin to co-elute due to an aploar interaction.

When purified phaseollin from the LH-20 column was re-chromatographed on alumina it was eluted with Et_2O:MeOH (95:5), (Fraction 8). Phaseollin was then routinely obtained by pooling fractions 5 and 8 from the alumina column prior to purification of Sephadex LH-20. The main advantages of the alumina column are the higher loading capacity for compounds than that of TLC plates (Section 4.3d) and the speed with which material is processed. The alumina and Sephadex LH-20 columns are of compatible capacity; extracts containing 5-10mg phaseollin were routinely applied to the columns and purified by this procedure.

4.3d Purification of Phaseollin and Kievitone (Method B)

The extracted residue (Section 4.3a) was dissolved in EtOAc (10ml) and applied to silicia GF UV_{254} thin-layer chromatography plates (0.75mm). The chromatograms were developed in an equilibrated tank with $CHCl_3$:EtOH (100:3) as solvent. After drying and vizualization under short-wave UV light (254nm) the bands containing phaseollin (R_f 0.60) and kievitone (R_f 0.12) were scrapped off, eluted with EtOH and further purified by Sephadex LH-20 column chromatography (Section 4.3c). [Note: This work was carried out by Dr. P.M. Dewick and M.J. Steele in the Department of Pharmacy, University of Nottingham].

4.3e Yields

The yields of pure phytoalexins were: Unlabelled phaseollin, 60mg (Method A); Unlabelled kievitone, 80mg (Method B); ^{13}C-enriched

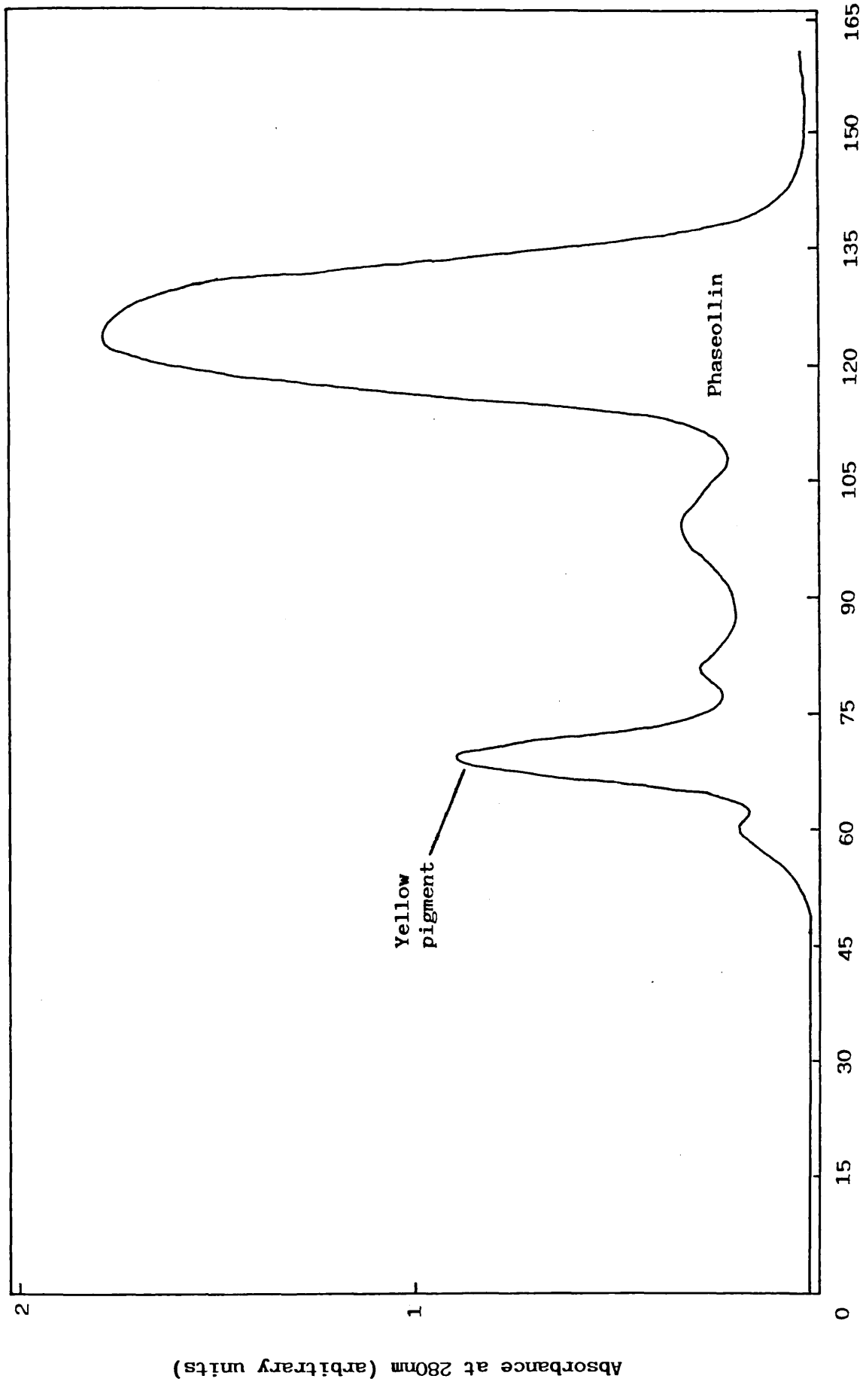


Figure 4.3 Purification of phaseollin by gel-filtration on Sephadex LH-20.

phaseollin, 49mg (Method B) and ^{13}C-enriched kievitone, 89mg (Method B).

4.4 Results

Signals in the ^{13}C-NMR of phaseollin (Figures 4.4, 4.5 and 4.6) and kievitone (Figures 4.7 and 4.8) were assigned on the basis of characteristic chemical shifts, off-resonance decoupling multiplicities, comparison with other isoflavonoid derivatives (Pelter et al., 1976; Wenkert and Gottlieb, 1977; Chalmers et al., 1977; Bailey et al., 1977 and Pelter et al., 1978) and where necessary, analysis of carbon-carbon coupling constants in spectra of the enriched samples. The assignments are listed in Table 4.1.

The 62.9 MHz spectrum of phaseollin derived from [1,2-$^{13}C_2$]-acetate showed four signals to be flanked by satellites (C-1, C-1a, C-2 and C-4; Figures 4.4 and 4.5). However, the corresponding signals for C-3 and C-4 were not visible due to the low intensities of the parent signal. These signals were shown to be flanked by satellites with the increased sensitivity of a 100.6 MHz spectrometer (Figure 4.6). From the relative intensities of the satellite peaks to the central signal, the enrichment level was estimated to be 0.3% at each acetate position.

In the spectrum of enriched kievitone (Figures 4.7 and 4.8), six signals were flanked by satellites. Two of these sets were plainly resolved as pairs of satellite signals, whereas the other four at both 62.9 MHz (Figure 4.8) and 100.6 MHz (not shown) were unresolved broad peaks. From the relative intensities of the satellite peaks to the central signal, the enrichment level was about 0.7%, more than double the figure for phaseollin. [Note: Dr. P.M. Dewick of the Department of Pharmacy, University of Nottingham, interpreted all of the ^{13}C-NMR spectra and assigned the carbon signals].

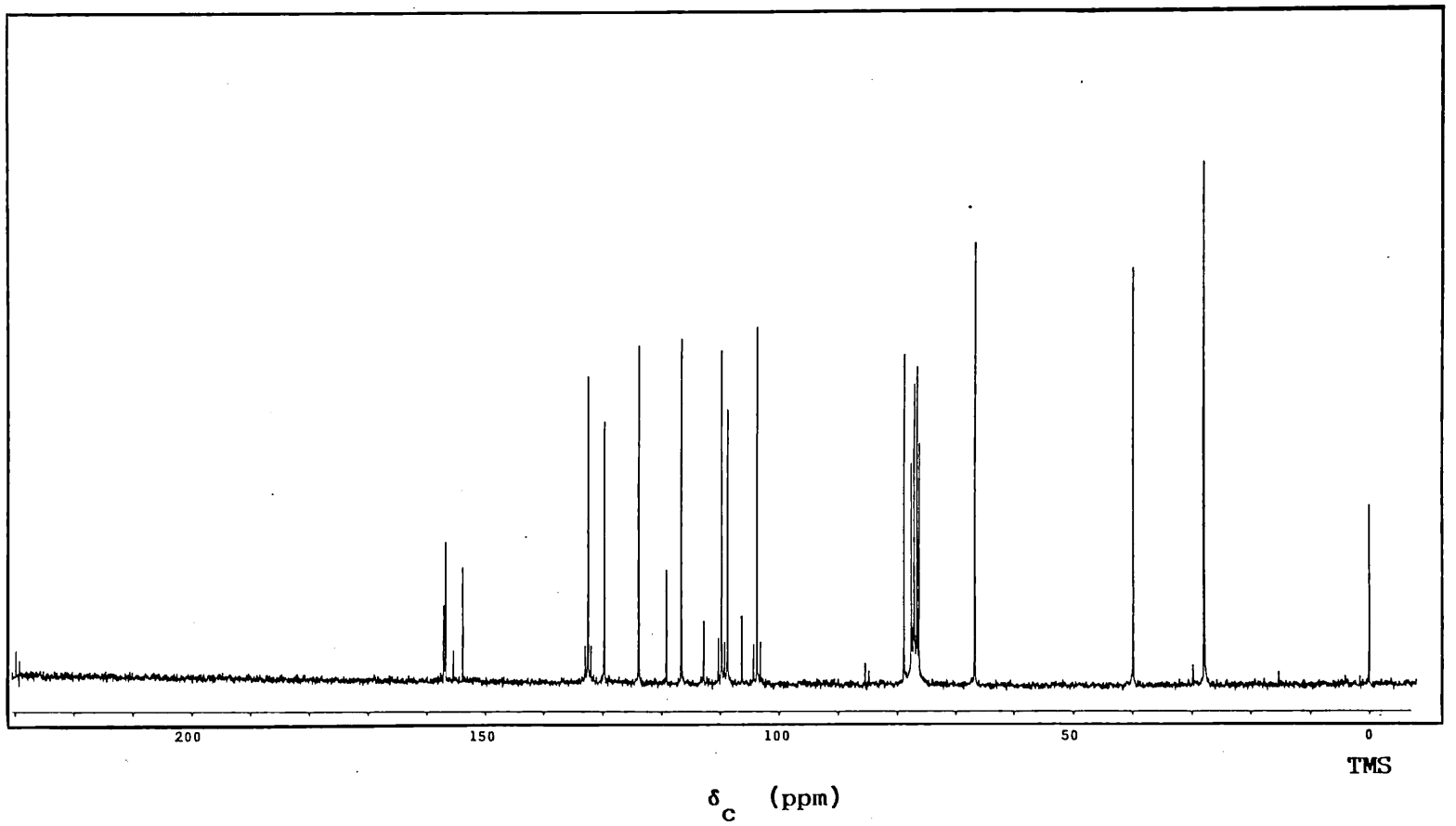


Figure 4.4

Complete ^{13}C-NMR spectrum of enriched phaseollin (62.9 MHz)

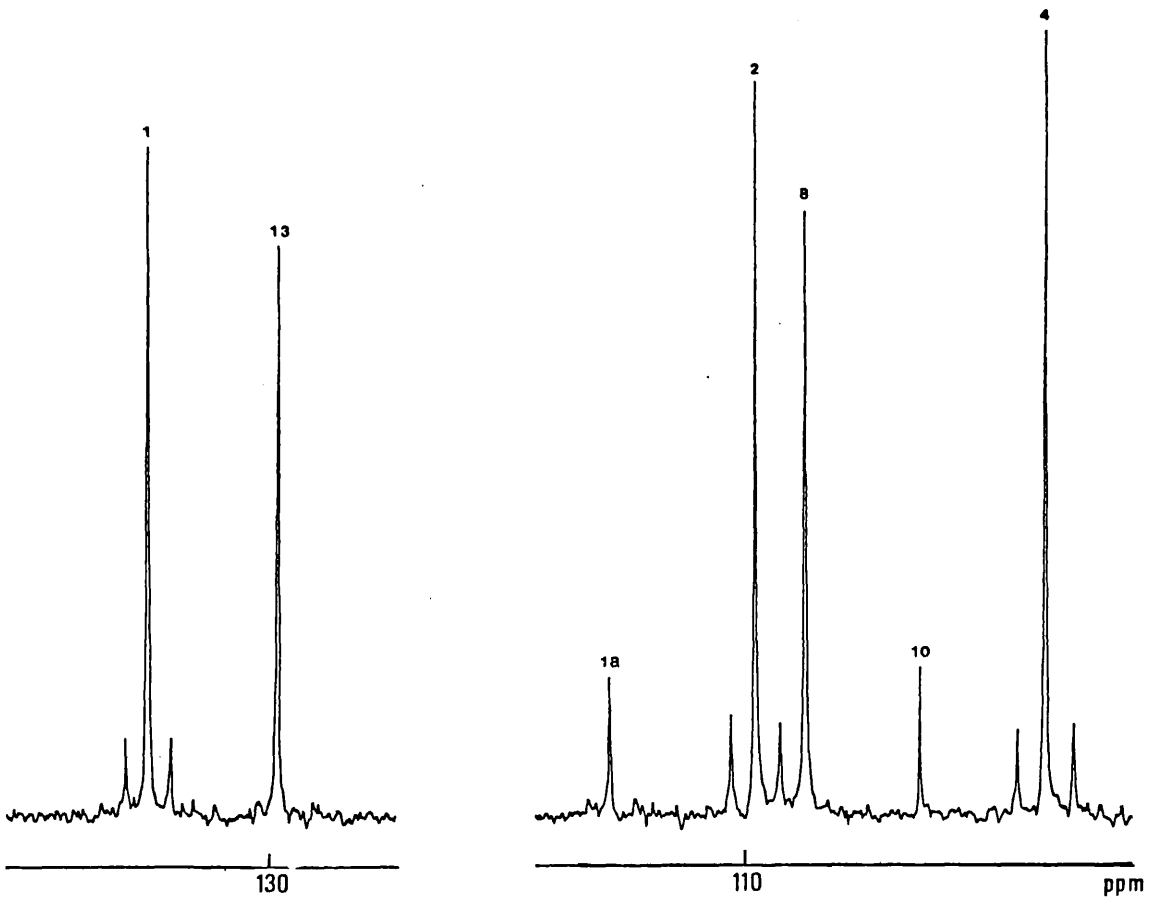


Figure 4.5

Enlarged ^{13}C-NMR spectrum of C-1, C-1a, C-2 and C-4 of phaseollin (62.9 MHz)

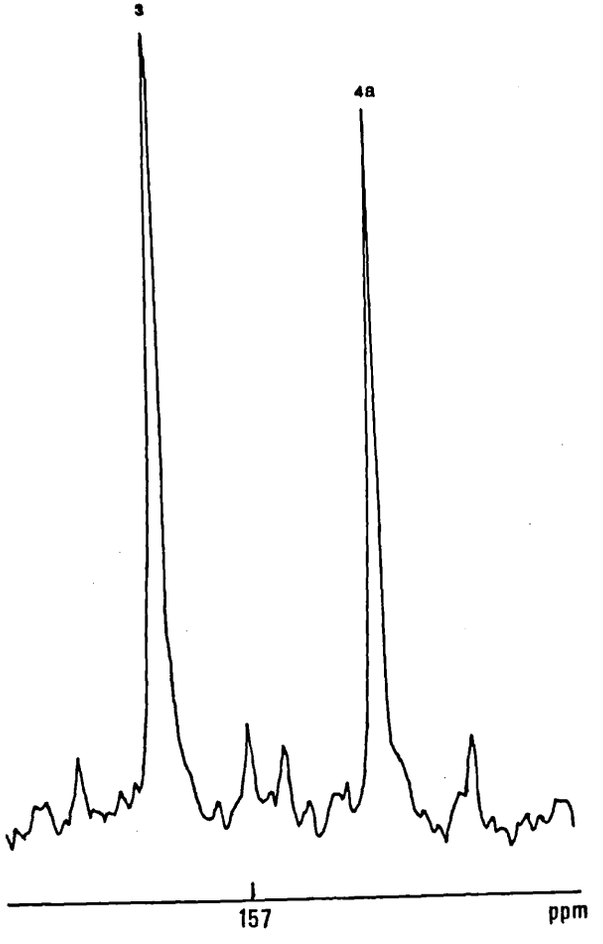


Figure 4.6

Enlarged ^{13}C-NMR spectrum of C-3 and C-4a of phaseollin (100.6 MHz)

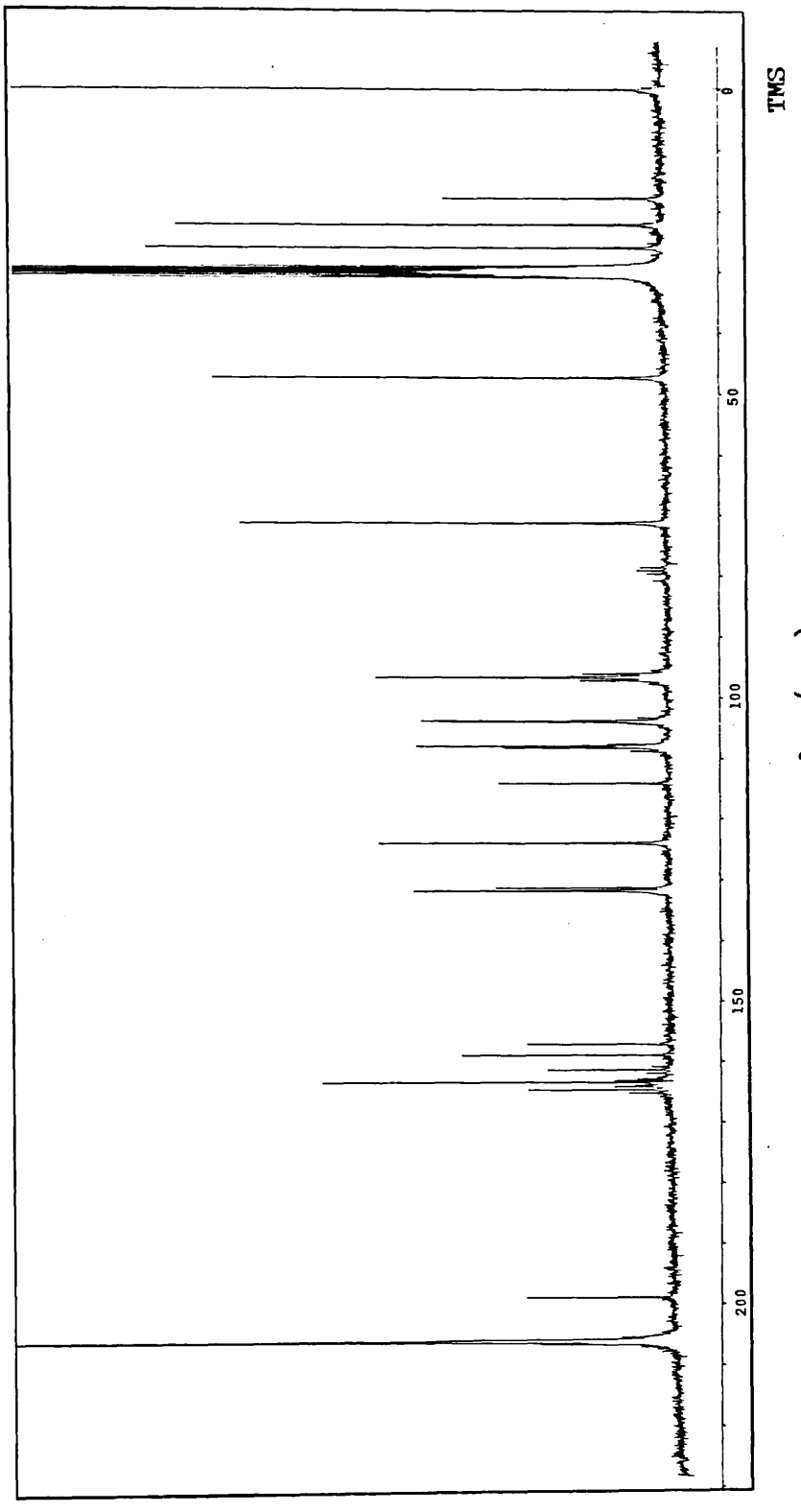


Figure 4.7

Complete ^{13}C-NMR spectrum of enriched kievitone (62.9 MHz)

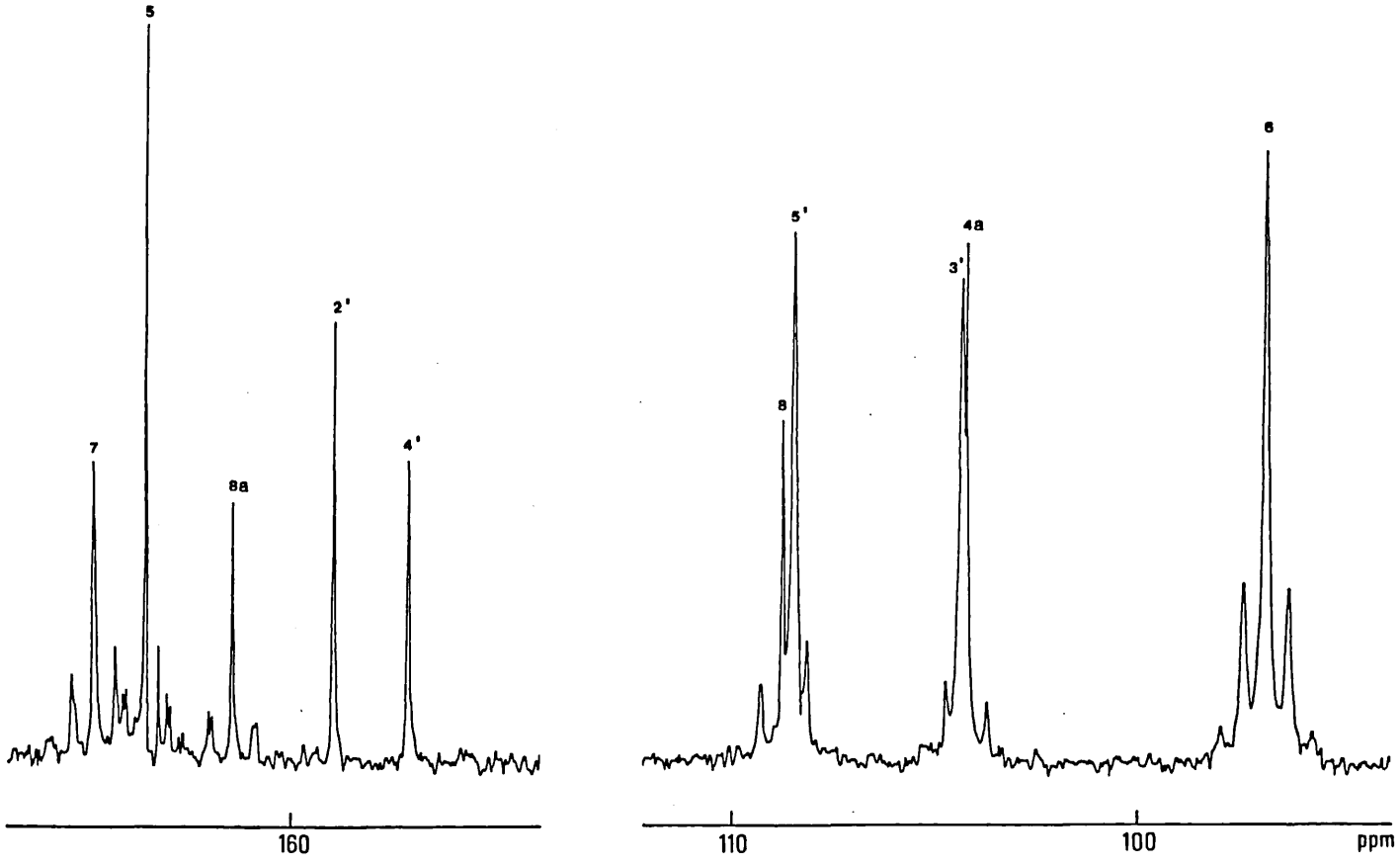


Figure 4.8

Enlarged ^{13}C-NMR spectrum of C-4a, C-5, C-6, C-7, C-8, and C-8a of kievitone (62.9 MHz)

Table 4.1 ^{13}C-NMR Chemical Shifts[a] and Coupling Constants for Phaseollin and Kievitone

Phaseollin				Kievitone			
Carbon	δ_c[ppm]	J_{cc}	(Hz)	Carbon	δ_c[ppm]	J_{cc}	(Hz)
1a	112.7	J_{1a-1}	61.0	4a	103.7	J_{4a-5}	62.0
1	132.3			5	163.4	J_{5-6}	72.0
2	109.7	J_{2-3}	64.7	6	96.5	J_{6-7}	ca.68.0
3	157.1			7	164.7	J_{7-8}	ca.68.0
4	103.7	J_{4-4a}	72.1	8	108.1	J_{8-8a}	73.0
4a	156.8			8a	161.3	J_{8a-4a}	63.0
6	66.6			2	71.2		
6a	39.7			3	47.2		
6b	119.1			1'	114.0		
7	123.8			6'	131.6		
8	108.6			5'	107.9		
9	155.4[b]			4'	157.0		
10	106.3			3'	103.8		
10a	153.8[b]			2'	158.8		
11a	78.7			4	198.8		
12	116.5			9	22.1		
13	129.6			10	123.9		
14	76.1			11	131.2		
14a	27.8			12	17.8		
14a'	27.8			13	25.8		

[a] ^{13}C-NMR spectra were obtained on a Bruker WM250 spectrometer operating at 62.90 MHz, or a Bruker WH400 at 100.62 MHz. Phaseollin was dissolved in $CDCl_3$, and kievitone in $(CD_3)_2CO$, and chemical shifts were recorded relative to TMS.

[b] Tentative assignments

4.5 Discussion

The $^{13}C - ^{13}C$ coupling constants of the six satellite signals in the enriched spectrum of phaseollin (Table 4.1) indicate that intact acetate units are incorporated into the A-ring in exactly the same manner as Stoessl and Stothers observed with pisatin, i.e. into carbons 1-1a, 2-3 and 4-4a. In contrast the pattern observed in the enriched spectrum of kievitone, where similiar coupling constants between four pairs of satellite signals caused the satellite signals to broaden (Table 4.1), is clearly analogous to that reported by Light and Hahlbrock for kaempferol, and is the result of two types of labelled kievitone being produced.

The biosynthetic pathway to phaseollin thus involves specific folding of the polyketide chain (Figure 4.1), and the "missing" oxygen function must therefore be reduced before the formation of the aromatic A-ring. In contrast, during the synthesis of kievitone, the aromatic A-ring is formed without the removal of oxygen, and either the folding process of the polyketide chain occurs in a non-specific and random manner, or at some stage the newly formed aromatic ring is free to rotate (Figure 4.1). Thus in *P. vulgaris*, the biosynthetic routes to 5-hydroxy and 5-deoxy flavonoids/isoflavonoids diverge prior to chalcone formation.

The results presented here are of particular significance as they show that both types of mechanism leading to chalcone formation occur in the same plant at the same time and although this situation has been observed previously in *Cicer arietinum* (with the use of ^{14}C-labelled precursors during the biosynthesis of the isoflavones formononetin and biochanin A, Grisebach and Bradner, 1961) they support the results presented in Chapter 3 where the differential accumulation of 5-hydroxy and 5-deoxy isoflavonoids in *P. vulgaris* cotyledons strongly suggested that the two pathways were independently controlled. The present results indicate

that phaseollin is synthesised by an as yet, uncharacterised, 6'-deoxychalcone synthase, which catalyses the formation, reduction, dehydration and possibly cyclisation of the polyketide intermediate, whilst kievitone is presumably synthesised via the 6'-hydroxychalcone synthase, which has been characterised from several plant tissues (Chapter 1). Thus, two independently regulated enzyme activities appear to control the formation and accumulation of 5-hydroxy and 5-deoxy isoflavonoid phytoalexins in *P. vulgaris*. The existence of a 6'-deoxychalcone synthase and the analogy of its reaction mechanism to that of 6-methylsalicylic acid synthase from *Penicillium patulum* (Dimroth et al., 1970) has been discussed in Chapter 1. However, the possibility remains that the "polyketide synthesising activity" leading to the triacyl intermediate is common to both enzymes.

In the next Chapter, 6'-hydroxychalcone synthase is purified from cell suspension cultures of *P. vulgaris* to enable the properties and activities of the enzyme to be studied in more detail and a 'screening experiment' to detect 6'-deoxychalcone synthase activity is reported.

CHAPTER 5

Purification and Properties of Chalcone Synthase from Cell Suspension Cultures of *Phaseolus vulgaris. L.*

5.1 Introduction

The enzyme chalcone synthase (CHS) catalyses the condensation of three molecules of malonyl CoA with one molecule of 4-coumaroyl CoA to yield 2',4,4',6'-tetrahydroxychalcone (**6**, Figure 5.1)(Heller and Hahlbrock, 1980). The chalcone may, however, undergo spontaneous isomerisation *in vitro* to yield the corresponding flavanone naringenin (**7**, Figure 5.1).

Such enzyme activity has been isolated from several sources including cell suspension cultures of parsley (*Petroselinum hortense*) (Heller and Hahlbrock, 1980; Kreuzaler and Hahlbrock, 1975a; Kreuzaler et al., 1979), *Haplopappus gracilis* (Saleh et al., 1978), and anthers from *Tulipa* c.v. Appledorn (Sütfeld et al., 1978; Sütfeld and Wiermann, 1980).

Chalcone formation is believed to occur via head to tail condensation of acetate units resulting in the formation of a polyketide intermediate (Kreuzaler and Hahlbrock, 1975b). Unlike other similiar reactions catalysed by fatty acid synthase, the immediate substrate for the first partial reaction of CHS appears to be the malonyl CoA thiol ester rather than a malonyl unit linked to an enzyme -SH group or an acyl carrier protein; the malonyl CoA is decarboxylated prior to condensation (Kreuzaler et al., 1978)(Figure 5.1).

A further characteristic of CHS, at least from *P. hortense* and *H. gracilis*, is the formation of "release" or "derailment" products as

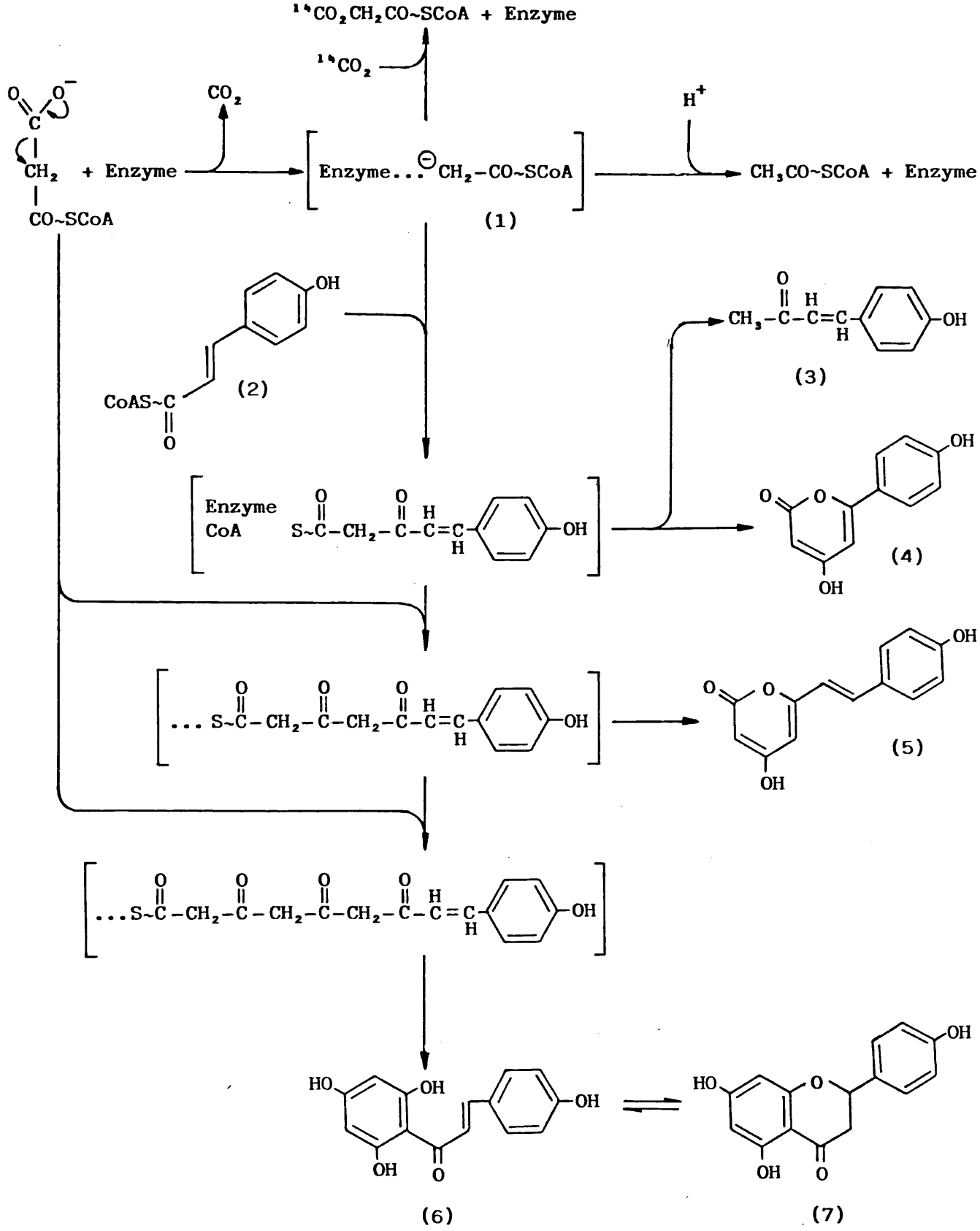


Figure 5.1 Summary of the initial reactions and formation of release products catalysed by chalcone synthase. (1) Hypothetical carbanion intermediate; (2) 4-Coumaroyl CoA; (3) 4-Hydroxybenzalacetone. (4) 4-Hydroxy-5,6-dihydro-6-(4-hydroxyphenyl)-2-pyrone; (5) Bis-noryangonin; (6) 2',4,4',6'-Tetrahydroxychalcone; (7) Naringenin (After Hrazdina et al., 1976 and Kreuzaler et al., 1978).

a result of release from the enzyme of partially completed polyketide chains (Kreuzaler and Hahlbrock, 1975a and 1975b; Saleh et al., 1978; Hrazdina et al., 1976). Such release products, which are formed *in vitro* but not *in vivo*, include the styrylpyrone bis-noryangonin (Kreuzaler and Hahlbrock, 1975b) and 4-hydroxybenzalacetone (Hrazdina et al., 1976) (Figure 5.1, **5** and **3** respectively).

P. vulgaris synthesises both 5-hydroxy and 5-deoxy isoflavonoid phytoalexins in response to microbial infection or abiotic treatments (Chapter 1). Evidence from both ^{14}C-labelling and ^{13}C-incorporation studies (Chapters 1 and 4) have indicated that chalcones are key intermediates in the biosynthesis of such compounds.

6'-Hydroxychalcone synthase (previously 5-hydroxyflavanone synthase) is a key enzyme in the synthesis of kievitone; this compound, and phaseollin, constitute the two main phytoalexins which accumulate in *P. vulgaris*. The differentiation of the two pathways at the level of the chalcone suggests that CHS may be an important regulatory site.

The results of experiments showing a) the temporal differences in the accumulation patterns of the two classes of isoflavonoid compounds (Chapter 3) and b) ^{13}C-acetate incorporation patterns into phaseollin and kievitone which indicate loss of the chalcone 6'-hydroxyl prior to ring closure during the biosynthesis of phaseollin (Chapter 4) strongly suggest that two separate chalcone synthases exist in *P. vulgaris*.

In cell suspension cultures of *P. vulgaris* 6'-hydroxy CHS activity undergoes a rapid transient increase in response to elicitor macromolecules isolated from the cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* (Lawton et al., 1983a). *In vivo* labelling studies with ^{35}S-methionine (Lawton et al., 1983a) and *in vitro* translation of polysomal mRNA using a message-dependent reticulocyte lysate system (Lawton et al., 1983b) have indicated that elicitor induces de novo

synthesis of 6'-hydroxy CHS subunits by bringing about an increase in the activity of specific CHS mRNA as a function of total cellular mRNA activity. These studies relied upon immunoprecipitation of bean CHS using anti-parsley CHS antiserum.

The purification of 6'-hydroxy CHS is important for several reasons. Firstly, as discussed in the previous chapter, there is the possibility that the "polyketide synthesising activity" is common to both 6'-hydroxy and 6'-deoxy CHS. Indeed it is possible that 6'-hydroxy CHS is the condensing enzyme of 6'-deoxy CHS. Secondly, once purified, the properties of 6'-hydroxy CHS from *P. vulgaris* can be compared with 6'-hydroxy CHS from other sources; for example *P. hortense*, a plant which only synthesises 5-hydroxylated flavonoids. Thirdly, purified CHS could be used to raise antibodies for immunological work to investigate rates of synthesis and appearance of the enzyme and extend work previously performed with anti-parsley CHS antiserum (Lawton et al., 1983b).

Previous efforts to detect 6'-deoxy CHS in *P. vulgaris* were unsuccessful (Dixon and Bendall, 1978b). The present work includes a 'screening experiment' which attempts to locate the activity *in vitro*.

5.2 Materials and Methods

The source of all chemicals has been described in Chapter 2.

5.3 Buffers

The main buffers used were (A) 100mM KH_2PO_4, pH 8.0; (B) 50mM KH_2PO_4, pH 8.0; (C) 25mM KH_2PO_4, pH 8.0; (D) 100mM KH_2PO_4, pH 7.0 and (E) 100mM Tris-HCl, pH range 7.0-8.5. All of the above buffers contained 1.4mM 2-mercaptoethanol.

5.4 Source of Enzyme

CHS activity was induced in cell suspension cultures (Section 2.1) by treatment with a crude *C. lindemuthianum* (CMI isolate IMI 112166) elicitor preparation (final elicitor concentration 100μg glucose equivalents/ml culture). After 5h in the presence of elicitor the cells were harvested by suction filtration, frozen in liquid N_2, and stored at -70°C until required for enzyme extraction. No significant loss of total CHS activity was observed in frozen cells over a period of several months.

The optimal elicitor concentration for CHS induction was established in a dose-response experiment (Figure 5.2). The optimal period of elicitation was similiarly established in a time-course experiment (Figure 5.3).

5.5 Purification of the Enzyme

All steps were carried out at 4°C. 300g of frozen cells were thawed and homogenised with 300ml of buffer A in a Waring blendor. The homogenate was passed through four layers of muslin to remove cell debris and the extract was centrifuged for 15min. at 15,000 x $g_{av.}$. The resulting supernatant was stirred for 20min. with 30g Dowex AG 1-X8 (phosphate form, equilibrated with buffer A),(0.1g/g was shown to be optimal, see Section 2.10) after slowly readjusting the pH to 8.0 with 1.0M KOH. After removal of the resin by filtration through glass wool the supernatant (475ml) was made up to 50% $(NH_4)_2SO_4$ saturation by the addition of 167g of the salt over a 30min. period, the pH being kept constant with 1M KOH. The precipitate was removed by centrifugation for 15min. at 15,000 x $g_{av.}$. A second precipitate was obtained in the same manner from 550ml of supernatant made up to 75% $(NH_4)_2SO_4$

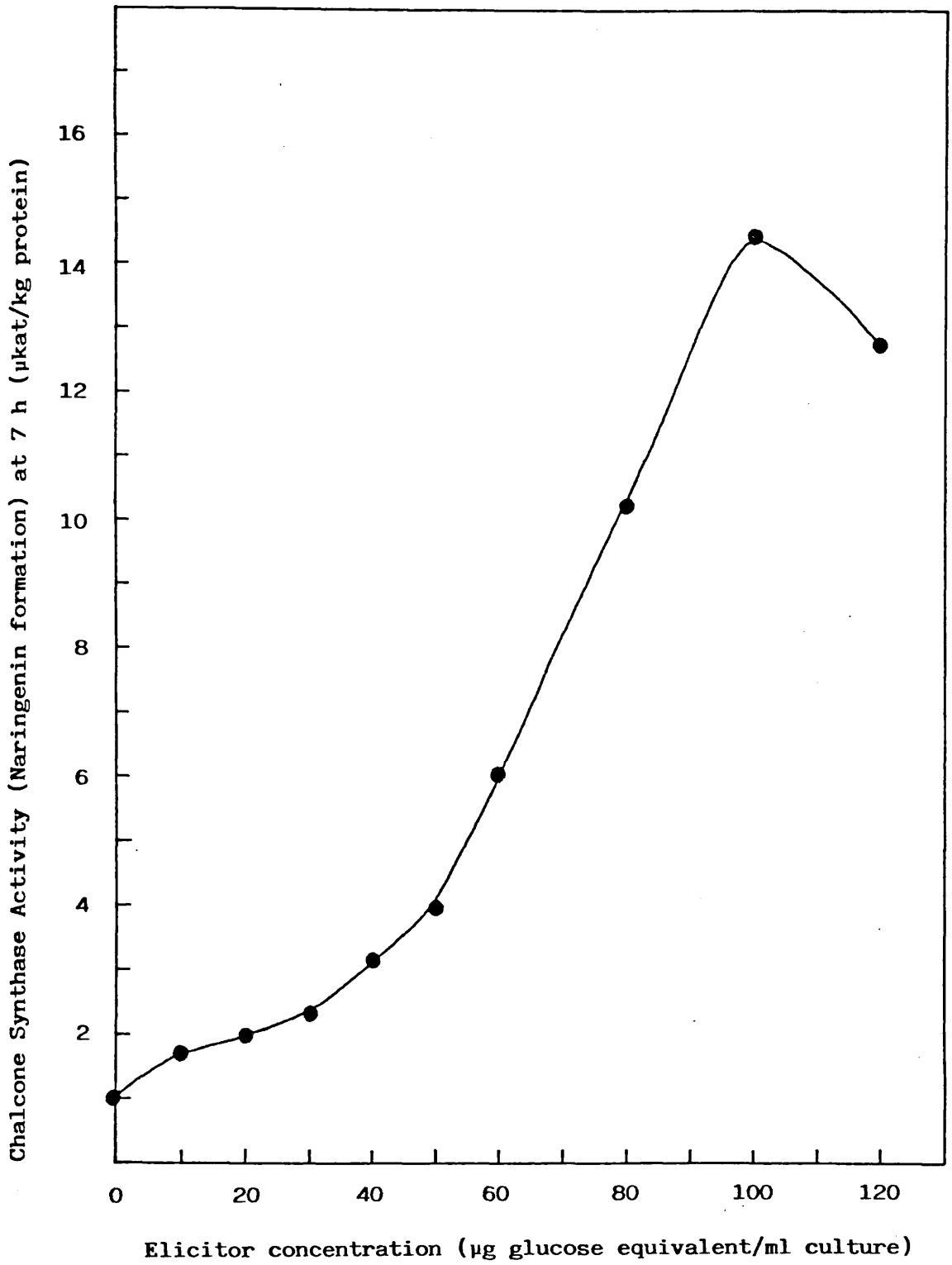


Figure 5.2

Dose-response curve for the effects of crude *Colletotrichum* elicitor concentration (CMI isolate IMI 1122166) on the induction of chalcone synthase in *Phaseolus vulgaris* cell suspension cultures.

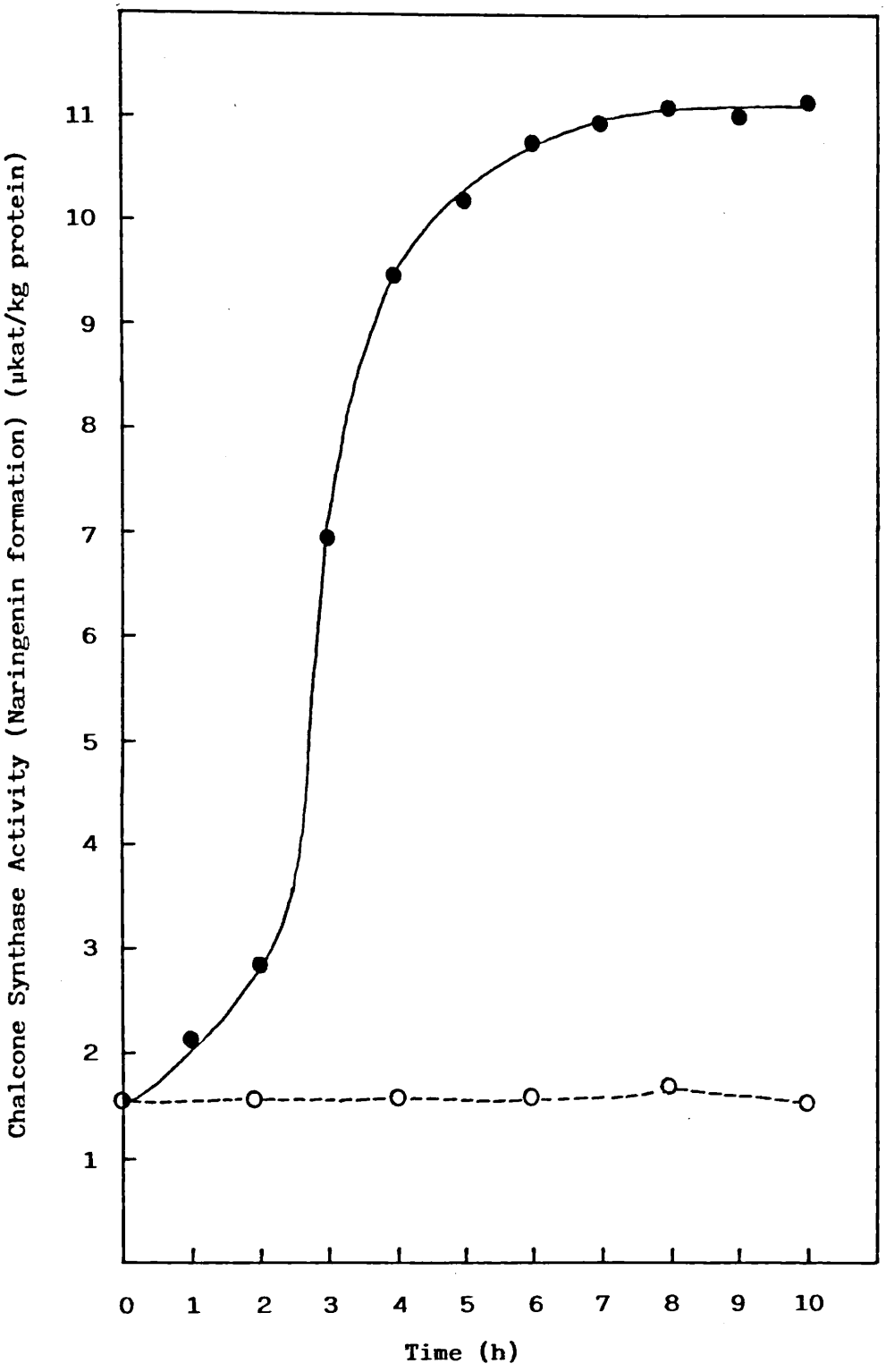


Figure 5.3 Time Course of Chalcone Synthase Activity in *Phaseolus vulgaris* cell suspension cultures. (●—●) Activity elicited with 100 µg glucose equivalents CMI elicitor/ml culture. (○—○) Untreated control values.

saturation with 77.5g of the salt. The fractionation of CHS by $(NH_4)_2SO_4$ was established in a preliminary experiment (Table 5.1).

The precipitate was dissolved in 5ml of buffer A, applied to a Sephadex G-100 column (90 x 2.5cm) and eluted with buffer B at a flow rate of 20ml/h. Fractions (4ml) were collected and those containing the highest CHS activity (Fractions 68-80, Figure 5.4, rapid assay method) were pooled, diluted to 25mM KH_2PO_4 with distilled water and immediately applied to a Whatman DE-52 DEAE-cellulose column (27 x 0.8cm) which had been equilibrated with buffer C. The column was eluted at a flow rate of 50ml/h first with 40ml buffer C and then with a linear gradient of 500ml of this buffer increasing in molarity from 25 to 150mM KH_2PO_4. Fractions (4.4ml) were collected and those containing the highest CHS activity (Fractions 36-53, Figure 5.5, rapid assay method) were combined (approximately 75ml) and concentrated to 7.1ml by ultrafiltration (Amicon UM-10 membrane, 75psi). The resulting enzyme solution was stored in 1ml aliquots with 30% glycerol and 14mM 2-mercaptoethanol at -70°C.

Table 5.1

Fractionation of CHS Activity from a Crude Supernatant of Elicited *P. vulgaris* Cell Suspension Cultures

$(NH_4)_2SO_4$ saturation (%)	CHS activity in precipitate (pkat)
0-40	0.11
40-50	0.41
50-60	2.29
60-70	2.66
70-80	0.85

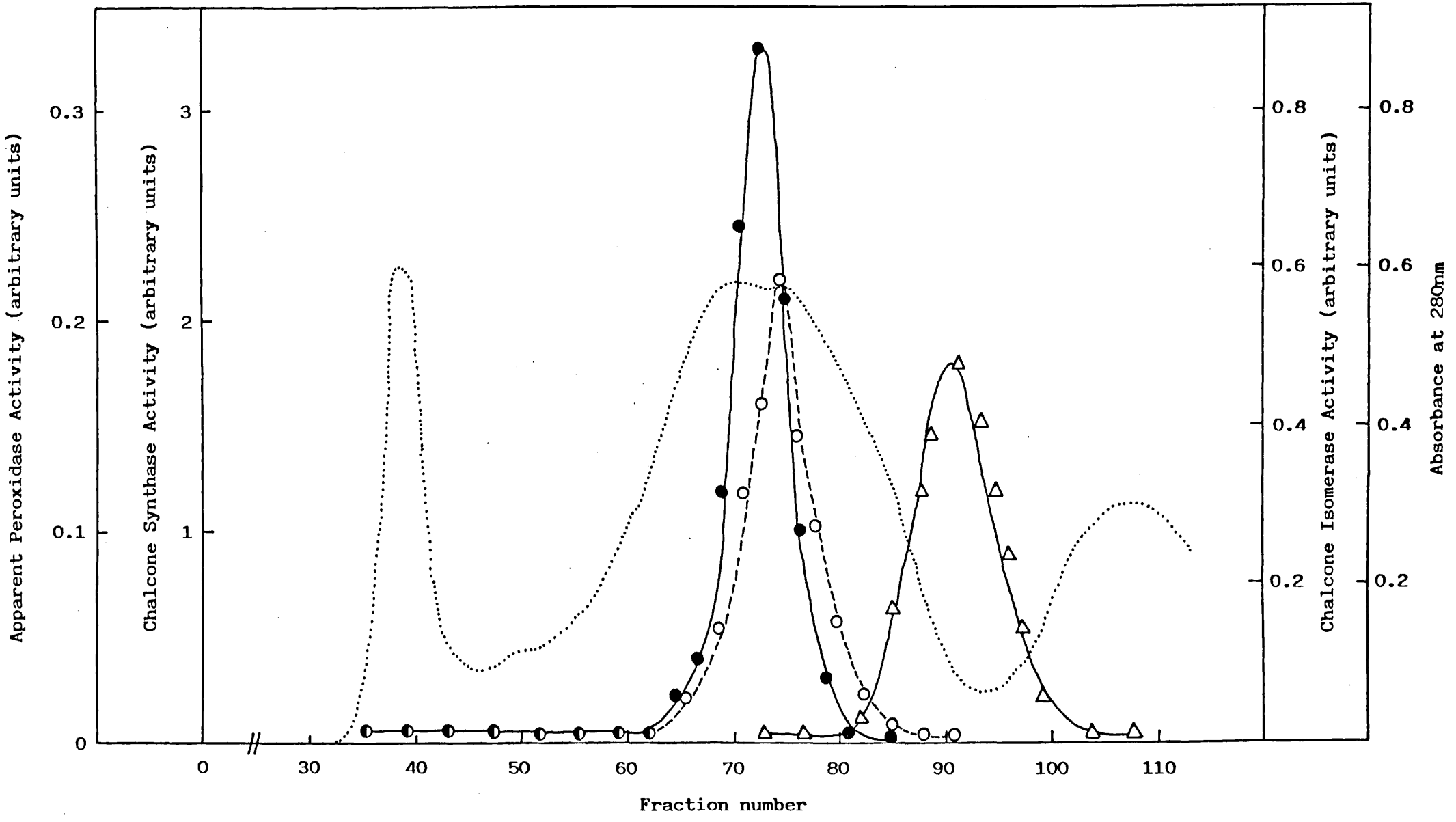


Figure 5.4 Purification of chalcone synthase by gel-filtration on Sephadex G-100. (●—●) Total chalcone synthase activity; (△—△) Chalcone isomerase activity; (O--O) Apparent peroxidase activity; (.....) Absorbance at 280 nm.

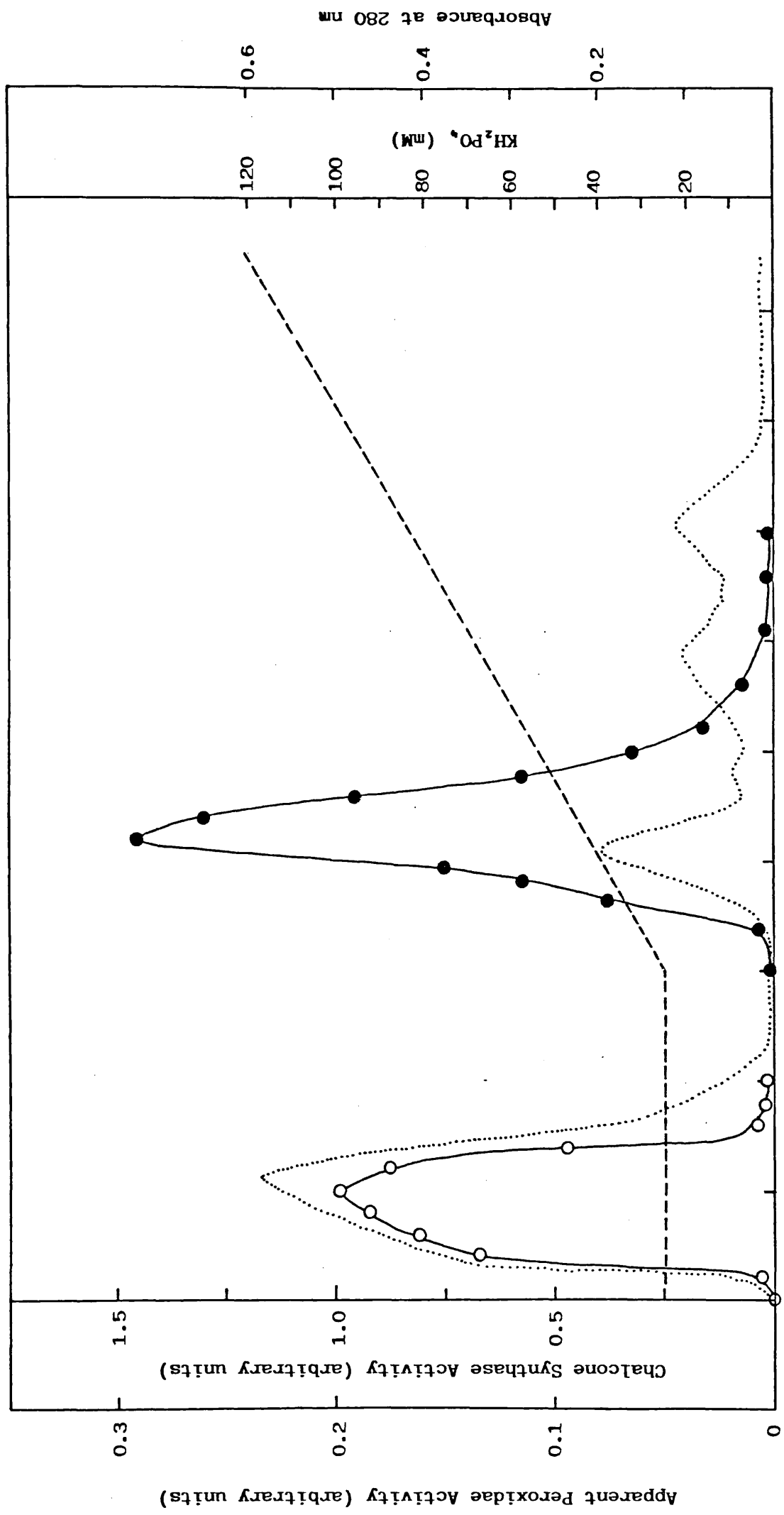


Figure 5.5 Purification of chalcone synthase on DEAE-cellulose. (●—●) Total chalcone synthase activity; (○—○) Apparent peroxidase activity; (·····) Absorbance at 280 nm; (- - - -) Potassium phosphate gradient.

5.6 Assays of Enzyme Activity

For experiments using stored, purified CHS enzyme, samples were thawed and dialysed overnight at 4°C against buffer A to remove glycerol and high 2-mercaptoethanol prior to assay. The assay of total chalcone synthase activity, naringenin formation, the CO_2 exchange reaction, CHI and apparent peroxidase activities have been described (Sections 2.9b,c,d and e).

5.7 Protein Determination

Protein was determined as described previously (Section 2.11).

5.8 Results

5.8a Purification Procedure

The purification procedure shown in Table 5.2 resulted in only a 32-fold increase in specific activity of the enzyme from crude, in spite of the removal of a large percentage of the total protein. The actual purificaton was approximately only half this value, as the Dowex treatment activates the synthase by removal of inhibitory flavonols and therefore increases total activity. This low purification value reflects the instability of the synthase, with approximately 60% of the activity being lost at each purification stage. The enzyme from the DEAE-cellulose step was free from both chalcone isomerase (converts chalcones to flavanones) and peroxidase (converts chalcones to a variety of oxidation products)(Wong and Wilson, 1976; Chapter 6). At each stage in the purification, the products formed in the synthase reaction were monitored by TLC (Cellulose, 30% HOAc). The ratio of naringenin to other 4-coumaroyl-CoA-dependent products decreased with increasing purification of the enzyme (Table 5.2). Enzyme assays

Table 5.2 Partial Purification of Chalcone Synthase from Cell Suspension Cultures of *Phaseolus vulgaris*

Fraction	Total activity (pkat)	Total protein (mg)	Specific activity[1] (μkat/kg)	Relative purity (-fold)	Recovery (%)	Ratio of naringenin to release products[2]	
						(a)	(b)
Crude extract	275	302.60	0.91	1.0	100.0	1:1.55	1:0.00
Supernatant after Dowex AG1-X8	367	213.80	1.72	1.9	133.0	1:1.30	1:0.03
50-75% $(NH_4)_2SO_4$ fractionation/ Sephadex G-100	117	13.60	8.60	9.5	42.5	1:1.36	1:0.12
DEAE-cellulose/ ultrafiltration	45	1.53	29.40	32.3	16.4	1:2.24	1:0.26

[1] Total activity (malonyl CoA converted to EtOAc-soluble products).

[2] Determined by TLC of EtOAc extractions from reaction mixtures incubated in the presence (a) and absence (b) of 4-coumaroyl CoA. Column (a) gives ratio of naringenin to total 4-coumaroyl CoA release product formation, column (b) the ratio of naringenin to products formed from malonyl CoA alone.

(total activity) were also carried out in the absence of 4-coumaroyl CoA. With the crude extract, no EtOAc soluble products were formed under these conditions; however, products dependent upon the presence of malonyl CoA alone were found in increasing amounts as the enzyme was purified (Table 5.2, last column).

5.8b Molecular Weight

The molecular weight of the synthase was estimated to be about 77,000 following gel-filtration on a Sephadex G-100 column calibrated according to the method of Andrews, 1964 (Figure 5.6).

5.8c Stability of the Enzyme Activity

The synthase activity was very unstable at all stages during the purification procedure when stored at 4°C. 200µl aliquots of the enzyme solution obtained form the DEAE/Amicon step were stored with various additives for 40 days at -70°C. The results are presented in Table 5.3 .

5.8d Linearity of the Reaction

The synthase reaction was linear with respect to time over a period of at least 30 min. at 35°C for both total activity and naringenin formation (Figure 5.7).

5.8e Dependence on Temperature

The highest rate of total product formation was observed at 40°C, the enzyme being routinely assayed at 35°C (Figure 5.8).

5.8f Dependence on Ionic Strength

Varying the ionic strength of the assay between 10 and 300mM KH_2PO_4 at pH 8.0 did not significantly affect the enzyme activity (Fig. 5.9).

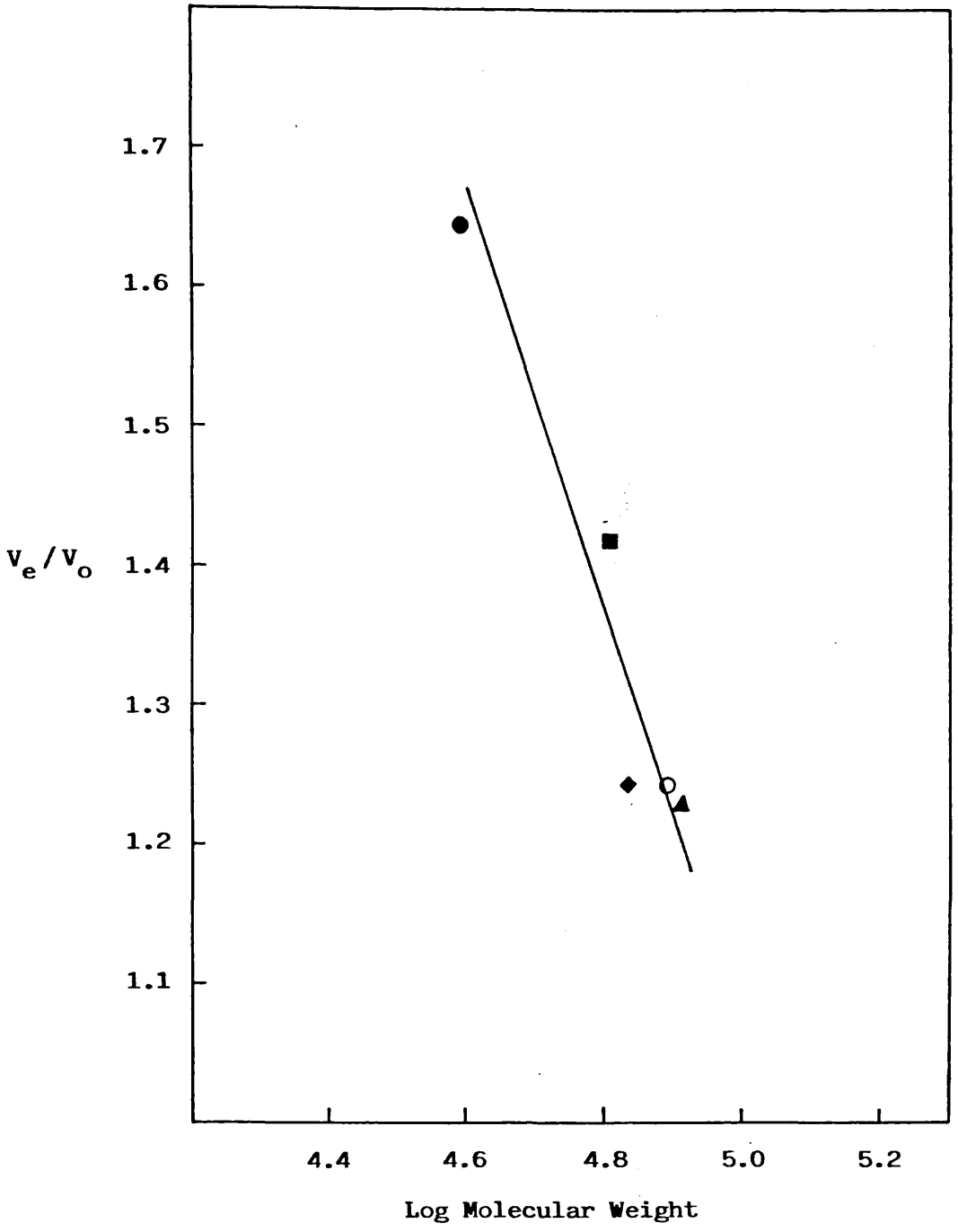


Figure 5.6

Estimation of the molecular weight of chalcone synthase (○) by column chromatography on Sephadex G-100. The column was calibrated according to the method of Andrews (1964) with ovalbumin (●), haemoglobin (■), the monomeric form of BSA (◆), creatine kinase (▲) and blue dextran. V_e = elution volume; V_o = void volume.

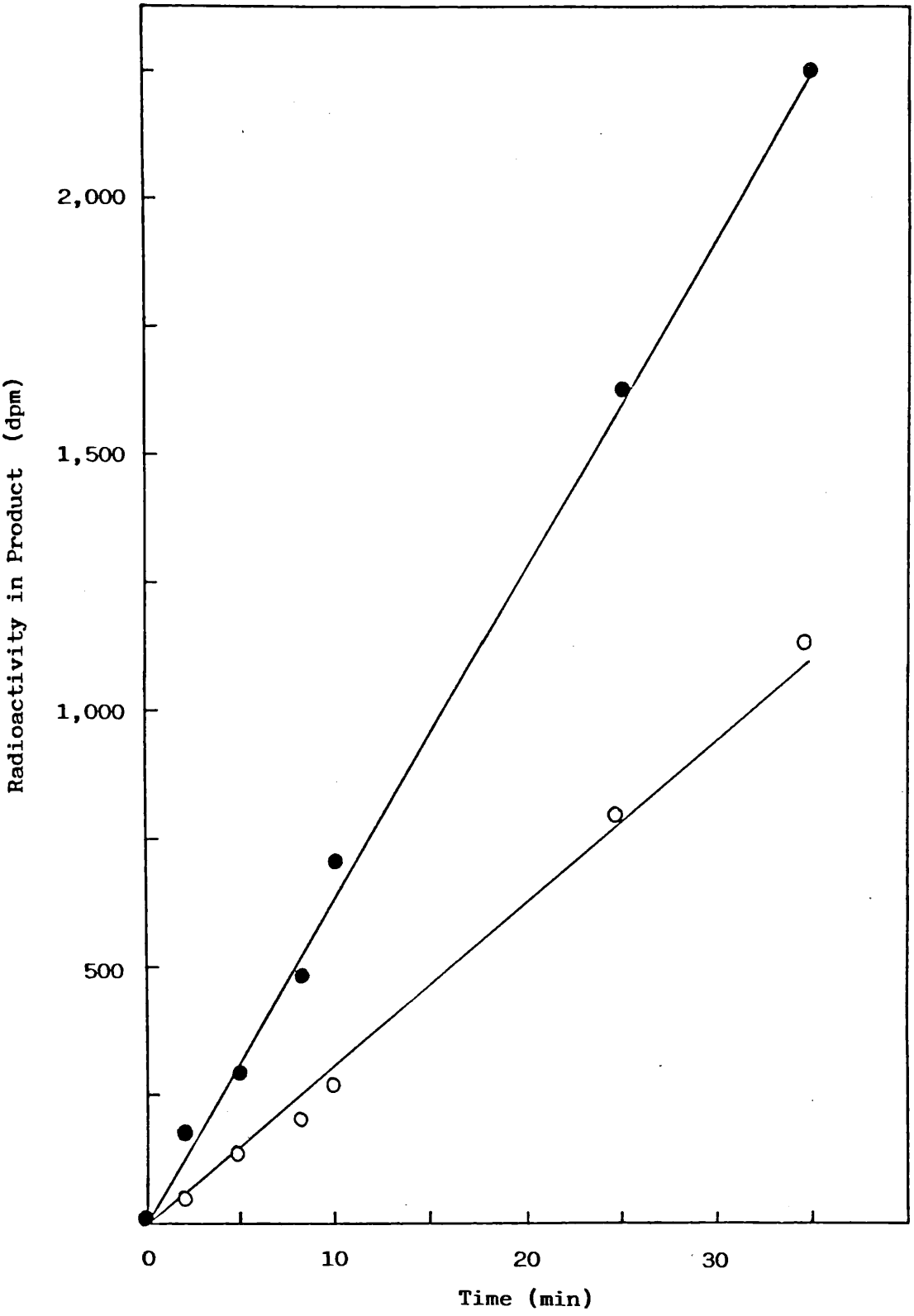


Figure 5.7

Linearity of chalcone synthase activity with respect to time.

(●—●) Total activity; (○—○) Naringenin formation.

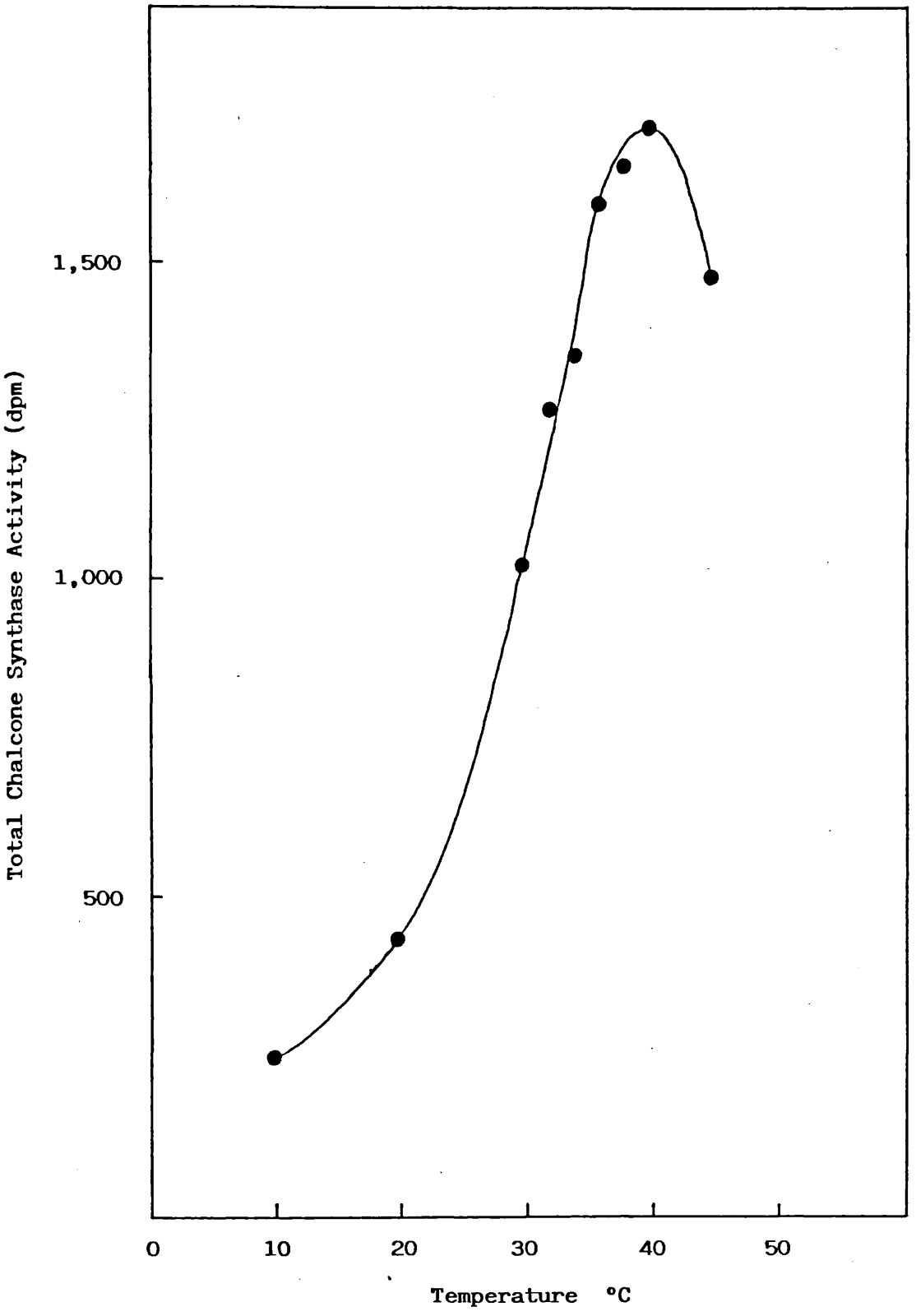


Figure 5.8

Total chalcone synthase activity as a function of temperature.

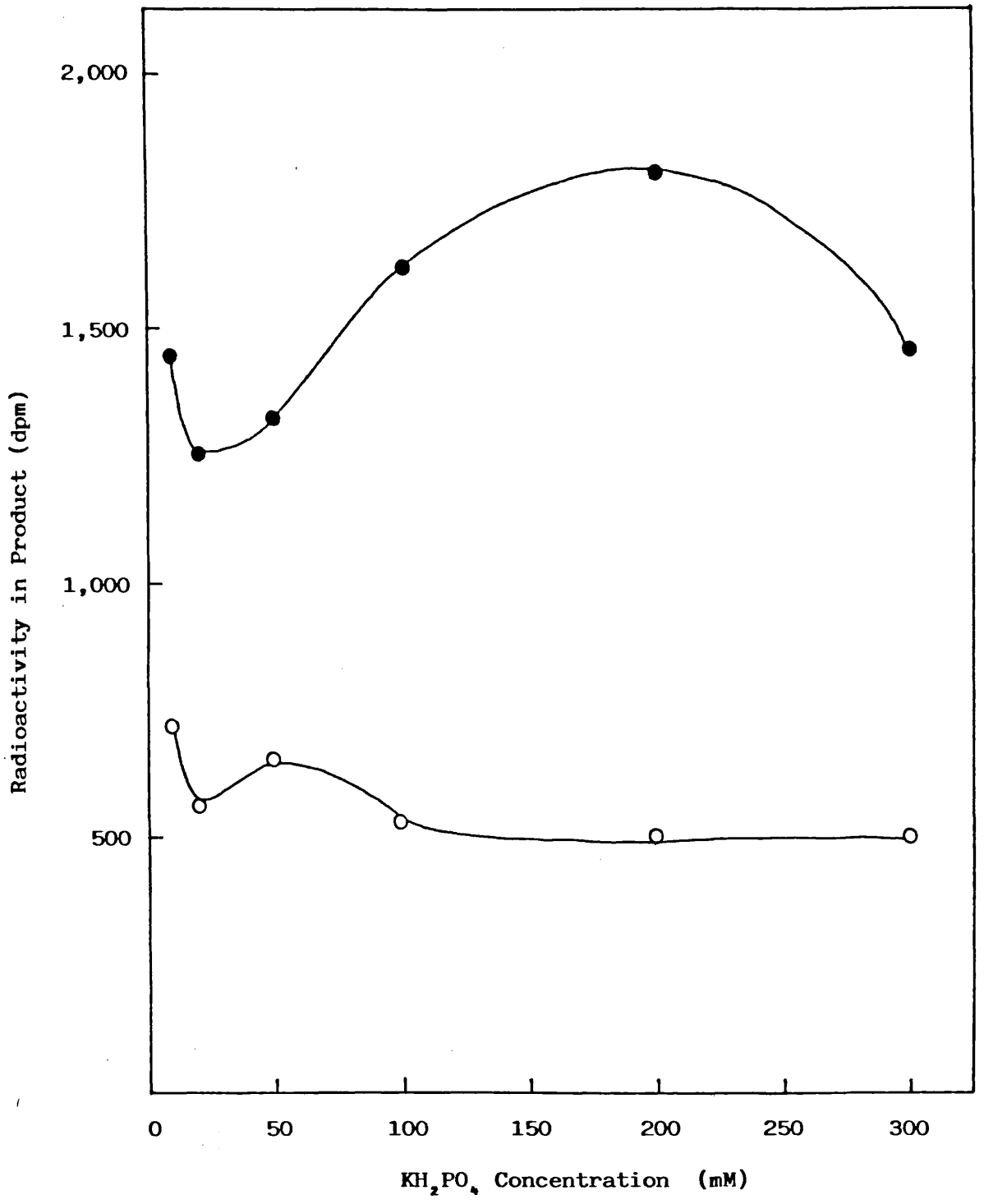


Figure 5.9

Effect of ionic strength on chalcone synthase activity at pH 8.0. (●——●) Total activity; (○——○) Naringenin formation.

Table 5.3

Stability of CHS Activity[1] in an Enzyme Solution from the DEAE/Amicon Purification Step Stored for 40 Days at -70°C with Various Additives

Addition[2]	Pre-storage S.A.[3]	Post-storage S.A.[3]	S.A.[3] retained (%)
None	69,006	0	0.0
30% glycerol	62,128	30,419	49.0
14mM 2-mercapto-ethanol	117,288	9,187	7.8
30% glycerol/ 14mM 2-mercapto-ethanol	116,248	49,102	42.2
2mg/ml BSA	58,316	5,924	10.2
2mg/ml BSA/ 14mM 2-mercapto-ethanol	174,852	17,590	10.1

[1]Total activity.
[2]2-Mercaptoethanol increases total CHS activity (see section 5.8h)
[3]S.A. = Specific activity (dpm/min./mg).

5.8g Effects of pH

The pH optimum of the synthase was approximately 8.0 with respect to both total product formation and naringenin production (Figure 5.10). However, the partially purified enzyme appeared to be more stable at around pH 7.0; when 100µl aliquots of stored enzyme were first dialysed at 4°C for 12h against buffers (100mM KH_2PO_4, containing 1.4mM 2-mercaptoethanol) to pH's between 6.5-8.5, and then back, under similiar conditions, to pH 8.0 for assay, the highest activity remained in preparations dialysed at pH 7.0, in spite of this pH being below the optimum for catalytic activity (Figure 5.10, inset). However attempts to purify the enzyme using buffers at pH 7.0 did not result in increased recovery.

[Note: The effect of pH on the stability of the enzyme necessitated that use of an alternative method to dialysis be employed when attempting to assess the pH optimum of the synthase. In a preliminary experiment

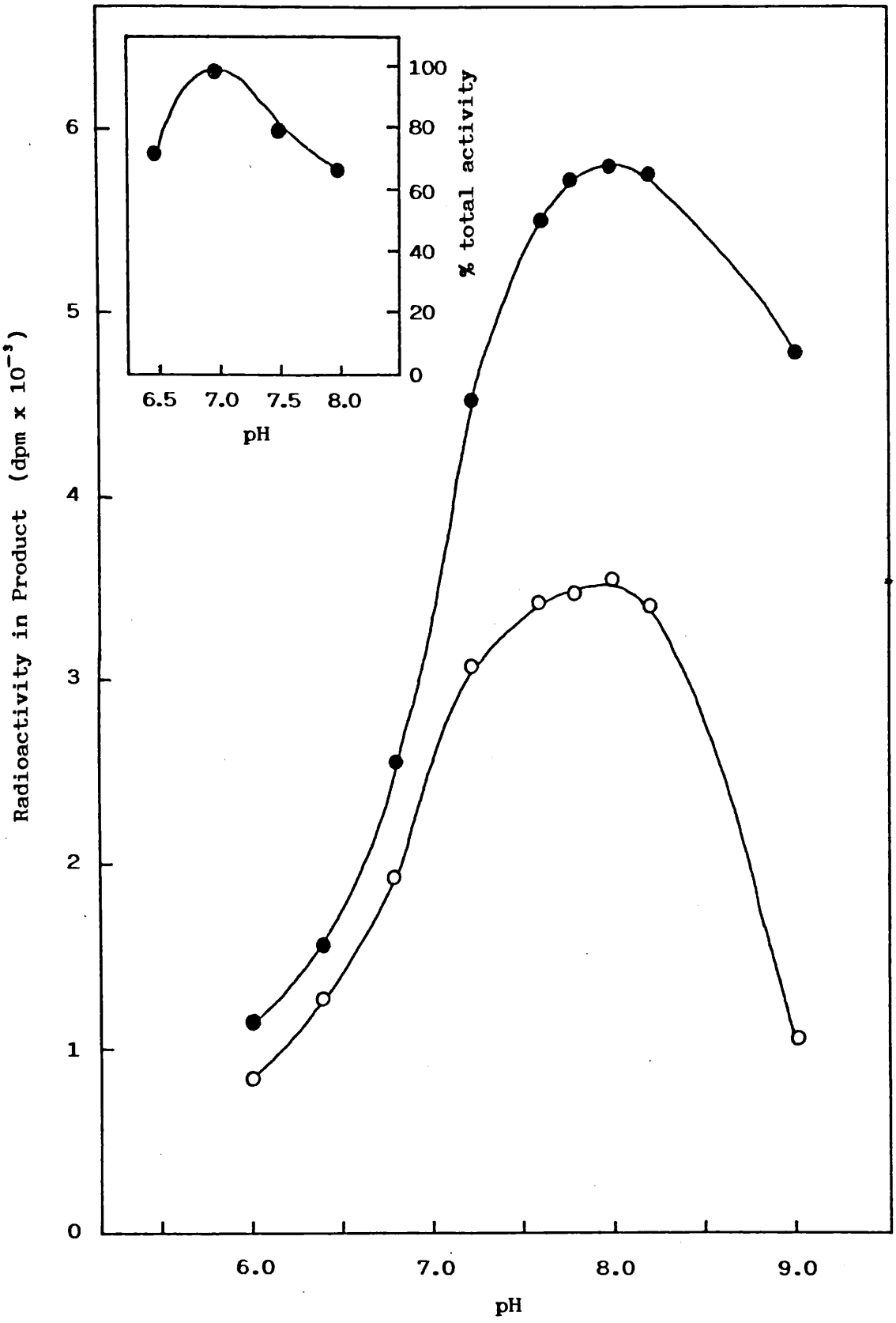


Figure 5.10

pH profile for chalcone synthase activity. (●——●) Total activity for malonyl CoA conversion to EtOAc soluble products; (○——○) Naringenin formation. Inset shows pH profile obtained for total activity after dialysis of enzyme samples for 12h at the pH of assay.

it was determined that 10mM KH_2PO_4, dilute HCl pH 3.5, and 271.4mM KH_2PO_4, in the pH range 6.0-8.0 when mixed together in the ratio of the assay mix ie. 50:15:35, produced buffers of unaltered pH's at a final concentration of 100mM KH_2PO_4. (This was also established for Tris-HCl buffers in the range 7.0-9.0). Thus 400µl of stored CHS enzyme was dialysed overnight against 100ml, 10mM KH_2PO_4 (or Tris-HCl), pH 7.0. The enzyme was then assayed by addition to the assay vials prepared as previoulsy described (Section 2.10) except that they contained 271.4mM buffers at the required pH. By this method, it was possible to remove the effect of pH on the stability of the enzyme when assessing the pH optimum].

5.8h Effects of Thiol Reagents

Total synthase activity was measured in the presence and absence of varying concentrations (0-20mM) of DL-dithiothreitol (DDT), 2-mercaptoethanol, L-cysteine and glutathione. The rate of reaction increased with increasing concentrations of DDT (a 5-fold increase at 20mM) and 2-mercaptoethanol (a 3-fold increase at 20mM) whereas cysteine and glutathione showed no effect (Figure 5.11). Analysis (by TLC) of products formed at varying concentrations of DTT indicated that, in the absence of DTT, all products formed were dependent upon the presence of 4-coumaroyl CoA in the reaction mixture. However, two non-coumaroyl CoA dependent products (R_f 0.61 and R_f 0.83) appeared on Silica gel G thin layer chromatograms developed in toluene: $CHCl_3$:Me_2CO (40:25:35) in increasing amounts over the range 0-5mM DTT (Figure 5.12A). Naringenin formation was optimal at 1.4mM DTT; further increases caused naringenin formation to be inhibited, although production of other 4-coumaroyl CoA dependent products was only maximal at 20mM DTT (Figure 5.12B). The major 4-coumaroyl CoA dependent band was tentatively assumed, from R_f measurements (cellulose, 30% HOAc; measured R_f 0.79, reported R_f 0.81 (Kreuzaler and Hahlbrock, 1975b) , to be the styrylpyrone bis-noryangonin which is a release product of the parsley synthase (Kreuzaler and Hahlbrock, 1975a,1975b). Of the two products requiring only malonyl CoA for their formation ,

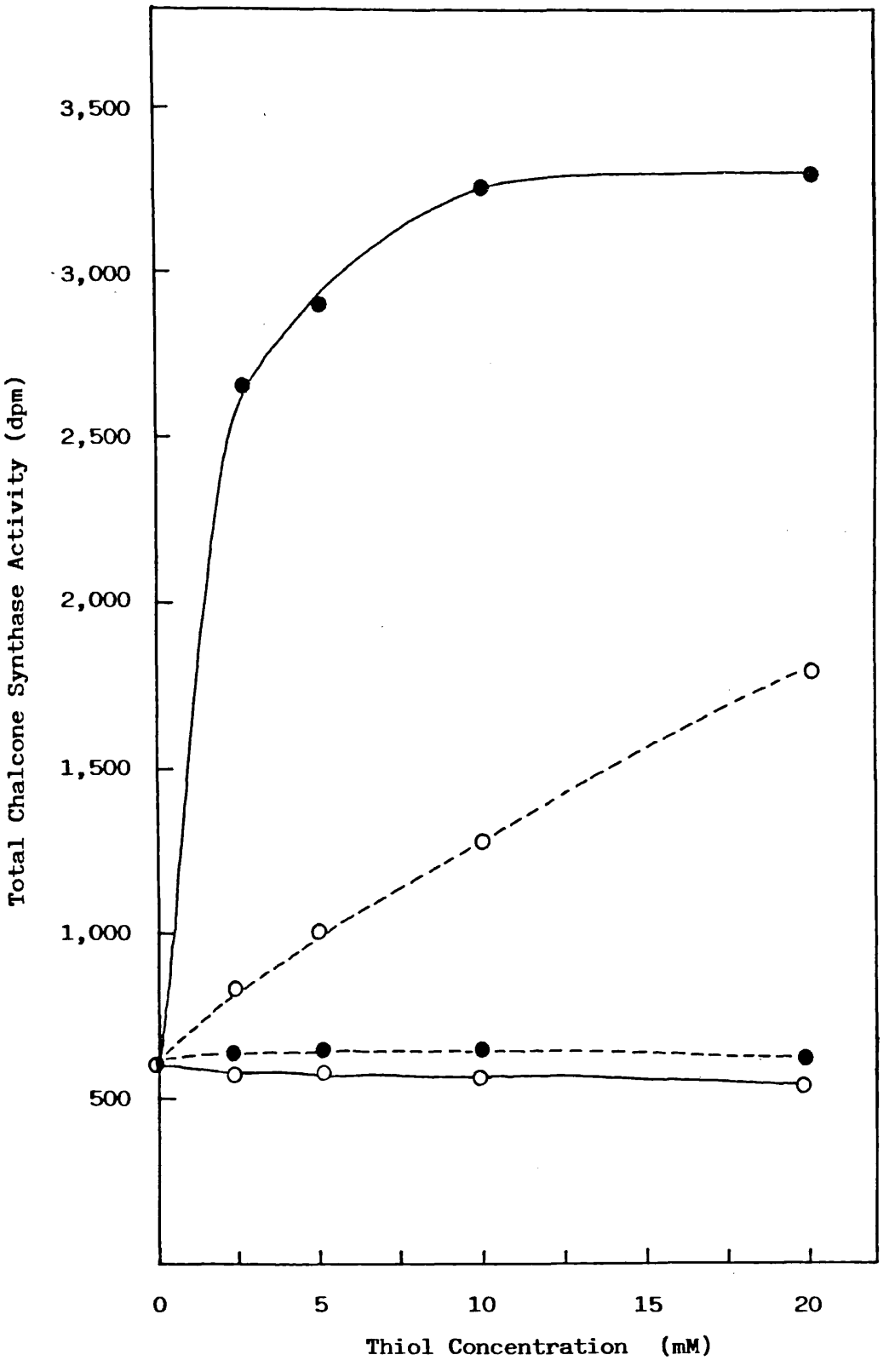


Figure 5.11

The effect of thiol reagent concentration on total chalcone synthase activity. (●——●) Dithiothreitol; (O---O) 2-Mercaptoethanol; (●---●) L-Cysteine; (O——O) Glutathione.

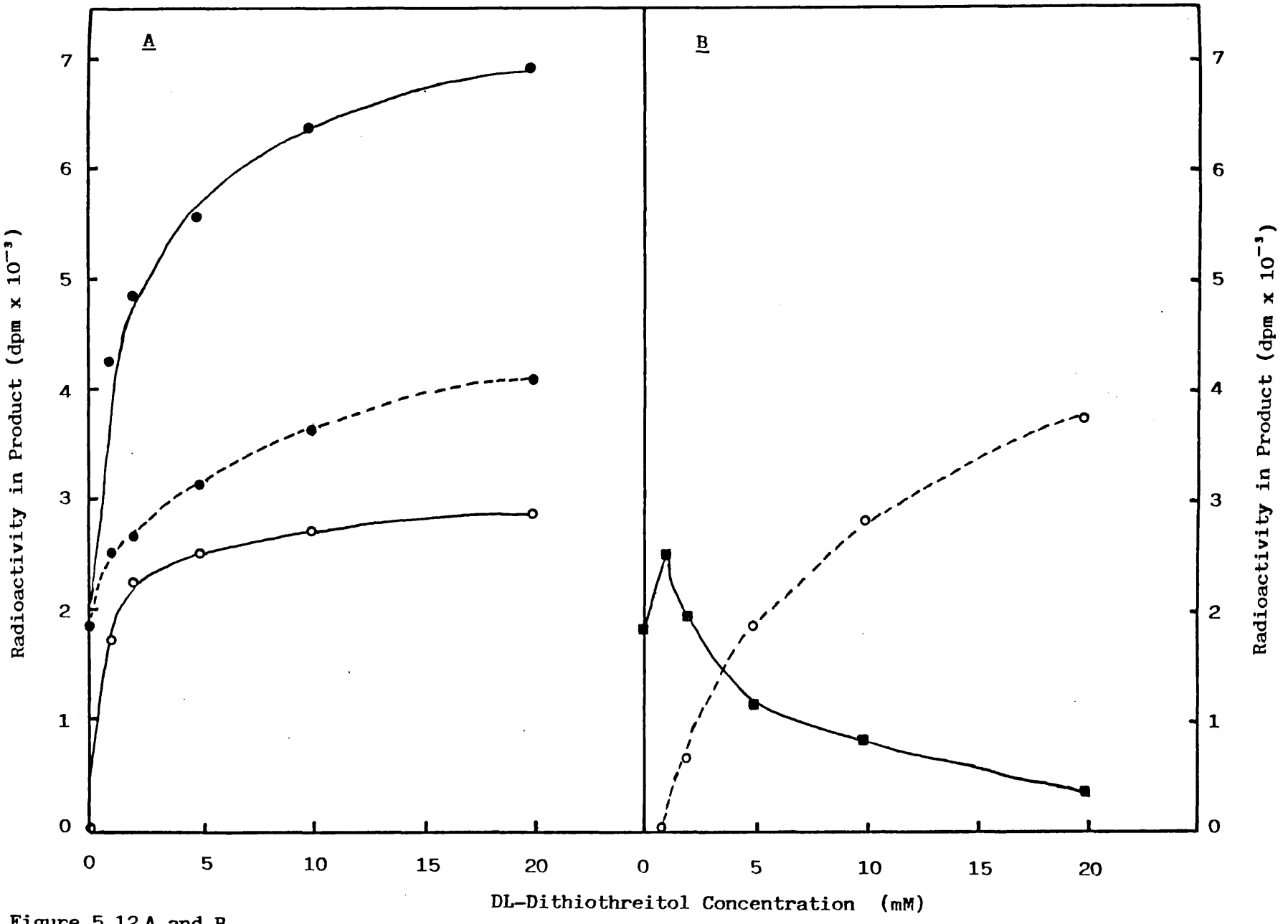


Figure 5.12 A and B

Effects of DL-dithiothreitol concentration on chalcone synthase activity. (●—●) Total activity for malonyl-CoA conversion to EtOAc-soluble products; (O—O) Product formation in absence of 4-coumaroyl CoA; (●---●) Total 4-coumaroyl-dependent products; (■—■) Naringenin; (O--O-) 4-Coumaroyl-CoA-dependent products other than naringenin.

neither co-chromatographed on cellulose TLC plates with a sample of triaceticacid lactone, a release product of the 6-methylsalicylic acid synthase from *Penicillium patulum* (Dimroth et al., 1970).

5.8i CO_2 Exchange Reaction

Stored CHS enzyme (50µl) was incubated with malonyl CoA (20nmol) and $NaH^{14}CO_3$ (173nmol, 10µCi) in buffer A (75µl), at 35°C. The reaction was terminated by the addition of HOAc (50µl)[in a fume cupboard as $^{14}CO_2$ is released]. Three radioactive products were formed. The major product co-chromatographed on cellulose TLC plates with an authentic sample of malonyl CoA (R_f 0.53, isobutryric acid: NH_3: H_2O, 66:1:30; R_f 0.50, 30% HOAc; R_f 0.00, 1M HOAc pH 3.8: 96% EtOH, 3:7). Two minor products (R_f 0.00 and R_f 0.72, 30% HOAc) were observed but not identified (Figure 5.13).

5.8j Decarboxylation of Malonyl CoA

Stored CHS enzyme (50µl) was incubated with malonyl CoA (1.52nmol, 0.05µCi) in buffer A (50µl) for 1h at 35°C. A control reaction employed buffer A (50µl) in place of the enzyme.

The reactions were terminated by the addition of MeOH:HOAc (1:1, 20µl) to each vial. The aqueous phase was extracted with EtOAc (3 x 100µl) and then applied to a cellulose TLC plate and developed with 1M HOAc pH 3.8: 96% EtOH (3:7). Figure 5.14 shows the separation of malonyl and acetyl CoA on the TLC plates. The presence of CHS in the reaction vial (B) does not increase the amount of acetyl CoA above the 10% present as non-enzymatic (A) degradation of malonyl CoA under the conditions of the experiment.

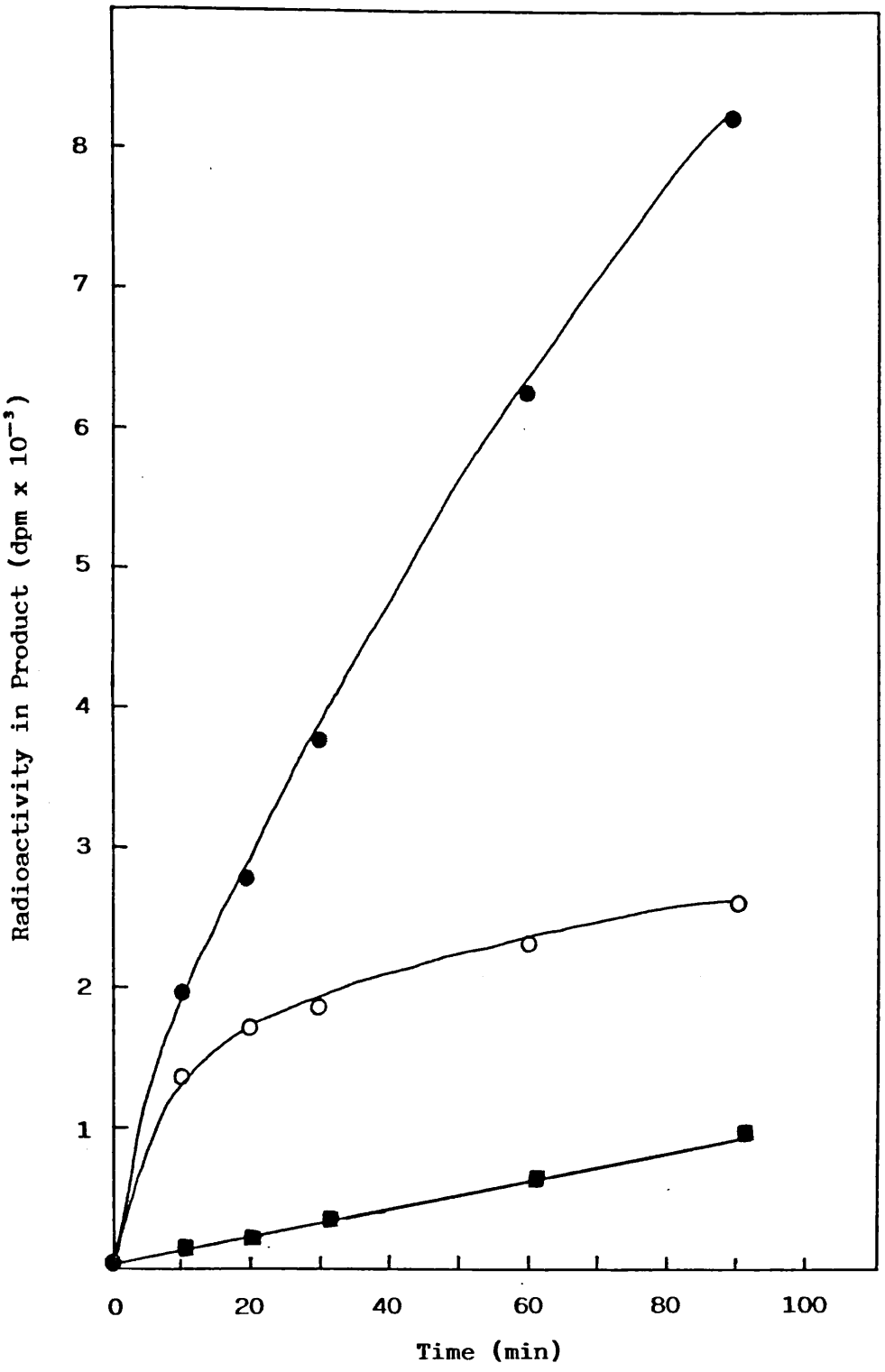


Figure 5.13

The CO_2 exchange reaction between $NaH^{14}CO_3$ and unlabelled malonyl CoA catalysed by chalcone synthase. (●——●) ^{14}C-Malonyl CoA; (○——○) and (■——■) unidentified products at R_f 0.72 and R_f 0.00 respectively.

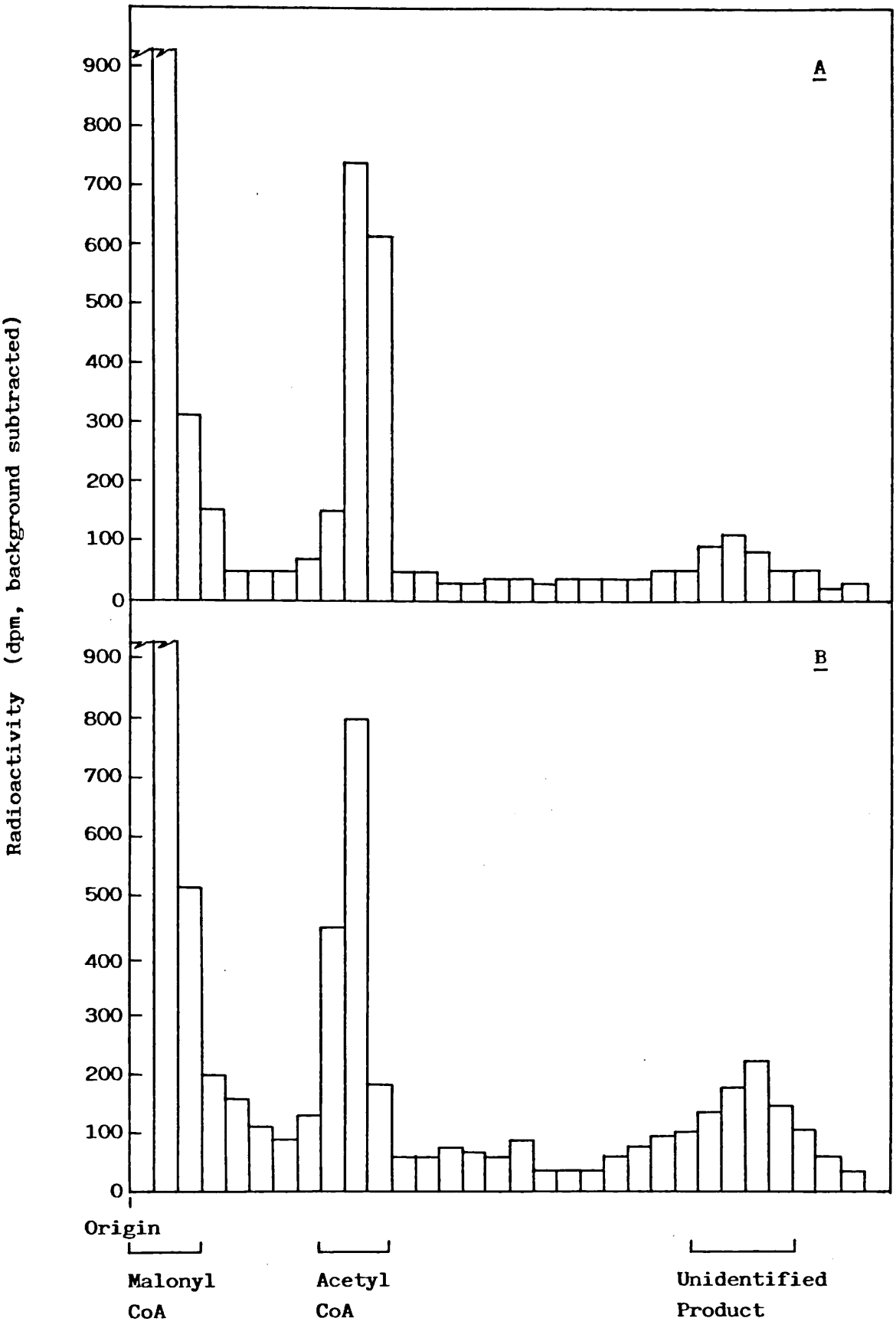


Figure 5.14 A and B

Separation of malonyl CoA and acetyl CoA on cellulose TLC (Solvent: 1M HOAc pH 3.8: 96% EtOH, 3:7). In both the absence (A) and presence (B) of chalcone synthase, acetyl CoA represents 10% of the total radioactivity. Chalcone synthase does not appear to decarboxylate malonyl CoA to acetyl CoA under the conditions of the experiment (pH 7.0, 35°C, 30 min.).

5.8k Substrate Specificity

The activity of the synthase with caffeoyl and feruloyl CoA as substrates was investigated at pH 7.0 and pH 8.0. For both substrates, formation of total EtOAc products was higher at pH 8.0. However, no radioactivity co-chromatographed with the flavanones eriodictyol or homoeriodictyol, the extractable activity possibly representing unidentified release products (Figure 5.15).

5.8l Effect of Inhibitors

Acetyl CoA and free CoA appeared to slightly stimulate activity at low concentrations (10μM) but were slightly inhibitory at higher concentrations (200μM) (Figure 5.16 A). The antibiotic cerulenin (an inhibitor of the condensing enzyme of fatty acid synthase) reduced enzyme activity by 50% at concentrations above 200μM (Figure 5.16 B). Preincubation of the synthase preparation with cerulenin (200μM) or kievitone (100μM) for 30 min. prior to assay did not significantly increase their inhibitory effect (Figures 5.16 B and C).

A range of isoflavonoids and related compounds was tested as inhibitors of the synthase (Figure 5.17). The 5-deoxy compounds (flavonoid/isoflavonoid A-ring numbering) liquiritigenin, 3,9-dihydroxypterocarpan and phaseollin were not inhibitory at the concentrations tested; the chalcone isoliquiritigenin was only slightly inhibitory. In contrast, the 5-hydroxy derivatives naringenin, the isoflavone 2'-hydroxygenistein and the isoflavanone dalbergioidin (the latter two being the direct precursors of the phytoalexin kievitone), were more potent inhibitors. Kievitone itself was the most potent inhibitor tested, reducing both total synthase activity and naringenin formation by 50% at a concentration around 10μM. [Note: All compounds tested were added to the reaction vials

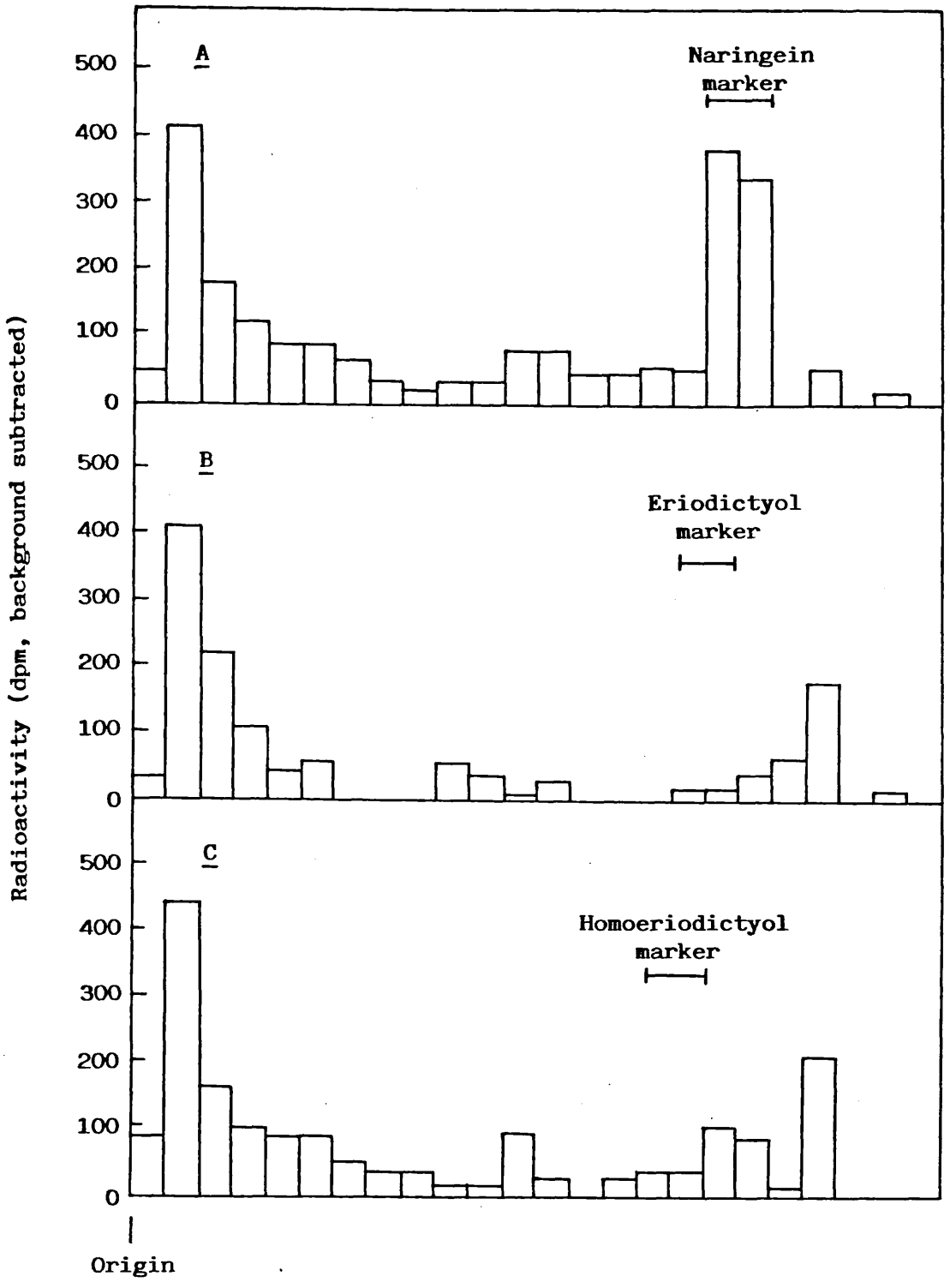


Figure 5.15 A – C

Separation of EtOAc soluble reaction products formed by chalcone synthase from *P. vulgaris* at pH 8.0 with (A) 4-coumaroyl CoA, (B) caffeoyl CoA and (C) feruloyl CoA on cellulose TLC (solvent 30% HOAc).

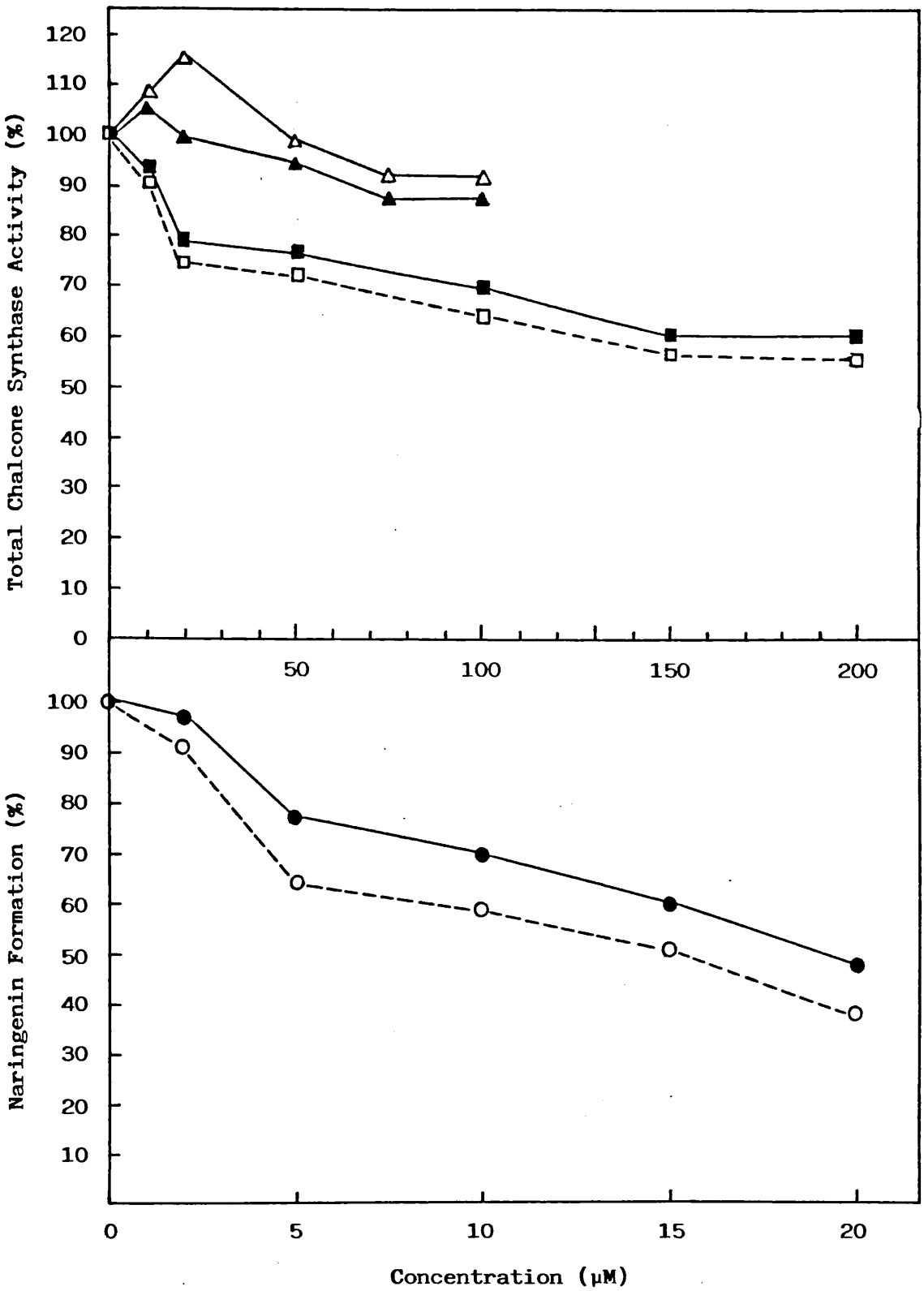


Figure 5.16 A and B

(A) Effects of (△—△) acetyl CoA, (▲—▲) free CoA and (■—■) cerulenin concentration on total chalcone synthase activity. (B) Effect of (●—●) kievitone concentration on naringenin formation by chalcone synthase. Preincubating chalcone synthase with either (□--□) cerulenin (A) or (○---○) kievitone (B) did not significantly alter the inhibitory effect.

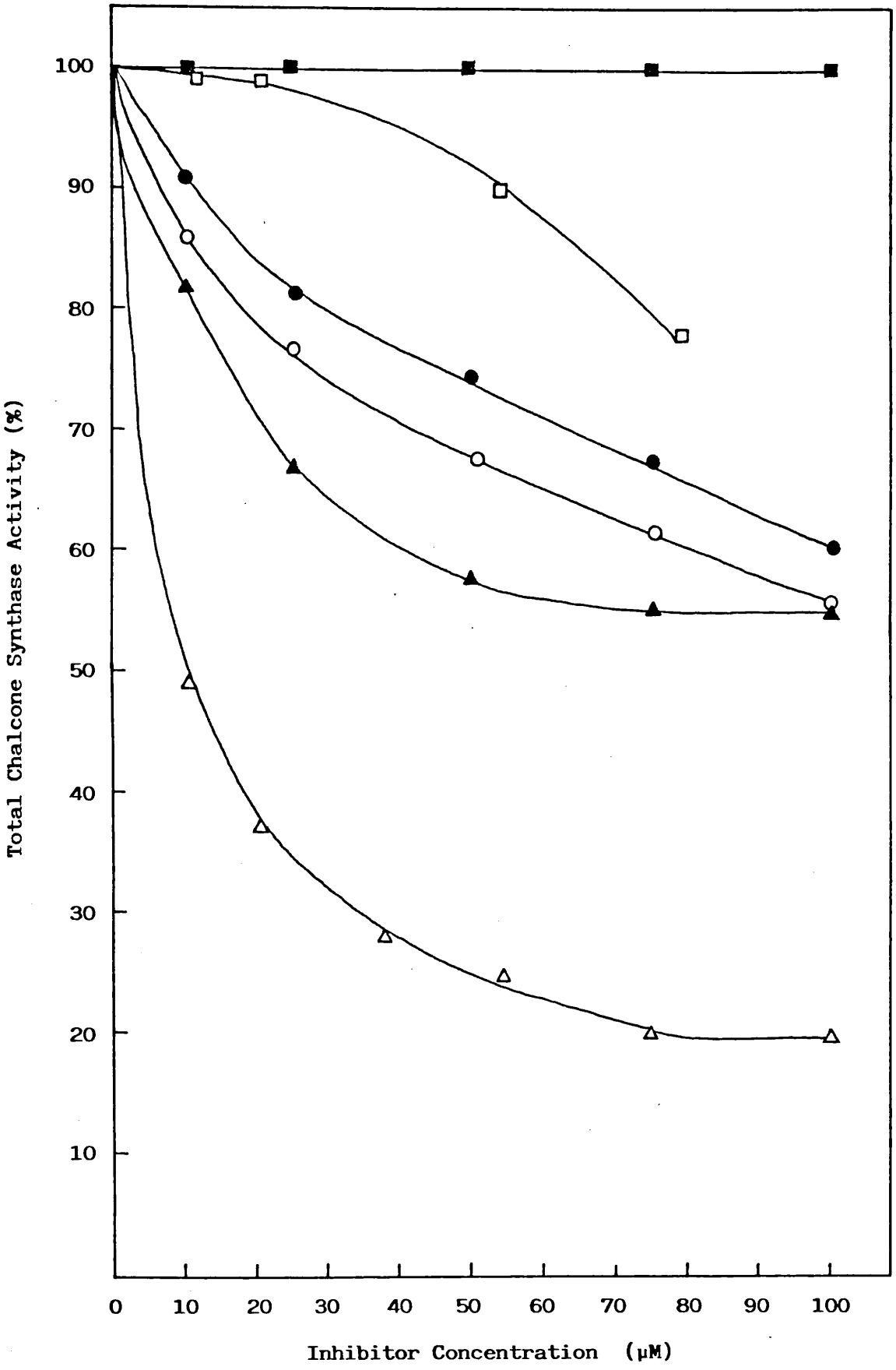


Figure 5.17

Effects of varying concentrations of flavonoid and isoflavonoid derivatives on total chalcone synthase activity (30 min incubation; 100% activity = 0.022pkat). (■——■) Liquiritigenin, 3,9-dihydroxypterocarpan or phaseollin; (□——□) Isoliquiritigenin; (●——●) 2'-Hydroxygenistein; (○——○) Dalbergioidin; (▲——▲) Naringenin; (△——△) Kievitone.

in 5µl EtOMe to ensure solubility. Only 40% of the original activity was retained when this compound was present, and the results are expressed as a percentage of this control value.

5.8m Kinetics

Initial rate measurements for the synthase reaction were made in the presence and absence of kievitone (10µM) by varying one substrate with the other saturating and *vice versa*. Double reciprocal plots of the initial rate data were constructed for both total synthase activity and naringenin formation (Figures 5.18 and 5.19). From plots in the absence of kievitone, the apparent K_m for 4-coumaroyl CoA was calculated to be 2.3µM for both total activity and naringenin formation. The apparent K_m for malonyl CoA was 21.4µM when total activity was measured. However, both v vs. [S] and double reciprocal plots for naringenin formation were sigmoidal, suggesting positive cooperativity (Figure 5.19A). With 4-coumaroyl CoA as variable substrate, kievitone inhibition of both total activity and naringenin formation appeared to be of the non-competitive type (Figures 5.18 B and 5.19 B). The K_i value for the inhibition of naringenin formation was 20.7µM. With malonyl CoA as variable substrate, kievitone inhibition of total activity appeared to be uncompetitive (Figure 5.18 A).

5.9 Screening Experiment for 6'-Deoxychalcone Synthase

In this experiment, three different types of *P. vulgaris* plant material were elicited. Enzyme samples were prepared and tested from both supernatant and pellet fractions, at two pH values and in five reducing systems.

5.9a Materials and Methods

The following reductants were added in 10µl buffer A to each

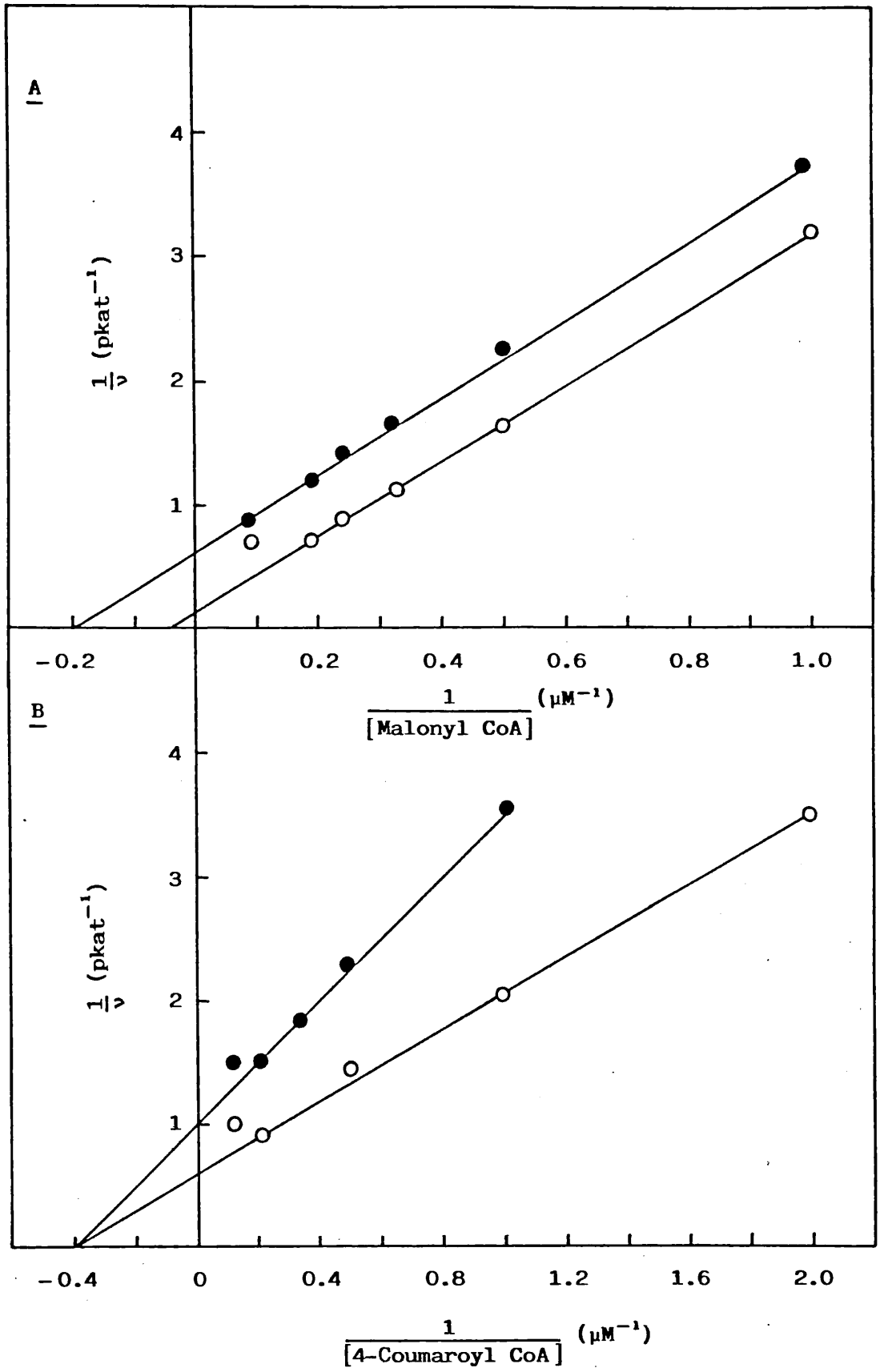


Figure 5.18 A and B

Double reciprocal plots for total chalcone synthase activity in the presence (●—●) and absence (○—○) of 10μM kievitone. (A) 4-Coumaroyl CoA saturating (5.3μM); (B) Malonyl CoA saturating (30μM).

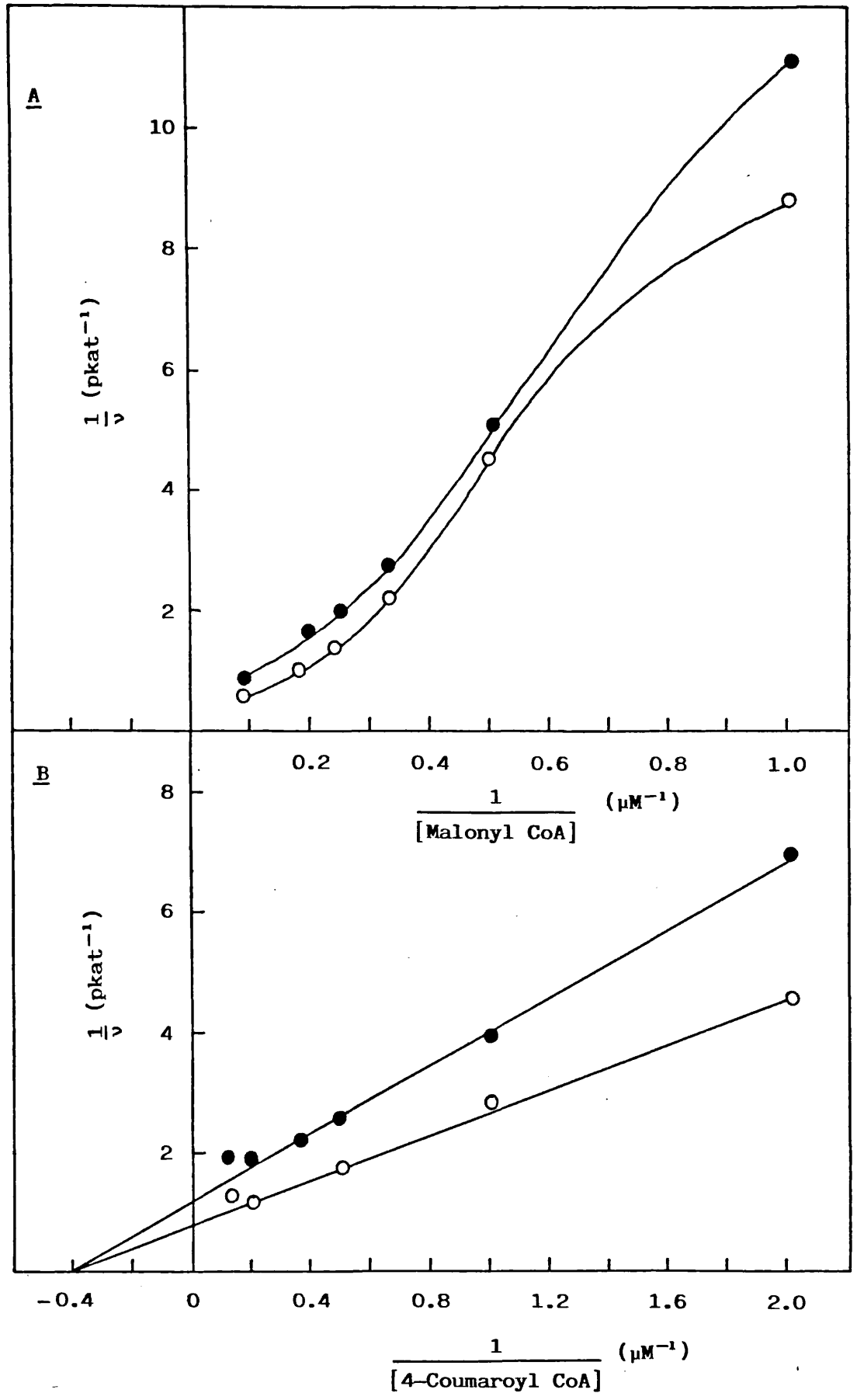


Figure 5.19 A and B

Double reciprocal plots for naringenin formation in the presence (●—●) and absence (○—○) of 10μM kievitone. (A) 4-Coumaroyl CoA saturating (5.3μM); (B) Malonyl CoA saturating (30μM).

reaction as indicated (Table 5.5). (A) An NADPH generating system containing 133nmol NADPH, 133nmol glucose-6-phosphate and .02 units glucose-6-phosphate dehydrogenase; (B) 150nmol NADH; (C) 15nmol L-ascorbic acid; (D) A plus C; (E) B plus C.

[Note: The NADPH generating system was prepared by dissolving NADP (10mg) and D-glucose-6-phosphate (3.75mg) in 1ml of buffer A at pH 6.8 and pH 8.0. The system was activated by the addition of glucose-6-phosphate dehydrogenase (.02 units in 10µl buffer. 1 unit will oxidize 1µmole glucose-6-phosphate to 6-phosphogluconate with the production of 1µmole NADPH per minute at 25°C, pH 7.4 in the presence of NADP). The production of NADPH was monitored by the increase in absorbance at 340nm. Addition of extra NADP restored the original rate; addition of extra glucose-6-phosphate did not increase the rate and thus the quantity used in the assays was not limiting].

5.9b Buffers

The main buffers used were (A) 100mM KH_2PO_4, pH 6.8 and 8.0; (B) 100mM HEPES, pH 6.8 and (C) 100mM HEPES, pH 8.0. Both B and C contained sucrose (10% w/v), EDTA (2mM) and 2-mercaptoethanol (2mM).

5.9c Source of Enzyme

Plant material was prepared as described previously (Section 2.1) and elicited as described below in Table 5.4.

Table 5.4

Elicitation of *P. vulgaris* Tissue Samples for 6'-Deoxychalcone Synthase Screening Experiment

Tissue sample	Method of elicitation[1]	Time (h)
(1) Tissue cultures	CMI elicitor (100µg/ml)	24
(2) Hypocotyls	Wounding	24
(3) Cotyledons	Wounding	66

[1] See section 2.4 .

5.9d Preparation of Extracts

All steps were carried out at 4°C. Plant material (7g, 1-3 of Table 5.4) was homogenised in a Waring blendor for 15 sec. in buffers B and C (50ml each). The homogenates were filtered through four layers of muslin and centrifuged for 15 min. at 1,000 x g_{av}. The resulting supernatants were assayed directly. Pellets were resuspended in the respective buffer (B or C) and then assayed.

5.9e Enzyme Assays

Reaction vials were prepared as previously described for the assay of 6'-hydroxychalcone synthase (Section 2.10c-1). The respective buffer (25µl), reductant (10µl) and enzyme extract (50µl) were added at the start of the assay to give a total volume of 100µl. Assays were incubated at 35°C for 30 min. and the reaction stopped by the addition of MeOH (20µl) containing naringenin/liquiritigenin/isoliquiritigenin (1.5mg/ml each). The products were partitioned into EtOAc (100µl) and separated on cellulose TLC plates with 30% HOAc (R_f Isoliquiritigenin: 0.24; naringenin: 0.47; liquiritigenin: 0.78). Radioactivity was determined as described previously (Section 2.10c-1).

5.9f Time Course Study

To investigate the results of the screening experiment in more detail (Table 5.5), a time course study was performed using an enzyme supernatant sample prepared from 66h elicited cotyledon material at pH 8.0 in the presence and absence of NADPH.

5.9g Results

The results of the screening experiment are presented in Table

Table 5.5 Results of Screening Experiment for 6'-Deoxychalcone Synthase in Tissues of *Phaseolus vulgaris*

Tissue Sample		Total EtOAc Soluble dpm					% of Total dpm in Isoliquiritigenin					% of Total dpm in Naringenin					% of Total dpm in Liquiritigenin				
		A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
Control[1]		5,720					1					73					6				
Tissue Culture pH 6.8	S	788	472	646	941	728	0	0	1	0	0	53	45	51	50	56	11	11	9	6	11
	P	675	754	461	721	565	1	1	0	3	2	18	15	25	17	16	6	1	5	2	3
Tissue Culture pH 8.0	S	907	783	741	864	728	0	0	0	0	0	31	26	43	53	41	3	3	3	3	2
	P	1,117	1,058	700	838	1,057	2	1	0	0	0	10	9	16	12	10	2	4	1	1	0
Hypocotyl pH 6.8	S	1,736	895	1,359	2,145	1,577	0	0	2	0	0	70	69	71	64	75	15	18	13	8	15
	P	1,434	1,646	866	1,559	1,141	1	1	0	4	3	25	20	37	22	23	8	1	8	3	5
Hypoctoyl pH 8.0	S	2,053	1,723	1,611	1,938	1,578	0	0	0	0	0	40	35	58	69	56	4	4	4	4	3
	P	2,616	2,458	1,503	1,870	2,454	3	1	1	0	0	12	11	22	15	12	3	5	1	2	1
Cotyledon pH 6.8	S	7,197	6,067	6,103	6,697	5,945	1	1	0	0	1	37	38	40	43	44	6	7	7	7	8
	P	2,066	2,372	1,318	2,309	1,489	1	2	3	1	0	22	18	37	29	49	5	5	4	4	6
Cotyledon pH 8.0	S	7,483	6,210	5,000	7,844	7,904	1	1	0	1	1	56	67	60	63	85	5	5	4	4	6
	P	2,660	2,931	1,881	3,394	1,844	0	3	0	1	2	47	25	62	24	52	8	7	5	3	5

[1] Control is cotyledon supernatant, pH 8.0 with no reductant. S = Supernatant, P = Pellet.

A = NADPH; B = NADH; C = Ascorbate D = NADPH + Ascorbate; E = NADH + Ascorbate.

5.5. Radioactivity in the three products is shown as a percentage of the total EtOAc extractable activity. In all tissues the supernatant fraction catalysed the formation of a minor radioactive product which co-chromatographed with the liquiritigenin marker in this system. In the presence of reductants, the formation of naringenin was reduced by about 30% in some cases.

The results of the time course study showing the formation of naringenin and the minor radioactive product 'X', are shown in Figure 5.20. Both products accumulate in a similiar fashion and their formation is slightly inhibited by the presence of the NADPH reductant. No radioactivity was detected in the area of the isoliquiritigenin marker.

5.10 Discussion

The 6'-hydroxychalcone synthase from *P. vulgaris* exhibited several properties in common with the parsley enzyme, including a similiar molecular weight (Kreuzaler et al., 1979); the same pH optimum for naringenin formation (Kreuzaler and Hahlbrock, 1975a); an identical concentration dependence for naringenin formation as a function of thiol concentration (Kreuzaler and Hahlbrock, 1975b); the formation of release products at high thiol concentrations (Kreuzaler and Hahlbrock, 1975a and 1975b; Hrazdina et al., 1976) and the ability to catalyse CO_2 exchange between $H^{14}CO_3^-$ and malonyl CoA (Kreuzaler et al., 1978). Differences include the relative apparent K_m values for malonyl and 4-coumaroyl CoA; the apparent production of non-coumaroyl CoA dependent products; the more restricted substrate specificity; the lack of effect of ionic strength on naringenin formation; the weaker inhibition by cerulenin, acetyl CoA and free CoA; the absence of a malonyl decarboxylating activity and the enzyme's apparent instability, a property in common with the synthase from *H. gracilis* (Saleh et al., 1978).

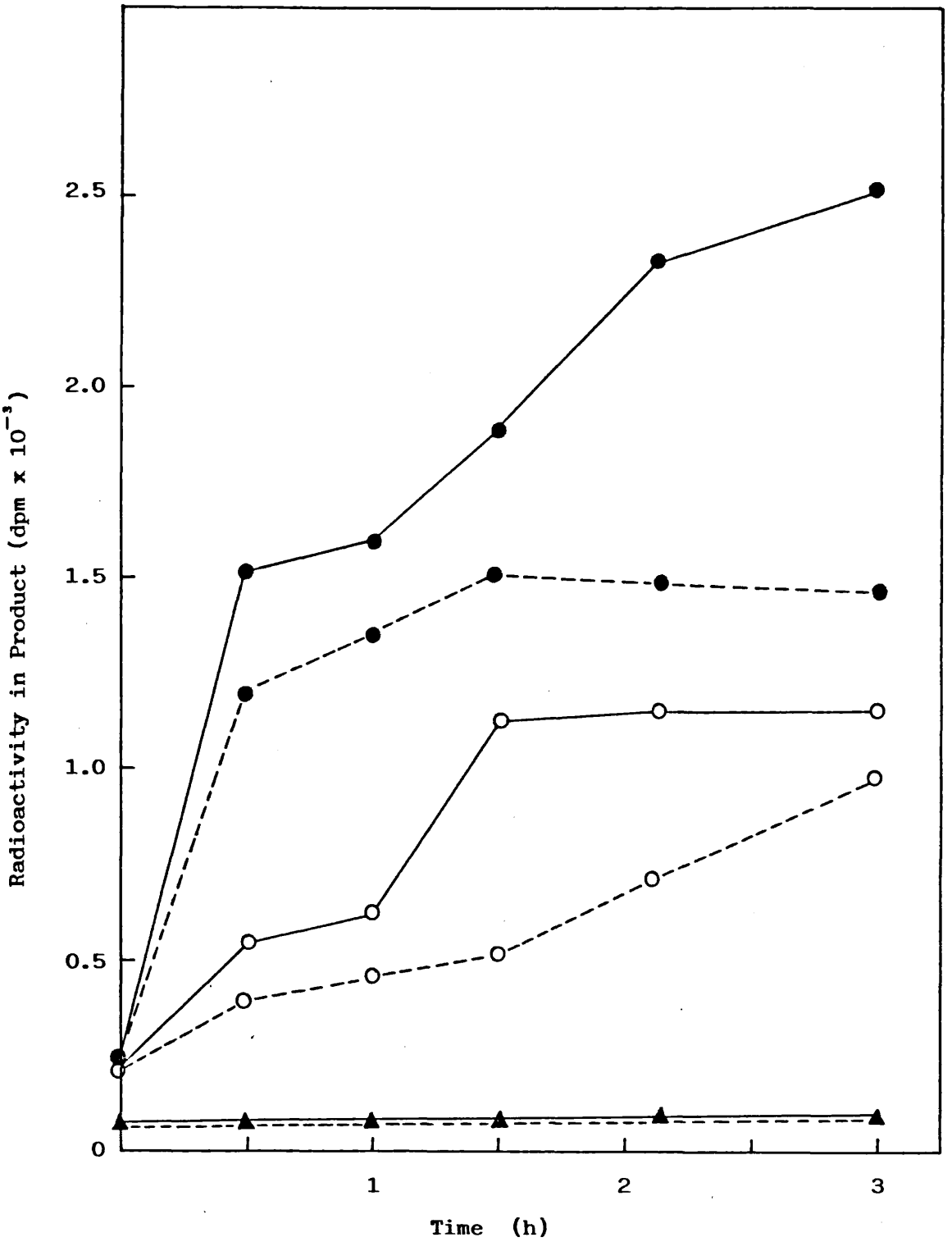


Figure 5.20

Time course of naringenin (●), 'X' (○) (an unidentified 4-coumaroyl CoA dependent product) and isoliquiritigenin (▲) accumulation in both the presence (----) and absence (——) of an NADPH generating system in wounded cotyledons of *P. vulgaris*.

The molecular weight of 77,000 for the 6'-hydroxy CHS from *P. vulgaris* is similiar to that of the enzyme from *P. hortense*, a plant which only synthesizes 5-hydroxy isoflavonoids. In a separate study (Lawton et al., 1983a), the molecular weight of the *P. vulgaris* CHS subunits, as precipitated from extracts of elicitor-induced cells by anti-(parsley CHS) serum and subjected to SDS PAGE, was 42,500. This fact coupled with the present molecular weight of the native enzyme determined by gel-filtration, indicates that the bean enzyme, like the parsley enzyme (Kreuzaler et al., 1979) is a dimer, probably with identical subunits. In contrast, the enzyme from *Tulipa* c.v. Apeldorn, a plant which only synthesizes 6'-hydroxychalcone glycosides, has a molecular weight of 55,000 and may be composed of only a single subunit (Sütfeld et al., 1978).

The effect of thiol reagents, in particular dithiothreitol, stimulated the total activity of CHS, producing not only naringenin but other unidentified EtOAc soluble release products. High thiol concentrations actually inhibit naringenin formation and this is consistent with the situation observed in *P. hortense* where high thiol concentrations cause the formation of bis-noryangonin and benzalacetone release products resulting from the condensation of one molecule of 4-coumaroyl CoA with either one or two acetate units from malonyl CoA molecules (Kreuzaler and Hahlbrock, 1975b; Hrazdina et al., 1976). The decrease in naringenin formation and concomitant increase in 4-coumaroyl CoA dependent release products has been ascribed to thiol compounds in the solvent acting as acceptors for partially completed enzyme bound intermediates, or alternatively in terms of altered enzyme structure (resulting from purification) leading to the stabilisation and therefore cyclisation of these intermediates. In contrast, no release products were formed by the CHS from *Tulipa* (Sütfeld et al., 1978).

The formation of release products dependent upon the presence

of malonyl CoA alone, was observed in both standard assays of the partially purified enzyme and also during the formation of ^{14}C-malonyl CoA by CO_2 exchange. This type of side reaction has not been reported for any of the chalcone synthases studied to date. Formation of these products did not occur in the absence of DTT, and was saturated at a lower concentration (5mM) than the production of 4-coumaroyl CoA dependent release products. This result and the absence of a demonstrable decarboxylating activity with the *P. vulgaris* enzyme may be explained by considering the hypothetical mechanism proposed by Kreuzaler et al. (1978) where the initial reaction catalysed by CHS was thought to be the decarboxylation of a malonyl CoA molecule to give an enzyme-bound acetyl CoA carbanion. The reaction could then proceed in a number of ways. Firstly, the carbanion could react with a molecule of 4-coumaroyl CoA (or a subsequent intermediate of chain elongation) resulting in the formation of the tetrahydroxy chalcone or other 4-coumaroyl CoA dependent products. Alternatively, under the artificial conditions of the assay, the carbanion could react with either a) a CO_2 molecule to regenerate malonyl CoA (CO_2 exchange) or b) a proton to form acetyl CoA (decarboxylation)(see Figure 5.1).

The results obtained here with the enzyme from *P. vulgaris* concur with the mechanism postulated above by demonstrating the essential CO_2 exchange reaction which argues for the existence of an acetyl CoA carbanion which acts as an intermediate in all of the observed reactions. The two differences observed with the *P. vulgaris* enzyme may be explained by postulating that a) the rate constant for the reaction of the carbanion with a proton is very low compared to those of the other possible alternative reactions, thus explaining the lack of a demonstrable decarboxylating activity and b) that the acetyl CoA carbanion can react with a malonyl CoA molecule(s) to give the observed non-4-coumaroyl CoA dependent

products in a reaction similiar to that observed with the enzyme 6-methyl-salicylic acid synthase from *Penicillium patulum* (Dimroth et al., 1970). It is also possible that the 'malonyl' release products could have arisen from the action of a second, contaminating enzyme in the preparation. Although there is no direct evidence which excludes this possibility, it is considered unlikely on the basis of the following: a), malonyl release products were not formed with crude enzyme preparations, b), the formation of malonyl release products increased with increasing purification of the synthase in a similiar manner to the 4-coumaroyl CoA dependent release products, c), on storage of purified synthase preparations, the total activity of both malonyl release product and 4-coumaroyl CoA dependent release product formation increased as a function of length of storage, this increase corresponding to a loss in naringenin formation capacity, d), the formation of malonyl release products was inhibited by kievitone and e), the most likely alternative system capable of co-purifying with the bean CHS and producing condensation products from malonyl units is the condensing enzyme of fatty acid synthase (Schuz et al., 1982); however, this enzyme requires malonyl-acyl carrier protein as substrate (Schuz et al., 1982). Although double reciprocal plots for total CHS activity with malonyl CoA as variable substrate produced a single straight line, this does not necessarily rule out the possibility of the presence of two enzymes, even if they had different K_m and V_{max} values (Segel, 1975). Unfortunately, attempts to further purify the bean synthase and assess its catalytic capabilities were unsuccessful.

The bean CHS exhibited a more restricted substrate specificity for the hydroxycinnamoyl CoA moiety than did the enzymes from parsley, *Happlopappus* and *Tulipa*. In the latter case naringenin, eriodictyol and homoeriodictyol were readily formed, with similiar K_m values, from 4-coumaroyl-, caffeoyl- and feruloyl CoA respectively (Sütfeld et al.,

1978). The enzymes from parsley and *Haploppus* formed both naringenin (pH optimum 8.0) and eriodictyol (pH optimum 6.5-7.0), although only small amounts of homoeriodictyol were formed from feruloyl CoA (Saleh et al., 1978). The *P. vulgaris* enzyme produced neither eriodictyol nor homoeriodictyol, although the formation of release products with caffeoyl and feruloyl CoA as substrate was greater at pH 8.0 than at pH 7.0. The formation of flavonoids with di- and tri- hydroxy substitution of the B-ring in *P. vulgaris* probably occurs by hydroxylation of naringenin. Enzymes catalysing this process have been reported from other sources (Fritsch and Grisebach, 1975; Forkmann and Stotz, 1981).

The presentation of kinetic analyses of CHS in terms of double reciprocal plots is perhaps over-simplistic in view of a) the complexity of the reaction sequence due to the binding of four separate substrate molecules and b) the formation of both malonyl dependent and 4-coumaroyl CoA dependent release products. The result of such analyses must therefore be interpreted with caution. The apparent K_m values for the bean CHS of 2.3μM (4-coumaroyl CoA) and 21.4μM (malonyl CoA)compare with values of 35μM and 1.6μM respectively for the parsley enzyme (Hrazdina et al., 1976), and around 1.7μM with respect to both substrates for the enzyme from *Tulipa* (Sütfeld et al., 1976). The sigmoidal kinetics observed in the present work with respect to the effects of varying malonyl CoA concentrations on naringenin formation suggest positively cooperative malonyl binding which may work to facilitate the formation of tri-acyl intermediates, thus favouring chalcone formation.

In a similiar manner to the effects of isoflavonoids on the *P. vulgaris* CHI (Chapter 6), the prenylated isoflavanone phytoalexin kievitone was a more potent inhibitor of the synthase than were its immediate isoflavone and isoflavanone precursors 2'-hydroxygenistein and dalbergioidin. It has recently been demonstrated that kievitone inhibits cellulase, polygalacturonase and polymethylgalacturonase from

culture filtrates of *Rhizoctonia solani* (Bull, 1981), and it has been suggested that such inhibition may be the result of non-specific enzyme inactivation by the tetrahydroxy-phenolic molecule (Smith, 1982). However, the inhibition by kievitone of both CHI and CHS from *P. vulgaris* is reversible (by dialysis) and occurs at much lower concentrations than required for a similiar % inhibition of the *Rhizoctonia* enzymes. Furthermore, the apparent uncompetitive inhibition of bean CHS by kievitone with malonyl CoA as variable substrate suggests that the phytoalexin binds to the enzyme-substrate complex (enzyme-malonyl complex) in order to inhibit the reaction, whereas the inhibitory effect of kievitone on CHI is apparently competitive and suggests that kievitone binds at the active site of CHI (Chapter 6). These observations tend to rule out non-specific binding as a cause of enzyme inhibition.

The kinetics of kievitone inhibition suggest that the phytoalexin acts at a site other than the malonyl CoA or 4-coumaroyl CoA binding sites. It is therefore possible that, *in vivo* , kievitone acts as a feedback inhibitor of its own synthesis in the classical manner. For such a hypothesis to be tenable, further confirmation would be required concerning the sites of kievitone synthesis and accumulation. In wounded bean cotyledons, large increases are observed in tissue which has already accumulated high levels of kievitone (Chapter 3). Assuming cotyledons to be 70% water by weight, the concentration of kievitone reaches 20-30 times the CHS K_i value. However, analysis of *Colletotrichum*-infected bean hypocotyls has indicated that elevated CHS activities occur both in the infected area and in apparently healthy tissue surrounding the infection site, although such healthy tissue may later show symptoms of infection (Lamb et al., 1983). Synthase activity and high concentrations of kievitone may therefore, to some extent, be spatially separated in infected tissue. A possible role for kievitone inhibition of CHS

may be in protecting the cells producing the phytoalexin and its precursors from phytotoxic concentrations of kievitone. The validity of this hypothesis would depend upon kievitone finally accumulating in cells other than those involved in its synthesis at any given time.

In the screening experiment for 6'-deoxychalcone synthase the inclusion of the chalcone marker which would be expected to be the first product formed was for the sake of completion. Unlike the tetrahydroxychalcone formed by 6'-hydroxychalcone synthase, the trihydoxychalcone is stable. However in crude preparations, the high activites of chalcone isomerase would probably isomerise any isoliquiritigenin formed to the flavanone, liquiritigenin. Thus, no radioactivity would be expected in isoliquiritigenin and the results (Table 5.5) are in agreement with this assumption.

The formation of the minor radioactive product 'X' is 4-coumaroyl CoA dependent and this compound is most probably the styrylpyrone, bis-noryangonin, and not the flavanone liquiritigenin, which co-chromatographs in this system. The formation of a 4-coumaroyl CoA dependent release product by purified 6'-hydroxychalcone synthase with an identical R_f to bis-noryangonin in this system has already been observed with high concentrations of DTT (Section 5.8h, Figure 5.12B) and has previously been reported (Kreuzaler and Hahlbrock, 1975a, 1975b). The formation of liquiritigenin is expected to be reductant dependent. However, 'X' accumulates in the presence and absence of the NADPH reductant. Although it is possible that some endogenous reductants(s) may be present in the crude extracts, it is probable that the supply would be quickly exhausted. Thus, as 'X' accumulates over a period of several hours, it is unlikely that the formation of this compound is reductant-dependent. Unfortuately, an authentic sample of bis-noryangonin was not available to enable further characterisation of 'X' by comparative TLC.

The production of naringenin is slightly inhibited by the reducing system. Naringenin formation could be expected to decrease if either malonyl CoA or 4-coumaroyl CoA became limiting as a result of utilization by a deoxychalcone synthase but this clearly has not happened. The greatest level of naringenin formation (cotyledon supernatant, pH 8.0), only accounts for approximately 10% of the total malonyl CoA (66,000 dpm) available. If an active, reductant-dependent, 6'-deoxychalcone synthase was present in the extracts it would be reasonable to expect an incorporation of greater than 1% (660 dpm) of the available malonyl CoA (unless the K_m for malonyl CoA of the 6'-deoxychalcone synthase is high). Furthermore, there is no concomitant production of a reductant-dependent product as would be expected. The most likely explanation is that some component of the reducing system inhibits the activity of the 6'-hydroxychalcone synthase in a non-specific manner similiar to that observed with EtOMe (Section 5.81).

The results of the screening experiment for the 6'-deoxychalcone synthase, although negative, do allow two conclusions to be drawn. Firstly, the production of naringenin is not significantly affected by the reductants employed in this experiment and this supports the previous results (Chapters 3 and 4) which suggest that the 6'-hydroxy and 6'-deoxy chalcone synthases are two separate enzymes. The possibility remains however, that the condensing enzyme for deoxychalcones is the 6'-hydroxychalcone synthase. Secondly, if the formation of 6'-deoxychalcones occurs by a typical sequence involving a reductase and dehydratase acting in concert with a condensing enzyme (analogous to type II fatty acid synthase, non-aggregated), then it is probable that the enzymes involved are either in an enzyme complex or are organised sequentially, bound to a membrane. In either case, the *in vitro* assay of 6'-deoxychalcone synthesis might be difficult to achieve, as is apparently the case (this work, and H. Grisebach, personal communication).

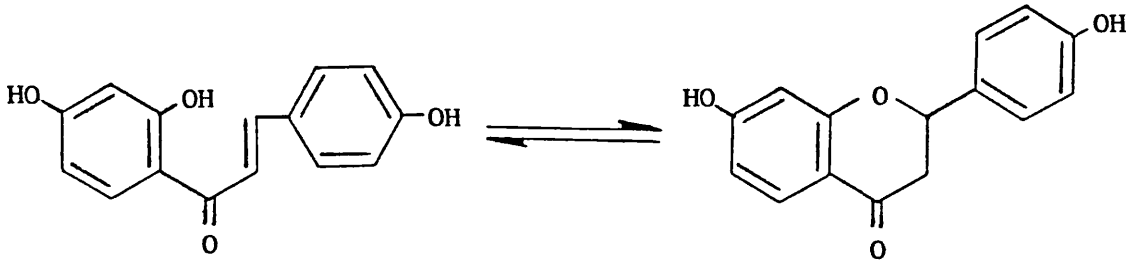
CHAPTER 6

Purification and Properties of Chalcone Isomerase from Cell Suspension Cultures of *P. vulgaris* L.

6.1 Introduction

Chalcone isomerase (CHI) (EC. 5.5.1.6) was first isolated from seeds of *Glycine max* (Moustafa and Wong, 1967), and was shown to catalyse the cyclisation of 2',4,4'-trihydroxychalcone (**1**) to 4',7-dihydroxyflavanone (2) (Figure 6.1). In *Phaseolus aureus*, *Petroselinum hortense* (parsley) and *Cicer arietinum* (garbanzo bean) the isomerase has been reported to exist in a number of isoenzymic forms (Hahlbrock et al., 1970a and 1970b).

Figure 6.1



1 **2**

A more detailed study of the soybean isomerase indicated the presence of only one form of the enzyme, with a molecular weight of about 16,000 (Boland and Wong, 1975). The enzyme catalysed the isomerization of both 6'-hydroxy and 6'-deoxy chalcones to their corresponding flavanones, and was competitively inhibited by a wide range of flavonoid derivatives.

The exact role of chalcone isomerase in flavonoid metabolism has for some time been in doubt. However it has recently been confirmed

that the first C_{15} compound formed from malonyl CoA and 4-coumaroyl CoA is the chalcone (Heller and Hahlbrock, 1980; Sütfeld and Wiermannn, 1980) and not the flavanone as previously believed (Kreuzaler and Hahlbrock, 1975a). This finding is supported by genetic evidence (Kuhn et al., 1978; Forkmann and Kuhn, 1979) and is consistent with ^{13}C-labelling (Stoessl and Stothers, 1979; Light and Hahlbrock, 1980; Sutter et al., 1975) which support a role for the isomerase in the synthesis of flavonoid derivatives. Labelling experiments (Wong, 1968) and chemical studies (Pelter et al., 1971) had suggested that isoflavonoids were formed directly from chalcones. However, recent evidence has shown that isoflavonoids are derived from flavonoids via an enzyme catalysed phenyl migration step (Hagmann and Grisebach, 1984). This new evidence has now established the role of CHI in the biosynthesis of flavonoids/isoflavonoids and all other related compounds such as flavones, flavonols, anthocyanins and proanthocyanidins.

In *P. vulgaris*, CHI is induced in response to both elicitor treatment and wounding (Chapters 1 and 3) and, although the increase in isomerase levels is less rapid than either PAL or CHS (Chapter 3), the basal activity is higher; for these reasons isomerase induction is probably not a major controlling factor in the phytoalexin response (Dixon and Lamb, 1979), although its status as an inducible enzyme makes it a suitable subject for further studies associated with the control of its levels.

The purification of CHI is important for several reasons. Firstly, there may be isoenzymic forms of the enzyme as in *P. aureus*, *P. hortense* and *C. arietinum*. If 6'-hydroxy and 6'-deoxy chalcones are each isomerised to their respective flavanones by specific isoenzymes of CHI then the isoenzymes may exert a controlling influence on the accumulation of the two different classes of isoflavonoid phytoalexin. However, if only a single form of the enzyme exists, as in soybean (Boland and Wong, 1975),

then the isomerase probably has no role in regulating the accumulation of phytoalexins in *P. vulgaris*. Secondly, an understanding of the regulation of CHI activity is important in itself, because apart from the prenyl transferase reported by Zähringer et al. (1979), CHI is the furthest enzyme down the pathway leading to isoflavonoid phytoalexins assayed to date. Thirdly, purified CHI could be used to raise antibodies for immunological studies on the rate of synthesis and appearance of the enzyme.

[Note: This chapter forms the basis of later work in which anti-(chalcone isomerase) serum was used to investigate the rates of synthesis and appearance of the enzyme. Robbins and Dixon, 1984].

6.2 Materials and Methods

The source of all chemicals and the assay of enzymes has been described in Chapter 2.

6.3 Buffers

The main buffers used were (A) 50mM Tris-HCl, pH 8.5, (B) 10mM KH_2PO_4, pH 8.0, (C) 30mM KH_2PO_4, pH 8.0, (D) 25mM Imidazole-HCl, pH 7.4 and (E) Pharmacia 'Polybuffer 74', pH 4.0, each containing 1.4mM 2-mercaptoethanol.

6.4 Source of Enzyme

Cell suspension cultures (Section 2.1) were harvested by suction filtration, frozen in liquid N_2, and stored at -70°C until required for enzyme extraction. No significant loss of CHI activity was observed in frozen cells over a period of several weeks.

6.5 Purification of the Enzyme

All steps were carried out at 4°C. 570g of frozen cells were thawed and homogenised with 600ml of buffer A in a Waring blendor. The

homogenate was passed through four layers of muslin and the residue re-extracted with a further 200ml of buffer A. The combined extracts were centrifuged for 30 min. at 23,000 x $g_{av.}$. The supernatant was adjusted to pH 4.0 over a 30 min. period by the addition of 1M HCl. The pH of the supernatant was then raised to 5.0 with 1M KOH, and the mixture left to stand for 10 min. before being centrifuged for 30 min. at 23,000 x $g_{av.}$. The pH of the supernatant was then slowly re-adjusted to pH 8.5 by further addition of 1M KOH. The supernatant was then fractionated using $(NH_4)_2SO_4$ as described previously (Section 5.5), the precipitate forming at 50-75% saturation (Table 6.1) being finally dissolved in 9.5ml of buffer A.

The solution was clarified by centrifugation at 23,000 x $g_{av.}$ for 15 min. and 9ml was applied to a Sephadex G-100 column (85 x 2.5cm) which was eluted with buffer B at a flow rate of 17ml/h, 4ml fractions being collected. Fractions containing the highest activity were pooled (Figure 6.2) and immediately applied to a Whatman DE 52 DEAE-cellulose column (27 x 0.8cm) which had been equilibrated with buffer B. The column was eluted at a flow rate of 30ml/h, first with 240ml buffer B, and then with a linear gradient of 500ml of this buffer, increasing in molarity from 10mM to 60mM. Fractions of 3.7ml were collected and those showing the highest activity were pooled (Figure 6.3) and concentrated to 5.9ml by ultrafiltration (Amicon UM-10 membrane, 75psi). The resulting enzyme solution was stored in 1ml aliquots with 5% glycerol at -70°C.

A sample (1.0ml) of the above enzyme preparation was applied to an affinity column (5 x 1cm) of 2',4'-dimethoxy-4-hydroxychalcone linked via a spacer to Sepharose 4B (Section 2.13). Buffer C (30ml) was passed through the column and the isomerase was then eluted with buffer C (minus 2-mercaptoethanol) containing 0.5mg/ml 2',4,4'-trihydroxychalcone. The fractions containing the enzyme decolourised rapidly; these were pooled, dialysed overnight against 500ml of buffer C and then concentrated

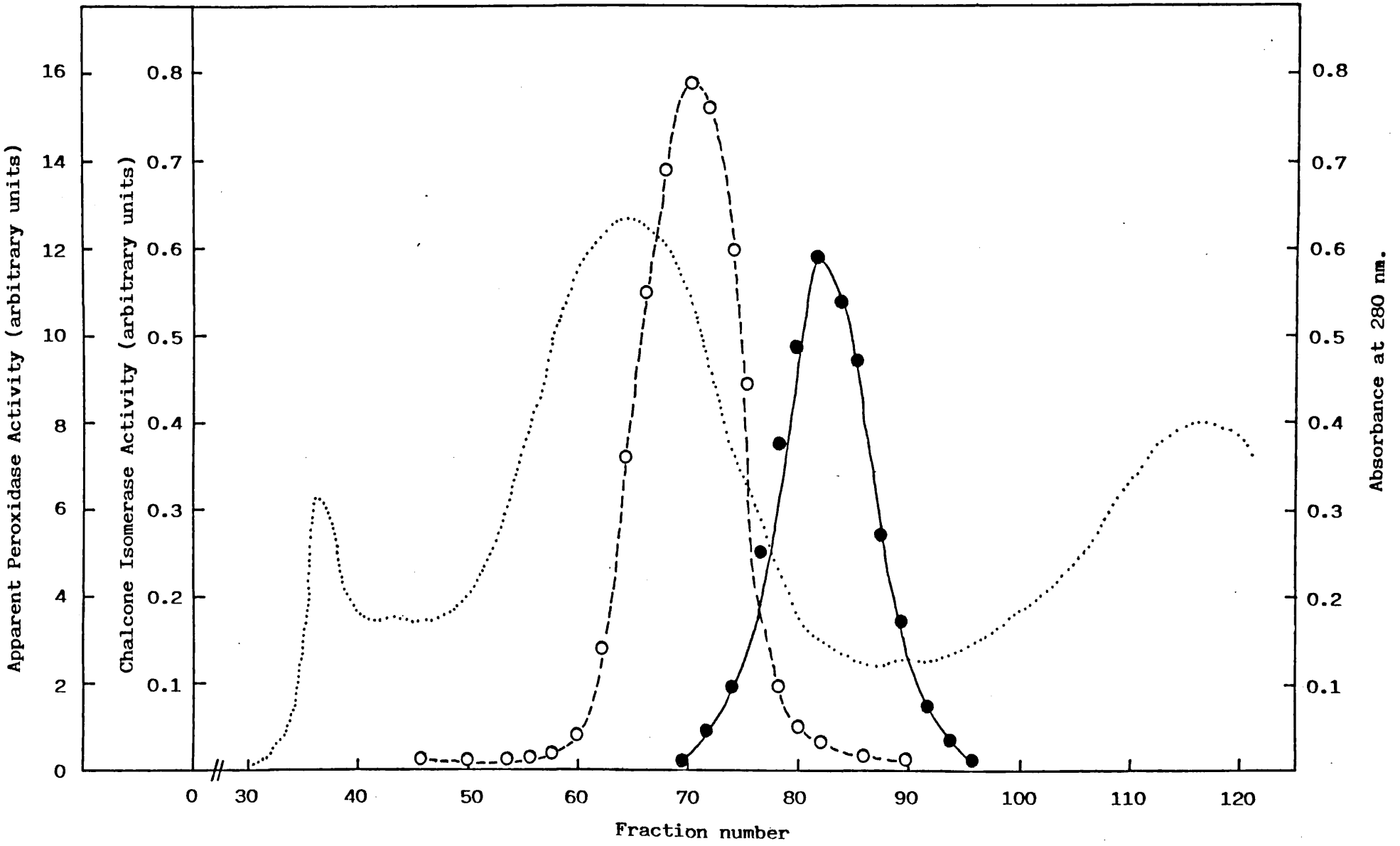


Figure 6.2 Purification of chalcone isomerase by gel-filtration on Sephadex G-100. (●—●) Chalcone isomerase activity; (○—○) Apparent peroxidase activity; (·····) Absorbance at 280 nm.

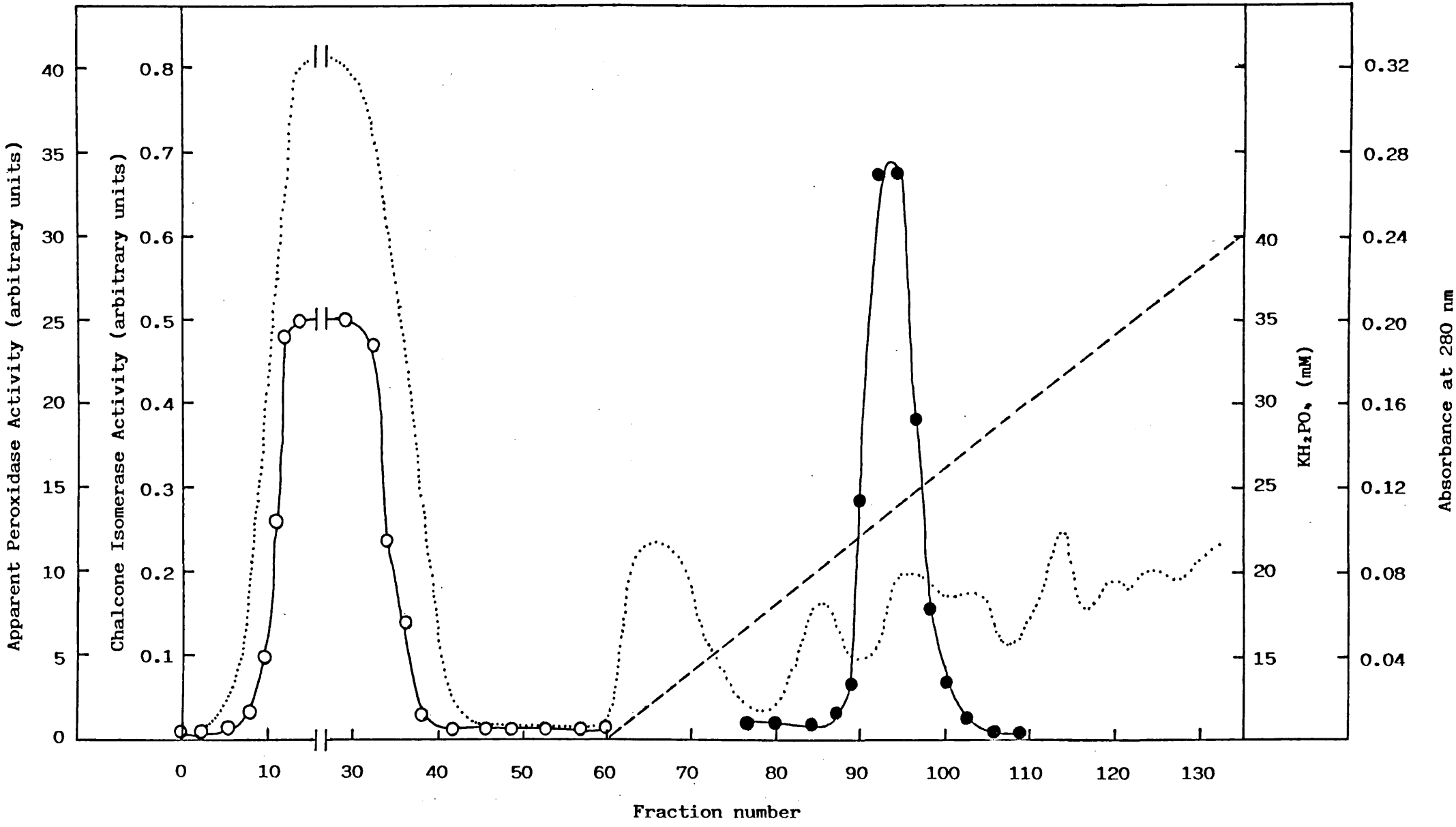


Figure 6.3 Purification of chalcone isomerase on DEAE-cellulose. (●—●) Chalcone isomerase activity; (O--O) Apparent peroxidase activity; (·····) Absorbance at 280 nm; (----) Potassium phosphate gradient.

to 1ml by ultrafiltration as described above.

Table 6.1

Fractionation of CHI Activity from a Crude Supernatant of *P. vulgaris* Cell Suspension Cultures

$(NH_4)_2SO_4$ saturation (%)	CHI activity in precipitate (μkat)
0-40	8.6
40-50	11.9
50-60	64.8
60-70	50.8
70-80	15.2

6.6 Kinetic Studies

The buffers used were 60mM KH_2PO_4 in the pH range 5.6-8.8, and 60mM Tris-HCl in the range 7.25-9.25. In experiments investigating substrate specificity or isomerisation of 2',4,4'-trihydroxychalcone at varying pH values, initial rates of disappearance of chalcone at 400nm were determined at six or more concentrations, correction being made for the different extinction coefficients of different substrates or the same substrate at different pH values.

A range of flavonoid and isoflavonoid inhibitors were tested by adding the compound (final fixed concentration between 0.75 and 1.5 times the K_i value) in 20μl EtOH to reaction mixtures containing varying concentrations of from 25 to 6.66μM 2',4,4'-trihydroxychalcone. K_m and K_i values were determined from double reciprocal plots of the initial rate data (Table 6.3, Figure 6.5).

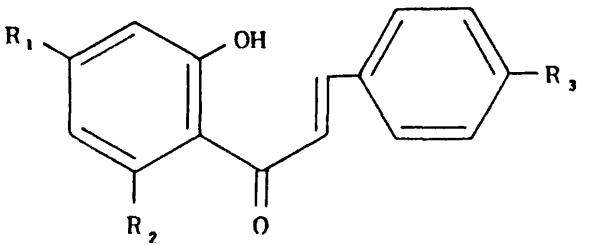
Table 6.2 Purification of Chalcone Isomerase from Cell Suspension Cultures of *Phaseolus vulgaris*

Fraction	Total activity (μkat)	Total protein (mg)	Specific activity (μkat/g)	Relative purity (-fold)	Recovery (%)
Crude extract	1.10	559	2	1	100
Supernatant from acid precipitation	1.05	256	4	2	95.2
50-75% $(NH_4)_2SO_4$ fraction	0.76	55	14	7	69.1
Sephadex G-100	0.57	13	44	22	51.8
DEAE-cellulose, ultrafiltration	0.51	0.76	671	336	46.4
Affinity chromatography, dialysis, ultrafiltration[1]	0.27	0.35	771	386	24.5

[1] The total volume after the DEAE step was 5.9ml; 1ml of this was applied to the affinity column. The figures above are calculated on the basis that the whole fraction was applied.

Table 6.3 Substrate Specificity of Chalcone Isomerase from *Phaseolus vulgaris*

V values refer to the 355-fold purified enzyme (129µg protein/ml)



(A) R_1= R_3= OH; R_2= H;

(B) R_1= OH; R_2= H; R_3= OCH_3;

(C) R_1= R_3= OCH_3; R_2= H;

(D) R_1= $OCH_2C_6H_5$; R_2=H; R_3= OCH_3

(E) R_1= R_2= R_3= OH.

Chalcone substitution pattern	K_m (µM)	V_{max} (nkat.ml^{-1})
2',4,4'-Trihydroxy (A)	8.9	23.25
2',4'-Dihydroxy-4-methoxy (B)	8.0	41.67
2'-Hydroxy-4,4'-dimethoxy (C)	No reaction	-
2'-Hydroxy-4'-benzyloxy-4-methoxy (D)	No reaction	-
2',4,4',6'-Tetrahydroxy (E)	13.3	80.65

6.7 Assay of Enzyme Activites

The assay of CHI and apparent peroxidase activities has been described previously (Section 2.10b).

6.8 Protein Determination

Protein was determined as described previously (Section 2.11).

6.9 Results

6.9a Purification Procedure

The purification procedure up as far as the DEAE-cellulose stage resulted in about a 350-fold purification of the isomerase with an approximate 50% loss in total activity (Table 6.2).

Peroxidase activity (chalcone oxidation) was also monitored throughout the purification procedure. The supernatant from the acid precipitation stage showed no assayable peroxidase activity, although such activity appeared in fractions from the gel-filtration step. H_2O_2 is probably destroyed during acid precipitation, but later produced endogenously in the still relatively crude preparation; on addition of H_2O_2 to assay mixtures (minus KCN) containing active isomerase at all stages up to the DEAE elutate, rapid decolourisation of added chalcone occured. Because measured rates of peroxidase activity in the fractions clearly depended on endogenous H_2O_2 levels, the term 'apparent peroxidase activity' is used.

Ion-exchange chromatography on DEAE-cellulose resulted in complete separation of the isomerase from peroxidase (Figure 6.2). The gel-filtration step had previously separated CHS from CHI (Figure 5.4).

The isomerase bound very strongly to the chalcone affinity column; an initial attempt to elute the enzyme with 50μM 2',4,4'-trihydroxychalcone was unsuccessful. The enzyme was finally eluted with approximately 2mM chalcone in 30mM KH_2PO_4, pH 8.0.

Chromatofocussing

Due to the disappointing result obtained with the affinity column (Table 6.2), a preliminary experiment with a chromatofocussing column was carried out; 2.5ml of enzyme solution (from the DEAE step) was dialysed for 5h against buffer D. This was applied to a Pharmacia 'Polybuffer Exchanger' PBE 74 column (10 x 1cm) which had been previously equilibrated with buffer D. The column was eluted with 240ml of buffer E, which generated a pH gradient from pH 7.0-4.0 on the column. The enzyme activity eluted as a single peak from the column in a total volume of 20ml at a pH corresponding to an apparent pI of 5.0 (Figure 6.4).

[Note: Unfortunately there was insufficient time available to calculate the purification obtained. However, recently published work using this method as the final purification step instead of the affinity column resulted in an apparently homogenous 595-fold purified enzyme (Robbins and Dixon, 1984)].

6.9b Stability of the Enzyme Activity

The 350-fold purified enzyme (129µg protein/ml) retained 95% of its original activity when stored for six days at 4°C; however, a 1:4 dilution of the same preparation only retained 53% activity under the same conditions. In the presence of 1% BSA, 65% of the original activity was retained after six weeks at 4°C. The preparation lost 35% of its activity on storage for six weeks at -20°C in the presence of 5% glycerol, whereas in the absence of glycerol 95% of the activity was lost.

6.9c Molecular Weight

From gel-filtration studies with a Sephadex G-100 column calibrated according to the method of Andrews (1964), the apparent molecular weight of the isomerase was estimated to be about 30,000.

This value corresponded well with the value of 28,000 obtained for the protein after affinity chromatography and SDS-gradient gel electrophoresis (Results of Richard A. Dixon and Prakash M. Dey).

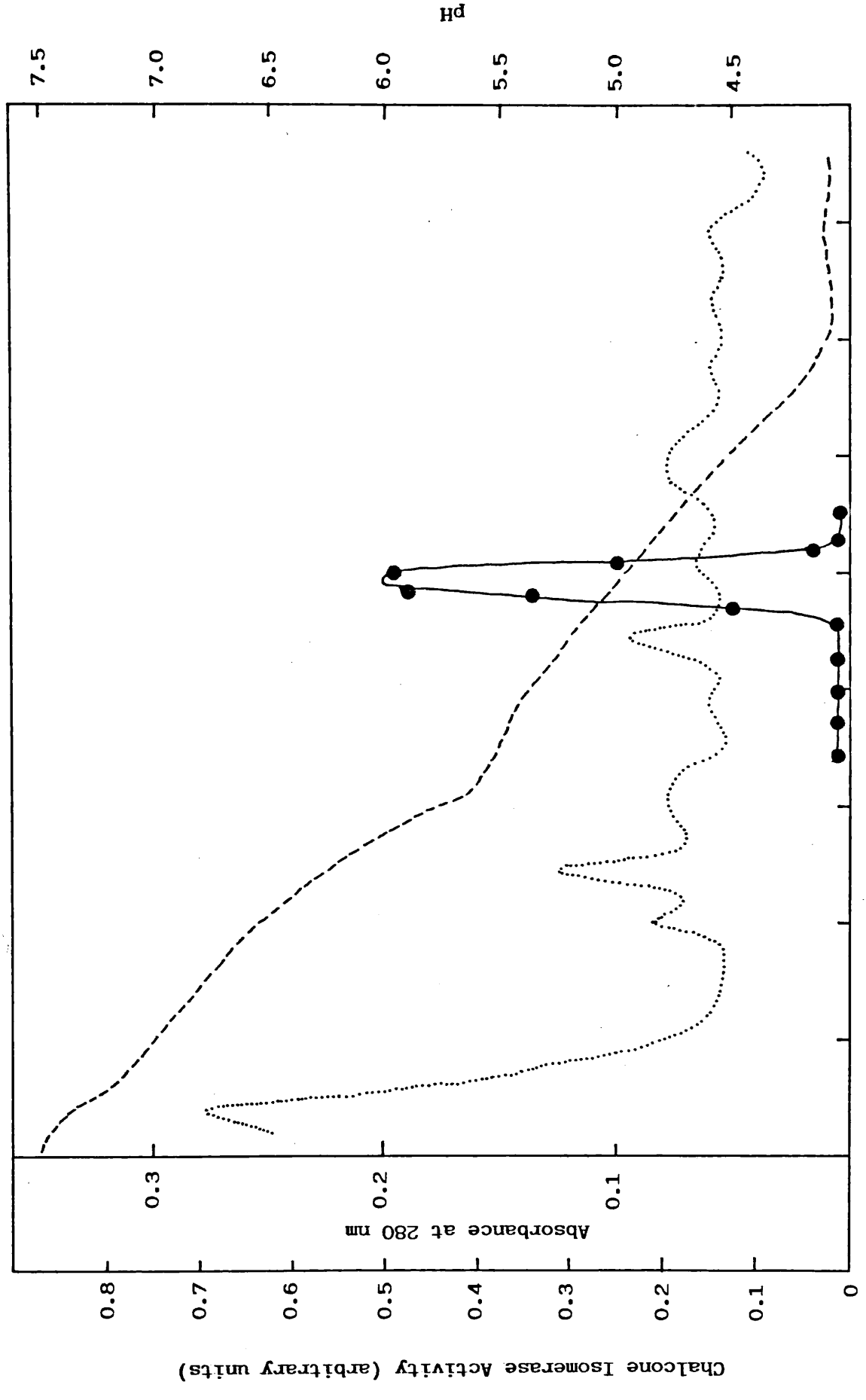


Figure 6.4 Chromatofocussing elution profile for chalcone isomerase. (●—●) Enzyme activity; (– – –) pH gradient; (.....) Absorbance at 280 nm.

6.9d Substrate Specificity

The enzyme catalysed the conversion of both 2',4,4'-trihydroxychalcone and 2',4,4',6'-tetrahydroxychalcone to their respective flavanones (Table 6.3). The K_m for the tetrahydroxychalcone is probably less accurate than those for the other chalcones, in view of the instability of this compound at pH 8.0 and the associated difficulty in assessing its purity. Unlike the isomerase from *Glycine max* (Moustafa and Wong, 1967), the *P. vulgaris* enzyme readily catalysed the isomerisation of the B-ring methoxylated chalcone (Table 6.3, compound B), although no activity was seen with chalcones substituted in the 4'-position.

Substrate inhibition was observed with both the 2',4,4'-trihydroxy and 2',4'-dihydroxy-4-methoxy chalcones at concentrations of 25μM and above, whereas double reciprocal plots for the tetrahydroxychalcone were still linear at high substrate concentrations (Results of Richard A. Dixon and Prakash M. Dey).

6.9e Competitive Inhibition of the Isomerisation of 2',4,4'-Trihydroxychalcone

Various flavonoid and isoflavonoid compounds were tested as inhibitors of the reaction (Table 6.4); in all cases inhibition was competitive as judged from increase in K_m, with no effect on V, observed from double reciprocal plots (Figure 6.5).

6.10 Discussion

The use of KCN in enzyme assay and gel-staining buffers ensures that no artefacts due to the presence of peroxidase are observed. Peroxidase catalyses the H_2O_2-dependent oxidation of 2',4,4'-trihydroxychalcone to a variety of products including the chalcone cyclic peroxide, the 4',7-dihydroflavonol and the 4',6-dihydroxyaurone (Rathmell and Bendall, 1971; Wong and Wilson, 1976; Wilson and Wong, 1976). The results in this

Table 6.4 Competitive Inhibition of Chalcone Isomerase by Flavonoid and Isoflavonoid Derivatives

Inhibitor	K_i (μM)
2',4'-dimethoxy-4-hydroxychalcone	3.4
4',5,7-trihydroxyflavanone (naringenin)	16.7
3,4',5,7-tetrahydroxyflavanol (kaempferol)	4.2
3,3',4',5,7-pentahydroxyflavone (quercetin)	4.1
4',5,7-trihydroxyisoflavone (genistein)	160.1
2',4',5,7-tetrahydroxyisoflavone (2'-hydroxygenistein)	37.5
2',4',5,7-tetrahydroxyisoflavanone (dalbergioidin)	57.0
2',4',5,7-tetrahydroxy-8-isopentenyl-isoflavanone (kievitone)	9.2
3,9-dihydroxypterocarpan[1]	25.9
3-hydroxy-2',2'-dimethylpyrano[6',5': 9,10]pterocarpan[1] (phaseollin)	50.0
4',7-dihydroxycoumestan[1] (coumestrol)	2.5

[1] For full systematic names see Appendix B.

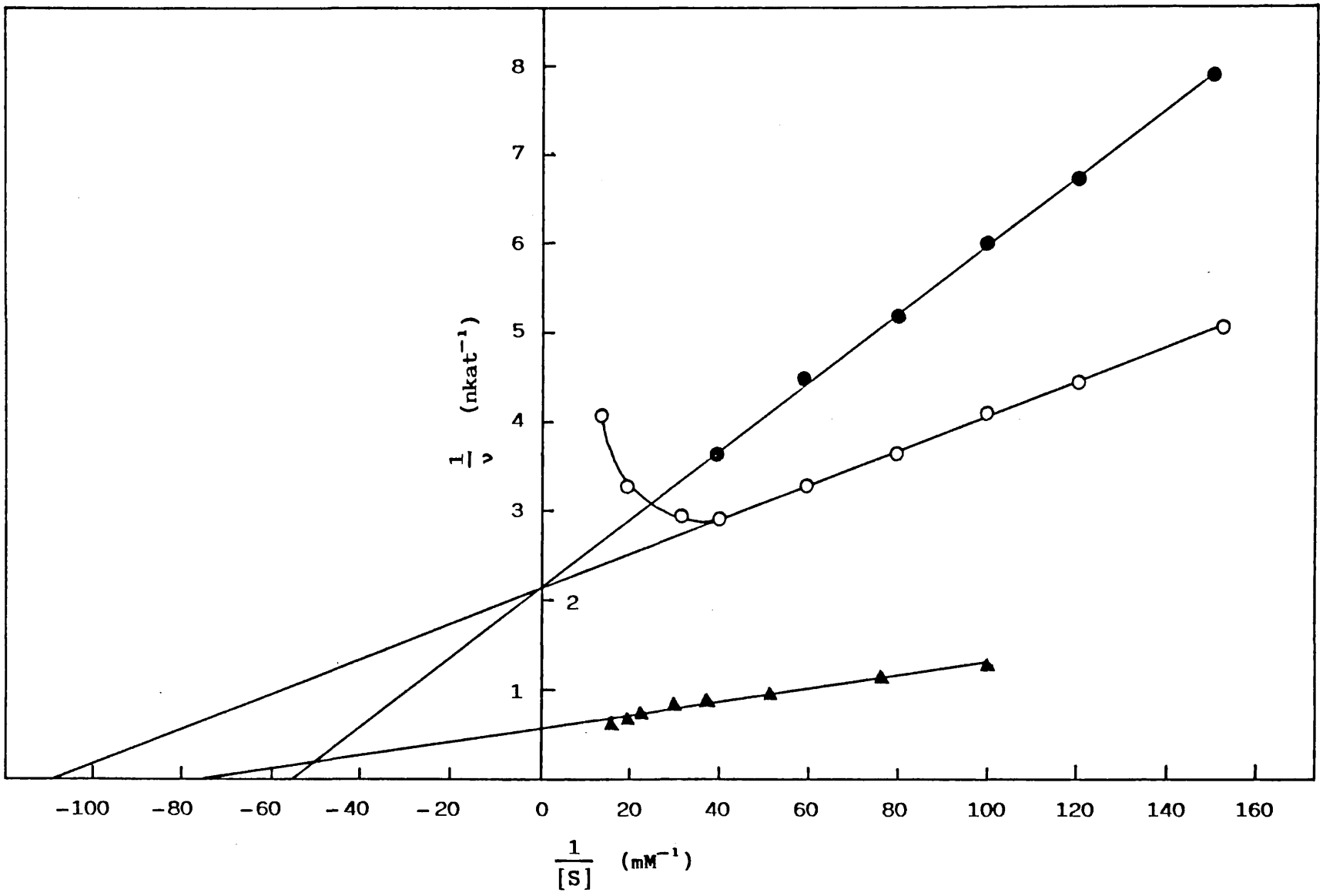


Figure 6.5 Lineweaver-Burk plots for the isomerisation of 2',4,4'-trihydroxychalcone (○—○), and 2',4,4',6'-tetrahydroxychalcone (▲—▲) at 30°C, pH 8.0. The isomerisation of the trihydroxychalcone in the presence of 9.55μM kievitone is also shown (●—●). Values for 1/v refer to 20μl of the 355-fold purified enzyme (2.6μg protein) in each case.

chapter indicate that care is needed in assessing peroxidase activity during the purification procedure, as H_2O_2 may be destroyed and then regenerated endogenously in the partially pure preparations.

The 386-fold purification of the *P. vulgaris* isomerase compares favourably with the 8,000-fold purification reported for the enzyme from soybean (Boland and Wong, 1975), where considerable amounts of storage protein are present; in spite of the seemingly higher purification, the actual specific activity achieved for the soybean enzyme was considerably less than that obtained for the *P. vulgaris* enzyme. The molecular weight of 28,000 lies between the previously reported values of 16,000 for the soybean enzyme (Boland and Wong, 1975) and 50,000 for the parsley enzyme (Kreuzaler and Hahlbrock, 1975a). The agreement between the molecular weights obtained by gel-filtration and SDS-gel electrophoresis suggests that the *P. vulgaris* isomerase consists of a single subunit. This observation is supported by the result of the chromatofocussing experiment which indicated that the isomerase exists in a single charged form. It is of interest that the bean isomerase, while existing in a single form, catalyses the isomerisation of both 6'-hydroxy and 6'-deoxy chalcones. This suggests that the differential regulation of the appearance of the 5-hydroxy and 5-deoxy substituted phytoalexins in bean (Chapter 3) is not regulated at the chalcone isomerase level.

The isomerase has low K_m values for both 2',4,4'-trihydroxy and 2',4,4',6'-tetrahydroxy chalcones; the K_m is similiar to that calculated for the parsley enzyme, although this enzyme is inactive against 6'-deoxy-chalcones (Hahlbrock et al., 1970a). The established correlation between isomerase specificity and the substitution patterns of the naturally occuring flavonoid compounds in the plant (Hahlbrock et al., 1970a)is again apparent with the enzyme from *P. vulgaris*, although 2',4'-dihydroxy-4-methoxychalcone can act as a substrate *in vitro*. The low K_m values

indicate that the enzyme binds substrates tightly and that the catalysis is very efficient.

The potent inhibition of the isomerase by the flavonols kaempferol and quercetin was similiar to that previously observed by Boland and Wong (1975) with the isomerase from *Glycine max*. Low K_i values were also observed for the 2'-methoxychalcone, as could be expected, and for the antimicrobial compounds, kievitone and coumestrol, which are both end products of the inducible isoflavonoid pathway. In contrast, the isoflavonoid precursors of kievitone (genistein, 2'-hydroxygenistein and dalbergioidin) were very poor inhibitors. It is therefore possible that, *in vivo*, kievitone and coumestrol act as feedback inhibitors of their own synthesis.

The increased inhibitory effect of kievitone, over genistein, 2'-hydroxygenistein and dalbergioidin appears to be directly due to the prenyl substituent. However, it is not immediately obvious, from a structual viewpoint, why kievitone and coumestrol act as such potent inhibitors.

CHAPTER 7

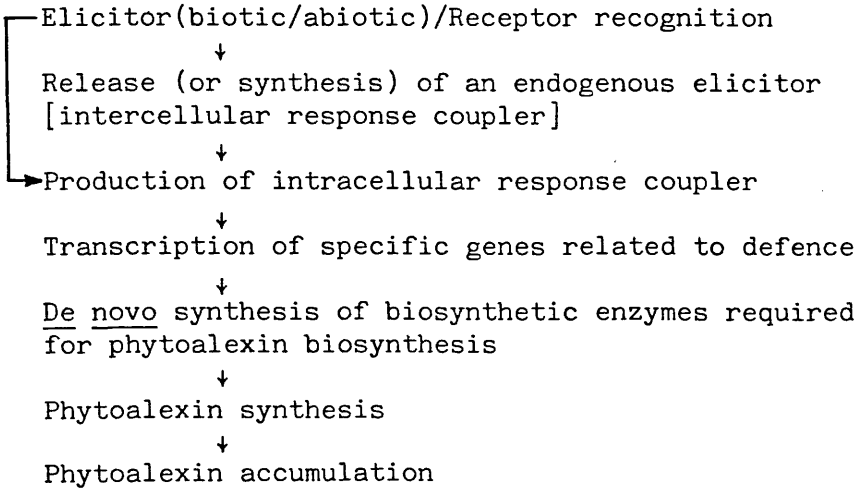
Final Discussion

The results presented in Chapters 3-6 have been specifically commented on in the discussion sections of the individual chapters. The purpose of this final chapter is to discuss general proposals related to the mechanism of accumulation of phytoalexins in the light of results presented in the earlier chapters. A further short summary of progress made in the field of biochemical reasearch into phytoalexins since the work for this thesis was completed, and of current questions still to be answered, is presented at the end.

7.1 Mechanisms of Phytoalexin Accumulation

The induction, synthesis and accumulation of phytoalexins is currently an area under intensive investigation at the biochemical, physiological and molecular genetic levels. A general consensus for the probable sequence of events which occur during the phytoalexin response is as follows (Scheme 7.1):

Scheme 7.1



In Chapter 3 it was observed that the two closely related classes of isoflavonoid phytoalexin (5-hydroxy and 5-deoxy) exhibited differential patterns of accumulation in response to wounding. A similiar situation was reported by Smith et al. (1975) who found that kievitone accumulated very rapidly in young lesions of *Rhizoctonia solani* on *Phaseolus vulgaris* hypocotyls. Within a few days the levels of kievitone had decreased whereas the concentration of phaseollin had increased during this period. One question to arise from these observations relates to how this later accumulation of 5-deoxy compounds i.e. phaseollin is induced.

A possible answer to this question may be obtained by considering the proposed sequence of events (Scheme 7.1) in reverse. The accumulation of phytoalexins is the result of de novo synthesis of the biosynthetic enzymes required for the formation of these compounds. This probably occurs after de-repression of the relevant structual genes by internal response couplers, whose action may or may not require prior formation of intercellularly acting endogenous elicitors (Hargreaves and Selby, 1978; Dixon et al., 1983). Although it was not possible to measure 6'-deoxy CHS activity it is assumed to be induced slightly prior to the accumulation of 5-deoxy compounds in a similiar manner to that observed with 6'-hydroxy CHS and 5-hydroxy compounds such as kievitone (Chapter 3) and, since 5-hydroxy and 5-deoxy isoflavonoid phytoalexins arise by pathways which diverge at the level of chalcone formation (Chapter 4), it may be necessary to propose the existence of at least two non-identical internal response couplers in order to explain the results observed in Chapter 3. The initial accumulation of kievitone is probably the result of wound damage/elicitor recognition (in the case of the *R. solani* interaction), which leads to the release of an internal elicitor which stimulates de novo synthesis of 6'-hydroxy CHS and results in the accumulation of 5-hydroxy compunds. Subsequently, cell death may cause the release of

a second non-identical elicitor which is specific for stimulating de novo synthesis of 6'-deoxy CHS which results in the observed increases in 5-deoxy compounds such as phaseollin and coumestrol (Chapter 3 and Smith et al., 1975). This proposal does not, however, explain the second increase in 5-hydroxy compounds which occurs concomitantly with the increases in 5-deoxy compounds, as observed in Chapter 3. This might be explainable if the second elicitor (i.e. that released upon cell death), stimulated both pathways.

Bailey (1982) has argued that cell death elicits phytoalexin accumulation. This was based on the original work into race-specific interactions (Bailey and Deverall, 1971, see Chapter 1) which showed that hypersensitivity (limited cell death) brought about the accumulation of phaseollin. Again, in susceptible interactions where the pathogen was at first able to grow biotrophically, phaseollin accumulation only occurred at a late stage, presumably after some cell death/membrane disruption had occured. Unfortunately, from the point of view of the present argument, this work did not monitor changes in the levels of 5-hydroxy compounds. Further indirect evidence for the existence of more than one type of response coupling mechanism was presented by Dixon et al. (1983) who demonstrated that different mechanisms operate for the overall transmission of the phytoalexin response in cell suspension cultures of *Phaseolus vulgaris* treated with biotic or abiotic elicitors.

If the accumulation of 5-hydroxy and 5-deoxy isoflavonoid phytoalexins in *P. vulgaris* is mediated by two different response coupling mechanisms then it is possible that different types of receptor linked to phytoalexin induction may exist on the surface of host cells. Although the existence of receptors and the identity of the secondary signals which are released/synthesized have yet to be established experimentally, a wealth of additional, albeit circumstantial, evidence exists to support

this hypothesis. For example, kievitone was the predominant phytoalexin in *P. vulgaris* tissue infected with *Rhizoctonia solani* (Smith et al., 1975) and also occured in tissues infected with *Colletotrichum lindemuthianum* (Bailey, 1974) or *Botrytis cinerea* (Fraile et al., 1980) but was not detected in lesions caused by *Thielaviopsis basicola* or *Fusarium solani* (Van Etten and Smith, 1975). These observed differences might possibly be explained in terms of elicitor activities with different receptors which are specific for the induction of each class of compound (i.e. 5-hydroxy and 5-deoxy).

The results of the inhibitor studies on 6-hydroxy CHS and CHI have already been discussed in Chapters 5 and 6. However, it is possible that these results may have a wider relevance to the processes of phytoalexin accumulation. Bailey (1982) suggests that phytoalexins accumulate in dead or dying cells which are themselves the stimulus for phytoalexin biosynthesis and that phytoalexin synthesis occurs in living tissue around the site of infection before such compounds accumulate in the dead or dying cells. The results in Chapter 3 are consistent with this proposal in that the extractable levels of kievitone from whole cotyledons after 48h are approximately 20-30 times the K_i for the inhibition of CHS and CHI by this compound , yet the levels of phaseollin and other 5-deoxy compounds in the tissue start to increase at this time. This would only be expected to occur if the sites of synthesis and accumulation of these compounds were different (as proposed by Bailey, 1982). Indeed, if phytoalexins accumulate in only dead or dying cells then the actual concentrations of phytoalexin in such tissue would be even higher than the values obtained from whole tissues. Phaseollin has been obtained from excised lesions of *P. vulgaris* at concentrations up to 3mg/g tissue (approximately 10mM, Van Etten and Pueppke, 1976).

No consideration has as yet been given to the possible mechanisms

involved in the accumulation of phytoalexins at specific sites, i.e. in dead or dying cells. Simple passive diffusion can be excluded since the concentrations of accumulating phytoalexins is so high at infection sites. Thus, it is necessary to postulate that phytoalexins are either adsorbed by some mechanism in dead or dying cells as suggested by Bailey (1982) or are actively transported into such cells. Adsorption would work by effectively reducing the concentration of 'free' phytoalexin molecules in the dead/dying cells and thus favour further passive diffusion into the cells. A mechanism involving active transport would require the integrity of plasma membranes of at least the living cells, and may not be possible in the light of reports that compounds such as phaseollin affect the permeability of such membranes (Shiraishi et al., 1975; Elnaghy et al., 1976).

The accumulation of phytoalexins in dead or dying cells would have at least three advantageous effects. Firstly, the concentrations of phytoalexins would increase more rapidly in the infected tissue. Secondly, the level of phytoalexins in living tissue synthesizing such compounds would be maintained at non-phytotoxic levels, thus enabling further synthesis; in this regard, the inhibitory effects of compounds such as coumestrol and kievitone on the activities of CHS and CHI may act as a 'fail-safe' mechanism by regulating the concentration of accumulating phytoalexins in the healthy cell independently of the rate of removal by transport/diffusion. Thirdly, the accumulation of phytoalexins in dead or metabolically inactive cells would prevent further metabolism of these compounds (at least by the host) and thus ensure that the high levels were maintained at the site of infection. The maintenance of inhibitory levels of phytoalexins at the site of infection is of importance in some interactions (Bailey, 1982) particularly if the effect of the phytoalexin is fungistatic and not fungitoxic.

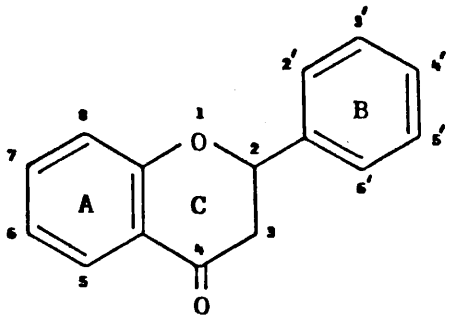
7.2 Recent, Current and Future Areas of Research

The elucidation of the biochemical pathways leading to phytoalexin formation and the characterisation of the enzymes involved in the biosynthesis of such compounds is only the first step towards achieving an understanding of how such induced responses are controlled at the molecular level. Although only a few key enzymes involved in the biosynthesis of isoflavonoid phytoalexins have been characterised from *Phaseolus vulgaris* (i.e. PAL, CHS and CHI), further work investigating the control and regulation of the activities of such enzymes has been published since the work described in this thesis was performed.

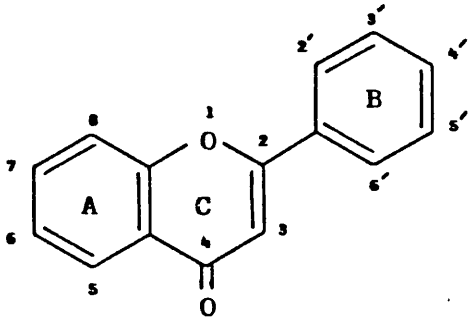
Density labelling studies (Dixon and Lamb, 1979) coupled with immunological studies (which require purified enzymes in order to raise specific antisera) (Lawton et al., 1983a) indicated that increases in the activities of the phytoalexin biosynthetic enzymes resulted from their increased de novo synthesis. Subsequent investigations showed that such de novo synthesis is brought about by increased activity of specific mRNA's encoding these enzymes (i.e. increased translational capacity) (Lawton et al., 1983b). Further work indicated that increases in CHS mRNA activity occured in a differential manner in compatible and incompatible plant-pathogen interactions (Bell et al., 1984). This suggested that the control of phytoalexin gene expression is of primary importance in determining the outcome of race-specific plant-pathogen interactions. This work was followed by investigations into the patterns of increased mRNA activities for the three enzymes under investigation (PAL, CHS and CHI) and the results showed that such increases occur in a highly co-ordinated manner which suggested that a similiar mechanism of induction (in response to elicitor) might operate for all three enzymes (Cramer et al., 1985).

The most recent work has measured rapid but transient increases in the actual amounts of PAL and CHS mRNA in response to elicitor treatment (Dixon, R.A. , personal communication). The results concur with the hypothesis that elicitor(s) causes a rapid but transient stimulation of transcription of PAL and CHS genes. Future work will obviously be directed at elucidating the mechanism(s) involved in the de-repression of such genes and should illustrate the role played by fungal elicitors in intiating such responses.

Appendix A Skeleton and Numbering Scheme



Flavanone



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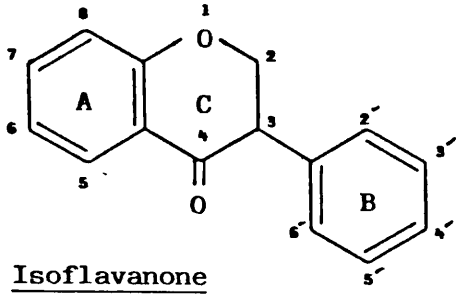
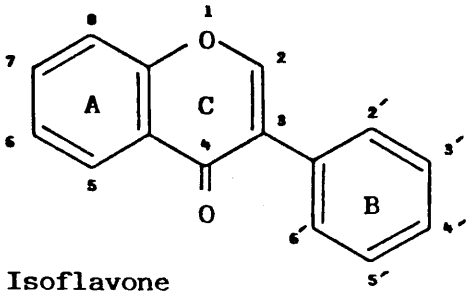
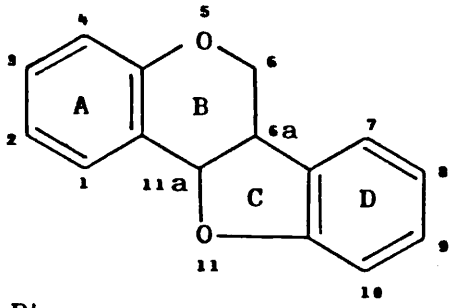


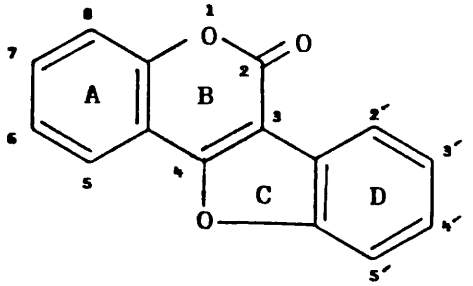
Isoflavanone



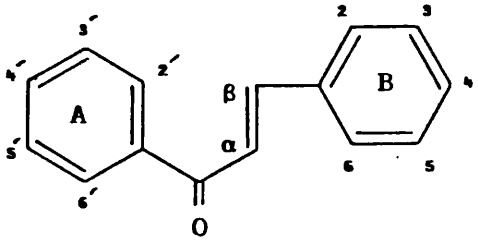
Isoflavone



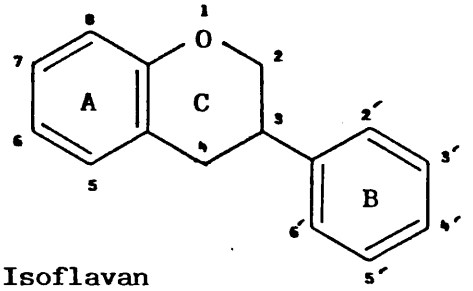
Pterocarpan



Coumestan



Chalcone



Isoflavan

Appendix B Compound Index

Trivial Name		Systematic Name
Apigenin		4',5,7-trihydroxyflavone
Biochanin A		5,7-dihydroxy-4-methoxyisoflavone
Bis-noryangonin		4-hydroxy-6[4-hydroxystyryl]-2-pyrone
Catechol		1,2-dihydroxybenzene
Chlorogenic acid		3-caffeoylquinic acid
Coumestrol		4',7-dihydroxycoumestan[1]
Daidzein		4',7-dihydroxyisoflavone
Dalbergioidin		2',4',5,7-tetrahydroxyisoflavanone
2,3-Dehydrokievitone		2',4',5,7-tetrahydroxy-8-isopentenylisoflavone
Demethylmedicarpin		3,9-dihydroxypterocarpan[2]
5-Deoxykievitone		2',4',7-trihydroxy-8-isopentenylisoflavanone
Equol		4',7-dihydroxyisoflavan
Eriodictyol		3',4',5,7-tetrahydroxyflavanone
Formononetin		7-hydroxy-4'-methoxyisoflavone
Genistein		4',5,7-trihydroxyisoflavone
Glyceollin (3 isomers)	G-I	6a,9-dihydroxy-2',2'-dimethylpyrano[5',6':2,3]-pterocarpan[2]
	G-II	6a,9-dihydroxy-2',2'-dimethylpyrano[6',5':3,4]-pterocarpan[2]
	G-III	6a,9-dihydroxy-2'-(2-methylethenyl)-2',3'-dihydro-furo[4',5':2,3]pterocarpan[2]
Homoeriodictyol		4',5,7-trihydroxy-3'-methoxyflavanone
2'-Hydroxygenistein		2',4',5,7-tetrahydroxyisoflavone
Isoliquiritigenin		2',4,4'-trihydroxychalcone
Kaempferol		3,4',5,7-tetrahydroxyflavanol
Kievitone		2',4',5,7-tetrahydroxy-8-isopentenylisoflavanone
Kinetin		6-furfurylaminopurine
Licoisoflavone A		2',4',5,7-tetrahydroxy-3'-isopentenylisoflavone
Liquiritigenin		4',7-dihydroxyflavanone
Maackian		3-hydroxy-8,9-methylenedioxypterocarpan[2]
Medicarpin		3-hydroxy-9-methoxypterocarpan[2]
Naringenin		4',5,7-trihydroxyflavanone
Naringenin chalcone		2',4,4',6'-tetrahydroxychalcone
Phaseollidin		3,9-dihydroxy-10-isopentenylpterocarpan[2]
Phaseollin		3-hydroxy-2',2'-dimethylpyrano[6',5':9,10] pterocarpan[2]
Phaseollinisoflavan		6',7-dihydroxy-2',2'-dimethylpyrano[6',5':4',5'] isoflavan

Appendix B (Continued)

Trivial Name	Systematic Name
Pisatin	6a-hydroxy-3-methoxy-8,9-methylenedioxy-pterocarpan[2]
Protocatechuic acid	3,4-dihydroxybenzoic acid
Psoralidin	4',7-dihydroxy-6-isopentenylcoumestan[1]
Quercetin	3,3',4',5,7-pentahydroxyflavone
Sativan	7-hydroxy-2',4'-dimethoxyisoflavan
Wighteone	4',5,7-trihydroxy-6-isopentenylisoflavone

Note: Several of the above named compounds are named in a semi-systematic manner to enable the reader to see more easily the relationship between certain classes of compound i.e. coumestans and pterocarpans which would otherwise be less obvious. For the sake of completion the full systematic names of these two classes of compound according to IUPAC rules is given below.

[1]	Coumestan	6H-benzofuro[3,2-c][1]benzopyran-6-one
[2]	Pterocarpan	6a,11a-dihydro-6H-benzofuro-[3,2-c]benzopyran

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Statement of Co-joint Work and Publications of Ian Michael Whitehead

Submitted as Subsidary Material in Support of Ph.D. Thesis Entitled:

'Enzymological Aspects of Phytoalexin Accumulation in *Phaseolus vulgaris*'

(1) Dixon, R.A., Dey, P.M., Murphy, D.C. and Whitehead, I.M. (1981)

Dose responses for *Colletotrichum lindemuthianum* elicitor-mediated induction in French bean cell suspension cultures.

Planta **151**, 272-280.

Contribution: Figure 2, page 275.

(2) Whitehead, I.M., Dey, P.M. and Dixon, R.A. (1982)

Differential patterns of phytoalexin accumulation and enzyme induction in wounded and elicitor-treated tissues of *Phaseolus vulgaris*.

Planta **154**, 156-164.

Contribution: Figure 1A-C, page 157; Figure 2A-C, page 158.

(3) Dixon, R.A., Dey, P.M. and Whitehead, I.M. (1982)

Purification and properties of chalcone isomerase from cell suspension cultures of *Phaseolus vulgaris*.

Biochim. et Biophys. Acta **715**, 25-33.

Contribution: Introduction, materials and methods, results and discussion, pages 25-33 (in part). Involved in all work except for experiments reported in Figures 4 and 5.

(4) Dewick, P.M., Steele, M.J., Dixon, R.A. and Whitehead, I.M. (1982)

Biosynthesis of isoflavonoid phytoalexins: Incorporation of sodium [1,2-$^{13}C_2$] acetate into phaseollin and kievitone.

Z. Naturforsch **37c**, 363-368.

Contribution: Experimental, pages 363 and 365.

(5) Whitehead, I.M. and Dixon, R.A. (1983)

Chalcone synthase from cell suspension cultures of *Phaseolus vulgaris*.

Biochim. et Biophys. Acta **747**, 298-303.

Contribution: Experimental, page 299; Results, pages 300-302; Discussion, page 302 (in part); Table 1, page 300; Figures 1A, 1B and 2, page 301; Figure 3, page 302.

Planta 151, 272–280 (1981)

Planta

Dose Responses for *Colletotrichum lindemuthianum* Elicitor-mediated Enzyme Induction in French Bean Cell Suspension Cultures

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Abstract. The induction of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) and flavanone synthase in French bean cell suspension cultures in response to heat-released elicitor from cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* is highly dependent upon elicitor concentration. The elicitor dose-response curve for PAL induction shows two maxima at around 17.5 and 50 μg elicitor carbohydrate per ml culture, whereas the flavanone synthase response shows one maximum at around 100 μg ml^{-1}. The PAL response is independent of the elicitor concentration present during the lag phase of enzyme induction; if the initial elicitor concentration is increased after 2 h by addition of extra elicitor, or decreased by dilution of the cultures, the dose response curves obtained reflect the concentration of elicitor present at the time of harvest. PAL induction is not prevented by addition of methyl sugar derivatives to the cultures; α-methyl-D-glucoside, itself a weak elicitor of PAL activity, elicits a multiphasic PAL response when increasing concentrations are added in the presence of *Colletotrichum* elicitor. Eight fractions with different monosaccharide compositions, obtained from the crude elicitor by gel-filtration, each elicit different dose-responses for PAL induction; the response to unfractionated elicitor is not the sum of the responses to the isolated fractions. There is no correlation between the ability of the fractions to induce PAL in the cultures and their ability to act as elicitors of isoflavonoid phytoalexin accumulation in bean hypocotyls.

Key words: Cell suspension culture – *Colletotrichum* – Elicitor – Flavanone synthase – *Phaseolus* – Phenylalanine ammonia-lyase – Phytoalexin.

Abbreviations: PAL = phenylalanine ammonia-lyase; PMS = *Phytophthora megasperma* var sojae

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Introduction

The accumulation of phytoalexins (host-synthesised, low molecular weight antimicrobial compounds) in plant cells in response to phytopathogenic fungi and bacteria is believed to be induced by fungal metabolites termed elicitors. Such molecules have been isolated from fungal culture filtrates and mycelial walls and appear to be either polysaccharides (Ayers et al. 1976; Anderson-Prouty and Albersheim 1975) or glycoproteins (Daniels and Hadwiger 1976; Stekoll and West 1978; Dow and Callow 1979a; De Wit and Roseboom 1980).

Treatment of French bean cell suspension cultures with a crude elicitor from cell walls of *Colletotrichum lindemuthianum*, the causal agent of anthracnose disease, results in the accumulation of the isoflavonoid-derived phytoalexin phaseollin, preceded by a rapid, transient increase in the activity of L-phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), this change resulting from increased de novo synthesis of the enzyme (Dixon and Lamb 1979). The extent of PAL induction in the cultures is highly dependent on elicitor concentration, and the complex dose-response curve observed results from differential effects of elicitor concentrations on PAL synthesis and removal (Lawton et al., 1980).

Elicitors may exert their effects by binding to sites in the host cell plasma membrane, and elicitor dose-response curves may possibly reflect binding phenomena (Albersheim and Valent 1978). Indirect evidence for cell surface binding comes from studies in which low molecular weight sugar derivatives have been shown to inhibit elicitor-mediated responses (Ayers et al. 1976; Marcan et al. 1979). In addition, the high molecular weight glucan elicitor from *Phytophthora infestans* can cause agglutination of potato protoplasts (Peters et al. 1978; Doke and Tomiyama 1980), and low molecular weight glucans from compatible

races of *P. infestans* inhibit elicitor activity by a process which may involve competition for membrane receptor sites (Doke et al. 1979). Radiolabelled glycopeptides from culture filtrates of *Cladosporium fulvum* have been shown to bind directly to isolated tomato leaf mesophyll cells (Dow and Callow 1979b). Dose-response profiles for elicitor activity vary from log-linear (hypersensitivity of potato protoplasts to *P. infestans* elicitor; Doke and Tomiyama 1980), linear saturating at higher concentrations (glyceollin accumulation in soybean hypocotyls in response to *Phytophthora megasperma* var. sojae (PMS) elicitor; Albersheim and Valent 1978), hyperbolic (PAL induction in soybean cell suspensions in response to PMS elicitor; Ebel et al. 1976) to more complex relationships (phaseollin accumulation or PAL induction in French bean cell suspension cultures in response to *C. lindemuthianum* elicitor; Dixon and Lamb 1979; Lawton et al., 1980).

In view of the value of suspension cultured material for elicitor binding studies, we have now investigated further the induction of PAL and flavanone synthase (the first enzyme unique to 5-hydroxyflavonoid/isoflavonoid biosynthesis) in response to varying elicitor concentrations in the French bean cell cultures. We conclude that the elicitor-mediated induction of PAL in the cultures may involve a complex interaction between various fractions present in the crude elicitor preparation, but that such phenomena may not be directly related to the induction of isoflavonoid phytoalexin accumulation in the whole plant.

Materials and Methods

Cell suspension cultures of dwarf French bean variety Canadian Wonder were initiated and maintained by regular subculture at 14-day intervals in a modified Schenk and Hildebrandt medium as previously described (Dixon and Fuller 1976). Unless otherwise stated, cultures were grown at 25° C in a Gallenkamp Model INR-401 orbital incubator at 110 rev min^{-1} and illuminated by a single 30 W fluorescent tube approximately 0.5 m above the cultures. All cultures used in the following experiments were in exponential growth phase (6–7 days after subculture).

Colletotrichum lindemuthianum (Commonwealth Mycological Institute, isolate IMI 112166) was maintained on a semi-solid glucose-neopeptone medium (Mathur et al. 1949) in a continuous black light incubator. Conidia were removed from the cultures by vigorous shaking with sterile distilled water, and transferred to 500 ml batches of the Mathur medium (modified by the addition of 15 g glucose l^{-1}) in 2 l conical flasks. These were incubated on an orbital shaker at 25° C and 120 rev min^{-1} for 14 days. Mycelium was harvested on 4 layers of muslin, and cell walls prepared as described elsewhere (Anderson-Prouty and Albersheim 1975). Walls were autoclaved (100 ml H_2O per g walls) at 120° C for 30 min to release elicitor, the preparation then being filtered through sintered glass, clarified by centrifugation, dialysed for 24 h against three 10 l changes of distilled H_2O and concentrated 5-fold under reduced pressure. This preparation is referred to as the crude elicitor. Elicitor (from 5 g of walls) for fractionation studies was lyophilized and re-dissolved in 5 ml of distilled H_2O prior to column chromatography.

Enzyme induction was measured in 20 ml batches of suspension (5 ml packed cell volume) incubated for 7 h with 1.5 ml elicitor solution under normal culture conditions. Controls (minus elicitor) were treated with 1.5 ml sterile distilled H_2O. Each individual experiment was performed with cells from the same culture batch. Cell samples for PAL assay were harvested by suction filtration on porous polythene filters, transferred to small stoppered vials and stored at −70° C until required. Extraction and assay of the enzyme were as described elsewhere (Dixon et al., 1980). Flavanone synthase activity, in extracts from freshly harvested cells, was determined by a radiochromatographic method using [2-^{14}C]malonyl coenzyme A (Radiochemical Centre, Amersham, U.K.) and p-coumaroyl coenzyme A as substrates, as previously described (Dixon and Bendall 1978b). p-Coumaroyl coenzyme A was sythesised from p-coumaric acid via the N-hydroxysuccinimide ester (Stöckigt and Zenk 1975) and purified from unreacted coenzyme A by chromatography on Sephadex G-10 (Lindl et al. 1973). Protein was measured by a modification of the method of Lowry et al. (Legett-Bailey 1962) and total carbohydrate by the α-napthol/sulphuric acid method (Fuller and Northcote 1956).

For determination of monosaccharide composition, crude and fractionated elicitor preparations were adjusted to a concentration of 100 μg glucose equivalents in 100 μl of distilled H_2O in small capped vials. Trifluoroacetic acid (2 M, 0.5 ml) was added, and the samples hydrolysed at 100° C for 6 h. Samples were then reduced to dryness over KOH in a vacuum dessicator for 24 h, taken up in 50 μl H_2O, and 4 μl aliquots applied to Merck No. 5748 silica gel 69 thin-layer sheets. The 10 cm sheets were developed three times in ethyl acetate:pyridine:acetic acid:H_2O (6:3:1:1, v/v), and the separated monosaccharides quantitatively determined by direct densitometric scanning of the plates (Menzies et al. 1978). Lactose was included in all samples as internal standard.

Phytoalexin induction by crude and fractionated elicitor preparations was determined by application of the elicitor to the wounded surfaces of excised, etiolated hypocotyls from 7-day old seedlings of French bean variety 'The Prince'. For each treatment, elicitor was applied to five 2 cm long hypocotyl sections as described elsewhere (Anderson-Prouty and Albersheim 1975). After 48 h incubation in the dark at 25° C, each batch of hypocotyl sections was extracted in a pestle and mortar with 20 ml ethanol. Samples were worked up for TLC analysis of isoflavonoids as previously described (Dixon and Bendall 1978a), except that diethyl ether was used in place of petroleum ether for partitioning against the aqueous phase. Chromatograms (Machery-Nagel silica gel G/UV_{254}) were developed in toluene:ethyl formate:formic acid (7:2:1 v/v). Isoflavonoids were eluted from chromatograms in 1 ml ethanol. Phaseollin (R_f 0.60) and kievitone (R_f 0.27) were quantitatively determined from their reported extinction coefficients at λ_{max} of 280 and 293 nm respectively (Bailey and Burden 1973). Kievitone was further characterised by the 21 nm bathochromic shift observed in the UV spectrum following addition of ethanolic $AlCl_3$ (Bailey and Burden 1973). Although traces of phaseollidin, phaseollinisoflavan and coumestrol were consistently observed on chromatograms from elicitor-treated material, the other major compound present was a 5-OH-isoflavone (R_f 0.32; λ_{max} (EtOH) 259, 280 (sh); λ_{max} (EtOH/$AlCl_3$) 270, 280 (sh)). It was eluted from chromatograms as above, and its concentration determined in arbitrary units from its absorption at 259 nm.

Results

The dose-response curve for PAL activity induced in the cultures by varying concentrations of the crude *Colletotrichum* elicitor showed two maxima at around

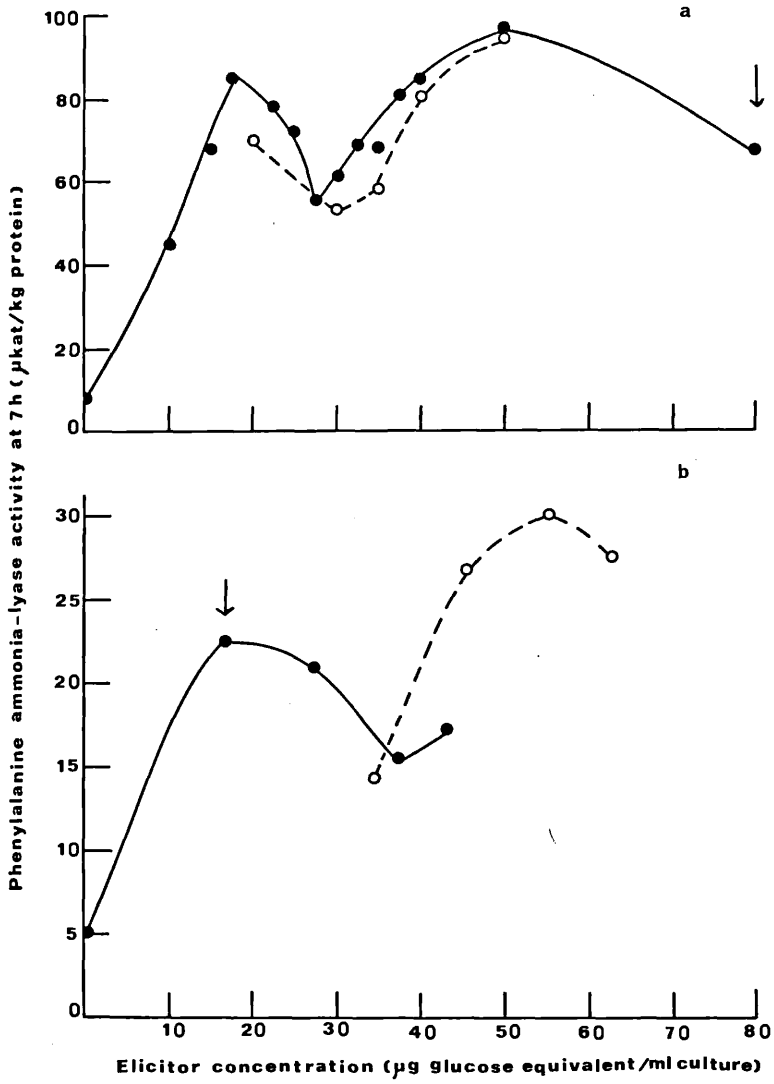

Fig. 1a, b. Dose response curves for the effects of crude *Colletotrichum* elicitor concentration on the induction of PAL in French bean cell suspension cultures. **a** Elicitor was added at zero time at the concentrations shown (●—●), or 80 μg ml^{-1} elicitor (arrow) was added at zero time and the cultures diluted with conditioned medium 2 h later to give the final concentrations shown (o—o). **b** Elicitor was added at zero time at the concentrations shown (●—●), or 17.5 μg ml^{-1} elicitor (arrow) was added at zero time and extra elicitor added 2 h later to give the final concentrations shown (o—o)

17.5 and 50 μg glucose equivalents ml^{-1} (Fig. 1a, filled-in circles). This response has been consistently observed in at least ten independent experiments; in all cases the activity induced by 27.5–40 μg carbohydrate per ml culture was less than that induced by 17.5 and 50 μg ml^{-1}. The absolute activity values of the maxima and minima vary with different elicitor preparations and different culture batches; in the first report of the phenomenon, the activity at 37 μg ml^{-1} was even lower than that obtained in controls minus elicitor (Lawton et al, 1980). Similar dose response curves for PAL induction have been observed with crude elicitor preparations from races γ and δ of *C. lindemuthianum* (D.L. Murphy, unpublished observations).

De novo synthesis of PAL in the cell cultures may be measured within 2 h following addition of *Colletotrichum* elicitor (Dixon and Lamb 1979). However the extent of the induced response is not finally determined by this time; treatment of cultures with elicitor at a concentration of 80 μg ml^{-1} for 2 h, followed by dilution of the cultures with conditioned medium to give various lower final elicitor concentrations resulted in a dose response curve of similar shape to that obtained if the final elicitor concentrations had been present throughout the 7 h induction period (Fig. 1a) The dose-response curve for cultures treated with 17 μg elicitor carbohydrate per ml, followed by addition at 2 h of extra elicitor to give various higher final concentrations (Fig. 1b) likewise in-

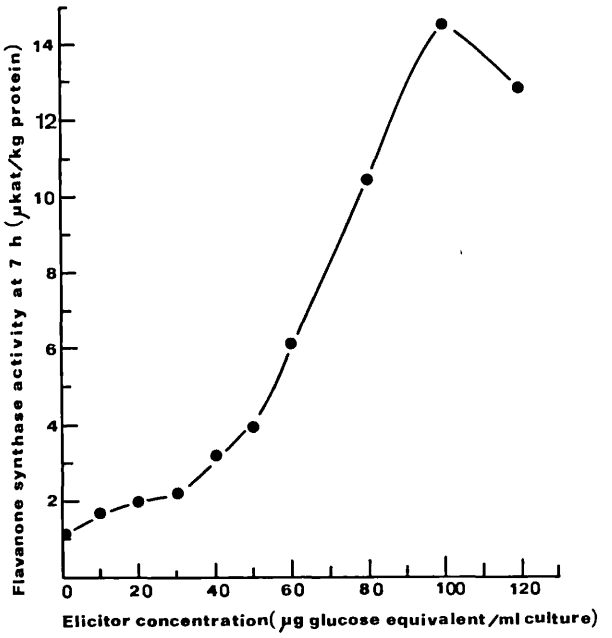

Fig. 2. Dose-response curve for the effects of crude *Colletotrichum* elicitor concentration on the induction of flavanone synthase in French bean cell suspension cultures

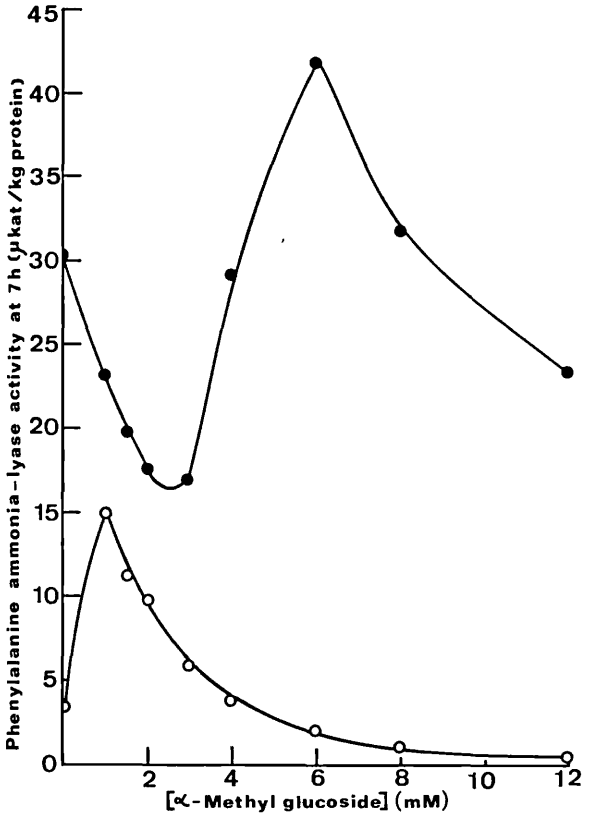

Fig. 3. PAL induction in French bean cell suspension cultures in response to varying concentrations of α-methyl D-glucoside alone (o—o) or in the presence of 20 μg ml^{-1} crude *Colletotrichum* elicitor (●—●)

dicates the reversibility of the elicitor-mediated response. The lower enzyme activities observed in Fig. 1 b are the result of using cultures grown under a lower level of illumination than those used in all other experiments.

The dose-response curve for elicitor induction of flavanone synthase showed only one maximum at around 100 μg carbohydrate per ml culture (Fig. 2). There was little induction of the enzyme over the range of elicitor concentrations which induced the first peak in PAL activity.

In soybean cotyledons, methyl α-D-mannoside inhibits phytoalexin accumulation in response to the elicitor from cell walls of *Phytophthora megasperma* var. sojae (Ayers et al. 1976), whilst in potato tuber discs *Phytophthora infestans* elicitor activity is inhibited by methyl β-D-glucoside and laminaribiose (Marcan et al. 1979). These low molecular weight sugar derivatives may possibly act as "haptens", competing with elicitor for binding sites at the host cell surface (Ayers et al. 1976; Marcan et al. 1979). If the complex elicitor dose-responses observed in the present work are due to the presence in the elicitor preparation of more than one active component, it may be possible, by the use of such compounds, to inhibit selectively only a part of the elicitor dose-response curve. Methyl α- and β-D-glucosides, methyl α- and β-D-galactosides and methyl α-D-mannoside were therefore tested at a range of concentrations for their effects on elicitor-mediated PAL induction in the French bean cultures. Of these derivatives, only methyl α-D-glucoside had significant effects on the PAL response. This compound acted as a weak elicitor of PAL activity when supplied to the cultures in the absence of elicitor (Fig. 3), although its effectiveness declined exponentially with concentrations above 1.0 mM. In contrast, when applied at the same time as crude elicitor (20 μg ml^{-1}), increasing concentrations of methyl α-D-glucoside first inhibited and then stimulated extractable PAL activity.

An alternative approach to investigate the possibility of multicomponent elicitor activity was to fractionate the crude elicitor on Biogel P-150 (Fig. 4). The elution profile for total carbohydrate showed much heterogeneity; the major carbohydrate-containing fraction eluted in the void volume of the column, but a further seven fractions of lower molecular weight were obtained. All fractions were lyophilized prior to further analysis. Fractions A and D contained the most protein, the high 280 nm absorption of fractions G and H being due to phenolic pigments. Each fraction was tested at five concentrations for its ability to induce PAL in the cultures (Fig. 5). Fractions A to H all induced significant levels of the enzyme, but each fraction produced a different dose response

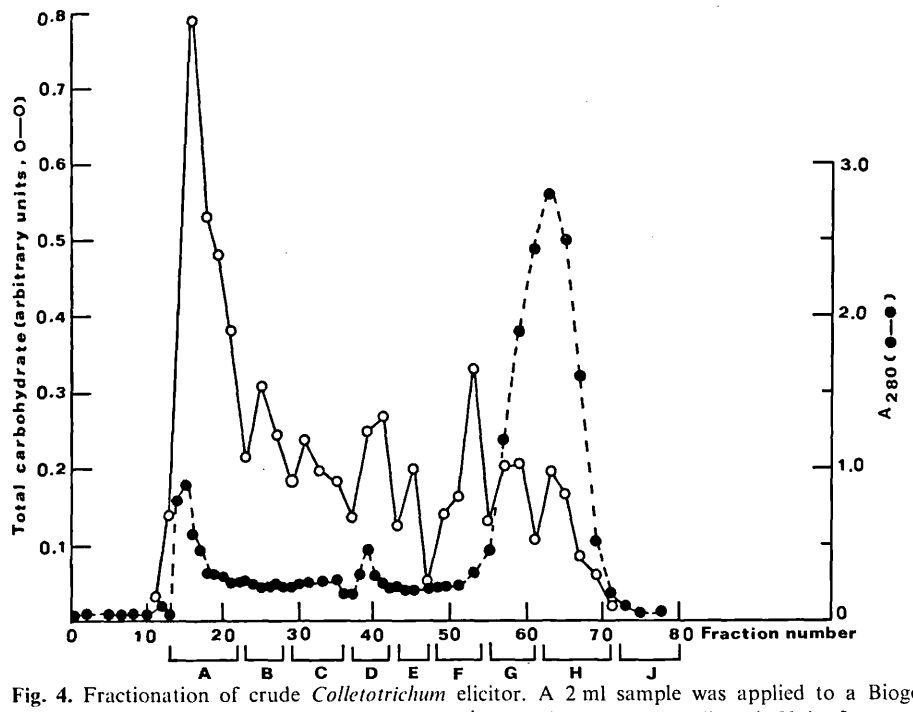

Fig. 4. Fractionation of crude *Colletotrichum* elicitor. A 2 ml sample was applied to a Biogel P-150 column (83·1.5 cm) and eluted with deionized H_2O at a flow rate of 12 ml h^{-1}; 3 ml fraction were collected. Units for total carbohydrate represent the absorbance at 555 nm obtained from a 20 µl aliquot of the fraction following reaction with α-naphthol/sulphuric acid reagent. Fractions corresponding to peaks A to J were pooled as shown, and freeze-dried prior to further analysis

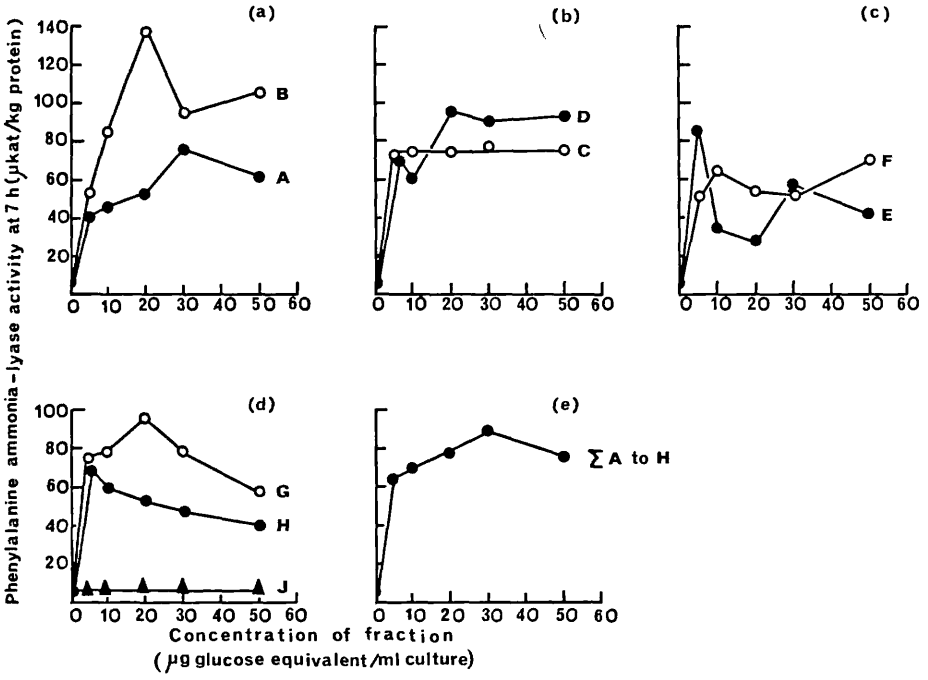

Fig. 5a–e. Dose-response curves for the effects of *Colletotrichum* elicitor fractions A to J as elicitors of PAL activity in French bean cell suspension cultures [graphs (a) to (d)]. Analysis of fractions A to J was performed in a single experiment using cells from the same culture batch. The sum of the curves for the individual fractions, weighted in accordance with the relative proportions of each fraction in the crude elicitor, is shown in graph (e)

curve. Fraction B (20 µg ml^{-1}) gave the highest level of enzyme induction, whereas the response to fraction C was saturated by concentrations above 5µg ml^{-1}. The only fraction to show a double peak in the dose-response was fraction E. The sum of the individual dose-response curves, when weighted for the relative proportions of each fraction in the crude elicitor, was not equivalent to that obtained for the unfractionated preparation (Fig. 5e).

Elicitor fractions A to H significantly differed in

Table 1. Monosaccharide composition of the crude *Colletotrichum* elicitor and the fractions obtained from it by chromatography on Biogel P-150. The figures in brackets after the fraction number are the percentage total carbohydrate content of the fraction with respect to the crude elicitor. The compounds x_1, x_2, x_3 and x_4 were unidentified pentose-containing oligosaccharides, showing low mobilities in the TLC system used (see Materials and Methods) and giving a pink colour with p-aminobenzoic acid reagent. Their presence may have resulted from incomplete hydrolysis of the fractions

		% Composition									
Monosaccharide		Galactose	Glucose	Mannose	Arabinose	Ribose	Rhamnose	x_1	x_2	x_3	x_4
R_G[a]		0.87	1.00	1.09	1.22	1.39	1.57	0.17	0.29	0.35	0.72
Fraction:											
Crude elicitor	(100)	15.5	17.0	22.9	25.8	14.8	2.9	0.36	–	–	0.74
A	(42.0)	9.2	17.6	21.3	23.5	14.1	4.4	0.91	3.10	–	–
B	(10.9)	11.0	15.3	21.3	27.3	12.95	4.2	0.60	0.84	0.84	5.75
C	(13.5)	24.1	14.5	40.4	12.2	2.5	6.2	–	–	–	–
D	(7.2)	23.2	11.9	38.8	9.3	10.3	6.5	–	–	–	–
E	(4.7)	13.0	13.5	17.6	29.3	15.6	3.2	0.87	0.92	0.92	5.40
F	(6.5)	14.0	12.7	21.0	29.3	11.0	4.0	–	–	1.9	6.01
G	(8.1	10.9	24.8	20.9	23.2	13.9	4.2	–	–	–	2.0
H	(5.9)	1.9	9.6	13.5	25.0	43.6	3.5	–	–	–	1.8

[a] TLC mobility relative to glucose

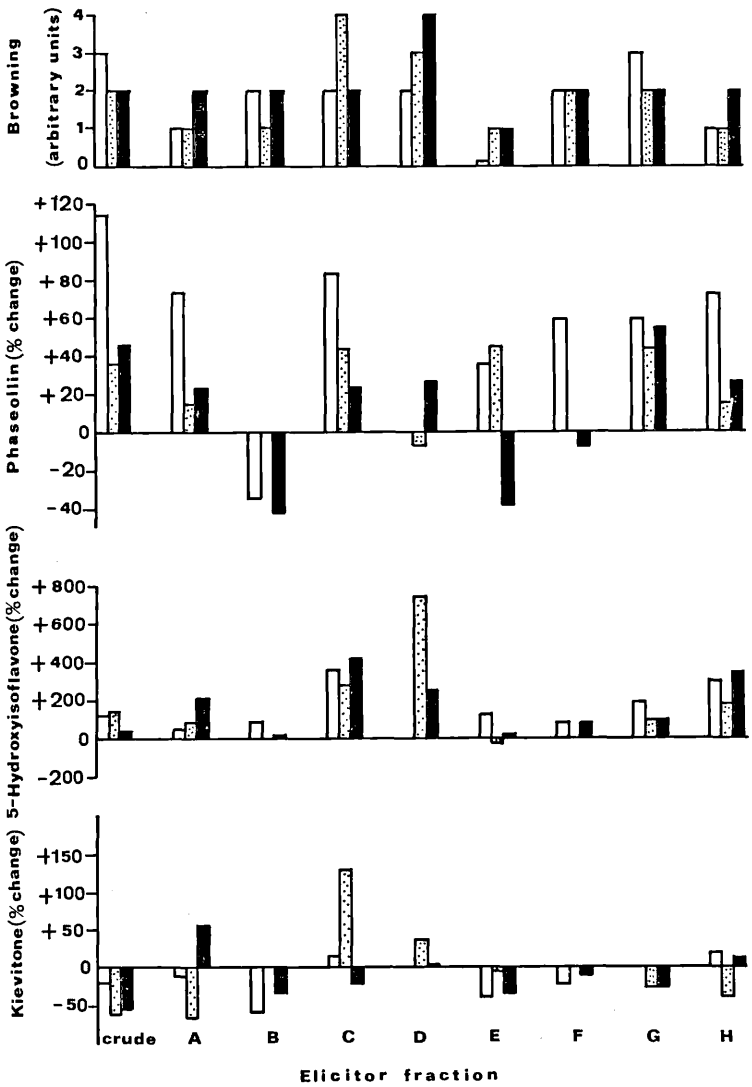

Fig. 6. Response of French bean hypocotyls to crude and fractionated *Colletotrichum* elicitor preparations. Elicitor samples (100 µl) containing 5, 20 or 50 µg glucose equivalents ml^{-1} (open, dotted and filled-in histograms respectively) were applied to the wounded surfaces of four 2 cm-hypocotyl segments, and the hypocotyls incubated in the dark at 25° C for 48 h. Browning was measured on an arbitrary scale of 0–4 (0 = no visible browning; 4 = very dark brown, streaky lesion). Isoflavonoid levels are expressed as the % change compared to control hypocotyls treated with 100 µl sterile distilled H_2O. Control values (per treatment) were: browning, 1.0; phaseollin, 55 µg; 5-hydroxyisoflavone, A_{259} of 0.30; kievitone, 26 µg

monosaccharide composition (Table 1). Fractions A and B appeared similar, with glucose, mannose and arabinose as the major monosaccharides present. Fractions C and D contained mannose as the major monosaccharide, with much lower levels of pentoses. Only fraction G contained glucose as the major monosaccharide. The results are in contrast to a previous report of the carbohydrate composition of the partially purified elicitor from the α race of *Colletotrichum lindemuthianum* (Anderson-Prouty and Albersheim 1975), where only glucose, galactose and mannose were observed following gas chromatography of acetylated elicitor preparations.

The fractionation of the predominantly glucan elicitor from the α race of *C. lindemuthianum* relied upon the bioassay of fractions by measurement of the induction of hypersensitive browning and phytoalexin accumulation in isolated bean hypcotyls and cotyledons (Anderson-Prouty and Albersheim 1975). Three concentrations of each of the present fractions were therefore applied to wounded French bean hypocotyls, and the extent of cellular browning and the levels of three isoflavonoid derivatives (the phytoalexins phaseollin and kievitone and a partially characterised 5-hydroxyisoflavone) were compared to controls treated with sterile distilled H_2O (Fig. 6). There was no constant direct correlation between cellular browning and tissue levels of all three isoflavonoids, but the crude elicitor and fractions A, C and D were the most consistent in inducing positive changes in all four parameters. Fraction B, although inducing slight cellular browning, inhibited phaseollin levels to below those found in wounded-only controls.

Discussion

The dose-response curve for the *Colletotrichum* elicitor-mediated induction of PAL in French bean cell suspension cultures is unusual in that it consists of two maxima separated by a relatively small increment in elicitor concentration. In soybean cell suspension cultures the dose response curves for PAL induction by two partially purified β-1-3-glucan elicitor fractions from *Phytophtora megasperma* var. sojae have single maxima at around 1 and 8 μg hexose equivalents ml^{-1} respectively (Ebel et al. 1976).

There are several possible explanations for the declines in elicitor-mediated responses observed at supra-optimal elicitor concentrations. Firstly, bell-shaped dose response curves of this are typically obtained for responses mediated by binding of lectins to animal cell surfaces (Novogrodsky and Ashwell 1977). If the functional units of fungal elicitors are purely carbohydrate in nature, as has been suggested for the *Colletotrichum lindemuthianum* and *Phytophthora megasperma* elicitors (Anderson 1978; Albersheim and Valent 1978), then the lectin component may be a part of the putative receptor for elicitor binding at the host cell surface. Secondly, β-1-3-glucans assume a helical conformation in solution and self-associate at high concentrations (Preston 1979); dose-response curves may therfore reflect elicitor-elicitor as well as elicitor-cell interactions. Another factor, which has received little attention, is the possible toxicity of elicitors at high concentrations. The dose response curves observed in the present work may result from one or more of the above phenomena, although the lowered response observed at intermediate elicitor concentrations would seem to imply the presence in the crude preparation of either two functionally distinct classes of elicitor or a further factor(s) with an inhibitory effect on elicitor-mediated PAL induction.

The elicitor dose-response curve for flavanone synthase induction closely resembles the shape of the curve for *Colletotrichum* elicitor-mediated induction of phaseollin accumulation in the French bean cultures (Dixon and Lamb 1979), although the flavanone synthase assayed in the present work is not believed to be directly involved in the synthesis of 5-deoxyisoflavonoid derivatives such as phaseollin (Dixon and Bendall 1978b). It is not yet clear whether the same or different elicitor components are responsible for the induction of PAL and flavanone synthase; the association of flavanone synthase induction with those elicitor concentrations which potentiate the second peak of the PAL dose-response curve may be coincidental.

Density labelling studies with ^2H from ^2H_2O have demonstrated that PAL induction in response to crude *Colletotrichum* elicitor involves rapid de novo synthesis of the enzyme, and that at intermediate and supra-optimal elicitor concentrations the rate constant for enzyme synthesis is lower than at the two observed optimum elicitor concentrations (Dixon and Lamb 1979; Lawton et al., 1980). Changes in PAL activity therefore reflect changes in PAL synthesis, and the present results suggest that elicitor induction of enzyme synthesis may be reversible. The dose-response curves obtained following dilution of cultures or addition of extra elicitor 2 h after initial treatment (Figs. 1a and 1b) would not be obtained if the overall rates of PAL synthesis during the 7 h induction period were fully determined during the period up to the appearance of newly synthesised active enzyme. Furthermore, if the elicitor dose-response curves reflect elicitor binding phenomena, elicitor must remain reversibly bound to the cells in an active state throughout the early stages of enzyme induction.

The inability of α-methyl sugar derivatives to inhibit all or part of the elicitor dose-response curve may reflect the need for a more complex saccharide structure to compete successfully with elicitor for host

cell recognition sites. High concentrations (>40 mM) of β-methyl-D-glucoside are required to inhibit browning of potato tuber discs in response to mycelial sonicates of *Phytophthora infestans* (Marcan et al. 1979). Similarly, concentrations of water soluble glucans from compatible races of *P. infestans* in the order of 10–100 mg ml^{-1} are required to suppress the hypersensitive response of potato protoplasts to *P. infestans* hyphal wall components (Doke and Tomiyama 1980). In the present work, α-methyl-D-glucoside concentrations above 4 mM inhibit extractable PAL activity in untreated bean cultures (Fig. 3), although stimulation of PAL levels is observed at α-methyl glucoside concentrations of between 4 and 8 mM in the presence of elicitor (20 μg ml^{-1}). There is clearly some functional interaction between α-methyl glucoside and elicitor, but in the present system this may be more complex than simple competition for elicitor binding sites, as has been proposed to explain the effects of methyl sugar derivatives on elicitor activity in other systems (Ayers et al. 1976; Marcan et al. 1979).

The monosaccharide composition of the crude elicitor from the CMI-isolate of *Colletotrichum lindemuthianum* here reported is compatible with the analysis previously reported for crude preparations from the α, β and δ races of the fungus, although significant differences in monosaccharide composition occur between different races (Anderson 1978). Fractionation of the crude elicitor on the basis of molecular size yielded 8 fractions which, on the basis of monosaccharide composition, could be divided into 4 groups; fractions A, B, E and F contained arabinose and mannose as the major monosaccharides, whereas fractions C and D contained approximately 40% mannose, with galactose as the next most abundant sugar and relatively low levels of pentoses. Fraction G was the fraction richest in glucose, while fraction H showed a preponderance of ribose. This fractionation pattern is different from that observed for the α, β and δ races of the fungus, where the highest molecular weight fraction eluted from a Biogel A-5 m column was predominantly glucan in nature and accounted for the major part of the total elicitor activity of the crude preparations (Anderson 1978). The presence of a relatively high proportion of pentose sugars in the hydrolysed crude elicitor agrees with previously published results (Anderson 1978). This pentose material is not the result of cytoplasmic contamination, as a similar amount could be extracted by reautoclaving the wall residue in fresh H_2O for a further 3 h.

In the present work, all 8 fractions obtained by gel-filtration showed elicitor activity as measured by their ability to induce PAL in the bean suspension cultures. The different dose-response curves for PAL induction exhibited by each fraction, and the fact that the sum of these curves does not show the characteristic double maximum observed in the response to the crude elicitor, suggests that heat-treatment of *Colletotrichum* cell walls releases a variety of polysaccharides or glycoproteins differing in molecular size, composition and biological activity which do not act independently when present in a crude mixture. Fractionation studies alone may not therefore resolve the mechanisms underlying the dose-response curves for enzyme induction.

The complexity of the elicitor-mediated response suggested by the above results is based solely upon studies of PAL induction in cell suspension cultures. As the increase in PAL activity generally precedes the accumulation of isoflavonoid phytoalexins in cell suspension cultures (Ebel et al. 1976; Dixon and Bendall 1978b; Dixon and Lamb 1979), such changes in enzyme levels may be a useful marker for elicitor activity. However, the most important criterion for the assessment of elicitor activity is the induced accumulation of phytoalexins in tissues of the whole plant. Assays of *Colletotrichum* elicitor activity in previous work have relied mainly upon the ability of fractions to elicit cellular necrosis when applied to French bean hypocotyls or cotyledons (Anderson-Prouty and Albersheim 1975; Anderson 1978; Theodorou and Smith 1979). Little data has been presented on the actual tissue concentrations of phytoalexins accumulating in response to elicitor preparations, presumably because this involves lengthy extraction procedures. In addition to PAL induction, all the elicitor fractions obtained by gel-filtration in the present work induced cellular necrosis when applied to the wounded surfaces of excised bean hypotocyls, although they did not all induce increased accumulation of phaseollin. Fraction B suppressed phaseollin levels below those observed in wounded-only controls, in spite of the fact that this fraction induced the highest PAL increases in the suspension cultures. Fraction B was also the least effective in inducing 5-hydroxyisoflavone accumulation, and was one of several fractions (including the crude extract) which appeared to suppress kievitone accumulation. Suppressors of hypersensitivity and phytoalexin induction have now been shown in culture fluids from several plant pathogens (Gnanamanickan and Patil 1977; Oku et al. 1977, Doke and Tomiyama 1980), although there have been no reports to date of their occurrence in the cell walls of the pathogen.

A full explanation of the complex dose-response phenomena associated with elicitor-mediated enzyme induction in the cell suspension cultures must await a more systematic study of the interactions between the various elicitor components. The present studies suggest, hower, that such phenomena may not be directly related to the induction of phytoalexin accumulation in the intact plant. Furthermore, attention

is drawn to the possibility that elicitor fractions may have different biological activities, and it may therefore be unwise to rely on a single parameter for the assessment of elicitor activity.

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Note Added in Proof

Separation of monosaccharides by TLC as described under Materials and Methods but using Schleicher and Schüll F1500 silica gel TLC plates in place of Merck No. 5748 silica gel 69 plates indicated the presence of xylose in the hydrolysates of elicitor preparations. This sugar co-chromatographed with ribose on the Merck TLC plates. Values for ribose in the present paper are therefore to be read as values for ribose plus xylose.

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Differential patterns of phytoalexin accumulation and enzyme induction in wounded and elicitor-treated tissues of *Phaseolus vulgaris*

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Abstract. In wounded cotyledons of *Phaseolus vulgaris* L. the accumulation of the 5-hydroxy isoflavonoids kievitone and 2′-hydroxygenistein precedes the major increases in the levels of the 5-deoxy compounds phaseollin and coumestrol. Increased phytoalexin levels are preceded by transient increases in the extractable activities of L-phenylalanine ammonia-lyase (EC4.3.1.5.), chalcone synthase and chalcone isomerase (EC5.5.1.6.). Accumulation of phytoalexins, above wounded control levels, is observed following treatment of excised cotyledons or hypocotyls with crude or fractionated elicitor preparations heat-released from the cell walls of *Colletotrichum lindemuthianum*. Chalcone synthase levels are also induced in cotyledons, although crude elicitor and all fractions suppress L-phenylalanine ammonia-lyase activity in both tissues. Kievitone is the major phytoalexin induced in cotyledons, whereas in hypocotyls phaseollin predominates. Patterns of phytoalexin accumulation have been studied in response to varying concentrations of the crude and fractionated elicitor; 5-hydroxy isoflavonoid accumulation is highly dependent upon elicitor concentration, the dose-response curves for kievitone accumulation showing maxima at around 1 μg glucose equivalents per cotyledon, minima at 2–3 μg equivalents and increasing induction at higher concentrations. Similar patterns are observed for L-phenylalanine ammonia-lyase and chalcone synthase levels, although the overall extent of these changes is masked by the high wound response. Accumulation of 5-deoxy isoflavonoids above control levels requires high elicitor concentrations; no experimental conditions were found under which phaseollin accumulated to higher levels than kievitone in cotyledons during the first 48 h after elicitation.

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Abbreviations: CHS = chalcone synthase; PAL = L-phenylalanine ammonia-lyase

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Key words: Chalcone synthase – *Colletotrichum* – Elicitor – *Phaseolus* – Phenylalanine ammonia-lyase – Phytoalexin.

Introduction

A variety of different isoflavonoid phytoalexins (host-synthesised antimicrobial compounds) and related metabolites accumulate in tissues of *Phaseolus vulgaris* in response to biotic or abiotic treatments (Bailey and Burden 1973; Smith et al. 1975; Woodward 1979a, b, 1980; Fraile et al. 1980). The recent identification of minor isoflavonoid components in endocarp tissues (Woodward 1979a, b), coupled with the results of labelling experiments in closely related plants (Dewick and Martin 1979a, b; Martin and Dewick 1980) has led to an understanding of the probable pathways involved in the formation of the phytoalexins kievitone and phaseollin from their initial isoflavonoid precursors (Woodward 1979b, 1980). Two separate pathways are believed to exist; a 5-OH pathway leading to kievitone formation from 2′,4,4′,6′-tetrahydroxychalcone, and a 5-deoxy pathway leading to phaseollin from 2′,4,4′-trihydroxychalcone (Dixon and Bendall 1978a, b). The differentiation of the two pathways at the level of the chalcone suggests the importance of the enzyme chalcone synthase in the regulation of isoflavonoid phytoalexin accumulation in *P. vulgaris*. Although much is now known concerning the regulation of the common phenylpropanoid pathway leading to chalcone formation (Dixon and Bendall 1978b; Dixon and Lamb 1979; Lawton et al. 1980) the only later enzymes of isoflavonoid phytoalexin biosynthesis so far detected are the 5-OH chalcone synthases from soybean and *P. vulgaris* (Zähringer et al. 1978; Dixon and Bendall 1978b) and the dimethylallylpyrophosphate: trihydroxypterocarpan

dimethylallyl transferase (5-deoxy pathway) from soybean (Zähringer et al. 1979). No evidence has yet been presented for the existence of a 5-deoxy chalcone synthase.

Various fractions obtained by gel filtration of an elicitor preparation, heat-released from cell walls of *Colletotrichum lindemuthianum*, induced L-phenylalanine ammonia-lyase (PAL EC 4.3.1.5) activity in *P. vulgaris* cell suspension cultures and phytoalexin accumulation in hypocotyl segments (Dixon et al. 1981); the induction of PAL was highly dependent upon elicitor concentration. We have now further investigated the accumulation of phytoalexins and changes in enzyme levels in *P. vulgaris* and conclude that the 5-OH and 5-deoxy pathways may be controlled independently. In addition, phytoalexin accumulation and enzyme induction in cotyledons show a similar dependence upon elicitor concentration to that observed for induction of PAL in the cell cultures.

Materials and methods

Authentic samples of 2'-hydroxygenistein and dalbergioidin were gifts from Dr. S. Antus, Budapest. 2',4,4'-Trihydroxychalcone was synthesised by alkaline condensation of resacetophenone with p-hydroxybenzaldehyde (Geissman and Clinton 1946); the same method was used for the synthesis of 2',4,4'-trimethoxychalcone from the dimethoxy acetophenone and p-methoxybenzaldehyde. Chalcones were purified by re-crystallization from aqueous ethanol. All other reagents were commercial "Analar" grade.

The maintenance and growth of *Colletotrichum lindemuthianum* (races β and γ), the preparation of crude wall-released elicitor and the analysis of monosaccharide composition of both crude and fractionated elicitor preparations were as previously described (Dixon et al. 1981). Total carbohydrate was determined by the α-naphthol/sulphuric acid method (Fuller and Northcote 1956).

Seeds of dwarf French bean cv. 'The Prince' were germinated in moist vermiculite for 7 d in the dark at 25° C. For experiments utilising aseptically grown material, seeds were first surface-sterilised with sodium hypochlorite solution (1–1.4% available Cl_2) for 30 min, soaked overnight in sterile tap H_2O, and re-sterilised with hypochlorite. They were then aseptically transferred to beakers of moist, sterile vermiculite, where they were allowed to germinate as above. Elicitor solutions, or sterile distilled H_2O for wounded controls, were applied as 50 µl droplets to surfaces of cotyledons or hypocotyls that had been cut by removal of the top 1 mm of cells with a sterile scalpel. At least 5 cotyledons, or 2 cm hypocotyl segments, were used per treatment; they were incubated in petri dishes on moist filter paper at 25° C in the dark. Samples for enzyme assay were frozen in liquid N_2 and stored at −70° C until required. Samples for phytoalexin determination were harvested by transfer to stoppered glass tubes containing 20 ml EtOH.

Extraction and assay of PAL were as described elsewhere (Dixon et al. 1980). Assay of chalcone synthase was essentially as described previously (Dixon et al. 1981) except that extracts prior to assay were stirred with 0.5 g Dowex AG1-X8 (equilibrated in 100 mM KH_2PO_4, pH 8.0) per g fresh weight of extracted material. These extracts were also used for determination of chalcone isomerase activity, with 2',4,4'-trihydroxychalcone as substrate, using a previously described method (Dixon and Bendall 1978b).

Isoflavonoid phytoalexins were extracted and quantitated essentially as described earlier (Dixon et al. 1981) but with the following modifications. Firstly, 20 µl of a 10 mM solution of 2',4,4'-trimethoxychalcone in EtOH was added to all samples at the initial stage of ethanol extraction of the plant material, and final values for isoflavonoid levels were corrected for the % recovery of this internal standard; in a preliminary experiment, the % recoveries of the chalcone, phaseollin and kievitone were 56.6, 54.8 and 55.1% respectively. Secondly, isoflavonoids were further purified on silica gel G thin-layer chromatography plates developed in either toluene: chloroform:acetone (40:25:35, by vol.) or chloroform:ethanol (100:5 or 100:7, v/v) prior to spectroscopic determination. They were quantitated on the basis of published extinction coefficients (Bailey and Burden 1973; Biggs 1975; Farkas et al. 1971). The control values for isoflavonoid levels in wounded cotyledons varied between cotyledon batches. Within a single batch the reproducibility was within ±7%, the same variation also being seen for elicited samples. In each experiment reported, all points were derived from material from the same batch of seedlings.

Results

Several isoflavonoid derivatives in addition to phaseollin, coumestrol and kievitone were detected on thin layer chromatograms (silica gel G; $CHCl_3$: EtOH 100:3) of extracts from wounded or wounded

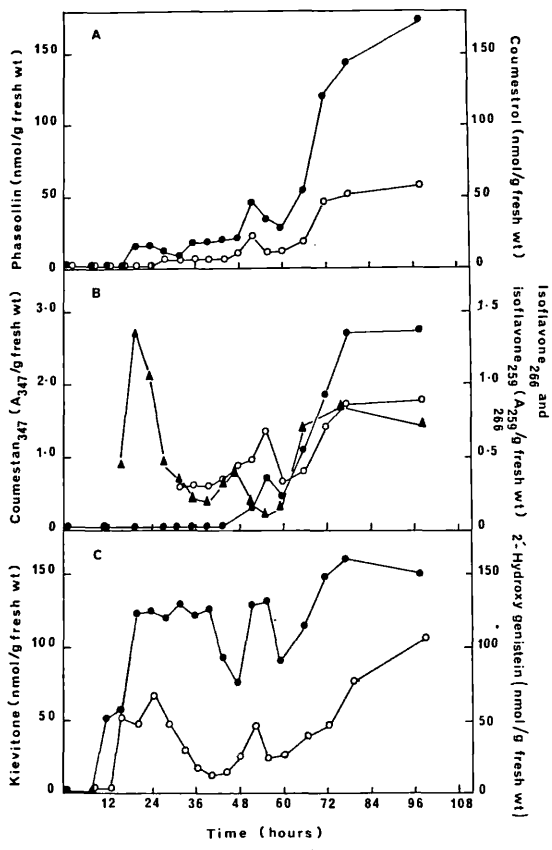

Fig. 1A–C. Accumulation of isoflavonoids in wounded cotyledons of *Phaseolus vulgaris*. **A** phaseollin (●—●), coumestrol (○—○); **B** coumestan λ_{max} 347 nm (●—●), isoflavone λ_{max} 266 nm (○—○), isoflavone λ_{max} 259 nm (▲—▲); **C** kievitone (●—●), 2'-hydroxygenistein (○—○)

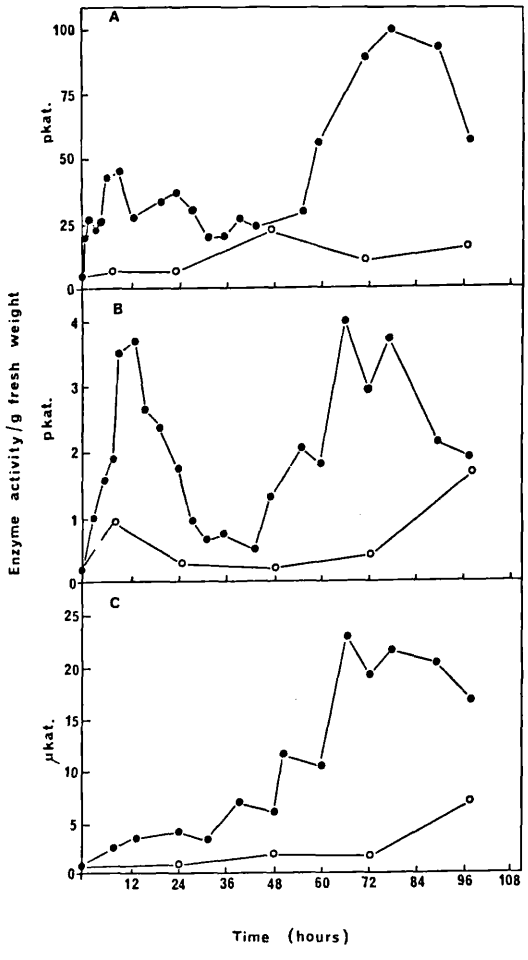

Fig. 2A–C. Changes in enzyme levels in wounded cotyledons of *Phaseolus vulgaris* (●—●) and non-wounded controls (○—○). **A** L-phenylalanine ammonia-lyase; **B** chalcone synthase; **C** chalcone isomerase

plus elicitor-treated French bean cotyledons. These included an uncharacterised coumestan (R_f 0.3, λ_{max} 347 nm), four isoflavones (R_f 0.18, λ_{max} 262 nm; R_f 0.21, λ_{max} 259 nm; R_f 0.37, λ_{max} 270 nm; R_f 0.39, λ_{max} 266 nm) and an isoflavanone (R_f 0.11, λ_{max} 288 nm). The isoflavone at R_f 0.18 was identified as 2′-hydroxygenistein (2′,4′,5,7-tetrahydroxyisoflavone; Biggs 1975) and the isoflavanone at R_f 0.11 as dalbergioidin (2′,4′,5,7-tetrahydroxyisoflavanone; Farkas et al. 1971) by comparison of chromatographic and spectroscopic properties with authentic samples. The isoflavone at R_f 0.21 was shown to be a 5-OH isoflavone by the 11 nm bathochromic shift observed in the U.V. spectrum following addition of ethanolic $AlCl_3$. No $AlCl_3$ shift was recorded for the isoflavone at R_f 0.39. Compounds with chromatographic or spectroscopic properties corresponding to phaseollidin and phaseolinisoflavan (Bailey and Burden 1973), licoisoflavone A (phaseoluteone), 5-deoxykievitone and 2,3-dehydrokievitone (Woodward 1979a, 1979b) were not detected.

A time course study of the accumulation of isoflavonoids in wounded cotyledons (Fig. 1) indicated that 5-OH substituted isoflavonoids (kievitone, 2′-hydroxygenistein and the isoflavone, λ_{max} 259 nm) appeared earlier than the 5-deoxy derivatives; near maximum levels of kievitone were attained by about 20 h after wounding, whereas phaseollin levels did not show their greatest increase until after 60 h. Major rates of increase of the 5-OH compounds occurred from 8–20 h and from 60 h onwards, this latter increase corresponding to the increase in the 5-deoxy compounds. Identical changes in phytoalexin levels were seen regardless of whether rigorous maintenance of

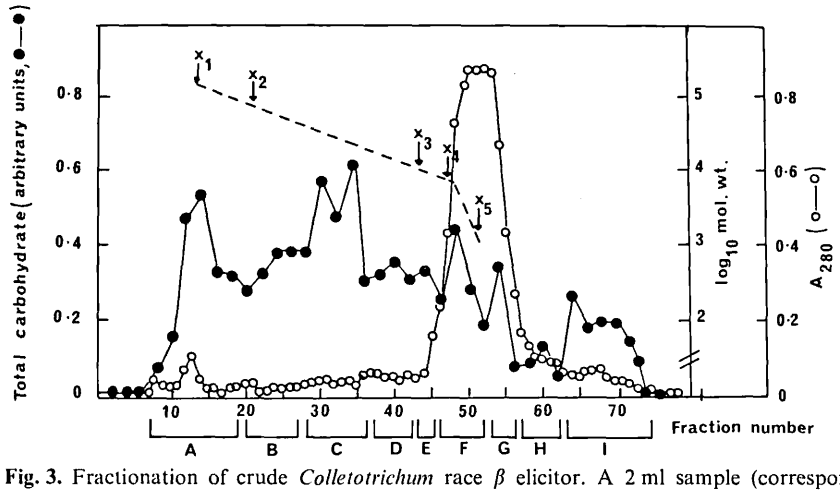

Fig. 3. Fractionation of crude *Colletotrichum* race β elicitor. A 2 ml sample (corresponding to the material released from 1.25 g cell walls) was applied to a Biogel P-150 column (83 × 1.5 cm) and eluted with deionized H_2O at a flow rate of 15 ml h^{-1}. 3.5 ml fractions were collected. The column was then calibrated with a range of molecular weight standards: X_1 = void volume (blue dextran exclusion volume); X_2 = bovine serum albumen, MW 66,500; X_3 = cytochrome C, MW 13,500; X_4 = insulin, MW 5,800 and X_5 = bromophenol blue, MW 670. Units for total carbohydrate represent the absorbance at 555 nm obtained from a 16 μl aliquot of the fraction following reaction with α-naphthol/sulphuric acid reagent. Fractions corresponding to peaks A–H were pooled as shown, and freeze-dried prior to further analysis

Table 1. Monosaccharide compositions of crude elicitor preparations from β and γ races of *Colletotrichum lindemuthianum* and fractions obtained from the crude β preparation by chromatography on Biogel P-150. Compounds X_1 and X_2 were unidentified pentose-containing oligosaccharides, showing low thin-layer chromatographic mobility and giving a pink colour with p-aminobenzoic acid reagent. Their presence probably resulted from incomplete hydrolysis of the fractions. Compound P was an unidentified pentose monosaccharide (possibly ribulose or xylulose)

	% Composition								
Monosaccharide	Galactose	Glucose	Mannose	Arabinose	Xylose/Ribose	Rhamnose	P	X_1	X_2
R_G[a]	0.87	1.00	1.09	1.22	1.39	1.57	1.42	0.17	0.29
Fraction:									
Crude β-race elicitor	11.0	18.0	22.5	26.0	15.5	1.5	3	1.5	1.25
A	13.2	21.3	23.4	29.5	10.1	3.1	–	–	–
B	12.3	15.9	27.5	29.7	14.5	–	–	–	1.4
C	13.9	15.6	25.8	17.7	22.6	4.3	–	–	–
D	19.6	18.5	39.1	15.5	7.2	–	–	–	–
E	13.0	20.7	29.3	23.3	13.8	–	–	–	–
F	6.0	32.8	–	29.8	20.9	–	10.4	–	–
G	–	12.8	–	58.5	11.2	1.6	11.2	4.8	–
H	–	31.7	–	43.9	19.5	–	2.4	–	–
Crude γ-race elicitor	30.6	19.0	18.2	24.0	8.3	–	–	–	–

[a] TLC mobility relative to glucose

Table 2. Phytoalexin and enzyme levels in etiolated cotyledons of *P. vulgaris* after treatment with crude β-race elicitor or elicitor fractions A–H. All treatments were applied at a concentration of 20 μg glucose equivalents ml^{-1} (=1.0 μg equivalents per cotyledon)

	Phytoalexin levels relative to control[a] ($nmol \cdot g^{-1}$ fresh wt.)				Enzyme levels relative to control[a] ($pkat \cdot g^{-1}$ fresh wt.)			
	Kievitone		Phaseollin		PAL		CHS	
Time	24 h (30)[b]	72 h (52)	24 h (6)	72 h (35)	8 h (100)	24 h (110)	8 h (1.23)	24 h (1.50)
Treatment:								
Crude elicitor	+ 6	+ 73	+4	+ 60	− 5	−65	+0.78	+0.55
A	− 2	+ 5	0	− 5	−55	−60	+0.61	+0.25
B	− 3	+ 83	+2	+107	−45	− 7	+0.87	+1.62
C	+ 6	+ 67	+1	+ 52	−14	+94	+0.35	+0.36
D	+ 2	+111	0	+ 91	−29	0	+0.54	+1.48
E	+ 8	+ 38	+1	+ 33	+13	+44	+0.26	−0.18
F	− 1	− 20	+1	+ 7	−55	−18	−0.11	−0.50
G	−11	+ 2	0	+ 17	−49	−35	+0.07	−0.35
H	−15	+ 2	+1	+ 11	−63	−28	−0.31	0

[a] Values at zero time (per g fresh wt.) were: phytoalexins, not detected; PAL, 10 pkat; CHS, 0.10 pkat
[b] Values in brackets are levels in wounded-only controls at the times shown

aseptic conditions was observed. No phytoalexin accumulation occurred in unwounded control cotyledons during the first 48 h; by 96 h, levels of kievitone and phaseollin had increased to approximately 20 and 30% of the wounded levels respectively.

The major changes in isoflavonoid levels were preceded by transient increases in the biosynthetic enzymes PAL, CHS and chalcone isomerase (Fig. 2); the first two enzymes, in particular the synthase, showed two distinct peaks of activity with respect to time.

The above results strongly suggest that the 5-OH and 5-deoxy pathways of isoflavonoid phytoalexin accumulation may be under separate control. It was therefore important to know whether differential accumulation patterns were solely time dependent, or whether different elicitor preparations, or different concentrations of the same elicitor preparation (cf. Dixon et al. 1981) could stimulate one pathway in the absence of the other. In order to investigate these possibilities, a crude elicitor preparation from *C. lindemuthianum* race β was firstly fractionated according to molecular size on a column of Biogel P-150 (Fig. 3). The various fractions obtained showed distinct differ-

Table 3. Phytoalexin and enzyme levels in 2 cm long etiolated hypocotyl segments of *P. vulgaris* after treatment with crude β-race elicitor or elicitor fractions A–H. All treatments were applied at a concentration of 20 μg glucose equivalents ml^{-1} (= 1.0 μg equivalents per hypocotyl segment)

	Phytoalexin levels relative to control[a] ($nmol \cdot g^{-1}$ fresh wt.)				PAL activity relative to control ($pkat \cdot g^{-1}$ fresh wt.)	
	Kievitone		Phaseollin			
Time	24 h (4)[b]	48 h (8)	24 h (24)	48 h (21)	8 h (185)	24 h (160)
Treatment:						
Crude elicitor	+ 7	+10	− 4	+38	− 85	−126
A	+ 6	+12	+12	+42	−110	− 75
B	0	+15	− 7	+38	−110	− 85
C	+ 3	+ 6	0	+38	− 77	− 79
D	+ 8	+ 5	+21	+32	− 91	− 70
E	+ 7	+13	+15	+46	− 93	− 62
F	+15	+10	+18	+20	−101	− 75
G	+12	+18	+19	+39	−122	− 80
H	+ 9	+ 7	− 5	+13	− 60	− 81

[a] Values at zero time (per g fresh wt.) were: phytoalexins, not detected; PAL, 4 pkat
[b] Values in brackets are levels in wounded-only controls at the times shown

ences in monosaccharide composition, and the composition of the crude β-race elicitor was different from that of a crude preparation from the γ-race (Table 1). Aliquots (50 μl, containing 1 μg glucose equivalents) of crude or fractionated elicitor were then applied to cotyledons or hypocotyl segments and both phytoalexin and enzyme levels measured at various times. In cotyledons (Table 2), kievitone was the major phytoalexin accumulating in wounded-only tissues, only fractions B, C, D and E gave significant phytoalexin induction above wounded control levels, and no treatment induced phaseollin at the earlier time (24 h). With two exceptions (fractions C and E at 24 h) all treatments resulted in suppression of PAL activity below control levels, whereas CHS was significantly induced by the crude elicitor and fractions A–D. In contrast to these findings, phaseollin was the major phytoalexin in wounded hypocotyl tissues (Table 3) and all fractions elicited phytoalexin induction above control levels, although PAL was again suppressed by all treatments.

In French bean cell suspension cultures, PAL induction by crude elicitor is highly dependent upon elicitor concentration (Lawton et al. 1980; Dixon et al. 1981), showing maximum enzyme induction at concentrations of around 20 and 60 μg glucose equivalents ml^{-1}, with a much diminished response at intermediate concentrations. Furthermore, different mechanisms of PAL accumulation operate at different elicitor concentrations (Lawton et al. 1980). In view of these findings, the response of phytoalexin accumulation to varying elicitor concentrations was investi-

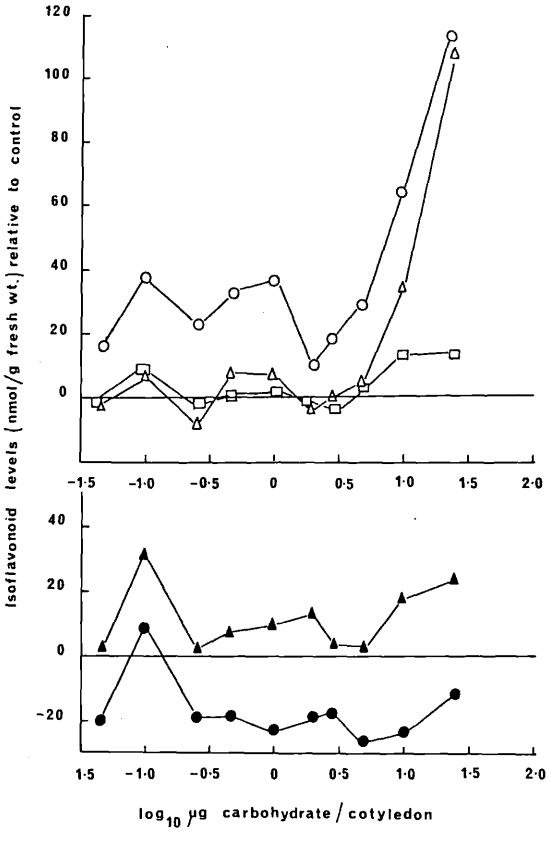

Fig. 4. Dose response curves for the effects of crude β-race elicitor on isoflavonoid levels at 48 h in wounded French bean cotyledons. Symbols and wounded-only control values (nmol g^{-1} fresh wt) are: o—o, kievitone (53); Δ—Δ, 2′-hydroxygenistein (30); □—□, dalbergioidin (31), ●—●, phaseollin (32); ▲—▲, coumestrol (7)

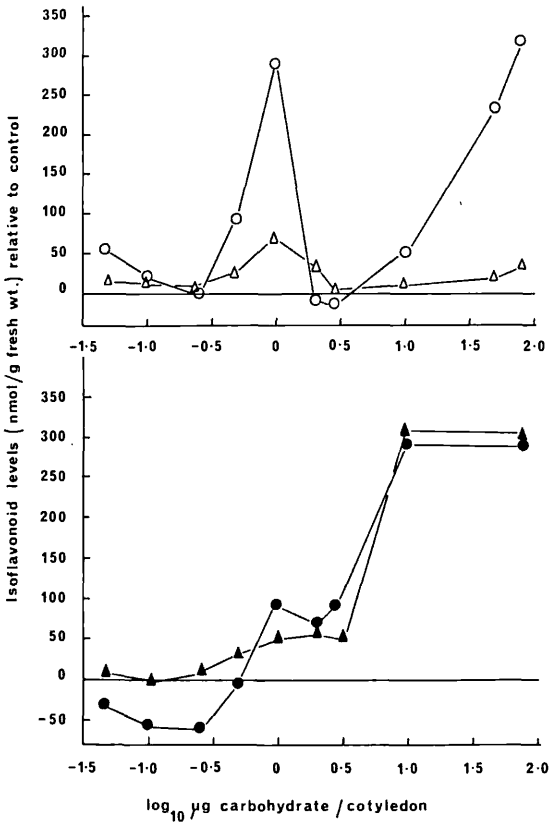

Fig. 5. Dose response curves for the effects of crude β-race elicitor on isoflavonoid levels at 72 h in wounded French bean cotyledons. Symbols and wounded-only control values (nmol g^{-1} fresh wt) are: o—o, kievitone (155); Δ—Δ, 2′-hydroxygenistein (17); ●—●, phaseollin (109); ▲—▲, coumestrol (60)

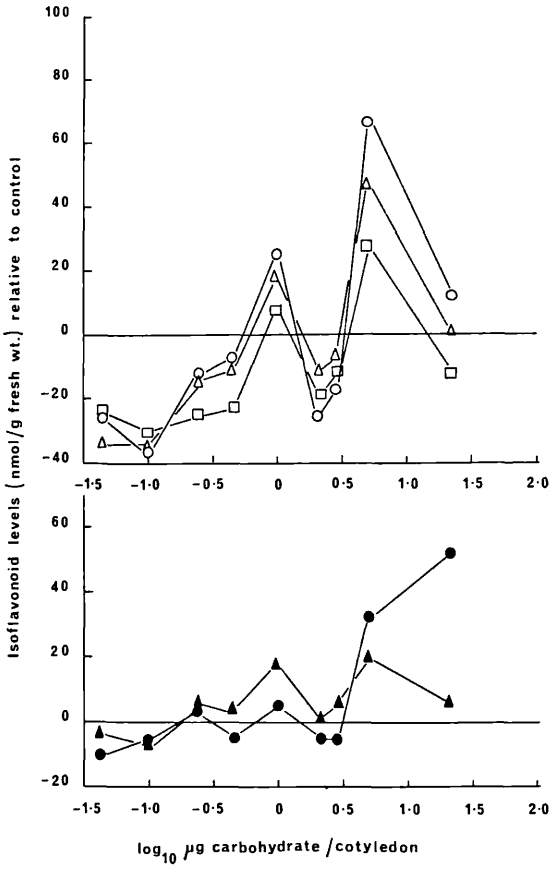

Fig. 6. Dose response curves for the effects of β-race elicitor fraction B on isoflavonoid levels at 48 h in wounded French bean cotyledons. Symbols and wounded-only control values (nmol g^{-1} fresh wt) are: o—o, kievitone (78); Δ—Δ, 2′-hydroxygenistein (43); □—□, dalbergioidin (46); ●—●, phaseollin (29); ▲—▲, coumestrol (12)

gated. At 48 h, the crude β-race elicitor suppressed phaseollin levels below the control value at nearly all concentrations (Fig. 4). Kevitone levels were always stimulated, especially at the highest concentrations tested, and 2′-hydroxygenistein levels followed a similar pattern. At 72 h (Fig. 5), the dose-response curve for kievitone accumulation showed a maximum at an elicitor concentration of 20 µg glucose equivalents ml^{-1} (1 µg equivalent per cotyledon), no induction at concentrations of 40 and 60 µg equivalents ml^{-1}, and increasing induction with higher concentrations. A small maximum at 20 µg equivalent ml^{-1} elicitor was also observed for 2′-hydroxygenistein. In contrast, maximum phaseollin and coumestrol accumulation only occurred at elicitor concentrations of 100 µg equivalents ml^{-1} and above. The same pattern as observed for kievitone accumulation in Fig. 5 was also seen when varying concentrations of a crude elicitor preparation from the γ-race of *C. lindemuthianum* was used (data not shown) although levels were lower than in response to the β-race elicitor and no induction of phaseollin above control values was observed.

The dose-response curve for kievitone induction at 48 h by fraction B of the β-race elicitor (Fig. 6) again showed a peak at 20 µg glucose equivalents ml^{-1}, followed by a sharp decline to below the control value at 40 and 60 µg equivalent ml^{-1}. 2′-Hydroxygenistein and dalbergioidin followed the same pattern, while phaseollin was only induced, at a low level, at concentrations of 100 µg equivalents ml^{-1} and above. Nearly identical dose-response curves were obtained for elicitor fraction D (data not shown).

L-phenylalanine ammonia-lyase and CHS induction by varying concentrations of elicitor fraction B (Fig. 7) showed similar qualitative patterns to the phytoalexin responses, although wounded control values were generally higher than those in the presence of elicitor.

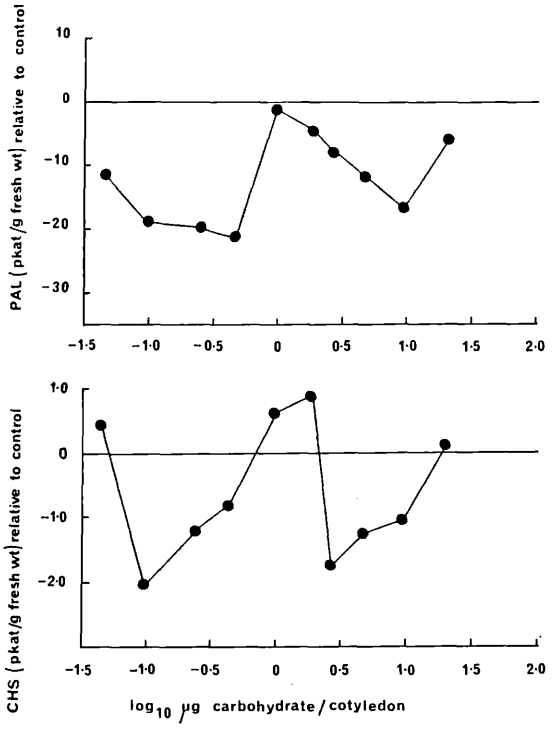

Fig. 7. Dose response curves for the effects of β-race elicitor fraction B on PAL and CHS levels at 24 h in wounded French bean cotyledons. Control values (pkat g^{-1} fresh wt) were PAL, 33.7 and CHS, 4.56

Discussion

The observed accumulation of phytoalexins in non-elicited, wounded cotyledons of *P. vulgaris* is in contrast to earlier reports (Anderson-Prouty and Albersheim 1975; Theodorou and Smith 1979), where control levels were very low in the absence of fungal preparations. Although it is possible that microorganisms already present on the cotyledon surface may in part be responsible for inducing phytoalexin accumulation, aseptically treated cotyledons which accumulated the full range of isoflavonoids did not appear to be contaminated when plated on semi-solid medium rich in sucrose. Differences in earlier workers' experiments include the bean variety used, the use of antibiotics, different times of sampling and the harvesting of only necrotic tissue (Anderson-Prouty and Albersheim 1975; Theodorou and Smith 1979). Any of these fractors may account for the above discrepancy.

The present results indicate a clear temporal differentiation between the accumulation patterns of the 5-OH and 5-deoxy isoflavonoid classes (Fig. 1). The more rapid initial decline in 2′-hydroxygenistein and isoflavone 259 levels than observed for kievitone is consistent with the intermediacy of 5-hydroxyisoflavones in the biosynthesis of kievitone (Woodward 1979a). The maintenance of a constant level of kievitone from 20–40 h, followed by a decline, may indicate turnover of the phytoalexin; in this respect, the half-life of the phytoalexin glyceollin in $HgCl_2$-treated cotyledons (Moesta and Grisebach 1980) has been calculated to be around 89 h, a value too long to account for the proposed role of turnover in the regulation of phytoalexin levels in this system (Yoshikawa et al. 1979). No information is yet available on the half-lifes of endogenous phytoalexins in *P. vulgaris*..

The changes in enzyme levels (Fig. 2) exactly preceded the changes in phytoalexin accumulation; the first peaks in PAL and CHS levels occurred approximately 10 h before the early maximum in 5-OH isoflavonoid levels was attained, with the second main increase in both enzymes and phytoalexins starting at around 60 h. The role of chalcone isomerase in the induced response is not clear; its appearance followed the same pattern as the 5-deoxy isoflavonoids, although K_m values for the *P. vulgaris* enzyme for 6′-OH and 6′-deoxy chalcones (corresponding to 5-substituted flavonoids/isoflavonoids) are very similar (Dixon et al. unpublished results) and the function of the enzyme in vivo appears to be associated with the flavonoid (Heller and Hahlbrock 1980) rather than the isoflavonoid pathway.

The monosaccharide compositions of the crude β-race elicitor and the fractions obtained from it by gel-filtration were similar to those reported previously for the Commonwealth Mycological Institute isolate IMI 112166 (Dixon et al. 1981), whereas the γ-race elicitor preparation had a different composition, containing high levels of galactose and no rhamnose. Although gel-filtration yielded fractions with significant differences in monosaccharide composition, it is nevertheless possible that a single class of polysaccharide showing extensive heterogeneity of molecular weight could be present in all fractions and be mainly responsible for elicitor activity; the hydrolysates almost certainly contain monosaccharides originating from non-elicitor wall components. The apparent lack of cytoplasmic contamination of these preparations has been shown elsehwere (Dixon et al. 1981).

The predominance of kievitone in cotyledons at 24 h and 48 h was observed irrespective of elicitor fraction or concentration, and this suggests that the timing of phaseollin accumulation is not affected by elicitor treatment; at later times when phaseollin is accumulating, the ratio of phaseollin to kievitone may be determined by elicitor concentration (Fig. 5).

In both cotyledons and hypocotyls, induction of phytoalexins by 20 µg equivalent ml^{-1} elicitor frac-

tions was associated with increased extractable CHS activities, although PAL was generally suppressed below the wounded control level. This appears to contradict the results obtained from *P. vulgaris* cell suspension cultures (Dixon and Lamb 1979; Lawton et al. 1980), where elicitor-mediated increases in phaseollin levels are preceded by rapid, transient increases in PAL activity. In elicitor-treated cell suspensions there is no wound-response; in cut cotyledons extensive PAL induction will occur in the cells in the vicinity of the wound, and this will tend to mask elicitor-mediated changes. In the present work, CHS induction also appears to be wound-stimulated, although the isomerase does not show a similar rapid, early increase. As enzyme levels in the wounded cotyledons are easily adequate to support phytoalexin production, the phenomena observed in the cotyledons do not weaken the argument that PAL and CHS induction are important regulatory steps in the induction of isoflavonoid phytoalexins in cell cultures or natural plant-pathogen interactions.

The above considerations do not, however, fully explain the observed suppression of both PAL and CHS at certain elicitor concentrations. In hypocotyls of cowpea (*Vigna sinensis*), kievitone accumulation in response to $CuCl_2$ only occurs in the top treated half, whereas PAL induction occurs throughout the hypocotyl (Munn and Drysdale 1975). Similarly, in damaged bean hypocotyls, phaseollin is believed to be synthesised in healthy tissue in close proximity to the site of damage, but to accumulate in the necrotic cells (Hargreaves and Bailey 1978). There may therefore be a spatial separation of the wound- and elicitor-mediated enzyme responses, which might suggest that the enzyme changes responsible for the shape of the dose-response curves observed in Fig. 7 are taking place in different cells from those responsible for the high wound-response levels. Release of an endogenous elicitor from wounded cells (Hargreaves and Selby 1978; Hargreaves and Bailey 1978) which will then act on healthy cells, may be affected by elicitor treatment; alternatively, at certain concentrations, competition may occur between endogenous and exogenous elicitors for a site of action. Such phenomena might explain the suppression of enzyme activities observed in the present work. An endogenous elicitor of PAL activity has recently been observed in *P. vulgaris* cell suspension cultures following treatments that cause cell damage (Dixon et al., unpublished observations).

Although dose-response curves showed distinct differences in the patterns of accumulation of 5-OH and 5-deoxy isoflavonoids, all components of a particular class responded in a similar manner. With respect to the 5-OH class of isoflavonoids, where the proposed pathway is believed to be genistein→2′-hydroxygenistein→dalbergioidin→kievitone (Woodward 1979a), this implies a high degree of coordination in the regulation of the individual steps of the pathway by elicitor. The negative slopes in the elicitor dose-response curves for PAL induction in cell suspension cultures in the narrow concentration range of 20–40 μg glucose equivalents ml^{-1} (Lawton et al. 1980; Dixon et al. 1981) are also seen in the dose-response curves for elicitor-mediated 5-OH isoflavonoid induction in the present work. This similarity in the behaviour of intact tissues and cultured cells provides support for the value of cell culture studies in investigations of the regulation of phytoalexin accumulation at the molecular level, and further underlines the subtle control mechanisms which may be involved.

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PURIFICATION AND PROPERTIES OF CHALCONE ISOMERASE FROM CELL SUSPENSION CULTURES OF *PHASEOLUS VULGARIS*

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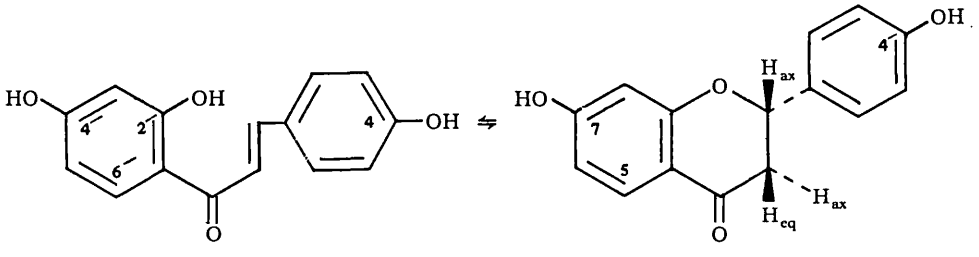
Key words: Chalcone isomerase; Peroxidase; Phytoalexin; Suspension culture; (Phaseolus vulgaris)

Chalcone isomerase (EC 5.5.1.6) from cell suspension cultures of *Phaseolus vulgaris* has been purified about 400-fold. The molecular weight, as estimated by gel-filtration and SDS-polyacrylamide gel electrophoresis, is approx. 28000. No isoenzymic forms are observed. The enzyme, which appears to require no cofactors, catalyses the isomerisation of both 6′-hydroxy and 6′-deoxy chalcones to the corresponding flavanones. Likewise, a range of both 5-hydroxy and 5-deoxy flavonoids and isoflavonoids act as competitive inhibitors. The most potent inhibitors include the naturally occurring antimicrobial compounds kievitone (K_i 9.2 μM) and coumestrol (K_i 2.5 μM). The kinetics of the isomerisation of 2′,4,4′-trihydroxychalcone to the flavanone liquiritigenin have been investigated at a range of pH values. The pH optimum was around 8.0 and K_m changed with pH in a manner consistent with control by groups which ionise with pK_a values of 7.05 and 8.7, respectively.At pH 8.0, the energy of activation was 17.56 kJ/mol in the range 25–40°C. The role of the enzyme in the induced accumulation of flavonoid/isoflavonoid derivatives in the French bean is discussed.

Introduction

Chalcone isomerase was first isolated from seeds of *Glycine max* [1], and was shown to catalyse the cyclysation of 2′,4,4′-trihydroxychalcone (I) to (−)4′,7-dihydroxyflavanone (II). The (−)(2*S*) configuration of the reaction product was demonstrated by NMR studies of flavanone production catalysed by either one of two isomerase isoenzymes partially purified from seedlings of *Phaseolus aureus* [2]. In addition to *P. aureus*, both parsley (*Petroselinum hortense*) and garbanzo bean (*Cicer arietinum*) isomerases have been reported to exist in a number of isoenzymic forms [3]. A more detailed study of the soybean isomerase indicated the presence of only one form of the enzyme, with a molecular weight of about 16000 [4]. The enzyme catalysed the isomerisation of both 6′-hydroxy and 6′-deoxy chalcones to their corresponding flavanones, and was competitively in-



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hibited by a wide range of flavonoid derivatives.

The exact role of chalcone isomerase in flavonoid metabolism has for some time been in doubt. However, it has recently been confirmed that the first C_{15} compound formed from malonyl coenzyme A and *p*-coumaroyl coenzyme A is the chalcone [5,6] and not the flavanone as previously believed [7]. This finding is supported by genetic evidence [8,9] and is consistent with ^{13}C-labelling [10] and enzymological data [5,11] which support a role for the isomerase in the synthesis of flavonoid derivatives such as flavones and anthocyanins. Labelling experiments [12] and chemical studies [13] have, however, suggested that isoflavonoids may be formed directly from chalcones.

The accumulation of isoflavonoid phytoalexins (host-synthesised, antimicrobial compounds) in French bean cell suspension cultures may be induced by treatment with abiotic elicitors such as denatured ribonuclease A [14] or biotic elicitor preparations from cell walls of *Colletotrichum lindemuthianum*, the causal agent of bean anthracnose disease [15]. In both cases, phytoalexin accumulation is preceded by changes in the levels of biosynthetic enzymes including L-phenylalanine ammonia-lyase (EC 4.3.1.5), chalcone synthase and chalcone isomerase [15,16]. The increase in isomerase levels is less rapid than that of the other two enzymes, and basal activities are higher; for these reasons the isomerase is probably not a controlling factor in the phytoalexin response [15], although its status as an inducible enzyme in the bean system makes it a suitable subject for further studies associated with the control of its levels. As a prelude to this work, we here report the purification of the French bean isomerase and its kinetic properties, and attempt to assess the function of the enzyme in vivo.

Materials and Methods

2′,4,4′,6′-Tetrahydroxychalcone was prepared from naringenin using the method of Moustafa and Wong [1]. All other chalcones were prepared by alkaline condensation of the appropriate hydroxyacetophenone with the appropriately substituted benzaldehyde derivative [17], and were recrystallised several times from aqueous ethanol. Kievitone, phaseollin and coumestrol were extracted and purified from wounded French bean cotyledons and hypocotyls as described elsewhere [18]. They were further purified by Sephadex LH-20 column chromatography using ethanol as solvent. Genistein and 3′,9-dihydroxypterocarpan were gifts from Dr. P.M. Dewick, Nottingham, and 2′-hydroxygenistein and dalbergioidin were gifts from Dr. S. Antus, Budapest. All other reagents were commerical "Analar" grade.

p-Aminobenzamidohexyl-Sepharose 4B was prepared from ω-aminohexyl Sepharose 4B by reaction with *p*-nitrobenzoyl azide and subsequent reduction with sodium dithionite [19]. 2′,4′-dimethoxy 4-hydroxychalcone was linked to the substituted Sepharose by diazonium coupling.

Buffers. The main buffers used were (A) 50 mM Tris-HCl, pH 8.5, (B) 10 mM potassium phosphate, pH 8.0 and (C) 30 mM potassium phosphate, pH 8.0, each containing 1.4 mM 2-mercaptoethanol.

Cell suspension cultures. Cell suspension cultures of dwarf French bean (Canadian Wonder variety) were grown in a modified Schenk and Hildebrandt medium as described elsewhere [18]. Cells in mid growth phase (6–7 days after subculture) were harvested by suction filtration, frozen with liquid nitrogen, and stored at −70°C until required for enzyme extraction. No significant loss of chalcone isomerase activity was observed in frozen cells over a period of several weeks.

Enzyme purification. All steps were carried out at 4°C. 570 g of frozen cells were thawed and homogenized with 600 ml of buffer A in a Waring blendor. The homogenate was passed through four layers of muslin and the residue re-extracted with a further 200 ml of buffer A. The combined extracts were centrifuged for 30 min at $23000 \times g$. The supernatant was adjusted to pH 4.0 over a 30 min period by the addition of 1 M HCl. The pH was then raised to 5.0 with 1 M KOH, and the mixture left to stand for 10 min before being centrifuged for 30 min at $23000 \times g$. The pH of the supernatant was then slowly re-adjusted to 8.5 by further addition of 1 M KOH. The supernatant was then fractionated using $(NH_4)_2SO_4$, the precipitate forming at 50–75% saturation being finally dissolved in 9.5 ml of buffer A. The solution was clarified by centrifugation at $23000 \times g$ for 15 min, and 9 ml was applied to a Sephadex G-100

column (85 × 2.5 cm) which was eluted with buffer B at a flow rate of 17 ml/h, 4 ml fractions being collected. Fractions containing the highest specific activity were pooled and immediately applied to a DEAE-cellulose column (27 × 0.8 cm) which had been equilibrated with buffer B. The column was eluted at a flow rate of 30 ml/h, first with 240 ml of buffer B, and then with a linear gradient of 500 ml of this buffer, increasing in molarity from 10 to 60 mM. Fractions of 3.7 ml were collected and those showing the highest activity were pooled and concentrated to 5.9 ml by ultrafiltration (Amicon UM-10 membrane, 75 lb/inch^2). The resulting enzyme solution was used for the kinetic experiments and for investigating stability under different storage conditions.

A sample (1.0 ml) of the above enzyme preparation was applied to an affinity column (5 × 1 cm) of 2′,4′-dimethoxy 4-hydroxy chalcone linked via a spacer to Sepharose 4B (see Materials). Buffer C (30 ml) was passed through the column and the isomerase was then eluted with buffer C (minus mercaptoethanol) containing 0.5 mg/ml 2′,4,4′-trihydroxychalcone. The fractions containing the enzyme decolourized rapidly; these were pooled, dialysed overnight against 500 ml of buffer C and then concentrated to 1 ml by ultrafiltration as described above.

Enzyme assays. The buffer used for enzyme assays was 60 mM potassium phosphate, pH 8.0. This contained 50 mM KCN when used for assay of isomerase activity in fractions containing peroxidase. Apparent peroxidase activity (chalcone oxidation dependent upon endogenous H_2O_2 levels) was estimated by assay in the presence and absence of 50 mM KCN; the rate in the presence of KCN (isomerase activity) was substracted from the rate in the absence of the peroxidase inhibitor.

For determination of chalcone isomerisation or oxidation, 20 μl of a 1.0 mg/ml solution of 2′,4,4′-trihydroxychalcone in ethanol was added to 2.5 ml of the relevant buffer followed by a suitable aliquot of enzyme (5–20 μl). The decrease in absorption at 400 nm was monitored at 30°C in a Unicam SP1800 recording spectrophotometer against a blank containing no chalcone. The initial rate of disappearance of chalcone was used to estimate enzyme activity. When using 2′,4,4′,6′-tetrahydroxychalcone as substrate, the rate of non-enzymic isomerisation was first measured; this was then subtracted from the rate in the presence of enzyme.

Protein determination. Protein was determined by the method of Lowry et al. [27] as modified by Leggett-Bailey [20].

Gel electrophoresis. Flat bed SDS-polyacrylamide gel electrophoresis was carried out following the method of Vaughan and Fliescher [21] using 0.05 M Tris-glycine, 0.1% SDS, 2 mM EDTA, pH 8.3. Gels (5–16% exponential gradient) were set in 0.375 M Tris-HCl, 0.1% SDS, 2 mM EDTA, pH 8.8. Separation of the fractions at various stages of purification, along with a range of molecular weight markers, was at 10 mA per gel (loading) followed by 3 h at 100 V. Non-gradient gels were run in the presence and absence of SDS under the same conditions.

Protein was stained with Coomassie brilliant blue. Enzyme activity on gels run in the absence of SDS was visualised by staining the gels in 60 mM KH_2PO_4, pH 8.0, containing 50 mM KCN and 0.5 mg/ml 2′,4,4′-trihydroxychalcone for 15 min at room temperature. The enzyme showed as a clear band against the yellow background. The presence of flavanone in the clear bands was shown by adding to the gels (immersed in 50 ml of 60 mM KH_2PO_4, pH 8.0) 5 ml of 1% (w/v) $NaBH_4$ in isopropanol. After 10 min, 5 ml of 2 M HCl was added followed 10 min later by 50 ml concentrated HCl. In all cases the colourless bands of isomerase activity stained bright pink.

Kinetic studies. The buffers used were 60 mM potassium phosphate in the pH range 5.6–8.8, and 60 mM Tris-HCl in the range 7.25–9.25. In experiments investigating substrate specificity or isomerisation of 2′,4,4′-trihydroxychalcone at varying pH values initial rates of disappearance of chalcone at 400 nm were determined at six or more substrate concentrations, correction being made for the different extinction coeffcients of different substrates or the same substrate at different pH values.

A range of flavonoid and isoflavonoid inhibitors of the enzyme were tested by adding the compounds (final fixed concentration between 0.75 and 1.5-times the K_i value) in 20 μl of ethanol to reaction mixtures containing varying concentrations of from 25 to 6.66 μM 2′,4,4′-trihydroxy-

chalcone. K_m and K_i values were determined from both double reciprocal and Eadie-Hofstee plots of the initial rate data.

Results

Purification procedure. The purification procedure up as far as the DEAE-cellulose stage resulted in about a 350-fold purification of the isomerase with an approx. 50% loss in total activity (Table I). Peroxidase activity (chalcone oxidation) was also monitored throughout the purification procedure. The supernatant from the acid precipitation stage showed no assayable peroxidase activity, although such activity reappeared in fractions from the gel-filtration step (Fig. 1). Hydrogen peroxide is probably destroyed during acid precipitation, but later produced endogenously in the still relatively crude preparation; on addition of H_2O_2 to assay mixtures (minus KCN) containing active isomerase at all stages up to the DEAE-eluate, rapid decolourization of added chalcone occurred. Because measured rates of peroxidase activity in the fractions clearly depended on endogenous H_2O_2 levels, the term 'apparent peroxidase activity' is used in this paper. Ion-exchange chromatography on DEAE-cellulose resulted in complete separation of the isomerase from peroxidase (Fig. 2) and from another enzyme of the flavonoid/isoflavonoid pathway, chalcone synthase (Whitehead, I.M., unpublished data).

The isomerase bound very strongly to the chalcone affinity column; an initial attempt to elute the enzyme with 50 μM 2',4,4'-trihydroxychalcone was unsuccessful. It was finally eluted with approx. 2 mM chalcone in 30 mM potassium phosphate, pH 8.0.

Gel electrophoresis under non-denaturing conditions followed by specific enzyme staining indicated, throughout the purification procedure, the presence of only one isomerase band. High resolution SDS gradient gels showed one major protein band, with eight to ten minor contaminants, by the DEAE-eluate stage. Even affinity chromatography did not yield a homogeneous protein, at least three minor contaminants being present.

Stability of the enzyme activity. The 350-fold purified enzyme (129 μg protein/ml) retained 95% of its original activity when stored for 6 days at 4°C; however, a 1:4 dilution of the same preparation only retained 53% activity under the same conditions. In the presence of 1% bovine serum albumin, 65% of the original activity was retained after 6 weeks at 4°C. The preparation lost 35% of its activity on storage for 6 weeks at −20°C in the presence of 5% glycerol, whereas in the absence of glycerol 95% of the activity was lost.

Molecular weight. From gel-filtration studies with a calibrated Sephadex G-100 column, the apparent molecular weight of the isomerase was estimated to be about 30000 ± 10%. This value corresponded well with the value of 28000 obtained for the protein after affinity chromatography and SDS-gradient gel electrophoresis.

TABLE I

PURIFICATION OF CHALCONE ISOMERASE FROM *PHASEOLUS VULGARIS*

All enzyme assays were carried out at 30°C, pH 8.0, with 25 μM 2',4,4'-trihydroxychalcone as substrate.

Fraction	Total activity (μkat)	Total protein (mg)	Specific activity (μkat/g)	Relative purity (-fold)	Recovery (%)
Crude extract	1.10	559	2	1	100
Supernatant from acid precipitation	1.05	256	4	2	95.2
50–75% $(NH_4)_2SO_4$ fraction	0.76	55	14	7	69.1
Sephadex G-100	0.57	13	44	22	51.8
DEAE-cellulose, ultrafiltration	0.51	0.76	671	336	46.4
Affinity chromatography, dialysis, ultrafiltration [a]	0.27	0.35	771	386	24.5

[a] The total volume after the DEAE step was 5.9 ml; 1 ml of this was applied to the affinity column. The figures above are calculated on the basis that the whole fraction was applied.

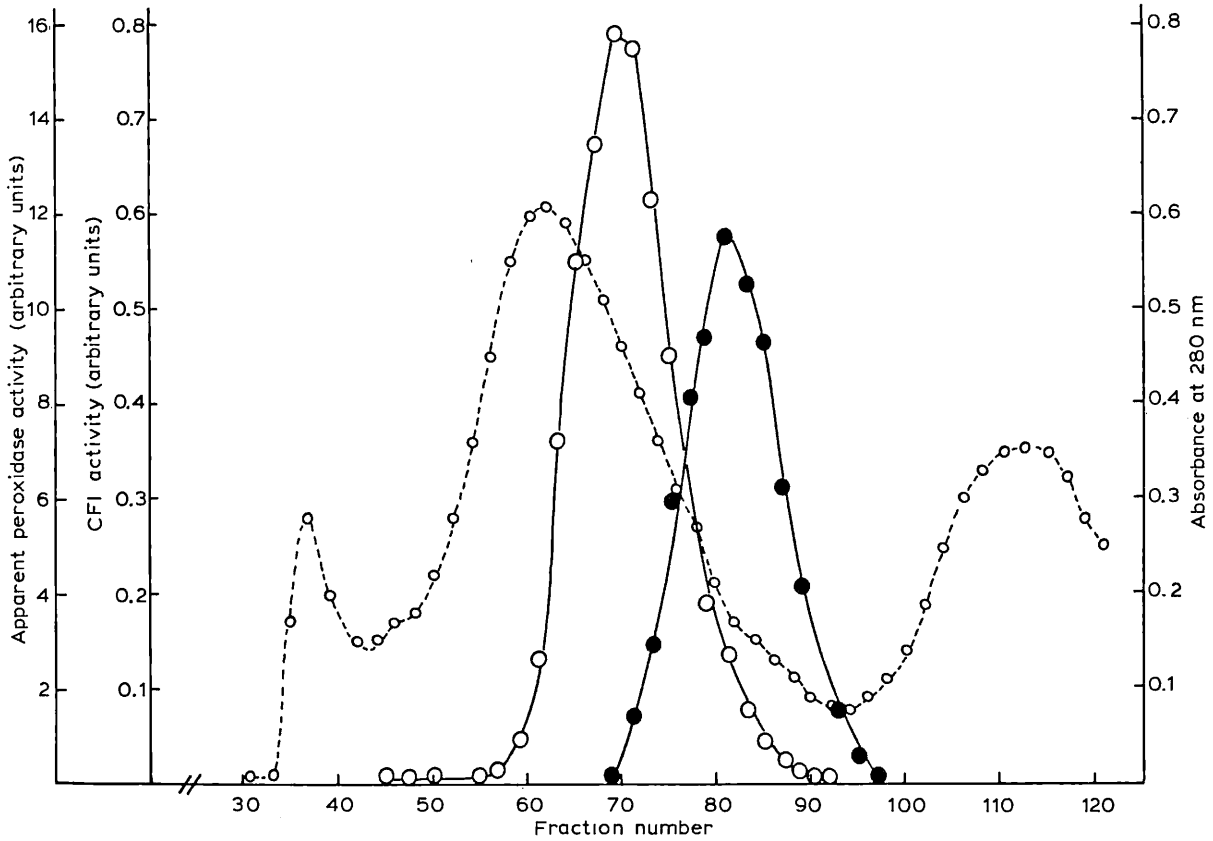


Fig. 1. Purification of chalcone isomerase by gel-filtration on Sephadex G-100. (●——●) chalcone isomerase activity; (○——○) apparent peroxidase activity; (○- - - - -○) absorbance at 280 nm.

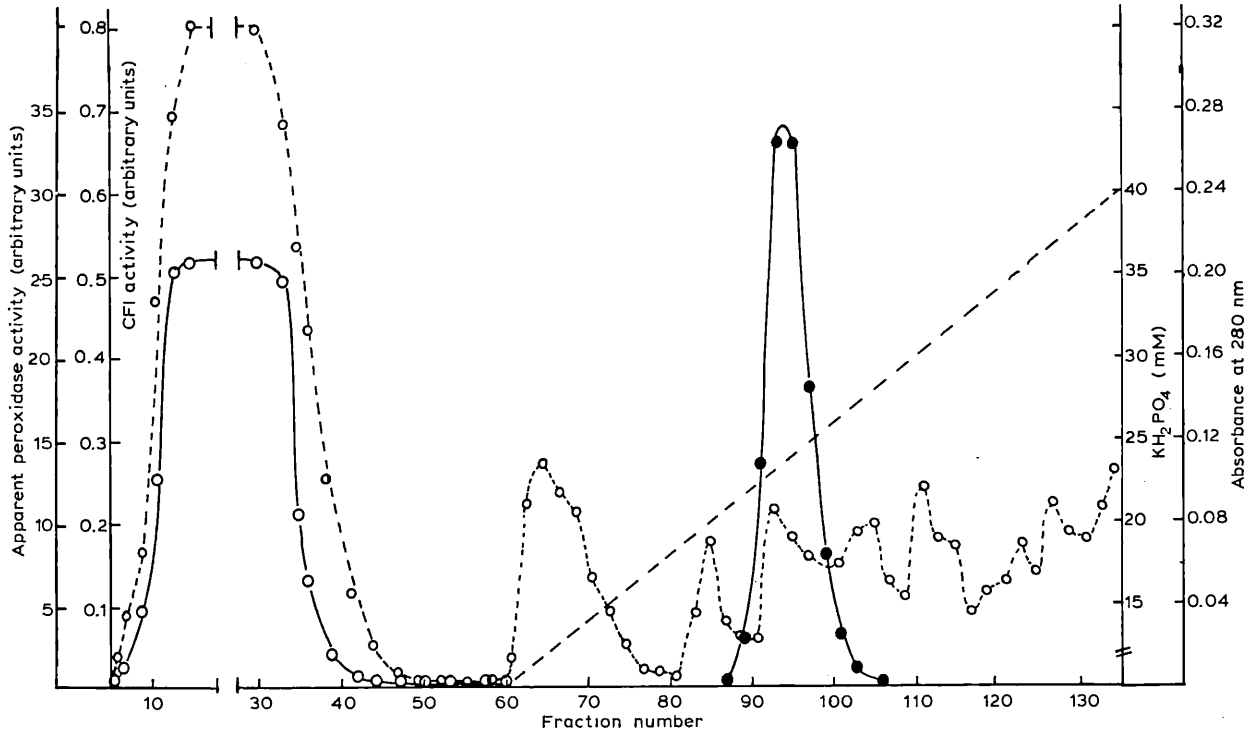


Fig. 2. Purification of chalcone isomerase on DEAE-cellulose. (●——●) chalcone isomerase activity; (○——○) apparent peroxidase activity, (○- - - - -○) absorbance at 280 nm, (- - - - -) potassium phosphate gradient.

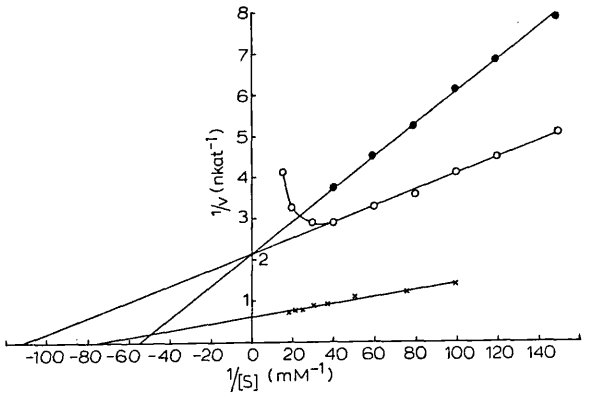


Fig. 3. Lineweaver-Burk plots for the isomerisation of 2',4,4'-trihydroxychalcone (O——O), and 2',4,4',6'-tetrahydroxychalcone (×——×) at 30°C, pH 8.0. The isomerisation of the trihydroxychalcone in the presence of 9.55 μM kievitone is also shown (●——●). Values for $1/v$ refer to 20 μl of the 355-fold purified enzyme (2.6 μg potein) in each case.

Substrate specificity. The enzyme catalysed the conversion of both 2',4,4'-trihydroxy and 2',4,4',6'-tetrahydroxy chalcones to their respective flavanones (Table II). The K_m for tetrahydroxychalcone is probably less accurate than those for the other chalcones in view of the instability of this compound at pH 8.0 and the associated difficulty of assessing its purity. Unlike the isomerase from *Glycine max* [1], the *P. vulgaris* enzyme readily catalysed the isomerisation of the B-ring methoxylated chalcone (B), although no activity

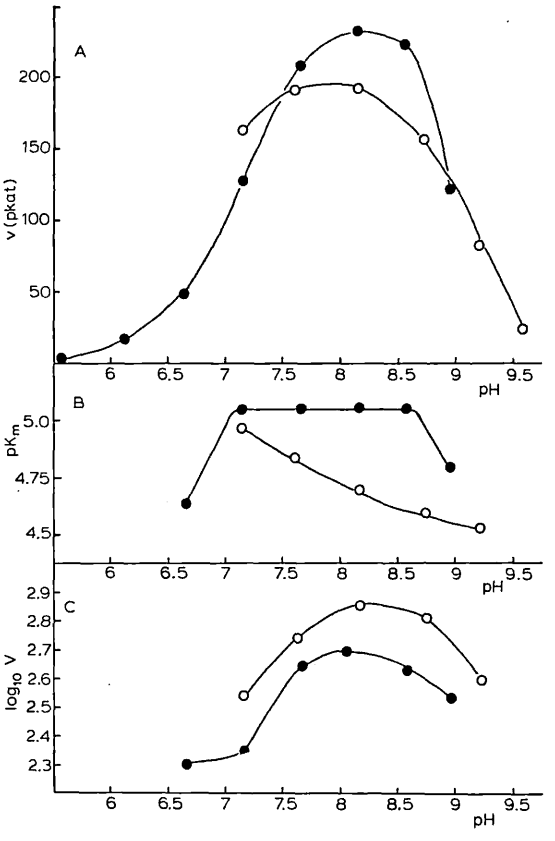
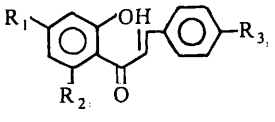


Fig. 4. pH Dependence of kinetic parameters for the isomerisation of 2',4,4'-trihydroxychalcone at 30°C. Values for v and V refer to 20 μl of the 355-fold purified enzyme in each case. v was determined at a substrate concentration of 25 μM. (●——●) potassium phosphate buffer; (O——O) Tris-HCl buffer.

TABLE II

SUBSTRATE SPECIFICITY OF CHALCONE ISOMERASE FROM *PHASEOLUS VULGARIS*

V values refer to the 355-fold purified enzyme (129 μg protein/ml).



(A) $R_1=R_3=OH$; $R_2=H$;
(B) $R_1=OH$; $R_2=H$; $R_3=OCH_3$;
(C) $R_1=R_3=OCH_3$; $R_2=H$;
(D) $R_1=OCH_2C_6H_5$; $R_2=H$; $R_3=OCH_3$;
(E) $R_1=R_2=R_3=OH$.

Chalcone substitution pattern	K_m (μM)	V_{max} (nkat·ml^{-1})
2',4,4'-Trihydroxy (A)	8.9	23.25
2',4'-Dihydroxy 4-methoxy (B)	8.0	41.67
2'-Hydroxy 4,4'-dimethoxy (C)	No reaction	—
2'-Hydroxy, 4'-benzyloxy 4-methoxy (D)	No reaction	—
2',4,4',6'-Tetrahydroxy (E)	13.3	80.65

was seen with chalcones substituted in the 4'-position.

Substrate inhibition was observed with both the 2',4,4'-trihydroxy and 2',4'-dihydroxy 4-methoxy chalcones at concentrations of 25 μM and above (Fig. 3), whereas double reciprocal plots for the tetrahydroxychalcone were still linear at high substrate concentrations.

Effects of pH on kinetic parameters. The pH optimum of the enzyme, as determined by initial velocity measurements made in phosphate buffers with 2',4,4'-trihydroxychalcone as substrate, was about 8.2 (Fig. 4a), although the value in Tris-HCl buffer was somewhat lower. K_m and V were determined from double reciprocal plots within the pH range 6.65–9.21; outside this range, rates were too low to obtain accurate kinetic parameters. In phosphate buffers, plots of K_m versus pH could be interpreted as showing the involvement of two groups with pK_a values of approx. 7.05 and 8.7 (Fig. 4b). This result is not completely inconsistent with the involvement of a histidine imidazole group [4], although it must be borne in mind that the present treatment of the data does not distinguish between ionizations in the free enzyme and the free substrate [22]. In Tris-HCl buffers, the dependence of K_m on pH does not show the expected pattern, the plot being a downward curve rather than consisting of straight line sections of unit or zero slope [22]. In Tris-HCl buffer at pH 8.0, the absorption maximum of the chalcone is shifted 9 nm towards the red as compared with phosphate buffer at the same pH; this may perhaps indicate some interaction between buffer and substrate. At all pH values tested, V was greater in Tris-HCl buffer than in phosphate (Fig. 4c).

Competitive inhibition of the isomerisation of 2',4,4'-trihydroxychalcone. Various flavonoid and isoflavonoid compounds were tested as inhibitors of the reaction (Table III); in all cases inhibition was competitive as judged by the increase in K_m, with no effect on V, observed from double reciprocal plots (see Fig. 3). In addition to the expected potency of flavonols [4] and the 2'-methoxylated chalcone, low K_i values were also observed for the isoflavonoid-derived antimicrobial compounds kievitone and coumestrol. In contrast, isoflavonoid precursors of kievitone (genistein, 2'-hydroxygenistein and dalbergioidin) were very poor inhibitors.

Energy of activation. V values were calculated from double reciprocal plots of initial rate versus 2',4,4'-trihydroxychalcone concentration at a range of temperatures. From the slope of the Arrhenius plot (Fig. 5) for values between 25 and 40°C, an activation energy of 17.56 kJ/mol was calculated.

Effects of metal ions and sulphydryl reagents. The enzyme was neither inhibited nor activated by the addition to the incubation mixture of Mg^{2+}, Ca^{2+}, Mn^{2+} or Fe^{2+} at concentrations from 2 to 20 mM. No inhibition was observed with EDTA or diethyldithiocarbamate at concentrations up to 10

TABLE III

COMPETITIVE INHIBITION OF CHALCONE ISOMERASE BY FLAVONOID AND ISOFLAVONOID DERIVATIVES

K_i values were determined at 30°C, pH 8.0.

Inhibitor	K_i (μM)
2',4'-Dimethoxy 4-hydroxy chalcone	3.4
4',5,7-Trihydroxy flavanone (naringenin)	16.7
4',5,6-Trihydroxy flavonol (kaempferol)	4.2
4',5,5',7-Tetrahydroxy flavonol (quercetin)	4.1
4',5,7-Trihydroxy isoflavone (genistein)	160.1
2',4',5,7-Tetrahydroxy isoflavone (2'-hydroxygenistein)	37.5
2',4',5,7-Tetrahydroxy isoflavanone (dalbergioidin)	57.0
2',4',5,7-Tetrahydroxy-8-(3,3-dimethylallyl) isoflavanone (kievitone)	9.2
3,9-Dihydroxy pterocarpan	25.9
7-Hydroxy-3',4'-dimethylchromanocoumaran (phaseollin)	50.0
7,12-Dihydroxy coumestan (coumestrol)	2.5

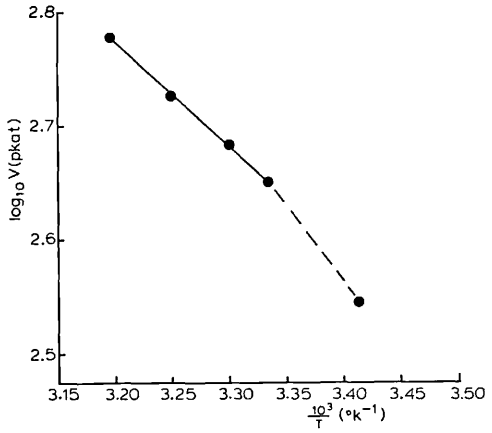


Fig. 5. Arrhenius plot for determination of the activation energy for the enzymic isomerisation of 2′,4,4′-trihydroxychalcone at pH 8.0. Values of V refer to 20 µl of the 355-fold purified enzyme.

mM. An inhibition of 50% was observed on addition of *p*-chloromercuribenzoate (75 µM) or mercuric chloride (150 µM) to the standard assay mixture. The enzyme was not inhibited by 5 mM potassium tetrathionate.

Discussion

During the purification of the *P. vulgaris* chalcone isomerase, only a single band of enzyme activity was detected on gels at any stage of increasing purity, thus suggesting that the enzyme does not exist in isoenzymic forms (cf. Ref. 3). The use of KCN in enzyme assay and gel-staining buffers ensures that no artefacts due to the presence of peroxidase are observed. Peroxidase catalyses the H_2O_2-dependent oxidation of 2′,4,4′-trihydroxychalcone to a variety of products including the chalcone cyclic peroxide, the 4′,7-dihydroflavonol and the 4′,6-dihydroxyaurone [23–25]. Our results indicate that care is needed in assessing peroxidase activity during the purification procedure, as H_2O_2 may be destroyed and then regenerated endogenously in partially pure preparations.

The 386-fold purification of the *P. vulgaris* isomerase compares favourably with the 8000-fold purification reported for the enzyme from soybean seeds [4], where considerable amounts of storage protein are present; in spite of the seemingly higher purification the final specific activity achieved for the soybean enzyme was considerably less than that reported here. The molecular weight of 28000 lies between the previously reported values of 16000 for the soybean enzyme [4] and 50000 for the parsley enzyme [7]. The agreement between the molecular weights obtained by gel-filtration and SDS-gel electrophoresis suggests that the *P. vulgaris* isomerase consists of a single subunit.

The isomerase has low K_m values for both 2′,4,4′-trihydroxy and 2′,4,4′,6′-tetrahydroxy chalcones; the K_m for the tetrahydroxychalcone is similar to that calculated for the parsley enzyme, although this enzyme is inactive against 6′-deoxy chalcones [3]. The correlation between isomerase specificity and the substitution patterns of the naturally occurring flavonoid compounds in the plant has already been pointed out [3]. The low K_m values and activation energy here reported indicate that the enzyme binds substrates tightly and that the catalysis is very efficient.

A mechanism for the isomerisation reaction involving an acid-base-catalysed formation of a flav-3-en-4-ol intermediate followed by a stereo-specific proton transfer to form the flavanone has been proposed [2], and the possible involvement of an imidazole or amino group has been suggested [4]. Our results suggest that the catalytic step may depend on groups which ionise with pK_a values of 7.05 and 8.7. The lower value is at the upper limit for the ionization of a histidine imidazole group. Another possibility is that this may represent substrate ionization; 2′,4,4′-trihydroxychalcone only becomes appreciably ionized at around pH 7.0, the macroscopic pK_a value being approx. 8.2 [23]. However, the 4-hydroxyl group would be expected to have the lowest pK value, and, unlike the enzyme from soybean [1], the *P. vulgaris* enzyme has a low K_m value for the isomerisation of chalcone substituted in this position. It is therefore difficult from our data to assess the possible role for substrate ionization; unfortunately, the chalcone with a single ionizable OH group (2′-OH; 4,4′-OCH_3) does not act as substrate. An ionized chalcone 2′-hydroxyl group, along with a histidine imidazole group, has, however, recently been implicated in the overall reaction mechanism of the soybean isomerase [28]. The change in the slope of the pK_a versus pH graph at pH 8.7 in the present work is

consistent with the involvement of an amino group, although again substrate ionization cannot be excluded. Amino group participation might explain the anomalous results observed using Tris-HCl buffers. The lack of effect of potassium tetrathionate suggests that the enzyme does not depend on an SH group for its activity, although it was inhibited by $HgCl_2$ and *p*-chloromercuribenzoate.

In *P. vulgaris*, accumulation of isoflavonoid phytoalexins appears to occur in the absence of significant changes in the levels of other classes of flavonoid compound. In cell suspension cultures, accumulation of phaseollin and isoflavones is only accompanied by increases in wall-bound phenolic material [14], whereas in hypocotyls phaseollin and coumestrol accumulate although no increases are seen in the levels of hydroxycinnamic acids, flavonols and proanthocyanidins [26]. The role of chalcone isomerase in the formation of the two latter classes of compound is now apparent [6,5,11]. The potent inhibition of the enzyme by kievitone and coumestrol, both end products of the inducible isoflavonoid pathway, might explain the lack of increase in flavonoid levels observed during phytoalexin accumulation in spite of the large increases in the levels of chalcone synthase [16]; the isomerase would therefore be seen as a branch point at the level of chalcone-derived inducible isoflavonoids and normal constitutive flavonoid compounds. Such a hypothesis awaits conclusive enzymological support for the direct formation of isoflavones from chalcones.

Acknowledgements

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Biosynthesis of Isoflavonoid Phytoalexins: Incorporation of Sodium [1,2-$^{13}C_2$]Acetate into Phaseollin and Kievitone

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Biosynthesis, ^{13}C-NMR, Phytoalexin, Phaseollin, Kievitone, Isoflavonoid

^{13}C-NMR analysis of the isoflavonoid phytoalexins phaseollin and kievitone produced by feeding sodium [1,2-$^{13}C_2$]acetate to wounded bean (*Phaseolus vulgaris*) cotyledons has demonstrated the incorporation of intact acetate units into the aromatic A rings. Phaseollin shows a specific folding of the polyketide chain, whereas kievitone exhibits a randomisation of label in accordance with the intermediacy of a 2′,4′,6′-trihydroxylated chalcone during its formation. In neither case was sufficient label incorporated into the isoprenoid substituents to allow further analysis.

Introduction

The A-ring of flavonoids and isoflavonoids is derived by condensation of acetate-malonate units, and recent studies [1, 2] using sodium [1,2-$^{13}C_2$]acetate have elegantly displayed significant differences in the pathways to the 5-hydroxy and 5-deoxy series of compounds. Thus, Stoessl and Stothers [1] observed the intact incorporation of double-labelled acetate units into carbons 1a–1, 2–3 and 4–4a of the 6a-hydroxypterocarpan pisatin (**1**) (an isoflavonoid) in *Pisum sativum* demonstrating a specific folding of the polyketide chain and reduction of the "missing" oxygen function prior to ring closure (Fig. 1). In contrast, Light and Hahlbrock [2], investigating the biosynthesis of the flavonol kaempferol (**2**) in *Petroselinum hortense* cell suspension cultures, noted that A-ring signals in the ^{13}C-NMR spectrum of the enriched material were flanked not by one pair of satellites (as with pisatin), but by two pairs of satellites where sufficiently different coupling constants enabled the signals to be resolved. This indicated a mixture of the two possible products (Fig. 1) and the randomisation of label was ascribed to free rotation of a chalcone intermediate. Similar results with the flavone apigenin were less certain, since chemical randomisation could have occurred during the work up [2].

Reprint requests to Dr. Dewick or Dr. Dixon.

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We have carried out a similar experiment using wounded bean (*Phaseolus vulgaris*) cotyledons which accumulate the isoflavonoid phytoalexins phaseollin (**3**) and kievitone (**4**), together with smaller amounts of other related isoflavonoids [3]. Phaseollin and kievitone are representative of the 5-deoxy and 5-hydroxy series of isoflavonoids in the same plant, and in addition contain isoprenoid substituents, which may also be expected to be derived in nature from acetate precursors.

The enzymic formation of 6′-hydroxychalcones (5-hydroxyflavonoid/isoflavonoid series) is now well understood [4, 5]. A comparison of the ^{13}C A-ring labelling patterns of phaseollin and kievitone will provide an insight into the nature of the as yet uncharacterised enzyme system catalysing the formation of 6′-deoxychalcones from 4-coumaroyl coenzyme A and acetate (malonyl CoA) units.

Experimental

Seeds of dwarf French bean (*Phaseolus vulgaris* cultivar The Prince) (0.5 kg) were germinated in the dark in moist vermiculite for seven days at 25 °C. Cotyledons were excised, wounded by removing the top 2 mm of cells from the inner surface with a razor blade, and arranged in petri dishes on moist filter paper. Distilled water (50 µl) was applied to the cut surface of each cotyledon, and the petri dishes were placed in a dark, humid chamber at

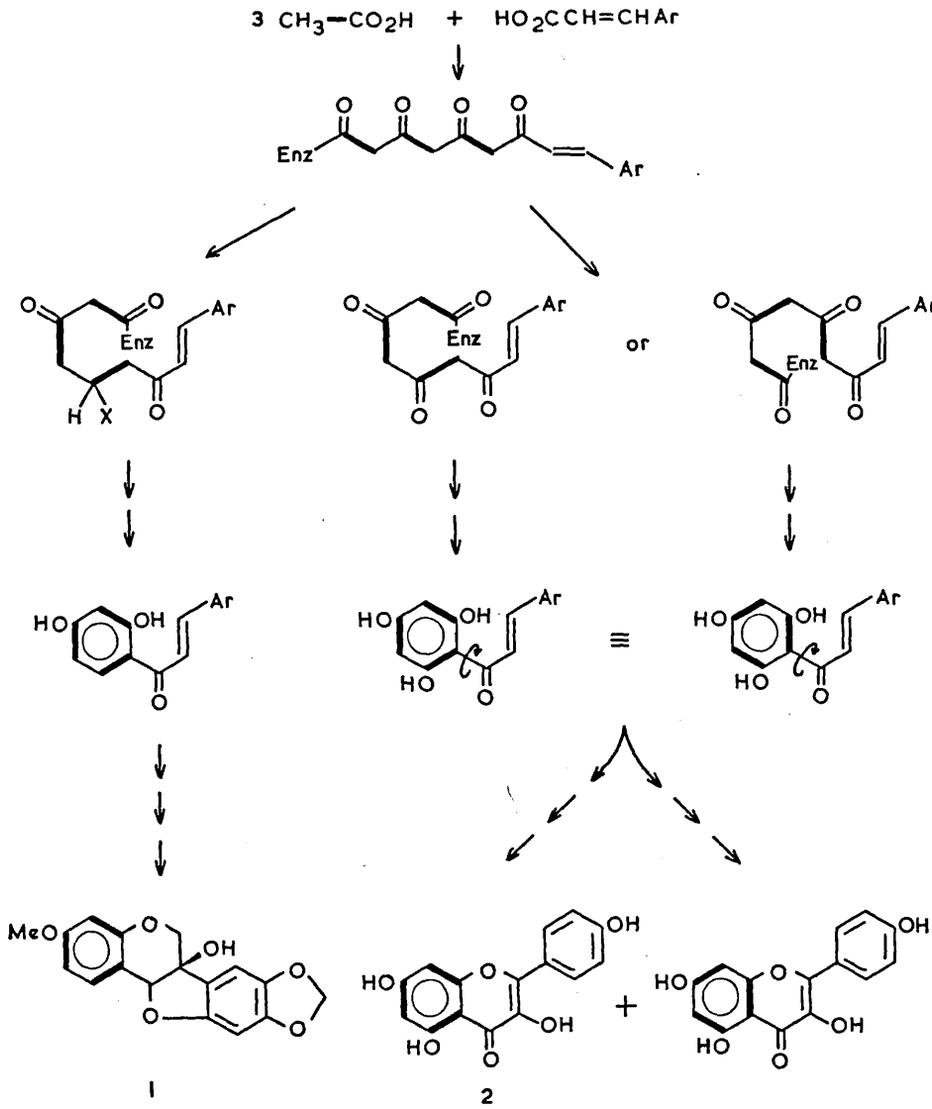
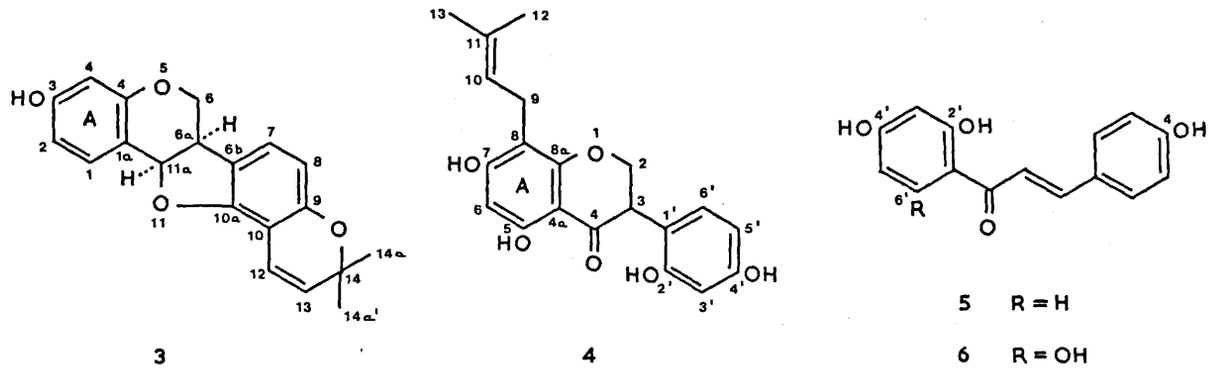


Fig. 1. Biosynthesis of pisatin (**1**) and kaempferol (**2**) from sodium [1,2-$^{13}C_2$]acetate [1, 2].



25 °C. After 48 h, 50 µl of an aqueous solution of [1,2-$^{13}C_2$]NaOAc (5 mg ml^{-1}; 90% labelled at each carbon atom; pH 7.6) was applied to the wounded surface of each cotyledon (total volume 100 ml). The cotyledons were incubated as above with the label for a further 48 h, and were then harvested by transfer to redistilled ethanol (500 ml). The precise timing and extent of the above labelling period were selected on the basis of previous experiments on the time courses of accumulation of phaseollin and kievitone in the cotyledons [3], and on a preliminary labelling experiment using [U-^{14}C]NaOAc (5 mg ml^{-1}, 5 µCi mg^{-1}, 50 µl per cotyledon). The ^{14}C-labelling experiment, under the conditions outlined above, resulted in the isolation of ^{14}C-labelled phaseollin which, after purification to constant specific activity, showed an incorporation of label of 5.7%, with an isotope dilution of 17.7.

The EtOH extracts were worked up, and the phytoalexins isolated and purified by TLC as previously described [11]. Yields of pure phytoalexins were: phaseollin, 49 mg; kievitone, 89 mg.

Results and Discussion

Signals in the ^{13}C-NMR spectra of phaseollin and kievitone were assigned on the basis of characteristic chemical shifts, off-resonance decoupling multiplicities, comparison with other isoflavonoid derivatives [6–10], and, where necessary, analysis of carbon-carbon couplings in spectra of the enriched samples. The assignments are listed in Table I.

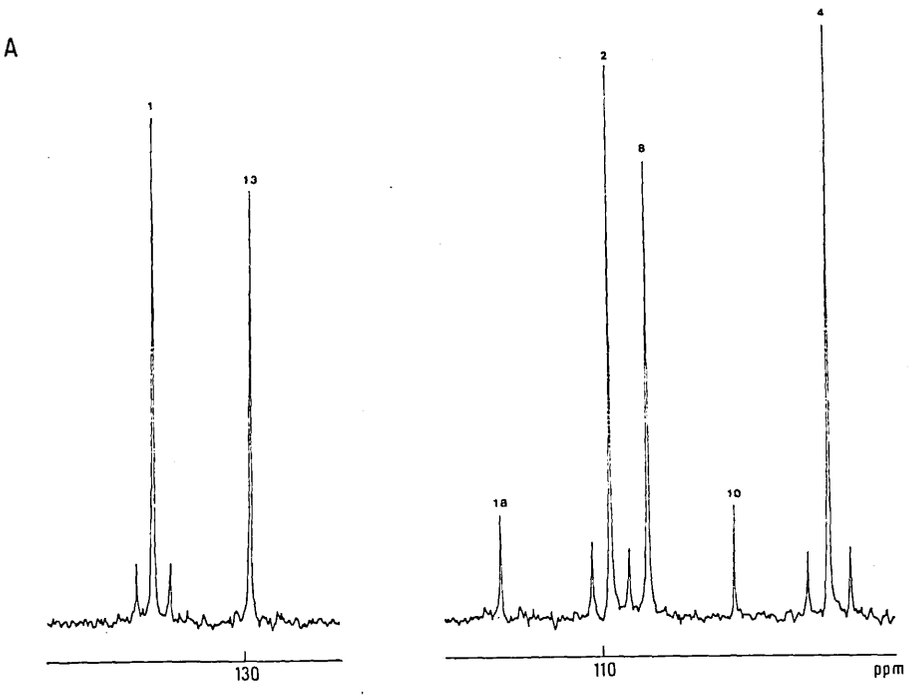
The 62.9 MHz spectrum of phaseollin derived from [$^{13}C_2$]acetate showed four signals to be flanked by satellites, and two more sets were observed with the increased sensitivity of a 100.6 MHz spectrometer (Fig. 2). These six signals correspond to the A ring carbons, and the ^{13}C-^{13}C coupling constants observed (Table I) indicate that intact acetate units are incorporated into the ring in precisely the same manner as Stoessl and Stothers observed with pisatin [1], *i.e.* into carbons 1a–1, 2–3 and 4–4a. From the relative intensities of the satellite peaks to the central signal, the enrichment level was estimated to be 0.30% at each acetate position.

In the spectrum of enriched kievitone, six signals were flanked by satellites. Two of these sets were plainly resolved as pairs of satellite signals, whereas the other four at both 62.9 MHz and 100.6 MHz were unresolved, broad peaks (Fig. 2). Clearly though, the pattern observed is analogous to that reported by Light and Hahlbrock for kaempferol [2], and is a result of two types of labelled kievitone

Table I. ^{13}C-NMR chemical shifts[a] and coupling constants for phaseollin (**3**) and kievitone (**4**).

Phaseollin (**3**)				Kievitone (**4**)			
Carbon	δ_c [ppm]	J_{cc}	[Hz]	Carbon	δ_c [ppm]	J_{cc}	[Hz]
1a	112.7	J_{1a-1}	61.0	4a	103.7	J_{4a-5}	62
1	132.3			5	163.4	J_{5-6}	72
2	109.7	J_{2-3}	64.7	6	96.5	J_{6-7}	ca. 68
3	157.1			7	164.7	J_{7-8}	ca. 68
4	103.7	J_{4-4a}	72.1	8	108.1	J_{8-8a}	73
4a	156.8			8a	161.3	J_{8a-4a}	63
6	66.6			2	71.2		
6a	39.7			3	47.2		
6b	119.1			1′	114.0		
7	123.8			6′	131.6		
8	108.6			5′	107.9		
9	155.4[b]			4′	157.0		
10	106.3			3′	103.8		
10a	153.8[b]			2′	158.8		
11a	78.7			4	198.8		
12	116.5			9	22.1		
13	129.6			10	123.9		
14	76.1			11	131.2		
14a	27.8			12	17.8		
14a′	27.8			13	25.8		

[a] ^{13}C-NMR spectra were obtained on a Bruker WM250 spectrometer operating at 62.90 MHz, or a Bruker WH400 at 100.62 MHz. Phaseollin was dissolved in $CDCl_3$, and kievitone in $(CD_3)_2CO$, and chemical shifts were recorded relative to TMS.
[b] Tentative assignments.



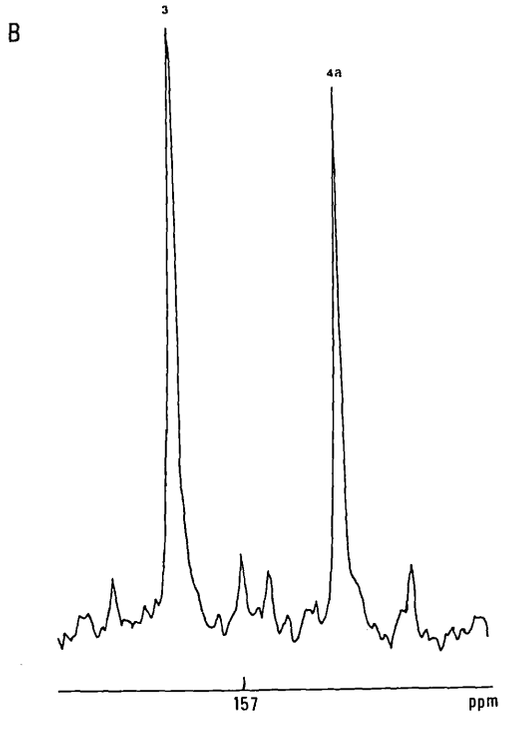


Fig. 2

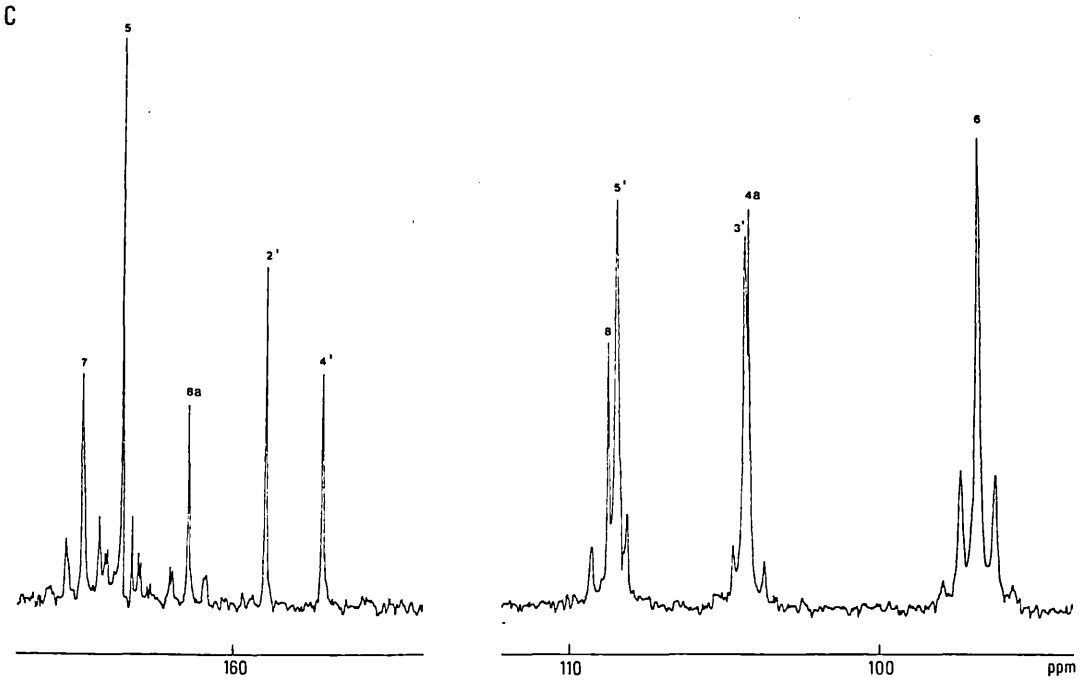


Fig. 2. ^{13}C-NMR spectra of enriched isoflavonoids: A, phaseollin, 62.9 MHz; B, phaseollin, 100.6 MHz; C, kievitone, 62.9 MHz.

being produced. From the relative intensities of the satellite peaks, the enrichment level was about 0.7% at each acetate position, more than double the figure for phaseollin.

The biosynthetic pathway to phaseollin thus involves specific folding of the polyketide chain, and the "missing" oxygen function must therefore be reduced before formation of the aromatic ring. This is in keeping with the role of 2′,4′,4-trihydroxychalcone (**5**) as an intermediate in phaseollin biosynthesis [11]. In contrast, for kievitone, the aromatic ring is formed without removal of oxygen, and if specific folding of the polyketide chain is again required, at some stage in the pathway this ring is free to rotate. A chalcone intermediate, most probably 2′,4′,6′,4-tetrahydroxychalcone (**6**) is thus implicated [12]. The biosynthetic routes to these 5-hydroxy- and 5-deoxy-isoflavonoids thus diverge prior to chalcone formation. A similar situation has also been demonstrated earlier with formononetin and biochanin A biosynthesis in *Cicer arietinum* using ^{14}C-labelled precursors [13]. The present results lend support to the involvement, in the biosynthesis of phaseollin, of a 6′-deoxychalcone synthase which catalyses the formation, reduction, dehydration and possibly cyclisation of a polyketide intermediate made from one molecule of *p*-coumaroyl coenzyme A and three molecules of malonyl CoA. The existence of such an enzyme, and the analogy of its reaction mechanism to that of 6-methylsalicylic acid synthase from *Penicillium patulum* [14], was pointed out earlier [4]. Kievitone is presumably synthesised via the normal "6′-hydroxy" chalcone synthase whose presence has been shown in French bean [15] and several other plant tissues [4, 16].

Although good incorporation of [$^{13}C_2$]acetate into the aromatic ring A was observed with both metabolites, neither spectrum gave evidence for any significant incorporation of acetate into the isoprenoid substituents, though these are undoubtedly acetate-mevalonate derived. Radiolabelling experiments have demonstrated that isoprenylation is a late stage in the biosynthesis of phaseollin [11] and the rotenoid amorphigenin [17], occurring after the basic pterocarpan or rotenoid skeletons have been built up. The same is probably true for kievitone. It is possible then that during the present experiment, the supply of precursor had been exhausted before isoprenylation occurred. However, it is more likely that there is

probably a significant difference in the efficiency of transport of acetate precursor into the acetate-malonate and acetate-mevalonate derived portions of the molecule. Mevalonate itself is poorly incorporated into phaseollin [18], and similarly into rotenoids [17] and other hemiterpenoid derivatives [19]. The incorporation of acetate into wholly terpenoid phytoalexins [20] is not affected in the same way. An enzyme catalysing the dimethylallylation of 3,6a,9-trihydroxypterocarpan has been isolated from soya bean cotyledons [21], and is possibly located in the plastid fraction. Apart from microsomal hydroxylation systems, the other known enzymes involved in the biosynthesis of isoflavonoids appear to be soluble cytoplasmic enzymes. Such subcellular compartmentation might explain the disappointing incorporation into the terpenoid fragment of the isoflavonoids in the present work.

Acknowledgements

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CHALCONE SYNTHASE FROM CELL SUSPENSION CULTURES OF *PHASEOLUS VULGARIS* L.

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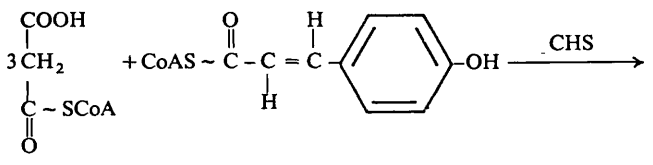
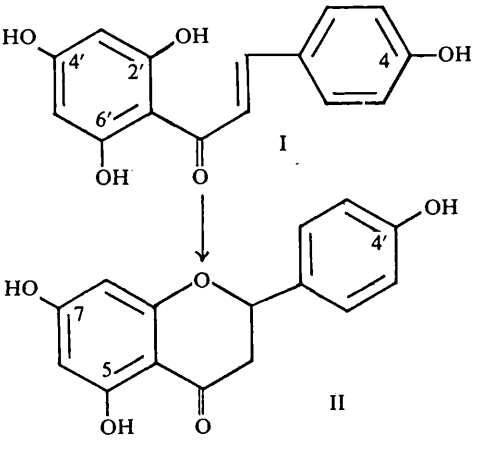
Key words: Chalcone synthase; Phytoalexin; Naringenin; (P. vulgaris)

Chalcone synthase from cell suspension cultures of *Phaseolus vulgaris* has been purified approximately 31-fold. The preparation formed the flavanone naringenin from malonyl-CoA and 4-coumaroyl-CoA. The *P. vulgaris* enzyme showed similarities to the enzyme recently purified from cell cultures of *Petroselinum hortense* with respect to molecular weight, pH optimum, ability to catalyse $^{14}CO_2$ exchange between $NaH^{14}CO_3$ and malonyl-CoA, and overall response of naringenin formation to varying thiol concentration. However, it appeared much less stable than the *P. hortense* synthase, exhibited significantly different apparent K_m values for the two substrates and produced different 'release products' following purification. The enzyme was strongly inhibited by the isoflavanone phytoalexin kievitone, but not by isoflavone and isoflavanone precursors of kievitone. The possible relevance of the inhibition of the enzyme by phytoalexins is discussed.

The enzyme chalcone synthase catalyses the condensation of three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA to yield 2′,4,4′,6′,-tetrahydroxychalcone (I) [1]. The chalcone may, however, undergo spontaneous isomerisation in vitro to yield the corresponding flavanone naringenin (II). Such enzyme activity has been isolated from several sources,





including cell suspension cultures of parsley (*Petroselinum hortense*) [1–3] or *Haplopappus gracilis* [4], and anthers from *Tulipa* c.v. Appledorn [5,6]. Chalcone formation is believed to occur via head-to-tail condensation of acetate units, resulting in formation of a polyketide intermediate [2]. Unlike similar reactions catalysed by fatty acid synthase, the immediate substrate for the first partial reaction of chalcone synthase appears to be the malonyl-CoA thiol ester rather than a malonyl unit linked to an enzyme -SH group or an acyl

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carrier protein; the malonyl-CoA is decarboxylated prior to condensation [7]. A further characteristic of chalcone synthase, at least from *P. hortense* and *H. gracilis*, is the formation of 'release' or 'derailment' products as a result of release from the enzyme of partially completed polyketide chains [2,4,8,9]. Such release products, which are formed in vitro but not in vivo, include the styrylpyrone bis-noryangonin [8] and *p*-hydroxybenzalacetone [9].

In cell suspension cultures of *P. vulgaris*, chalcone synthase activity undergoes a rapid transient increase in response to elicitor macromolecules isolated from the cell walls of the phytopathogenic fungus *Colletotrichum lindemuthianum* [10]. This increase precedes the accumulation of isoflavonoid-derived phytoalexins (low-molecular-weight host-synthesised anti-microbial compounds) in the cultures. In vivo labelling studies with [^{35}S]methionine [10] and in vitro translation of polysomal mRNA using a message-dependent reticulocyte lysate system [11] have indicated that elicitor induces de novo synthesis of chalcone synthase subunits by bringing about an increase in the activity of specific chalcone synthase mRNA as a function of total cellular mRNA activity. These studies relied upon immunoprecipitation of bean chalcone synthase using anti-parsley chalcone synthase antiserum. We report here some of the properties of the *P. vulgaris* chalcone synthase.

Cell suspension cultures of *P. vulgaris* c.v. Canadian Wonder were grown in a modified Schenk and Hildebrandt medium as described previously [12]. Chalcone synthase activity was induced by treatment of cells in mid-growth phase (6–7 days after subculture) with a crude elicitor preparation obtained from autoclaved cell walls of *C. lindemuthianum*, race β (final elicitor concentration 100 μg glucose equivalents per ml culture) [12]. After 5 h in the presence of elicitor the cells were harvested by suction filtration, frozen in liquid N_2, and stored at -70°C until required for enzyme extraction. No significant loss of chalcone synthase activity was observed in frozen cells over a period of several months.

The enzyme was purified from 300 g of frozen cells by: a, homogenization in 300 ml of 100 mM KH_2PO_4, pH 8.0, containing 1.4 mM 2-mercaptoethanol; b, stirring the supernatant with 30 g Dowex AG1-X8 (phosphate form); c, ammonium sulphate precipitation (with the enzyme activity precipitating between 50 and 75% saturation); d, gel-filtration on a Sephadex G-100 column (90 × 2.5 cm) eluted with 50 mM KH_2PO_4/1.4 mM 2-mercaptoethanol, pH 8.0; and e, dilution of the chalcone synthase peak to 25 mM KH_2PO_4 followed by ion-exchange chromatography on a DEAE-cellulose column (27 × 0.8 cm), eluting with a 500 ml gradient of 25–150 mM KH_2PO_4. Fractions containing the highest chalcone synthase activity were concentrated to 7.1 ml by Amicon ultrafiltration, and stored with 30% glycerol and 14 mM 2-mercaptoethanol at -70°C. The enzyme was assayed by either the 'rapid assay method' [13], which measures incorporation of 2-[^{14}C]malonyl-CoA into ethyl acetate-soluble material (total chalcone synthase activity), or by the thin-layer chromatographic separation of naringenin as described elsewhere [14]. The CO_2 exchange reaction was measured by determining the radioactivity in malonyl-CoA following TLC of incubation mixtures containing enzyme, unlabelled malonyl-CoA and $NaH^{14}CO_3$ [7].

The purification procedure shown in Table I resulted in only a 31-fold increase in specific activity of the enzyme from crude, in spite of the removal of a large percentage of the total protein. The actual purification was approximately only half this value, as the Dowex treatment activates the synthase by removal of inhibitory polyphenols, and therefore increases total activity. This low purification value reflects the instability of the *P. vulgaris* synthase, with approximately 60% of the activity being lost at each purification stage. Attempts to further purify the enzyme by chromatography on hydroxyapatite or by preparative gel electrophoresis resulted in near total inactivation. At each stage in the purification, the products formed in the synthase reaction were monitored by TLC (cellulose, 30% acetic acid). The naringenin band co-chromatographed with an authentic sample of the flavanone in the four TLC systems described previously [14]. The ratio of naringenin to other 4-coumaroyl-CoA-dependent products decreased with increased purification of the enzyme. Enzyme assays (total activity) were also carried out in the absence of 4-coumaroyl-CoA. With the crude extract, no ethyl acetate-soluble

TABLE I

PARTIAL PURIFICATION OF CHALCONE SYNTHASE FROM CELL SUSPENSION CULTURES OF *P. VULGARIS*

Fraction	Total activity (pkat)	Total protein (mg)	Specific activity [a] (μkat/kg)	Relative purity (-fold)	Recovery (%)	Ratio of naringenin to release products [b]	
						(a)	(b)
Crude extract	275	302	0.9	1.0	100	1:1.55	1:0.00
Supernatant after Dowex Ag1-X8	367	213	1.7	1.9	133	1:1.30	1:0.03
55–75% $(NH_4)_2SO_4$ fractionation/Sephadex G-100	117	13	8.6	9.5	43	1:1.36	1:0.12
DEAE-cellulose/-ultrafiltration	45	1.5	29.4	32.3	16	1:2.24	1:0.26

[a] Total activity (malonyl-CoA converted to ethyl acetate-soluble products).

[b] Determined by TLC of ethyl acetate extractions from reaction mixtures incubated in the presence (a) and absence (b) of 4-coumaroyl-CoA. Column (a) gives the ratio of naringenin to total 4-coumaroyl-CoA-dependent release product formation, column (b) the ratio of naringenin to products formed from malonyl-CoA alone.

products were formed under these conditions; however, products dependent upon the presence of malonyl-CoA alone were found in increasing amounts as the enzyme was purified (Table I, last column).

Recent enzymological [1,6] and genetical [15,16] evidence has pointed to the production of 2′,4,4′,6′-tetrahydroxychalcone as the initial synthase reaction product. It is most probable that this is also the case for the French bean synthase; in the present paper, although the partially purified preparation was free of chalcone isomerase, the assay conditions would favour spontaneous isomerisation of tetrahydroxychalcone to naringenin.

The *P. vulgaris* chalcone synthase had a molecular weight of 77 000 (assessed by gel-filtration on Sephadex G-100), a pH optimum of 8.0 for naringenin formation (although the partially purified enzyme was more stable at pH 7.0), exhibited optimum product formation at 40°C, catalysed the exchange of $^{14}CO_2$ between $NaH^{14}CO_3$ and malonyl-CoA, had apparent K_m values for malonyl-CoA and 4-coumaroyl-CoA of 21.4 and 2.3 μM, respectively, and was inhibited by naringenin and the antibiotic cerulenin. Apart from the K_m values, these results suggest a strong similarity to the enzyme purified from *P. hortense* cell suspension cultures [1–3,7–9]. However, important differences observed for the *P. vulgaris* synthase were: a, its apparent instability, a property in common with the synthase from *H. gracilis* [4]; b, the lack of effect of ionic strength (10–300 mM KH_2PO_4) on naringenin formation (cf. Ref. 2); c, the formation of 4-coumaroyl-CoA-independent release products as a function of dithiothreitol concentration in the assay mixture; d, the formation of release products alone (with no flavanone formation) on replacing 4-coumaroyl-CoA with either caffeoyl-CoA or feruloyl-CoA (cf. Ref. 4); and e, the only weak inhibitory effects of 200 μM acetyl-CoA or free CoA (cf. Ref. 2).

The bean chalcone synthase exhibited a more restricted substrate specificity for the hydroxycinnamoyl-CoA moeity than did the enzymes from parsley, *Haplopappus* and *Tulipa*. In the latter case naringenin, eriodictyol and homoeriodictyol were readily formed, with similar K_m values, from 4-coumaroyl-, caffeoyl- and feruloyl-CoA, respectively [5]. The enzymes from parsley and *Haplopappus* formed both naringenin (pH optimum, 8.0) and eriodictyol (pH optimum, 6.5–7.0), although only small amounts of homoeriodictyol were formed from feruloyl-CoA [4]. The *P. vulgaris* enzyme produced neither eriodictyol nor homoeriodictyol, and the formation of release products with caffeoyl- or feruloyl-CoA as substrate was greater at pH 8.0 than at pH 7.0. The formation of flavonoids with di- or trihydroxy substitution of the B-ring in *P. vulgaris* probably occurs by hy-

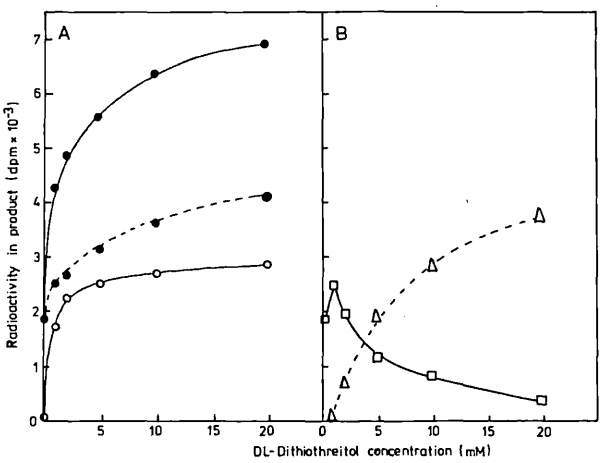

Fig. 1A and B. Effects of DL-dithiothreitol concentration on chalcone synthase activity. ●———●, Total activity for malonyl-CoA conversion to ethyl acetate-soluble products; ○———○, product formation in absence of 4-coumaroyl-CoA; ●- - - - -●, total 4-coumaroyl-CoA-dependent products; □———□, naringenin; △- - - - -△, 4-coumaroyl-CoA-dependent products other than naringenin.

droxylation of naringenin. Enzymes catalysing this process have been reported from other sources [17,18].

Synthase activity was measured in the absence and presence of varying concentrations (0–20 mM) of DL-dithiothreitol, 2-mercaptoethanol, L-cysteine and glutathione. The rate of reaction increased with increasing concentration of dithiothreitol (a 4-fold increase at 20 mM) and 2-mercaptoethanol (a 2-fold increase at 20 mM) whereas cysteine and glutathione showed no effect. Analysis (by TLC) of products formed at varying concentrations of dithiothreitol indicated that, in the absence of dithiothreitol, all products formed were dependent upon the presence of 4-coumaroyl-CoA in the reaction mixture. However, two non-coumaroyl-CoA-dependent products, (R_F 0.61 and R_F 0.83) appeared on silica gel G thin-layer chromatograms developed in toluene/ chloroform/ acetone (40 : 25 : 35) in increasing amounts over the range 0–5 mM dithiothreitol (Fig. 1A). Naringenin formation was optimal at 1.4 mM dithiothreitol, although production of other 4-coumaroyl-CoA-dependent products was only maximal at 20 mM dithiothreitol (Fig. 1B). The major 4-coumaroyl-CoA-dependent band was tentatively assumed, from R_F measurements (cellulose, 30% acetic acid; measured R_F 0.79, reported R_F 0.81 [8]), to be the styrylpyrone bis-noryangonin, which is a release product of the *Petroselinum* synthase [2,8]. Of the two products requiring only malonyl-CoA for their formation, neither co-chromatographed on cellulose thin-layer plates with a sample of triacetic acid lactone, a release product of the 6-methylsalicylic acid synthase from *Pencillium patulum* [19].

A range of isoflavonoids and related compounds were tested as inhibitors of the synthase (Fig. 2). The 5-deoxy compounds (flavonoid/ isoflavonoid A-ring numbering) liquiritigenin, 3,9-dihydroxypterocarpan and the phytoalexin phaseollin were not inhibitory at the concentrations tested, the chalcone isoliquiritigenin being slightly inhibitory. In contrast, the 5-hydroxy derivatives, naringenin, the isoflavone 2′-hydroxygenistein and the isoflavanone dalbergioidin (the

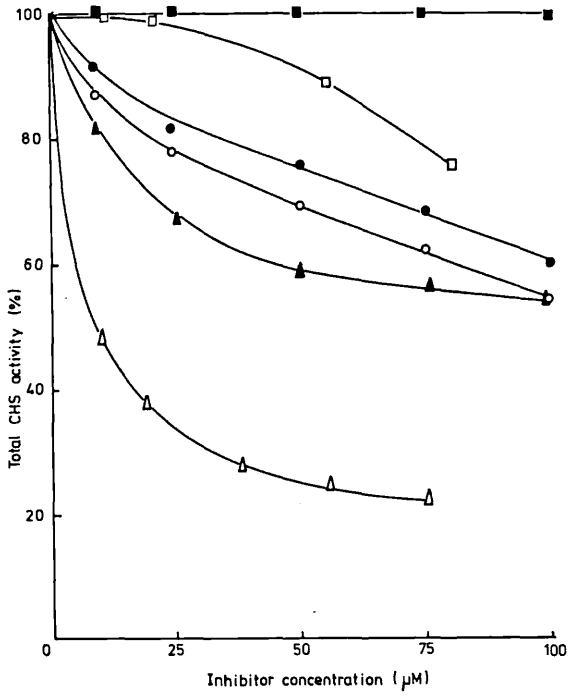

Fig. 2. Effects of varying concentrations of flavonoid and isoflavonoid derivatives on total chalcone synthase (CHS) activity (30 min incubation; 100% total activity = 0.022 pkat). ■, Liquiritigenin, 3,9-dihydroxypterocarpan or phaseollin; □, isoliquiritigenin; ●, 2′-hydroxygenistein; ○, dalbergioidin; ▲, naringenin; △, kievitone.

latter two being the direct precursors of the phytoalexin kievitone), were more potent inhibitors. Kievitone itself was the most potent inhibitor tested, reducing both total synthase activity and naringenin formation by 50% at a concentration of around 10 μM.

Initial rate measurements for the synthase reaction were made in the presence and absence of kievitone (10 μM) by varying one substrate with the other saturating and vice versa. Double-reciprocal plots of the initial rate data were constructed for both total synthase activity (data not shown) and naringenin formation (Fig. 3). With malonyl-CoA as variable substrate, both v vs. [S] and double reciprocal plots for naringenin formation were sigmoidal, indicating positive cooperativity (Fig. 3A). With 4-coumaroyl-CoA as variable substrate, kievitone inhibition of both total activity and naringenin formation appeared to be of the non-competitive type (Fig. 3B). The K_i value for inhibition of naringenin formation was 20.7 μM. With malonyl-CoA as variable substrate, kievitone inhibition of total activity appeared uncompetitive.

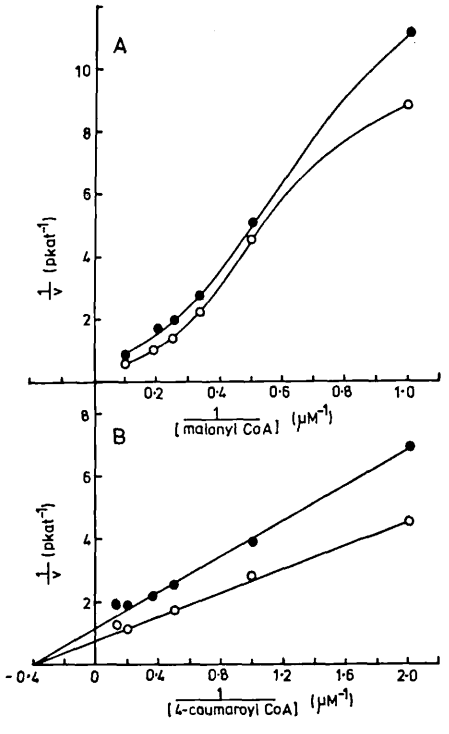

Fig. 3. Double-reciprocal plots for naringenin formation in the presence (●) and absence (○) of 10 μM kievitone. A, 4-Coumaroyl-CoA saturating (5.3 μM); B, malonyl-CoA saturating (30 μM).

It has recently been demonstrated that kievitone inhibits the cellulase, polygalacturonase and polymethylgalacturonase from culture filtrates of *Rhizoctonia solani* [20], and it has been suggested that such inhibition may be the result of non-specific enzyme inactivation by the tetrahydroxyphenolic molecule [21]. However, the inhibition by kievitone of both chalcone isomerase [22] and chalcone synthase from *P. vulgaris* is reversible, and occurs at much lower concentrations than required for a similar % inhibition of the *Rhizoctonia* enzymes. Furthermore, the apparent uncompetitive inhibition of bean chalcone synthase by kievitone with malonyl-CoA as variable substrate suggests that the phytoalexin binds to the enzyme-substrate complex (enzyme-malonyl complex) in order to inhibit the reaction. This would tend to rule out non-specific binding as a cause of enzyme inhibition.

The kinetics of kievitone inhibition suggest that the phytoalexin acts at a site other than the malonyl-CoA- or 4-coumaroyl-CoA-binding sites. It is, therefore, possible that, in vivo, kievitone may act as a feedback inhibitor of its own synthesis in the classical manner. For such a hypothesis to be tenable, further confirmation would be required concerning the sites of kievitone synthesis and accumulation. In wounded bean cotyledons, large increases in chalcone synthase activity are observed in tissue which has already accumulated high levels of kievitone [23]. However, analysis of *Colletotrichum*-infected bean hypocotyls has indicated that elevated chalcone synthase activities occur both in the infected area and in apparently healthy tissue surrounding the infection site, although such healthy tissue may later show symptoms of infection [24]. Therefore, synthase activity and high concentrations of kievitone may, to some extent, be separated spatially in infected tissue. A possible role for kievitone inhibition of chalcone synthase may be in protecting the cells producing the phytoalexin and its precursors from phytotoxic concentrations of kievitone. The validity of this hypothesis would depend upon kievitone finally accumulating in cells other than those involved in its synthesis at any given time.

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