PHYTOALEXINS: ENZYMIOLOGY AND MOLECULAR BIOLOGY


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Phytoalexins are host-synthesized, low molecular weight, broad-spectrum antimicrobial compounds whose synthesis from distant precursors is induced in plants in response to microbial infection or treatment of plant tissues with a wide range of naturally occurring or synthetic, artificial compounds (biotic or abiotic elicitors). Phytoalexins were first implicated in the protection of susceptible potato cultivars against infection with a virulent race of Phytophthora infestans (potato late blight) as a result of pre-inoculation of tuber tissue with a race of the fungus to which the potato cultivar was resistant (1,2). It was a further 20 years before the first reported phytoalexin, pisatin, was isolated from fungally infected pea pods and its pterocarpanoid structure determined (3,4). During the two subsequent decades, many new phytoalexins have been isolated and identified from other plant species, these compounds belonging to such structurally diverse chemical classes as isoflavonoid, terpenoid, stilbene, polyacetylene, and dihydrophenanthrene. The reader is referred to several excellent reviews dealing wholly, or in part, with the structure, occurrence, and biological activity of phytoalexins (5–14).

Two important factors are implied in the definition of a phytoalexin. First, phytoalexins are believed to be crucial components of the overall expression of disease resistance in the plant cells in which they accumulate; although the evidence supporting this view is strong, it is nevertheless indirect (see Section V), and the study of phytoalexin induction is still, therefore, of much interest to plant pathologists. Second, phytoalexins are
not found in healthy, uninfected tissues; as their induced synthesis has conclusively been demonstrated by radioisotopic labeling experiments, phytoalexin induction has recently become of great interest to the biochemist as a suitable experimental system for investigating the regulation of enzyme levels, and possibly de novo gene expression, in plant cells. A knowledge of the key enzymes of phytoalexin metabolism is therefore an important starting point for the study of the expression of disease resistance in plants at the molecular level.

Accumulation of phytoalexins is not the only induced biochemical defense mechanism conferring disease resistance to plants; the involvement of induced, physical, cell surface barriers (15-17), agglutinins (18), and protease inhibitors (18,19) has also been implicated in certain plant-pathogen interactions. It is only through detailed investigation of these and other possible resistance phenomena that we will eventually arrive at an understanding of the exact nature of the resistance genes whose manipulation by classical methods still plays an important role in plant breeding programs. With the advent of recombinant DNA technology, the opportunity now exists for significant advances to be made in the field of plant disease resistance in the coming years (20). In addition to its main theme of discussing the enzymological aspects of phytoalexin metabolism and its control, it is hoped that the present review will focus attention on areas where the tools of modern molecular biology can be most fruitfully applied.

There are many aspects of the phytoalexin induction process which appear outside the scope of this review, although they are so much a part of an integrated, temporal sequence of events that they cannot be wholly ignored in the discussions that follow. These areas include (a) initial recognition between host and pathogen, (b) the biochemical determination of race specificity, (c) the structure and mode of action of fungal elicitors, (d) the nature of the putative receptors for fungal elicitors and the sites of action of abiotic elicitors, and (e) possible inter- and intracellular transmission of elicitation. Detailed information on these and other aspects of the biochemistry of plant disease resistance is available in a number of recent review articles (10, 18, 21-26). In the present review, sufficient background information will be given to enable the reader who is approaching this field for the first time to place the discussions of biosynthesis, enzymology, and regulation in a wider plant pathological context.

As attention has only recently been turned to biosynthetic aspects of phytoalexin accumulation, it is unavoidable that in some cases the enzymic
reactions to be discussed have only been investigated in plant species other than those producing a particular class of phytoalexin; alternatively, in fewer cases, the enzymes may only have been well characterized in animal systems. It is hoped that the inclusion of information obtained from other organisms will be of value in directing attention to possible avenues for future research in the context of the phytoalexin pathways.

II. Isoflavonoid Phytoalexins

For historical and scientific reasons, the isoflavonoids are the most studied class of phytoalexins with respect to their occurrence and metabolism. Although potato, a producer of terpenoid phytoalexins, was the subject of the investigations that led to the formulation of the phytoalexin hypothesis (1,2), the first fully characterized phytoalexins were isolated from members of the Leguminosae; the garden pea (*Pisum sativum*), dwarf French bean (*Phaseolus vulgaris*), and soybean (*Glycine max*), therefore, soon became favored experimental material. Of equal importance is the fact that the biosynthesis of the isoflavonoid phytoalexins was known to share a common series of reactions with the general phenylpropanoid pathway leading to lignin formation, and much of the enzymology of this pathway had already been elucidated by the time chemists and biochemists turned their attention to phytoalexin biosynthesis. The following sections do not attempt to deal with all known isoflavonoid phytoalexins. The section on structure and occurrence presents only a general cross section of the different types of isoflavonoid compound encountered, whereas the discussion of enzymology and regulation is limited to those few systems where sufficient detailed work has been done to enable a coherent overall picture to be presented. As many reactions are common to the synthesis of different isoflavonoid phytoalexins, many general details of enzymology will be relevant to plants whose phytoalexin metabolism has not yet been investigated.

A. STRUCTURE AND OCCURRENCE

Most phytoalexins are chemically and biogenetically related to known classes of secondary products which may accumulate constitutively in the same, or taxonomically related, plant species. Table I summarizes the structures and sources of the most important postinfectionally formed isoflavonoid derivatives of the Leguminosae. Other strongly or weakly antifungal isoflavonoids may occur as preformed metabolites, for example,
the isoflavone formononetin (XVI) in *Medicago sativa*, formononetin and biochanin A (XVII) in *Cicer arietinum* and *Trifolium pratense*, and luteone (XVIII) in the leaves of *Lupinus albus* (27). Most of the compounds in Table I conform to the definition of phytoalexins as given earlier. Exceptions include genistein and 2'-hydroxygenistein which, although accumulating with phaseollin and kievitone in infected (28) and wounded (53) tissues of *Phaseolus vulgaris*, are only weakly antimicrobial. These two isoflavones also serve as precursors for the true phytoalexin kievitone (see Section II.B.4). Coumestrol is sometimes classed as a phytoalexin, although its biological activity is antibacterial rather than antifungal (51); it accumulates, along with related coumestans and true isoflavonoid phytoalexins, in several legumes including *Phaseolus vulgaris* (51, 52) and *Medicago sativa* (50). Maackiain occurs as a phytoalexin in *Pisum sativum* (43) and *Trifolium pratense* (41). It is, however, also present as the preformed glucoside trifolirhizin in heartwood, callus, or root tissue of several species including *Trifolium* and *Sophora* (7).

It will be seen from Table I that different plant species produce different groups of closely related phytoalexins; the main exception within the Leguminosae is the production, along with medicarpin, of the acetylenic
<table>
<thead>
<tr>
<th>Chemical class</th>
<th>Trivial name</th>
<th>Compound number</th>
<th>Structure</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflavone</td>
<td>Genistein</td>
<td>(I)</td>
<td><img src="image" alt="Structure" /></td>
<td>Phaseolus vulgaris</td>
<td>(28)</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>2'-Hydroxygenistein</td>
<td>(II)</td>
<td><img src="image" alt="Structure" /></td>
<td>Phaseolus vulgaris</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cajanus cajan</td>
<td>(29)</td>
</tr>
<tr>
<td>Isoflavone</td>
<td>Wighteone</td>
<td>(III)</td>
<td><img src="image" alt="Structure" /></td>
<td>Glycine wightii</td>
<td>(30)</td>
</tr>
<tr>
<td>Isoflavanone</td>
<td>Kievitone</td>
<td>(IV)</td>
<td><img src="image" alt="Structure" /></td>
<td>Phaseolus vulgaris</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vigna unguiculata</td>
<td>(32)</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Vigna sinensis</td>
<td>(33)</td>
</tr>
<tr>
<td>Isoflavan</td>
<td>Vestitol</td>
<td>(V)</td>
<td><img src="image" alt="Structure" /></td>
<td>Lotus corniculatus</td>
<td>(34)</td>
</tr>
</tbody>
</table>
Isoflavan  Sativan  (VI)  

Isoflavan  Phaseollinisoflavan  (VII)  

Isoflavan  2'-Methoxyphaseollinisoflavan  (VIII)  

Pterocarpan  Medicarpin  (IX)  

Pterocarpan  Maackiain  (X)  

Pterocarpan  Pisatin  (XI)  

Lotus corniculatus  (34)  
Medicago sativa  (34)  
Phaseolus vulgaris  (35)  
Phaseolus vulgaris  (36)  

Vicia faba  (37)  
Vigna unguiculata  (38)  
Canavalia ensiformis  (39)  
Medicago sativa  (40)  
Trifolium pratense  (41)  
Cicer arietinum  (42)  
Pisum sativum  (43)  
Trifolium pratense  (41)  
Cicer arietinum  (42)  
Pisum sativum  (3,4)
<table>
<thead>
<tr>
<th>Chemical class</th>
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<th>Compound number</th>
<th>Structure</th>
<th>Species</th>
<th>References</th>
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</thead>
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<tr>
<td>Pterocarpan</td>
<td>Phaseollidin</td>
<td>XII</td>
<td><img src="image" alt="Structure" /></td>
<td>Phaseolus vulgaris</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vigna sinensis</td>
<td>(45)</td>
</tr>
<tr>
<td>Pterocarpan</td>
<td>Phaseollin</td>
<td>XIII</td>
<td><img src="image" alt="Structure" /></td>
<td>Phaseolus vulgaris</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>Vigna sinensis</td>
<td>(45)</td>
</tr>
<tr>
<td>Pterocarpan</td>
<td>Glyceollin</td>
<td>XIV</td>
<td><img src="image" alt="Structure" /></td>
<td>Glycine max</td>
<td>(48, 49)</td>
</tr>
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<td>(Isomer I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coumestan</td>
<td>Coumestrol</td>
<td>XV</td>
<td><img src="image" alt="Structure" /></td>
<td>Medicago sativa</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Phaseolus vulgaris</td>
<td>(51, 52)</td>
</tr>
</tbody>
</table>
phytoalexin wyerone (see Section IV.A) by *Vicia faba* (54, 55). Also, the same phytoalexin may be found in a variety of different, but closely related species, this being particularly the case with medicarpin. The relative proportions of the different phytoalexins accumulating in a given plant species may depend upon the nature of the invading pathogen (56,57).

The reader is referred to the articles by Cruickshank (5,12), Van Etten and Peuppke (9), and Rizk and Wood (11) for further details of the structure, occurrence, and mode of action of isoflavonoid phytoalexins.

**B. BIOSYNTHESIS AND ENZYMEOLOGY**

1. **The Central Phenylpropanoid Pathway**

The central or “core” phenylpropanoid pathway results in the formation of hydroxycinnamoyl coenzyme A thiol esters from L-phenylalanine (Scheme 1). The esters of 4-coumaric and caffeic acids are precursors in the biosynthesis of flavonoids (including anthocyanins and condensed tannins), isoflavonoids, and chlorogenic acid, whereas the esters of ferulic and sinapic acids, after reduction to the corresponding side-chain alcohols, may undergo oxidative polymerization to yield lignin. Induced lignification is an important defense response in many plants including potato (58) and wheat (59,60). Furthermore, hydroxycinnamic acids have recently been shown to serve as precursors for the stilbene and dihydrophenanthrene phytoalexins (see Section IV); increased flux through the central phenylpropanoid pathway as a result of fungal infection is not, therefore, limited to those plants which produce isoflavonoid phytoalexins.

The operation and control of the phenylpropanoid pathway during the biosynthesis of lignin and flavonoids has been the subject of several excellent reviews (61-65). The role of this pathway in isoflavonoid phytoalexin biosynthesis, in addition to the provision of early evidence for induced *de novo* synthesis of these phytoalexins, has clearly been demonstrated by radioactive labeling experiments. In immature pea pods, [U-14C]phenylalanine and [1,14C]cinnamic acid were readily incorporated into pisatin following treatment of the tissues with the abiotic elicitor CuCl2 or spore suspensions of *Monilinia fructicola* (66,67). [14C]Phenylalanine was incorporated into glyceollin (XIV), the isoflavone daidzein (XIX), and the coumestans coumestrol (XV) and sojagol (XX) in soybean hypocotyls infected with an incompatible race of the fungal pathogen *Phytophthora megasperma* var *sojae* (*P. megasperma* f.sp.*glycinea*) (68), and into medicarpin (IX) in CuCl2-treated seedlings of *Trifolium pratense* (69). Phenyl-
Scheme 1. The central phenylpropanoid pathway.
alanine and cinnamic acid were similarly good precursors of phaseollin (XIII) in excised pods (70,71) and tissue cultures (72,73) of Phaseolus vulgaris.

L-Phenylalanine ammonia-lyase (PAL, E.C.4.3.1.5) catalyzes the first committed step in the biosynthesis of phenylpropanoid compounds: the elimination of ammonia from L-phenylalanine to yield trans-cinnamic acid (reaction 1).

\[
\begin{align*}
\text{H} & \text{C} & \text{C} & \text{COOH} & \leftrightarrow & \text{H} & \text{C} & \text{C} & \text{COOH} + \text{NH}_3 \\
\text{H} & \text{H} & \text{H} & \text{H} & & \text{H} & \text{C} & \text{C} & \text{COOH} + \text{NH}_3
\end{align*}
\] (1)

Since its discovery in 1961 by Koukol and Conn (74), a great deal of attention has been given to the regulation of the enzyme in response to light, plant growth substances, wounding, and fungal, bacterial, or viral infection. It has been the subject solely, or in part, of several useful reviews (61,75-77). In this section the general properties of the enzyme are summarized, and its role in the phytoalexin response is discussed. The
complex biochemical control mechanisms which may underlie changes in PAL levels or activity will be discussed in Section V.

PAL is a tetrameric enzyme; from most plant sources the molecular weight is around 330,000, the four subunits appearing identical with molecular weights of around 83,000 (Table II). Exceptions to this include the enzyme from mustard cotyledons, which is composed of four 55,000 molecular weight subunits (78), and the enzyme from wheat leaves, reported to consist of two subunits of molecular weight 75,000 and two of 85,000 (79). PAL possesses two functionally active sites per tetramer (80), and, like histidine ammonia-lyase, the active sites contain a dehydroalanine residue (81–83). This arises via post-translational modification, and it is not believed to be part of an orthodox peptide chain but rather to be present in a Schiff's base linkage at the active site (81). A mechanism of action for the enzyme has been proposed (81) in which the amino group of phenylalanine adds to the β-position of the dehydroalanyl double bond; this mechanism results in the formation of enzyme-ammonia and enzyme-cinnamate intermediates and can account for the correct stereochemical elimination of the pro-3S hydrogen of phenylalanine leading to trans-elimination of ammonia (81). The enzyme from all sources is product inhibited by trans-cinnamic acid. This results from product binding at the active site, since both cinnamic acid and phenylalanine can protect the enzyme from inactivation resulting from the reduction of the dehydroalanine residue by sodium borohydride (84) or its reaction with sodium cyanide (85).

PAL from many plant sources is negatively rate cooperative (Hill coefficient <1.0) with respect to L-phenylalanine (84,86,88,91). This fact, coupled with the competitive nature of the inhibition by trans-cinnamic acid (86,87), implies that the enzyme in vivo is far more sensitive to changes in cinnamic acid levels than to changes in phenylalanine pool size (92). Cinnamic acid is therefore an important component in the regulation of the flux through the phenylpropanoid pathway, and the regulatory role of this compound is further amplified by its effects on the maintenance of the level of the enzyme (see Section V). Detailed kinetic analysis of PAL from wheat seedlings (93) has indicated that the enzyme follows neither the classical allosteric model of Monod et al. (94) nor the simple sequential model of Koshland et al. (95). A partially concerted mechanism has been proposed (93) in which the binding of substrate induces a conformational change in the corresponding subunit as well as the appearance of a new conformation of the unliganded protomer. PAL
<table>
<thead>
<tr>
<th>Species</th>
<th>Molecular weight</th>
<th>Subunit molecular weight</th>
<th>pH optimum</th>
<th>$K_m$ (molar)</th>
<th>Hill coefficient</th>
<th>Activity with L-tyrosine</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><em>Cucumis sativus</em></td>
<td>316,000</td>
<td></td>
<td>8.8</td>
<td>$2.9 \times 10^{-4}$</td>
<td>0.65</td>
<td></td>
<td>(86)</td>
</tr>
<tr>
<td><em>Nicotiana tabacum</em></td>
<td>330,000</td>
<td>83,000</td>
<td>8.0-8.6</td>
<td>$2.2 \times 10^{-4}$</td>
<td>-</td>
<td></td>
<td>(87)</td>
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<td><em>Petroselinum hortense</em></td>
<td>240,000</td>
<td>55,000</td>
<td>8.6</td>
<td>$1.5 \times 10^{-4}$</td>
<td>1.08</td>
<td>-</td>
<td>(89)</td>
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<tr>
<td><em>Rhodotorula glutinis</em></td>
<td>275,000-300,000</td>
<td></td>
<td>8.5</td>
<td>$3.0 \times 10^{-4}$</td>
<td>+</td>
<td></td>
<td>(85)</td>
</tr>
<tr>
<td><em>Sinapis alba</em></td>
<td>330,000</td>
<td>83,000</td>
<td>8.75</td>
<td>$3.8 \times 10^{-5}$</td>
<td>-</td>
<td></td>
<td>(80,81,84)</td>
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<td><em>Solanum tuberosum</em></td>
<td>226,000</td>
<td></td>
<td>9.0</td>
<td>$2.6 \times 10^{-4}$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sporobolomyces pararoseus</em></td>
<td>275,000-300,000</td>
<td></td>
<td>8.5</td>
<td>$3.0 \times 10^{-4}$</td>
<td>+</td>
<td></td>
<td>(85)</td>
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<tr>
<td><em>Streptomyces verticillatus</em></td>
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<td></td>
<td>9.0</td>
<td>$2.7 \times 10^{-4}$</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Triticum aestivum</em></td>
<td>320,000</td>
<td>75,000(2)</td>
<td>8.5-9.2</td>
<td>$2.9 \times 10^{-5}$</td>
<td>+</td>
<td></td>
<td>(79)</td>
</tr>
<tr>
<td><em>Zea mays</em></td>
<td>330,000</td>
<td>83,000</td>
<td>8.7</td>
<td>$2.7 \times 10^{-4}$</td>
<td>-</td>
<td></td>
<td>(91)</td>
</tr>
</tbody>
</table>
preparations from monocots and microorganisms catalyze the deamination of tyrosine to yield 4-coumaric acid (82,85,91).

PAL is often regarded as a soluble cytoplasmic enzyme, existing in only one form in a given plant species. There are, however, reports of the existence of (a) PAL isoenzymes which may differ in their affinities for the substrate and the extents of their inhibition by phenolic compounds (96-98), (b) minor forms possibly associated with microsomal multi-enzyme complexes (99) or (c) forms found in subcellular organelles (100,101). Spinach leaves contain three forms of PAL separable by ion-exchange chromatography (101). The major form is the soluble cytoplasmic enzyme, and the other two forms are found in the chloroplast; one of the chloroplast forms in light activated via reduced thioredoxin (101). Little attention seems to have been paid to the possible role of different forms of PAL in plants whose phytoalexins are synthesized via this enzyme.

In most cases studied, the accumulation of isoflavonoid phytoalexins by either fungal infection or biotic and abiotic elicitors is preceded by increases in extractable PAL activity. In pea endocarp tissues, a wide range of abiotic treatments which induced pisatin (XI) accumulation also stimulated in vivo PAL activity; these treatments included heavy metal salts (102,103), polypeptides and polyamines (104,105), DNA intercalating compounds (106), antiviral, antimalarial, and antihistamine drugs (107), bromodeoxyuridine (108), low concentrations of RNA- and protein-synthesis inhibitors (102,109), and ultraviolet light (110). It has been proposed that all these treatments act directly at the level of the genome to cause de-repression of the genes coding for PAL and, presumably, other enzymes of pisatin synthesis (109). However, how such structurally diverse abiotic elicitors could be directly responsible for specific gene induction is still not clear; it is possible that enzyme induction and phytoalexin accumulation in response to these treatments result indirectly from the toxic effects of the chemicals applied or the ability of some of them to destabilize cell membranes.

Several authors have suggested that PAL may not be an important regulatory point in isoflavonoid phytoalexin induction. For example, the induction of kievitone (IV) accumulation in hypocotyls of *Vigna sinensis* in response to CuCl₂ or low concentrations of actinomycin D or cycloheximide was associated with increased PAL activity throughout the hypocotyls, whereas the phytoalexin only accumulated in the upper, treated half; this was interpreted as evidence against a regulatory role for
PAL in kievitone accumulation in *Vigna* (111). Similarly, in *Glycine max*, PAL, chalcone isomerase, and peroxidase induction was observed in wounded or fungally infected tissues, whereas glyceollin (XIV) accumulation only preceded infection (112). The role of PAL in the above systems may not be finally assessed until detailed information is available concerning (*a*) the actual cellular and subcellular sites of phytoalexin synthesis (as opposed to accumulation), and (*b*) the fluxes through the phenylpropanoid pathway, as assessed by qualitative and quantitative analysis of rates of total phenylpropanoid metabolism, under different experimental conditions.

In cell suspension cultures of *Glycine max* and *Phaseolus vulgaris*, PAL induction preceded the accumulation of isoflavonoid phytoalexins in response to biotic elicitor macromolecules heat-released from the cell walls of *Phytophthora megasperma* var *sojae* (*P. megasperma* f.sp.*glycinea*) and *Colletotrichum lindemuthianum*, respectively (113,114); enzyme induction in these systems is clearly independent of any wound response. Furthermore, in *P. vulgaris* cell suspensions, the extent of PAL induction was dependent on elicitor concentration in a manner suggesting complex regulatory phenomena (115,116; see Section V); the elicitor dose-response curves observed for enzyme induction in cell suspensions were of a similar pattern to the curves obtained for the variation of isoflavonoid levels with elicitor concentration in wounded cotyledons, in spite of the stimulation of phytoalexin accumulation by wounding in this system (117). Analysis of total phenolic levels in hypocotyls (118) and cell suspension cultures (52) of *P. vulgaris* during accumulation of phytoalexins in response to abiotic elicitors has indicated a specific stimulation of isoflavonoid accumulation in the absence of changes in the levels of many of the other classes of phenylpropanoid compound known to occur constitutively in *P. vulgaris*. It is therefore clear that further regulation of the isoflavonoid phytoalexin pathways occurs at points subsequent to the PAL reaction.

That PAL can be the main rate-limiting enzyme in flavonoid biosynthesis has been clearly demonstrated in parsley cell suspension cultures accumulating flavone glycosides in response to ultraviolet irradiation, where the time integrals for PAL activity and flavonoid accumulation are equal (65). Evidence from other systems is still indirect; in this respect, Margna (119) has argued that substrate supply (i.e., phenylalanine pool size) may determine the flux through the phenylpropanoid pathway. This view does not help explain the physiological role of the negative cooperativity exhibited by PAL; nor is it supported by the often constant phenylalanine
pool sizes measured during rapid fluctuations in the rates of phenylpropanoid synthesis (120,121) and the limited evidence against changes in the levels or extractable activities of enzymes of the shikimic acid pathway during phenylpropanoid biosynthesis (122). This latter area of metabolism has not been studied in relation to phytoalexin accumulation.

Conversion of trans-cinnamic acid to 4-coumaric acid (XXI) is catalyzed by the enzyme cinnamic acid 4-hydroxylase (CA4H, E.C. 1.14.13.11), a mixed function monooxygenase. The enzyme was first characterized in pea seedlings (123), where it appears specific for unsubstituted cinnamic acid and catalyzes the insertion of an atom of oxygen into cinnamic acid from molecular oxygen, with resultant oxidation of NADPH (reaction 2).

\[
\begin{align*}
\text{C} & - \text{C}=\text{COOH} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{HO}-\text{C} & - \text{C}=\text{COOH} + \text{H}_2\text{O} + \text{NADP}
\end{align*}
\]

The enzyme from parsley was shown to be absolutely specific for the trans-isomer of cinnamic acid, with cis-cinnamic acid acting as a competitive inhibitor (124). In in vitro assays, CA4H requires the presence of a thiol reagent; 2-mercaptoethanol is usually employed, although the enzyme from gherkin tissues is strongly inhibited by this compound, and requires reduced glutathione for maximal activity (125).

As with other enzymes catalyzing related aromatic hydroxylations in both animals and plants, CA4H is associated with cytochrome P450 and is located in the microsomal fraction. P450 involvement has been demonstrated by the light-reversible inhibition of cinnamic acid hydroxylation by carbon monoxide (123,126), and the absorption spectrum of the enzyme preparation changes, on binding of cinnamic acid, in a manner characteristic of P450-linked systems (127). Treatment of parsley microsomal preparations with phospholipase A2 completely removed CA4H activity, the activity being partially restored by addition of crude microsomal lipid (128).

The mechanism of action of CA4H is believed to follow the pattern elucidated for animal microsomal mixed function oxygenases; the retention of $^3$H at position 4 in 4-coumaric acid indicated that the pea enzyme catalyzed a reaction involving an "NIH shift" (129) in a manner similar to the microsomal hydroxylases from rat liver (130). Such reactions are believed to take place via an arene oxide intermediate (131). Further
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Details of the mechanism of P₄₅₀-linked monoxygenases, and their roles in plants, are available elsewhere (132, 133).

Most studies on the modulation of CA₄H in plants have concentrated on the appearance of the enzyme activity in wounded and/or illuminated storage tissues (134–136), there being few reports of its induction in systems producing phytoalexins. In illuminated potato tuber discs, the CA₄H activity accounts for a large proportion of the cytochrome P₄₅₀ detectable by difference spectrophotometry (127), although this is not the case with many other plant microsomal preparations; there is clearly a need for studies of P₄₅₀ levels in fungally infected plant cells. In *Phaseolus vulgaris* cell suspension cultures producing phaseollin in response to the abiotic elicitor denatured ribonuclease A, CA₄H activity increases linearly for up to 48 hr after elicitor addition (114). This response is slower than the associated transient increase in PAL activity, whereas parallel increases in the two enzymes are often observed in storage tissues (135, 137). Induction of CA₄H activity has been reported in cotyledons of *Glycine max* producing glyceollin in response to a polysaccharide elicitor heat-released from the cell walls of *Phytophthora megasperma* f.sp. *glycinea* (138).

The discovery that coenzyme A thiol esters of hydroxycinnamic acids were important intermediates in the biosyntheses of lignin and flavonoid derivatives was soon followed by the isolation, from various sources, of enzymes capable of catalyzing their formation (139–143). The hydroxycinnamoyl-CoA ligases (EC. 6.2.1.12) have been most studied in two systems: *(a)* differentiating shoots producing lignin and *(b)* cell suspension cultures producing either flavonoids and/or ligninlike material. The reaction catalyzed is 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).

![Chemical reaction](https://example.com/reaction3.png)

Enzyme + R–CH=CH–COOH + ATP $\xrightleftharpoons{Mg^{2+}}$ Enzyme[R–CH=CH⋅CO⋅AMP] + PP$_i$

Enzyme[R–CH=CH⋅CO⋅AMP] + CoA⋅SH $\xrightarrow{\text{}}$ R–CH=CH⋅CO⋅S⋅CoA + AMP + Enzyme.

The enzyme from most sources has a molecular weight of around 55,000–67,000. Cell suspension cultures of *Glycine max* contain two isoenzymic forms of the CoA ligase, separable by ion-exchange chromatography; these had different pH optima, but both were competitively inhibited by AMP (143). The enzyme from cell suspension cultures of *Petroselinum hortense*
exhibited non-Michaelis Menten kinetics (positive cooperativity, Hill coefficient = 1.5 for both ATP and CoA), thus suggesting the presence of more than one binding site for each of these substrates (144); however, normal Michaelis Menten kinetics were observed when only measuring the first half-reaction by [\(^{32}\)P]pyrophosphate exchange in the absence of CoA.

A comparison of the relative activities of the hydroxycinnamoyl CoA ligases from various sources with differentially substituted cinnamic acids as substrates has been given elsewhere (144). In plants possessing isoenzymic forms, substrate specificity studies have suggested that one form may be involved in lignin biosynthesis [generally active with ferulic (XXIII) and sinapic (XXIV) acids as substrates], whereas the other form may be involved in flavonoid/isoflavonoid biosynthesis [no activity with sinapic acid; highest activity with 4-coumaric (XXI) and caffeic (XXII) acids (143)]. The two isoenzymes from Glycine max, which showed different substrate specificity as noted above, were also inhibited to different extents by AMP, the extent of inhibition being dependent on ATP levels (143). 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 flavonoid pathways (145).

In cell suspension cultures and cotyledons of Glycine max (65,138) and cell suspension cultures of Phaseolus vulgaris (114), increases in hydroxycinnamoyl CoA ligase activity (measured vs. 4-coumaric acid) accompanied the formation of glyceollin (XIV) and phaseollin (XIII) in response to a biotic elicitor from Phytophthora megasperma f.sp.glycinea (G. mar) or denatured RNAase A (P. vulgaris). A detailed knowledge of the substrate specificities of the CoA ligases induced by elicitors may have important implications for iso flavonoid phytoalexin biosynthesis. All iso flavonoid phytoalexins are either hydroxylated or further substituted at position 4' on the B ring (iso flavonoid numbering); this corresponds to position 4 of the hydroxycinnamic acid precursor. 2'-Hydroxylation of the B ring is believed to occur at the iso flavonoid stage; however, phytoalexins such as pisatin (XI) and maackiain (X) also possess a 4',5'-methylene dioxy substituent on ring B (see Section II.B.4). The initial oxygenation pattern (4',5'-dihydroxy) may arise either at the level of the C₂₃C₃ precursor or, as suggested by radiolabeling experiments (146,147), at the C₁₅ stage; in the first case, the involvement of a CoA ligase with activity toward caffeic acid (XXII) would be predicted. Such substrate specificity would also be reflected in the next enzyme of the pathway, the chalcone synthase.
2. Enzymic Formation of Chalcones

The intermediacy of a chalcone in the biosynthesis of isoflavonoid phytoalexins has been demonstrated by radiolabeling experiments utilizing 2',4,4'-trihydroxychalcone (XXVI) in seedlings of *Medicago sativa* and *Trifolium pratense* treated with the abiotic elicitor CuCl₂ (148,69), in hypocotyls of *Glycine max* infected with *Phytophthora megasperma* f.sp.*glycinea* (68), and during coumestrol biosynthesis in *Phaseolus aureus* (149).

Chalcone synthase (CHS, formerly called flavonone synthase) was first detected in cell suspension cultures of *Petroselinum hortense* following irradiation with ultraviolet light (159), a treatment that results in the accumulation of flavone glycosides. The enzyme activity has since been observed in several plant systems including cotyledons of *Glycine max* (151), anthers of *Tulipa* c.v. "Apeldoorn" (152), cell suspension cultures and cotyledons of *Phaseolus vulgaris* (114,117), cell suspension cultures of *Haplopappus gracilis* (153,154), and in seedlings of *Pisum sativum*, *Brassica oleracea*, and *Spinacia oleracea* (155,156). Under the assay conditions originally employed (150), the *Petroselinum* enzyme catalyzed the formation of the flavanone naringenin (XXV) from one molecule of 4-coumaroyl CoA (XXIa) and three molecules of malonyl CoA, the C₆C₃ unit forming the B ring of the flavanone and the A ring being formed by head-to-tail condensation of acetate (malonyl) units, as originally predicted by Birch and Donovan (157) (reaction 4).
The formation of the flavanone naringenin rather than its isomeric chalcone (XXVII) was somewhat unexpected since the results of radiosampling experiments had suggested that the chalcone might be a more immediate precursor of flavonoid and isoflavonoid compounds than its corresponding flavanone (158). Also, mutants of Callistephus chinensis, which accumulated naringenin chalcone-2'-glucoside in their blossoms, were shown to be deficient in chalcone isomerase, the enzyme responsible for the interconversion between chalcones and flavanones (159). A similar situation was observed in mutants of Petunia hybrida, where naringenin chalcone accumulated in the pollen of recessive genotypes lacking the isomerase (160). These results clearly showed that the chalcone biosynthetically precedes the flavanone. In addition, $^{13}$C NMR experiments have indicated the direct intermediacy of the chalcone during the formation of kaempferol from $[^{13}$C$]$acetate in cell suspensions of P. hortense (161) and during kievitone (IV) formation from $[^{13}$C$]$acetate in cotyledons of Phaseolus vulgaris (162). Naringenin chalcone (XXVII) is unstable and spontaneously cyclizes to the flavanone at the usual pH of the CHS assay (pH 8.0). It has recently been confirmed that, under certain assay conditions, the enzymes from both P. hortense (163) and Tulipa (164) can produce naringenin chalcone as the main product. In the case of the Tulipa enzyme, the conditions involved a two-phase assay system with enzyme present in a viscous lower phase and substrates present in an upper phase (pH 4.0) where chalcone could accumulate without isomerization (164).

The properties of CHS from various sources are summarized in Table III. Of the three hydroxycinnamoyl-CoA-thiol esters [4-coumaroyl (XXIa), caffeoyl (XXIIa), and feruloyl (XXIIIa)] tested as substrates for the P. hortense enzyme, only 4-coumaroyl resulted in flavanone formation at pH 8.0 (165), although eriodictyol (XXVIII) formation was observed from caffeoyl CoA when either the P. hortense or Haplopappus gracilis enzymes were assayed at pH 6.0 (154). In contrast, the enzyme from
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Molecular weight</th>
<th>Number of subunits</th>
<th>pH optimum</th>
<th>Release products</th>
<th>$K_m$ (μM)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brassica oleracea</td>
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<td>8.0</td>
<td>6.5-7.0</td>
<td>None</td>
<td></td>
<td>(155)</td>
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<tr>
<td>Haplopappus gracilis</td>
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<td></td>
<td></td>
<td>Styrylpyrone</td>
<td></td>
<td>(154)</td>
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<td>(150,154,</td>
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<td></td>
<td></td>
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<td></td>
<td>Dihydropyrone</td>
<td>35</td>
<td>163,165,</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Benzalacetone</td>
<td></td>
<td>167</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Unidentified</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>2</td>
<td>8.0</td>
<td>21.4</td>
<td></td>
<td>(169,170)</td>
</tr>
<tr>
<td>Tulipa c.v. “Apeldoorn”</td>
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<td>1?</td>
<td>8.0</td>
<td>Approx. 1.7</td>
<td>1.7</td>
<td>(164,166)</td>
</tr>
</tbody>
</table>

**TABLE III**
Properties of Chalcone Synthase from Various Sources
Tulipa formed naringenin, eriodictyol, or homoeriodictyol (XXIX) with the substrates 4-coumaroyl-, caffeoyl-, or feruloyl CoA, respectively (166).

An interesting feature of CHS is the formation of "release" or "derailment" products: partially purified preparations of the synthase from P. hortense (but not the Tulipa enzyme) in the presence of high concentrations of 2-mercaptoethanol or dithioerythritol (5–12 mM) produce, in addition to naringenin, 4-OH-6[4-OH-styryl] 2-pyrone (bis-noryangonin, XXX), 4-OH-5,6-dihydro-6-(4-hydroxyphenyl)2-pyrone (XXXI), and p-hydroxybenzalacetone (XXXII) (165,167). 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 (167), whereas the other two products are released after condensation with one molecule of malonyl CoA (165). The reactions leading to these products are shown in Scheme 2. Similar release of a partially completed polyketide (as triacetic acid lactone) occurs with the enzyme 6-methylsalicylic acid synthase from Penicillium patulum (168).

Early results indicated several similarities between the P. hortense CHS and the enzyme fatty acid synthase (FAS). Both enzymes are strongly inhibited by sulphydryl reagents and the antibiotic cerulenin, which inhibits the β-ketoacyl-ACP synthetase, or "condensing enzyme," of the FAS complex (150); both catalyze the transfer of malonate from malonyl CoA to pantotheine (150) and both can decarboxylate malonyl CoA to yield acetyl CoA (171). These findings, coupled with the original report of a high molecular weight for CHS (120,000, as determined by gel-filtration), led to the proposal of a hypothetical mechanism of action based on the priming and condensing reactions of FAS and involving transfer of 4-
Scheme 2. Formation of release products by chalcone synthase (165).
coumaroyl, malonyl, or partially completed polyketide units between at least two active SH groups on the enzyme (150). More recently the molecular weight of the enzyme has been shown, by SDS PAGE and sedimentation equilibrium measurements, to be about 77,000, and analysis of the size of the CHS mRNA has strongly suggested that the enzyme consists of two identical subunits. Furthermore, amino acid analysis and bacterial growth bioassays have failed to show the presence of cysteamine, β-alanine, or pantothenate in hydrolysates of the purified enzyme (172). The demonstration of 14C exchange between 14CO₂ and malonyl CoA in the absence of 4-coumaroyl CoA has indicated that malonyl-CoA decarboxylation occurs prior to condensation (171). Minimum catalytic activities now ascribed to the enzyme are transfer of primer to the enzyme surface and condensation (172). Further activities, for example, thiolesterase, may not be needed if the chalcone is released from the enzyme by spontaneous cyclization of the final tri-β-ketoacyl derivative, and a free ACP or enzyme-bound pantetheinyl residue is not required as malonyl CoA itself is the immediate substrate in chalcone formation. This direct intermediacy of a CoA-thiol ester as substrate for the condensing reaction is the most striking difference between CHS and the related enzymes FAS and 6-methylsalicylic acid synthase. A scheme to account for this finding, and the malonyl-CoA decarboxylation and CO₂ exchange reactions, is shown in Scheme 3. 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 (172), and it has recently been suggested that CHS may have arisen via gene duplication (172).

CHS from all sources so far investigated catalyzes the formation of chalcones with phloroglucinol-type A-ring hydroxylation patterns, the three hydroxyl groups originating from the malonyl-CoA carboxyl groups involved in CoA-thiol ester linkage. This reflects the hydroxylation pattern of the flavonoids occurring in the particular plant source. However, most isoflavonoid phytoalexins lack the A-ring hydroxyl group at position 5 (see Table I). Exceptions include kievitone (IV) from *Phaseolus vulgaris* and wighteone (III) from *Glycine wightii*, and these phytoalexins are presumably synthesized via the normal “6'-hydroxy” CHS enzyme (“5-hydroxy,” flavanone/isoflavanone numbering). Radiolabeling experiments have demonstrated conclusively that 6'-hydroxy chalcones are not metabolically converted to 5-deoxy isoflavonoids (173), and it is therefore necessary to postulate the existence of a separate 6'-deoxy CHS to account for the formation of the majority of isoflavonoid phytoalexins. Indepen-
Scheme 3. Hypothetical scheme for the initial reactions catalyzed by chalcone synthase (171).
dent regulation of the 5-hydroxy and 5-deoxy pathways is also indirectly suggested by the different time courses for the appearance of 5-hydroxy and 5-deoxy classes of isoflavonoid in wounded cotyledons of *Phaseolus vulgaris* (117).

To date there has been no report of the direct characterization of a 6'-deoxy CHS in a cell-free system, although it has been suggested that formation of 5-deoxy isoflavonoids may be catalyzed by an enzyme system in some ways analogous to the 6-methylosalicylic acid synthase from *Penicillium patulum*. 6-Methylosalicylic acid is synthesized on a multi-enzyme complex from acetyl CoA (primer) and three molecules of malonyl CoA (168). The complex contains 4-phosphopantetheine (174), and is very similar to the type I (aggregated) FAS from yeast and from *P. patulum* itself (175); an NADPH-dependent reduction, and subsequent dehydration, of the enzyme-bound polyketide occurs at the triacetic acid level prior to the final condensation with malonyl CoA (168,174). A similar type of mechanism for 6'-deoxy chalcone formation has been deduced from a study of the incorporation pattern of \( ^{13}\text{C} \)acetate into the A ring of the pterocarpan phytoalexin pisatin (XI) in *Pisum sativum* (176) and into the A ring of phaseollin (XIII) in *Phaseolus vulgaris* (162). No details of the reductant involved are known; incubation of cell-free extracts from *P. vulgaris* with \( [\text{2-}\text{\textsuperscript{14}}\text{C}] \)malonyl CoA, 4-coumaroyl CoA, and NADH or NADPH generating systems only resulted in formation of labeled narigenin (XXV) (114).

Plants synthesize fatty acids via a type II (nonaggregated) FAS system. It is interesting to speculate that, in addition to the similarity between 6'-hydroxy CHS and \( \beta \)-ketoacyl-ACP synthetase, the 6'-deoxy CHS might make use of two further activities which are very similar to the separate \( \beta \)-ketoacyl-ACP dehydrogenase and \( \beta \)-hydroxyacyl-ACP dehydratase of type II FAS. Such a possibility awaits further investigation.

In spite of the important position of CHS as the first committed step in flavonoid/isoflavonoid biosynthesis, few studies have been made on its activity in plant cells producing phytoalexins. In cotyledons of *Glycine max*, CHS activity was induced by treatment with a cell wall suspension prepared from mycelium of *Phytophthora megasperma* f.sp.glycinea (151). The enzyme activity reached a maximum level by about 25 hr after treatment; this increase was subsequent to the induction of PAL, but it slightly preceded the accumulation of glyceollin (XIV). In cell suspension cultures of *Phaseolus vulgaris*, rapid transient increases in CHS activity preceded phaseollin (XIII) accumulation in response to both biotic
(Colletotrichum lindemuthianum cell wall-released) and abiotic (denatured RNAase A) elicitor preparations (116,114). In cotyledons of P. vulgaris, multiphasic changes in the levels of 5-hydroxy isoflavonoids in response to wounding were exactly paralleled by changes in CHS levels (117). Surprisingly, induction of CHS by light in cell suspension cultures of Phyttophthora hortense was totally inhibited by the elicitor from Phytophthora megasperma f.sp.glycinea (177). The molecular mechanisms which may underlie elicitor modulation of CHS are discussed in Section V.

In P. hortense, light induction of the enzymes of flavone glycoside formation is also associated with induction of acetyl-CoA carboxylase (EC 6.4.1.2), the enzyme directly responsible for provision of malonyl CoA (178). This enzyme has yet to be investigated in the context of isoflavonoid phytoalexin induction.

Chalcone isomerase (CHI, EC 5.5.1.6) catalyzes the stereospecific isomerization of chalcones to their corresponding (-) flavanones. The (-) (2S) configuration of the product was demonstrated by NMR studies of flavanone production catalyzed by either one of two isomerase isoenzymes partially purified from seedlings of Phaseolus aureus (179). The enzyme has also been purified from Glycine max (180,181), Petroselinum hortense (182), Cicer arietinum (182), and Phaseolus vulgaris (183). The G. max enzyme has a molecular weight of around 16,000 and consists of a single subunit, whereas the enzymes from P. vulgaris and P. hortense are larger, having molecular weights of around 28,000 and 50,000, respectively (183,150). The lack of inhibition of the G. max and P. vulgaris enzymes by tetrathionate (181,183) argues against the involvement of essential SH groups for activity as suggested earlier (180). A mechanism for the isomerization reaction involving an acid-base catalyzed formation of a flav-3-en-4-ol intermediate followed by a stereospecific proton transfer to form the flavanone was proposed (179), and the possible involvement of an enzyme imidazole or amino group was suggested (181). More recently it was shown that the existence of the chalcone 2'-hydroxyl group in an ionized form was essential for activity (184), this requirement being observed in profiles for the variation of \( V_{\text{max}} \) with pH for the forward reaction catalyzed by the G. max enzyme in the presence of 20% formamide. In view of these findings, a mechanism of action was proposed which involved nucleophilic addition of an imidazole group at the active site to the chalcone double bond followed by nucleophilic attack by the 2'-phenolate group, resulting in ring closure with inversion of configuration at C-2 (Scheme 4). The properties of CHI from several sources are
Scheme 4. Proposed mechanism for chalcone isomerase (184).
summarized in Table IV. The substrate specificities of the enzyme generally reflect the aromatic substitution patterns of the flavonoids found in the particular plant source (182). In this respect, CHI from Petroselium hortense is inactive against chalcones lacking the A-ring hydroxyl group corresponding to position 5 of the flavanone (182). A chalcone cyclase (isomerase) with very different properties has been isolated from Citrus paradisi. In order to act as substrate, the chalcone requires an unhindered B-ring hydroxyl group [unlike the enzyme from P. vulgaris (183)] and the 4' position must be glycosylated via β-linked neohesperidose (185).

Chemical models for the synthesis of isoflavonoids have predicted direct formation of isoflavones from chalcones (186,187), and this would imply no direct role for CHI in the synthesis of isoflavonoid phytoalexins. The enzyme does, however, increase in activity in Glycine max following infection by Phytophthora megasperma f.sp.glycinea (112), and in Phaseolus vulgaris cell suspension cultures treated with biotic (188) or abiotic (114) elicitors. In P. vulgaris the slow response of the enzyme to elicitor, and its high basal level, suggested the lack of any key regulatory role (188); CHI from this source was, however, competitively inhibited by low concentrations of kievitone (IV) and coumestrol (XV), whereas isoflavone and isoflavanone precursors of kievitone were much less inhibitory (183).

3. Formation of Isoflavones

The intermediacy of isoflavones in the formation of isoflavonoid phytoalexins and related compounds has been conclusively demonstrated by radiolabeling experiments. For example, in Phaseolus aureus, daidzein (XIX) is a good precursor of coumestrol (XV) (149), whereas in P. vulgaris, levels of daidzein increased along with the phytoalexins phaseolin (XIII) and kievitone (IV) following infection of endocarp tissue with spore suspensions of Monilinia fructicola (189). Similarly, formononetin (XVI) was effectively incorporated into the phytoalexins medicarpin (IX), sativan (VI), and vestitol (V) in CuCl₂- or ultraviolet light-treated seedlings of Medicago sativa (148), and the presence of low levels of formononetin in infected leaves of Trifolium was recently demonstrated by GLC/mass spectrometry with single ion monitoring (190). The formation of isoflavones from chalcones during phytoalexin accumulation has been demonstrated in hypocotyls of Glycine max infected with Phytophthora megasperma f.sp.glycinea, where radiolabeled daidzein was isolated after
<table>
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<th>pH Optimum</th>
<th>Activity with chalcone glycosides</th>
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<td>Cicer arietinum</td>
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<td>16</td>
<td>No activity</td>
<td>(182)</td>
</tr>
<tr>
<td>Citrus paradisi</td>
<td>7.0</td>
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<td>No activity</td>
<td>(185)</td>
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<tr>
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feeding \([^{14}\text{C}]2',4',4''\text{-trihydroxychalcone} \ (\text{XXVI}) \ (68)\). Evidence for the incorporation of chalcones into isoflavonoid phytoalexins has been given in the previous section, and radiolabeling experiments in \textit{Cicer arietinum} and \textit{Trifolium subterraneum} \ (158) have suggested that the chalcone may be a more immediate precursor of isoflavones than the corresponding flavanone.

Early radiolabeling experiments on the formation of isoflavones from phenylalanine have been reviewed by Grisebach \ (191). Evidence for an aryl migration during the formation of either formononetin \ (XVI) or biochanin A \ (XVII) was obtained by following the incorporation of DL-phenylalanine labeled in either the 1, 2 or 3 position \ (192-194), the labeling pattern obtained \ (Scheme 5) being consistent with the migration of the B ring from position 2 to position 3 \ (isoflavone numbering) during isoflavone formation. Early proposals for the mechanism of such a reaction \ (191) were based on the \textit{in vitro} rearrangement of suitably substituted chalcone epoxides in the presence of BF\(_3\) and AlBr\(_3\) \ (Scheme 6). Later, it was proposed that oxidation of a flavanone enolic tautomer could lead to isoflavone formation \ (191).

![Scheme 5. Labeling evidence for aryl migration during the formation of isoflavones.](image)

![Scheme 6. Formation of an isoflavone via a chalcone epoxide.](image)
Scheme 7: Formation of an isoflavone via a spirodienone intermediate.
Another model, again based on chemical oxidation analogies, involves the intermediacy of a spirodienone intermediate (195). This model (Scheme 7) has several attractive features; in particular, it explains the requirement, in chemical studies, for a free 2- or 4-hydroxyl group (chalcone numbering) on the migrating aryl unit. Labeling experiments in CuCl₂- and ultraviolet light-treated seedlings of *Medicago sativa* have shown that neither 2',4'-dihydroxy-4-methoxy chalcone (XXXIII) nor daidzein (XIX) is incorporated into medicarpin (IX), sativan (VI), or vestitol (V), whereas 2',4',4'-trihydroxychalcone (XXVI) and formononetin (XVI) are good precursors (148). These results confirm the requirement for a free chalcone 4-hydroxyl group in the enzyme-catalyzed reaction, and strongly suggest that methylation of this group is an integral part of the aryl migration process in *M. sativa*. A model incorporating electron withdrawal from the spirodienone to the positively charged sulphur atom of S-adenosyl methionine has been presented (9) (Scheme 7). Addition of a proton in place of a methyl group would account for the synthesis of the 4'-hydroxy series of isoflavonoids, as methylation of the 4' position of isoflavones occurs after aryl migration in some species (196).

The above models have not yet found direct support from enzymic studies. A spirodienone-type compound was tentatively identified as a product of the oxidation of 2',4,4'-trihydroxychalcone (XXVI) by two isoenzymes of peroxidase from infected hypocotyls of *Glycine max* (112). More specifically, extracts from *Cicer arietinum* and *Phaseolus vulgaris* catalyzed the oxidation of 2',4,4'-trihydroxychalcone to yield hydrated aurone and dihydroflavonol derivatives (197,198). These reactions were shown to be catalyzed by peroxidase (198,199) and to take place via a cyclic peroxide intermediate which could be converted to a benzoxygenone-spiro-cyclohexadienone isomeric with 7,4'-dihydroxyflavonol (197,199). It was pointed out that such reactions,
although occurring by a free radical mechanism and thus unlikely to result in either stereospecific formation of products or account for the often limited number of specific flavonoid derivatives formed under a particular set of conditions, may, in vivo, take place in a more strictly controlled manner (198). Although the spiro-intermediate in the above work was different from that required by Scheme 7, it is nevertheless possible that the immediate substrates for aryl migration may be formed from chalcones by the controlled action of a peroxidase.

Very few reports exist concerning the cell-free formation of isoflavones from chalcones. In crude extracts from Cicer arietinum, incorporation of chalcone into isoflavone was extremely low compared with incorporation into flavanone or flavanonol derivatives (200). More work is clearly needed in this area.

4. Conversion of Isoflavones to Pterocarpans and Further Elaboration of the Isoflavonoid Skeleton

Schemes 8, 9, and 10 summarize our present understanding of the biosynthetic pathways leading from chalcones to isoflavonoid phytoalexins (isoflavones, isoflavanones, isoflavans, and pterocarpans) in Medicago sativa and Phaseolus vulgaris. In M. sativa (Scheme 8) the pathway was elucidated by radioactive feeding experiments using CuCl₂- or ultraviolet light-treated seedlings (148, 201, 202); further confirmation for the pathway to medicarpin has come from the isolation of formononetin (XVI), the 2'-hydroxy isoflavone (XXXIV), and the 2'-hydroxy isoflavanone (XXXV) from fungally infected leaves of Trifolium repens (190). As compounds XVI, XXXIV, XXXV, and XXXVI were all excellent precursors of medicarpin (IX) (148), a metabolic grid is probably involved as shown, with the enzymological implication that 2'-hydroxylation can occur at either the isoflavone or isoflavanone level. Hydroxylases involved in isoflavonoid biosynthesis have not yet been isolated; the nearest analogous system may be the hydroxylations of the flavanone naringenin (XXV) to yield eriodictyol (XXVIII), dihydrokaempferol (XL), and dihydroquercetin (XLI) during the biosynthesis of cyanidin in cell suspension cultures of Haplopappus gracilis (203). These reactions appeared to involve microsomal, mixed-function oxygenase activity.

An important finding concerning pterocarpan and isoflavan biosynthesis was the fact that radiolabeled medicarpin (IX) and vestitol (V) were interconvertible in M. sativa (201). This, coupled with kinetic labeling
experiments suggesting that they were synthesized from a common precursor (201), led to the proposal of the intermediacy of a carbonium ion intermediate XXXVIII, formed from the putative isoflavanol intermediate XXXVII. As 7-hydroxy,2',4'-dimethoxy isoflavone (XXXIX) was a very poor precursor of sativan, this latter phytoalexin may be formed by methylation of vestitol (201).
The biosynthesis of the phytoalexins in *Phaseolus vulgaris* may proceed by a very similar route to that elucidated in *M. sativa*, the main differences between Schemes 8, 9, and 10 being the existence of the 5-hydroxy isoflavonoid pathway and the predominance of isoprenylation rather than methylation as the major substitution process after the initial Cl$_5$ stage in *P. vulgaris*. Most of the evidence for the reaction sequences in Schemes 9 and 10 has come from studies on the isolation of minor isoflavonoid components from *P. vulgaris* endocarp tissue challenged with *Monilinia fructicola* (189,204–206). This work has led to the proposal of a pathway to kievitone involving 2'-hydroxylation of genistein (I), oxidation to the isoflavanone [to yield dalbergioidin (XLIIa)], and finally isoprenylation of the A ring (205). Alternatively, isoprenylation could take place at the level of 2' -hydroxygeinistein, with 2,3-dehydrokievitone (XLIII) then being reduced to kievitone (205). It is possible that a metabolic grid may operate at the level of 2'-hydroxygeinistein, with an isoflavanone intermediate lacking a 2'-hydroxyl group was not isolated (204). As in Scheme 8, the hypothetical sequence of reactions leading to pterocarpan and isoflavan phytoalexins (Scheme 10) may similarly proceed via an isoflavanol intermediate (XLIV) (189).

In addition to the possible association of methylation with the aryl migration system in some plants, O-methylation of isoflavonoid derivatives may also occur at or after the isoflavone stage. In cell suspension cultures and seedlings of *Cicer arietinum*, a specific isoflavone 4'-O-methyl transferase has been isolated (196). This enzyme had a molecular weight of around 110,000, a pH optimum of 9.0, and required SH-protecting agents
Scheme 10. Biosynthesis of 5-deoxy isoflavonoid phytoalexins in *Phaseolus vulgaris* (189).
for activity. It was specific for the methylation of 4'-hydroxy isoflavonoids, $K_m$ values for daidzein (XIX) and S-adenosyl methionine (SAM) being 80 $\mu$M and 0.16 mM, respectively. The enzyme was inhibited by S-adenosyl homocysteine (SAH) ($K_i$ 0.3 mM with SAM saturating, 30 $\mu$M with daidzein saturating). The reaction was shown to follow an ordered BiBi mechanism with SAM and SAH as leading reaction partners. This transferase is clearly involved in the final stages of biosynthesis of formononetin (XVI) and biochanin A (XVII) in C. arietinum.

In cell suspension cultures of Glycine max, two distinct classes of O-methyl transferase enzymes have been characterized (207). The enzymes were distinguished by differences in stability upon storage, gel chromatographic properties, changes in specific activity during the growth cycle, and substrate specificity. One enzyme, the S-adenosyl methionine:caffeic acid O-methyl transferase (CMT), catalyzed the formation of ferulic (XXVIII) and sinapic (XXIV) acids from caffeic (XXII) and 5-hydroxyferulic acids, respectively (208). It had no activity against flavonoids or isoflavonoids, this being consistent with its role in the lignin pathway (208). The other enzyme methylated flavonoids, but was inactive against caffeic acid and the isoflavone daidzein (XIX) (209). As the activity of this latter enzyme was induced concomitantly with PAL, preceding the accumulation of flavone glycosides, its role in the flavonoid pathway was reinforced. It should be noted that the isoflavonoid phytoalexins of G. max are not O-methylated. In plants where O-methylation does occur, detailed substrate specificity studies may be necessary before implicating O-methyltransferase activity in the phytoalexin response; in callus cultures of Canavalia ensiformis producing medicarpin (IX) in response to attack by Pithomyces chartarum, O-methyltransferase activity with caffeic acid or naringenin as substrate was not induced, although three- to fourfold increases in transferase activity were observed with 2',4,4'-trihydroxychalcone (XXVI), daidzein (XIX), or genistein (I) as substrates (210).

The phytoalexins pisatin (XI) and maackiain (X) possess a B-ring 4',5'-methylene-dioxy substituent. Radiolabeling experiments in Trifolium pratense (146) have demonstrated the formation of maackiain via 7,3'-dihydroxy-4'-methoxyisoflavone (XLVI), and the possibility of a metabolic grid in which 2'-hydroxylation occurred either before or after the cyclization of the methylene-dioxy ring was suggested (146) (Scheme 11). Both XLVI and 7-hydroxy,3',4'-methylene-dioxy isoflavone (XLVII) have been isolated from T. pratense (211). A similar pathway to pisatin was
Scheme 11. Biosynthesis of maackiain in Trifolium pratense (146).
proposed from labeling experiments in *Pisum sativum* (147). The possibility of the determination of a 4'-hydroxy 5'-O-methyl substitution pattern prior to the chalcone level (although perhaps not in these systems) has already been discussed.

Many isoflavonoid derivatives have C₅ prenyl groups attached to their aromatic rings. These occur in both induced phytoalexins and constitutive isoflavonoids. There may be one free prenyl group as in kievitone (IV), phaseollidin (XII), or wighteone (III), two free groups as in the di-prenyl genisteins (212), or prenyl groups cyclized to o-hydroxy groups resulting in the 2,2-dimethylchromen ring structures of, for example, phaseollin (XII) or glyceollin (XIV). It has been suggested several times that prenylation increases the antifungal activity of isoflavonoids by making them more lipophilic. The prenyl group clearly arises by the classical mevalonic acid pathway (see Section III.B.1), as evidenced by the incorporation of [2-¹⁴C]mevalonic acid into glyceollin in elicitor-treated cotyledons of *Glycine max* (151).

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 (XLII) or 2,3-dehydrokievitone (XLIII)], isoflavanone [= kievitone (IV) or 5-deoxylievitone (XLV)], prenyltransferase involving the biosynthesis of the glyceollins (214). The enzymes from both *R. graveolens* and *G. max* appear to be located in the plastids, and the activity of the *G. max* enzyme is induced by the elicitor treatment. The latter enzyme catalyzed the formation of the putative precursor of glyceollin isomer 2 (XLIX) (reaction 5):
Small amounts of product prenylated at C-4 were also detected [corresponding to the major glyceollin isomer I (XIV)]. The significance of the proportions of C-2 and C-4 prenylation, and the number of transferases involved, remains to be elucidated.

C. DEGRADATION OF ISOFLAVONOID PHYTOALEXINS

1. Host Metabolism

Many of the phenolic compounds which accumulate in plants are not simply end products of metabolism, but can be further metabolized by degradation and/or polymerization (215, 216). For example, pulse-labeling experiments in Cicer arietinum have clearly demonstrated that the levels of the isoflavone formononetin (XVI) and the flavanol kaempferol can be regulated via increases or decreases in their turnover rates (217, 218), and studies on the metabolism of the isoflavone daidzein (XIX) exogenously supplied to cell suspension cultures of Phaseolus aureus have shown the operation of pathways of degradation, polymerization, glucosylation, and further metabolism to coumestrol (XV) (219, 220). Polymerization reactions may follow hydroxylation by mixed function oxygenase, phenolase, or peroxidase activities (215, 216), and plant degradation pathways for flavonoids and hydroxycinnamic acids involving peroxidative ring cleavage have also been discussed (215, 216).

Studies on host metabolism of isoflavonoid phytoalexins fall into two clearly defined categories: (a) experiments in which the fate of exogenously added phytoalexins has been investigated, and (b) pulse-labeling experiments in which the turnover of endogenous phytoalexin pools is
monitored. It may not be possible to extrapolate from one type of study to the other.

The pterocarpanoid phytoalexin phaseollin (XIII) is highly toxic to cell suspension cultures of *Phaseolus vulgaris*, concentrations over 30 μg ml⁻¹ causing nearly complete cell death within 30 min (221,222). Lower concentrations of phaseollin (26.5 μg ml⁻¹) resulted in inhibition of culture growth for around 21 days, after which time growth resumed; of the initial phaseollin added, more than 50% was taken up by the cells within 24 hr, although only 9% was recovered after 21 days (221). In an independent study, the half-life for phaseollin when added to *P. vulgaris* suspension cultures at a concentration of 4 μg ml⁻¹ was around 4 hr, ¹⁴C label from phaseollin being recovered in ethyl acetate- and H₂O-insoluble products associated with cell debris (222). No ¹⁴C-labeled phaseollin-like products were recovered. Pretreatment of cell cultures with 4 μg ml⁻¹ phaseollin did not protect them from the toxic effects of higher concentrations of the phytoalexin (222), as might have occurred had the host turnover system been inducible. Metabolism of exogenous phaseollin has also been observed in cell suspension cultures of *Phaseolus aureus*, which are not themselves able to synthesize this phytoalexin (222). In contrast, no evidence was obtained for the metabolism of exogenously added [¹⁴C]pisatin (XI) by leaf or endocarp tissue of *Pisum sativum* (67).

In wounded cotyledons of Glycine max, incorporation of radiolabel from [¹⁴C]phenylalanine suggested the presence of glyceollin (XIV) synthetic activity, although no net accumulation of the phytoalexin was observed; exogenously applied glyceollin was rapidly metabolized (223). Glyceollin accumulation in this system was stimulated by a biotic elicitor preparation (cell walls from *Phytophthora megasperma* f.sp.glycinea), and this treatment had no effect on the rate of metabolism of added glyceollin. However, the abiotic elicitor HgCl₂ appeared to inhibit exogenous glyceollin turnover while having little effect on the rate of synthesis of the phytoalexin. Comparison of the behavior of a wide variety of biotic and abiotic elicitors confirmed these findings and resulted in the proposal of a role for regulation of glyceollin turnover as a key factor in the induced response to abiotic elicitors (223). Further studies suggested that rates of glyceollin synthesis (as assessed by incorporation of label from [¹⁴C]phenylalanine) were identical in compatible and incompatible interactions of *G. max* c.v. Harosoy 63 with races of *Phytophthora megasperma* f.sp.glycinea, whereas pulse-chase experiments were interpreted as indicating that turnover of the phytoalexin in incompatible interactions
was less than in compatible interactions or wounded-only controls (224). Concentrations of blasticidin S which inhibited glyceollin synthesis had little effect on degradation of the phytoalexin, thereby suggesting that the turnover system was constitutive (224).

As different elicitor treatments may differentially affect \(^{14}\)C phenylalanine uptake, and degradation of exogenously supplied phytoalexins may occur in different pools to that of the \textit{in vivo} synthesized compounds, the above work was reexamined using cotyledons of \textit{Glycine max} pulse-labeled with \(^{14}\)CO\(_2\) (225,226). The kinetics of incorporation of \(^{14}\)C into glyceollin and its precursor 3,6a,9-trihydroxypterocarpan (XLVIII) were shown to be very similar with abiotic (HgCl\(_2\)) and biotic (cell wall glucan from \textit{Phytophthora megasperma} f.sp.\textit{glycinea}) elicitors, and pulse-chase experiments allowed the calculation of a half-life for glyceollin of 100 hr with either elicitor (226). It was therefore concluded that rates of glyceollin accumulation are mainly regulated via elicitor effects on phytoalexin synthesis.

Studies on the metabolism of exogenously added phaseollin by \textit{Phaseolus vulgaris}, coupled with results indicating the synthesis of phaseollin in living cells and its ultimate accumulation in neighboring, dead cells (221,227), have led to the proposal that phytoalexin turnover may have an important physiological role in protecting the cells that initially synthesize the phytoalexin from its phytotoxic action. If rapid turnover of endogenous phaseollin pools can be shown to occur, this may act as a stimulus for further work on the enzymology of this aspect of host metabolism. The demonstration of phaseollin incorporation into insoluble cellular material (222) suggests that oxidative attack and subsequent polymerization may be one important metabolic fate, at least for exogenously added phaseollin.

\section*{2. Fungal Metabolism}

Over the past 10 years, many reports have been published concerning the ability of fungi to metabolize isoflavonoid phytoalexins to less fungitoxic products. This ability is generally, although not exclusively, limited to fungi which are pathogenic on leguminous species. In certain cases the ability to detoxify phytoalexins may be a major determinant of fungal pathogenicity (228,229), and it is therefore unfortunate that so little attention has been paid to the enzymology of fungal detoxification of phytoalexins. Identification of detoxification products has arisen from two types of study: experiments in which novel compounds have been isolated from fungally infected tissues, and studies on the degradation products of specific phytoalexins in fungal cultures.
A variety of different biochemical modifications have been observed during the detoxification of isoflavonoid derivatives; these include demethylation, aromatic hydroxylation, ring opening, methylation, hydration, and oxidation. Most of these are illustrated in Schemes 12 and 13, which show the fungal metabolism of medicarpin and three phytoalexins from *Phaseolus vulgaris*, respectively. The 9-demethylation of medicarpin

![Scheme 12. Fungal metabolism of medicarpin (IX). Fungi bringing about the transformations are (1) Botrytris cinerea, (2) Colletotrichum lindemuthianum, (3) C. coccodes, and (4) C. coffeum.](image-url)
Scheme 13. Fungal metabolism of phytoalexins from *Phaseolus vulgaris*. Fungi bringing about the transformations are (1) *Fusarium solani* f.sp.*phaseoli*, (2) *Stemphylium botryosum*, (3) *Colletotrichum lindemuthianum*, and (4) *Septoria nodorum*. 
(IX) to yield 3,9-dihydroxypterocarpan (L) is brought about by several fungi including *Colletotrichum coccodes* (230), *C. lindemuthianum* (231), *Botrytis cinerea* (231), and *Fusarium proliferatum* (232). The latter fungus can also demethylate pterocarpan at the 3 position (232), a property in common with other *Fusarium* spp. (233–235), *Nectria haematococca* (229), and *Ascochyta pisi* (236), which are all able to convert pisatin (XI) to 3,6a-dihydroxy-8,9-methylenedioxy pterocarpan (LI) (reaction 6).

*Colletotrichum coccodes* may possess a very specific pterocarpan demethylase enzyme; under the conditions used to measure pterocarpan 9-demethylation, homopterocarpin (3,9-dimethoxy pterocarpan, LII) was not demethylated in either position (230), a surprising result in view of the ready metabolism of homopterocarpin to 3-methoxy-9-hydroxy pterocarpan (LIII), 3,9-dihydroxy pterocarpan (L), and 7,2',4'-tri hydroxyisoflavans (LIV) by *Fusarium proliferatum* (232). The exact nature of the fungal demethylating enzymes involved in phytoalexin transformations is not known. The ability of *Fusarium oxysporum f.sp. pisi* to readily demethylate pisatin without prior exposure to the phytoalexin (233) suggests that the enzyme(s) involved may be constitutive, whereas the 2-hr lag phase observed in cultures of *Botrytis cinerea* prior to medicarpin detoxification (231) has been interpreted as reflecting the production of inducible detoxifying enzymes.

Conversion by *B. cinerea* of medicarpin to its corresponding isoflavan
vestitol (V) by cleavage of the benzyl ether linkage of the dihydrofuran ring (231) is an example of another commonly occurring fungal modification. Analogous reactions are the conversions of phaseollin (XIII) and 3-hydroxy-8,9-methylenedioxy pterocarpan (maackiain, X) and 3,6a-dihydroxy-8,9-methylenedioxy pterocarpan (LI) to their respective isoflavans by Stemphylium botryosum (237,238) and Fusarium oxysporum f.sp.pisi (233), respectively. The interconvertibility of medicarpin and vestitol via a carbonium ion intermediate has already been discussed with respect to the biosynthesis of medicarpin (201) (Scheme 9). During the degradation of pisatin by F. oxysporum f.sp.pisi, the isoflavan product is further metabolized to CO₂ and other, unknown products (233). Similarly, phaseollinisoflavan (VII) disappears from cultures of Fusarium solani (239).

Fungal hydroxylation of pterocarpons can occur at a variety of positions. Colletotrichum coffeanum, Botrytis cinerea, and C. lindemuthianum have all been shown to bring about the 6a-hydroxylation of medicarpin (231) (Scheme 12), the two latter fungi also hydroxylating phaseollin in this position (240) (Scheme 13c); 6a-hydroxylation by Colletotrichum species may also be followed by hydroxylation at the 7 position (240) (Schemes 12 and 13). The appearance of 6a,7-dihydroxy medicarpin (LV) only after a period 50 hr in C. coffeanum-infected leaves of Melilotus alba, whereas the 6a-hydroxy derivative (LVI) appears by 25 hr, suggests that C. coffeanum requires long exposure to 6a-hydroxy medicarpin before producing enzymes capable of further metabolizing this compound (231). Several phytoalexins [e.g., pisatin (XI) and glyceollin (XIV)] are 6a-hydroxylated during their biosynthesis. No attempts have yet been made to characterize and compare the plant and fungal enzyme systems bringing about this reaction.

Hydroxylation of phaseollin in the 2,2-dimethylchromen ring, resulting in cis- and trans-isomers of 12,13-dihydrodihydroxy phaseollin (LVIIa,b,c, and d), is brought about by cultures of Septoria nodorum (241), whereas Fusarium solani f.sp.phaseoli and Cladosporium herbarum convert phaseollin to 1a-hydroxyphaseollone (LVII) (239,242,243), an unusual metabolic conversion that has not yet been observed for other ptero-carpanoid phytoalexins.

Medicarpin (IX) and 3,9-dihydroxypterocarpan (L) may be o-hydroxylated in the A ring by Botrytis cinerea (331) (Scheme 12). Attention has been drawn (231) to the similarity of this reaction to the hydroxylation of taxifolin (LIX) to dihydrogossypetin (LX) by a soil pseudomonad (reaction 7). This reaction, which requires molecular oxygen
and NAD(P)H and is followed by A-ring cleavage, is catalyzed by a non-microsomal flavoprotein (244). The enzyme system was induced by growing the cells in the presence of catechin (244).

Cultures of *Fusarium solani* f.sp.*phaseoli* are able to convert kievitone (IV) and phaseollidin (XII) into less toxic compounds by hydration of their isopentenyl groups (245, 246) (Scheme 13a,b). An enzyme system catalyzing the formation of kievitone hydrate (LXI) has been isolated from cell-free culture filtrates of the fungus (247), and limited purification was obtained by gel-filtration. The enzyme had a molecular weight of around 175,000, and could also detoxify phaseollidin (XII) (247). The more rapid rate of kievitone and phaseollidin disappearance in cultures pretreated with kievitone suggested that the enzyme system was inducible (247).

### III. Terpenoid Phytoalexins

Terpenoids have vital and varied functions in the general metabolism of living organisms. As constituents of membranes, sterols play an important role in both animal and plant cells; on the other hand, other steroids are well known as hormones and vitamins. The realization of the involvement of terpenoids in the biochemical defense mechanisms of plants against fungal infection arose from the pioneering work of Muller and Borger (1,2) on the interaction of potato cultivars with *Phytophthora infestans*.

Among the known phytoalexins that can be classified as terpenes are sesquiterpenoids, diterpenoids, triterpenoids, and possibly some steroid-derived glycoalkaloids. The poly-isoprene carbon skeletons of terpenes provide the biosynthetic flexibility of varying chain-length and chain-folding patterns, thus offering a wide variety of structurally related products (248). It seems unlikely, therefore, that only a single terpenoid phytoalexin would be responsible for resistance of a plant exposed to a
variety of potential pathogens. In addition to qualitative and quantitative variations in infective agent inoculum, localized conditions of incubation may also greatly influence the terpenoid accumulation in the host (249). The level at which the terpenoid phytoalexins accumulate in host tissue is likely to depend on the relative rates of their synthesis and degradation. Thus only a low level of rishitin (LXII) is observed in potato tubers 120-144 hr after inoculation with Phytophthora infestans or treatment with a cell-free extract from the fungus (250,251).

Further general details concerning terpenoid phytoalexins can be found in a number of recent reviews (12-14,252-258).

A. STRUCTURE AND OCCURRENCE

A list of terpenoid phytoalexins and other postinfectionally formed plant terpenoid compounds is presented in Table V. Detailed studies have been made of the metabolism of only some of these compounds. Not all of the compounds in Table V can strictly be termed phytoalexins; some appear to be precursors of true phytoalexins, whereas others appear as symptoms of infection and have not yet been allocated a precise biological function.

B. BIOSYNTHESIS AND ENZYMEOLOGY

The basic unit from which terpenoids are synthesized is the five-carbon isoprene unit; monoterpenes are derived from two such units, sesquiterpenes from three units and diterpenes from four units. A carbon atom may be lost in some terpenoid phytoalexins during the process of biosynthesis; for example, the norsesquiterpenoid rishitin (LXII) has 14 instead of 15 carbon atoms. A general scheme relating the synthesis of terpenoid phytoalexins with other areas of tissue metabolism is depicted in Scheme 14. Expression of the whole, or specific parts, of the pathway will depend on the particular metabolic conditions resulting from infection or wounding of the tissue, thus leading to numerous qualitative and quantitative variations in the terpenoids produced.

1. Formation of Precursors

The primary precursor for the biosynthesis of terpenoids is mevalonic acid (MVA). This compound if formed from acetyl CoA through a multi-step pathway (Scheme 15), catalyzed by known, specific enzymes. Two molecules of acetyl CoA are first condensed to form acetoacetyl CoA, the reaction being catalyzed by acetoacetyl-CoA thiolase (EC 2.3.1.9). The
### TABLE V

Structures and Sources of Terpenoid Phytoalexins and Related Postinfectional Compounds

<table>
<thead>
<tr>
<th>Source (Plant species)</th>
<th>Compound</th>
<th>Compound number</th>
<th>Structure</th>
<th>Fungal species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato (<em>Solanum tuberosum</em>)</td>
<td>Rishitin</td>
<td>(LXII)</td>
<td><img src="image" alt="Structure of Rishitin" /></td>
<td><em>Phytophthora infestans</em></td>
<td>(252, 259-261)</td>
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<tr>
<td></td>
<td>Rishitinol</td>
<td>(LXIII)</td>
<td><img src="image" alt="Structure of Rishitinol" /></td>
<td><em>P. infestans</em></td>
<td>(259)</td>
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<tr>
<td></td>
<td>Rishitinone</td>
<td>(LXIV)</td>
<td><img src="image" alt="Structure of Rishitinone" /></td>
<td><em>P. infestans</em></td>
<td>(262)</td>
</tr>
<tr>
<td></td>
<td>Phytuberin</td>
<td>(LXV)</td>
<td><img src="image" alt="Structure of Phytuberin" /></td>
<td><em>P. infestans, Ceratocystis fimbriata</em></td>
<td>(263, 264)</td>
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<tr>
<td></td>
<td>Phytuberol</td>
<td>(LXVI)</td>
<td><img src="image" alt="Structure of Phytuberol" /></td>
<td><em>Glomerella cingulata, P. infestans</em></td>
<td>(254, 265, 266)</td>
</tr>
<tr>
<td>Source</td>
<td>Compound</td>
<td>Compound number</td>
<td>Structure</td>
<td>Fungal species</td>
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</tr>
<tr>
<td>Lubimin</td>
<td>(LXVII)</td>
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<td>P. infestans</td>
<td>(267,268)</td>
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</tr>
<tr>
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<td>(LXVIII)</td>
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<td>P. infestans</td>
<td>(259,267)</td>
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</tr>
<tr>
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<td>(LXIX)</td>
<td><img src="image3" alt="Structure" /></td>
<td>G. cingulata</td>
<td>(254)</td>
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<td>lubimin</td>
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<td></td>
</tr>
<tr>
<td>Epilubimin</td>
<td>(LXX)</td>
<td><img src="image4" alt="Structure" /></td>
<td>G. cingulata Monilinia, fructicola</td>
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<tr>
<td>Hydroxyepilubimin</td>
<td>(LXXI)</td>
<td><img src="image5" alt="Structure" /></td>
<td>P. infestans</td>
<td>(269)</td>
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15-Dihydro-epilubrin
Isolubrin
Solavetivone
Anhydro-rotunol
Tobitin
Lubimin
Auberginone

G. cingulata, M. fructicola
P. infestans, G. cingulata
P. infestans
P. infestans
M. fructicola
M. fructicola

Tomato (Lycopersicon esculentum)
Eggplant (Solanum melongena)
<table>
<thead>
<tr>
<th>Source</th>
<th>Compound</th>
<th>Compound number</th>
<th>Structure</th>
<th>Fungal species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jimson weed</td>
<td>Lubimin</td>
<td>(LXVII)</td>
<td>—</td>
<td>M. fructicola</td>
<td>(267,274,278)</td>
</tr>
<tr>
<td></td>
<td>Hydroxy-lubimin</td>
<td>(LXVIII)</td>
<td>—</td>
<td>M. fructicola</td>
<td>(267,278,279)</td>
</tr>
<tr>
<td></td>
<td>Capsidiol</td>
<td>(LXXVI)</td>
<td><img src="image1.png" alt="structure" /></td>
<td>M. fructicola</td>
<td>(228,280,281)</td>
</tr>
<tr>
<td></td>
<td>2,3-Dihydroxy-germacrene</td>
<td>(LXXVIII)</td>
<td>—</td>
<td>M. fructicola</td>
<td>(278,279)</td>
</tr>
<tr>
<td>Tobacco</td>
<td>Phytuberin</td>
<td>(LXV)</td>
<td>—</td>
<td>Tobacco necrosis virus and bacteria</td>
<td>(282–286)</td>
</tr>
<tr>
<td>(Nicotiana spp.)</td>
<td>Capsidiol</td>
<td>(LXXVII)</td>
<td><img src="image2.png" alt="structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glutinosone</td>
<td>(LXXIX)</td>
<td><img src="image3.png" alt="structure" /></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sweet pepper</td>
<td>Capsidiol</td>
<td>(LXXVII)</td>
<td>—</td>
<td>M. fructicola</td>
<td>(281,287,288)</td>
</tr>
<tr>
<td>(Capsicum frutescens)</td>
<td>Capsinone</td>
<td>(LXXX)</td>
<td><img src="image4.png" alt="structure" /></td>
<td>F. oxysporum</td>
<td>(279,289)</td>
</tr>
</tbody>
</table>
Sweet potato (Ipomea batatas)

- Ipomeamarone (LXXXI)
- Ipomeamaronol (LXXXII)
- Dehydro-ipomeamarone (LXXXIII)

Castor (Ricinus communis)

- Casbene (LXXXIV)

Rice (Oryza sativa)

- Momilactone A (LXXXV)
- Momilactone B (LXXXVI)

Cotton (Gossypium spp.)

- Gossypol (LXXXVII)
<table>
<thead>
<tr>
<th>Source</th>
<th>Compound</th>
<th>Compound number</th>
<th>Structure</th>
<th>Fungal species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-Methoxygossypol</td>
<td>(LXXXVIII)</td>
<td>As above, R = OCH₃, R' = OH</td>
<td></td>
<td>(Verticillium)</td>
<td>(304)</td>
</tr>
<tr>
<td>6,6'-Dimethoxygossypol</td>
<td>(LXXXIX)</td>
<td>As above, R,R' = OCH₃</td>
<td></td>
<td></td>
<td>(304)</td>
</tr>
<tr>
<td>Hemigossypol (R = OH)</td>
<td>(XC)</td>
<td><img src="image" alt="Structure" /></td>
<td></td>
<td></td>
<td>(303,305)</td>
</tr>
<tr>
<td>6-Methoxyhemigossypol</td>
<td>(XCI)</td>
<td>As above, R = OCH₃</td>
<td></td>
<td>(Verticillium)</td>
<td>(303,305)</td>
</tr>
<tr>
<td>Desoxyhemigossypol (R = OH)</td>
<td>(XCII)</td>
<td><img src="image" alt="Structure" /></td>
<td></td>
<td></td>
<td>(303,305)</td>
</tr>
<tr>
<td>Desoxy-6-methoxyhemigossypol</td>
<td>(XCIII)</td>
<td>As above, R = OCH₃</td>
<td></td>
<td>(Verticillium)</td>
<td>(303,305)</td>
</tr>
</tbody>
</table>
molecular properties of this enzyme, mainly from animal sources, have been widely studied (306-313). The mitochondrial enzyme has been shown to possess a cysteine residue at the active site (306) and can be alkylated by reactive halide-alkylating reagents (307-312). However, less reactive reagents such as alka-3-ynoyl-CoA esters were also able to inactivate the enzyme (312). It was found that these compounds were initially converted by the enzyme into alka-2,3-dienoyl-CoA which was a potent inhibitor. Inactivation mechanisms involving a primary enzymic isomerization of the acetylenic bond to an allene had been reported earlier (313-317).

Acetoacetyl CoA is condensed with a further molecule of acetyl CoA to yield β-hydroxymethylglutaryl CoA (HMG CoA), the reaction being catalyzed by HMG-CoA synthetase (EC 4.1.3.5); this step is relatively irreversible (318). Studies employing the purified enzyme from yeast (319-321) and animal tissues (322-324) led to the suggestion that the reaction proceeded via the formation of an acetylated enzyme intermediate, and the involvement of a cysteinyl-SH group at the active site.
Scheme 15. The biosynthesis of mevalonic acid.
was demonstrated (323) (Scheme 16). This observation supported the earlier view (319) that the CoA moiety of acetyl CoA, and not that of acetoacetyl CoA, is cleaved during the reaction. It was possible to trap the acetyl-S-enzyme intermediate when the reaction was terminated at \(-25^\circ\) in the presence of 25\% alcohol (324).

An alternative pathway for the formation of HMG-CoA was proposed in which enzyme-bound malonate condenses with acetyl CoA (through its enzyme-bound form) yielding enzyme-bound acetoacetate. This could then further condense with another molecule of acetyl CoA to yield enzyme-bound HMG (325). However, although \[^{14}\text{C}\]malonyl CoA was incorporated into isoprenoids by enzyme preparations from yeast, pigeon, and rat liver, and *Hevea brasiliensis* latex, the incorporation was directly related to the presence of malonyl-CoA decarboxylase activity in the preparations (326). Moreover, the labeling pattern of ergosterol formed by the yeast preparation was also indicative of decarboxylation prior to incorporation (326). HMG-CoA can also be formed via a pathway involving leucine and valine (327-331); the physiological significance of this pathway in plants is not known.

HMG-CoA reductase (EC 1.1.1.34) catalyzes the NADPH-linked conversion of HMG-CoA to mevalonic acid through a hemiacetal intermediate (Scheme 15). This reaction is considered rate limiting in cholesterol biosynthesis in a wide variety of mammalian cells (332-334). However, its regulatory role during induced terpenoid accumulation in plants is not well understood (335-338). The major studies on the enzyme have been carried out with preparations from mammalian tissues (334,339-341) and microorganisms (342-347), and several reviews of this work are available (334,339,348-350).

As in some animal tissues, HMG-CoA reductase from seedlings of *Pisum sativum* was found to be associated with the microsomal fraction (335). Some activity was also found in the fractions containing plastids and mitochondria. It is, however, not known whether these activities belong to identical proteins or isoenzymes responsible for the synthesis of different products. No details are available concerning the activity modulation of these enzyme species by metabolites. The microsomal fraction from roots of sweet potato (*Ipomea batatas*) that were either infected with the black rot fungus, *Ceratocystis fimbriata*, or treated with the abiotic elicitor HgCl\(_2\), displayed high levels of HMG-CoA reductase activity. This activity was related to the synthesis of the furanoterpenoid phytoalexin ipomeamarone (LXXXI) (337,338,351,352). The pH optima of the micro-
somal reductases from pea (336) and sweet potato (337) were 6.8 and 7.3-7.5, respectively; the apparent $K_m$ values for HMG-CoA being 100 $\mu$M and 6.5 $\mu$M. The enzymes had an absolute requirement for NADPH, no activity being observed with NADH (336). Thiol protecting reagents such as 2-mercaptoethanol, glutathione, or dithiothreitol were found necessary for optimal activity (336,337). The enzyme is generally inhibited by free CoA and HMG, but MVA had no effect at concentrations up to 1 $mM$ (336, cf. Ref. 353). Any in vivo importance of the inhibitory compounds is not clear; free HMG is not an intermediate in terpenoid biosynthesis.

Studies carried out over the last decade with HMG-CoA reductase from animal tissues have revealed the existence of intricate control mechanisms (354-371). Under physiological conditions, a major proportion of the enzyme seems to exist in a phosphorylated, inactive form. Dephosphorylation of this form leads to an active enzyme, which can again be inactivated in vitro by an ATP-Mg$^{2+}$-dependent kinase. Reactivation is achieved by treatment with a phosphatase. The activity of the kinase can similarly be modulated by phosphorylation/dephosphorylation. In this case, the phosphorylated form is active, with dephosphorylation reducing the activity. An ATP-Mg$^{2+}$-dependent kinase is also involved in this phosphorylation. No details are available regarding the identity/nonidentity of the two phosphatases involved in the dephosphorylation of the reductase and the kinase, respectively.

There are indications that some form of multivalent feedback regulatory mechanism may exist in mammalian cells to control the activity of HMG-CoA reductase (372). Using the inhibitor compactin (373) as a tool, it was demonstrated that at least two feedback inhibitors might be involved, namely cholesterol and a nonsterol product derived from mevalonate (372). A membrane-mediated control mechanism for HMG-CoA reductase has also been put forward (374); however, its validity is in doubt (375). In this model, the regulation of the enzyme was associated with the fluidity of the supporting microsomal membrane.

Mevalonic acid is readily phosphorylated in vivo in the presence of ATP and Mg$^{2+}$, the reaction being catalyzed by MVA kinase (EC 2.7.7.36) and yielding mevalonic acid 5-phosphate (MVAP). The activity of this kinase has been demonstrated in a number of plants (376-389). Table VI shows some of the properties of the enzyme (378,389,390). A comparative study of the molecular weights of the enzyme from various plant and animal sources has been presented by Gray and Kekwick (390). The enzymes from Phaseolus vulgaris (384), Hevea brasiliensis latex (378), and Pinus
TABLE VI
Some Properties of Plant Mevalonate Kinase (378,387,389,390)

<table>
<thead>
<tr>
<th>Properties</th>
<th>Phaseolus vulgaris</th>
<th>Hevea brasiliensis</th>
<th>Cucumis melo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Etiolated cotyledons</td>
<td>Green leaves</td>
<td></td>
</tr>
<tr>
<td>$K_m (M)$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mevalonate</td>
<td>$4.26 \times 10^{-5}$</td>
<td>$4.55 \times 10^{-5}$</td>
<td>$0.13 \times 10^{-4}$</td>
</tr>
<tr>
<td>ATP</td>
<td>$1.54 \times 10^{-3}$</td>
<td>$1.75 \times 10^{-5}$</td>
<td>$2.0 \times 10^{-3}$</td>
</tr>
<tr>
<td>pH Optimum</td>
<td>7.0</td>
<td>7.0</td>
<td>7.5</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>100,000</td>
<td>100,000</td>
<td>99,500</td>
</tr>
<tr>
<td>Inhibition (with respect to ATP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVA 5-P</td>
<td>40% at 0.3 mM</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ADP</td>
<td>Not inhibited</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IPP</td>
<td>Not inhibited</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>GPP</td>
<td>30.8</td>
<td>32.5</td>
<td>17.8</td>
</tr>
<tr>
<td>FPP</td>
<td>7.1</td>
<td>7.5</td>
<td>5.2</td>
</tr>
<tr>
<td>GGPP</td>
<td>$K_i (\mu M)$</td>
<td>48.6</td>
<td>48.8</td>
</tr>
<tr>
<td>Phytyl PP</td>
<td>3.6</td>
<td>3.5</td>
<td>3.9</td>
</tr>
<tr>
<td>Metal ion requirement (maximum activity at, mM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>9.0</td>
<td>9.0</td>
<td>4.0</td>
</tr>
<tr>
<td>(no inhibition at higher conc.)</td>
<td></td>
<td></td>
<td>(inhibition at higher conc.)</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>2.0</td>
<td>2.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(inhibition at higher conc.)</td>
<td></td>
<td></td>
<td>(inhibition at higher conc.)</td>
</tr>
</tbody>
</table>

Abbreviations: MVA 5-P, mevalonic acid 5-phosphate; ADP, adenosine diphosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; Phytyl PP, phytol pyrophosphate.
*Pinus pinaster* (391) have been partially purified. In earlier studies, two forms of the enzyme were demonstrated in *P. vulgaris* (381,382), one form with a pH optimum of 7.5 being located in the chloroplast, and the second, with an optimum of pH 5.5, being of extra-chloroplast origin (see also Ref. 336). Since the chloroplast membrane is impermeable to mevalonic acid, it was suggested that two sites of terpenoid biosynthesis may exist (381, 392,393). The occurrence of two isoenzymes was, however, later disputed (387). The relatively acidic pH optimum displayed by the second enzyme was probably due to the use of a crude enzyme preparation which also possessed phosphatase activity. A partially purified preparation which could not be resolved into isoenzyme forms by gel-filtration displayed a pH optimum of 7.0 (Table VI). Mevalonate kinases from other plant sources show pH optima of 6.5 or above (378,384,386,387). The kinase generally requires Mn$^{2+}$ or Mg$^{2+}$ for activity and is in some cases activated by thiol compounds (384,386) and inhibited by some of the products of the acetate-mevalonate pathway (Table VI). This inhibition may be important as a possible control mechanism. The detailed mechanism of action of a homogeneous kinase preparation from hog liver has been studied (394).

Two forms of mevalonate kinase differing in molecular weight have been separated from *Pinus pinaster* and *Agave americana* (388), whereas two ionically differing forms exist in *Hevea* latex. The substrate specificities of these forms have not been studied in detail (376).

The transformation of MVA into further metabolites is illustrated in Scheme 17. Mevalonic acid 5-phosphate is further phosphorylated to yield MVA 5-pyrophosphate; the reaction is catalyzed by 5-phosphomevalonate kinase (EC 2.7.4.2). This reaction is freely reversible in animal systems (395,396,397). The product of the reaction has been demonstrated in a number of higher plants (377,380,384-386); however, the specific kinase has only been detected in the latex of *Hevea brasiliensis* (398) and in leaves of *Nepeta cataria* (399). The kinase has been isolated from yeast (400) and some animal tissues (396,397,401), although most studies have been carried out with relatively crude preparations (348). The enzyme is generally specific for ATP, however, the kinase from latex can also utilize UTP and ITP, although at lower rates (398). A divalent metal cation is also required for activity, with preference being shown for Mg$^{2+}$. The apparent $K_m$ values with respect to MVA 5-phosphate for the pig liver and the *Hevea* enzymes are $3 \times 10^{-4} \text{ M}$ and $4.2 \times 10^{-5} \text{ M}$, respectively (396,398), and both enzymes appeared to require -SH groups for activity. The pig liver enzyme has recently been purified to homogeneity, and a molecular
weight of 21,000-22,500 was estimated (402). The amino acid composition shows a high content of acidic amino acids and one cysteine residue per molecule. Evidence was presented for the participation of cysteine and lysine groups in the enzymic catalysis (403).

A concerted decarboxylation-dehydration of MVA 5-pyrophosphate catalyzed by mevalonic acid 5-pyrophosphate anhydrodecarboxylase (pyrophosphomevalonate decarboxylase, EC 4.1.1.33) produces isopentenyl pyrophosphate (IPP), the biogenetic isoprene unit (see Scheme 17). This reaction is highly stereospecific and involves trans-elimination of water and CO₂ from the substrate (404,405). The enzyme has been isolated from the latex of Hevea brasiliensis (406), seeds of Marah macrocarpus (407), roots of Ipomea batatas (408), yeast (400), and pig liver (409,410), and has also been detected in other animal tissues (397,411). The presence of IPP or its immediate reaction products has been demonstrated in various plant and microbial sources (412-418), thus indirectly implying the presence of the anhydro-decarboxylase.

It was suggested (419,420) that 3-phospho-5-pyrophosphomevalonate was an intermediate during the enzymic reaction, this then undergoing decarboxylation and β-elimination of the 3-phosphate group to finally yield IPP. An alternative mechanism (420) envisaged a direct intermolecular displacement of the hydroxyl group at C-3 of the substrate with that of the terminal phosphate of ATP accompanied by decarboxylation. Details of the regulation of the anhydrocarboxylase in the seeds of Marah macrocarpus and roots of Ipomea batatas have been presented (407,408,421).

Isopentenyl pyrophosphate isomerase (EC 5.3.3.2) catalyzes the reversible stereospecific transformation of IPP to dimethylallyl pyrophosphate (DMAPP) through isomerization of the double bond from position 3 to position 2 of the substrate molecule (Scheme 17). This enzyme has been isolated, and in some cases extensively purified, from various plant (422-428), microbial (429-432), and animal (433-438) sources. The presence of IPP isomerase was demonstrated in the proplastid and mitochondrial fractions of Ricinis communis endosperm (426); however, it is not known whether one or more enzymic forms are involved here. Evidence has been presented for the existence of two forms of the enzyme in pumpkin (437) with apparent Kₘ values for IPP of 22 μM and 45 μM. IPP isomerase from pig liver was strongly activated by mevalonic acid and inactivated by ATP (436), the inactivation showing sigmoidal dependence on ATP concentration (436). These observations may have relevance for the in vivo regulation of the enzyme.
Scheme 17. The formation of dimethylallyl pyrophosphate from mevalonic acid.
The stereospecificity of the reaction catalyzed by the isomerase and its mechanism of action have been reviewed (348,439). The enzyme seems to require a sulphhydryl group for its activity (348,436). During the catalysis a proton from C-2 of the substrate (2-pro-R hydrogen atom of IPP) is removed, forming the double bond of DMAPP in which the new methyl group (formed from the terminal methylene group) is trans to C-1. The addition of the proton to the double bond of IPP is to the re face.

Enzymic condensation (via prenyl transfer reaction) of DMAPP with IPP is the basis of the formation of a variety of terpenoids. In this way the first C\textsubscript{10} terpenoid precursor, geranyl pyrophosphate (GPP), is formed. A further prenyl transfer from IPP to GPP produces the C\textsubscript{15} compound, farnesyl pyrophosphate (FPP), which is the precursor of sesquiterpenoids.

2. Sesquiterpenoid Phytoalexins

In this and the following sections, only those phytoalexins and stress metabolites will be discussed for which sufficient data have been presented to help explain the pathways of biosynthesis. Some general reviews are available (252-254,256,440). In view of the often close metabolic interrelationships between the various terpenoid phytoalexins of a given plant species, the biosynthesis of these compounds will be discussed under the heading of the species of origin rather than the precise chemical class.

a. Phytoalexins of Potato (Solanum tuberosum). Rishitin (LXII), lubimin (LXVII), and phytuberin (LXV) are the main phytoalexins of potato. However, a number of other stress compounds have also been isolated (Table V). All of these compounds appear to be closely related biogenetically (254-256), although little is known about the possible phytoalexin nature of the stress compounds.

Rishitin accumulates in potatoes as a result of infection with an incompatible race of Phytophthora infestans. Similar accumulation may be induced by other fungal species including Monilinia fructicola, Glomerella cingulata, Fusarium solani, F. avenaceum, Ceratocystis fimбриata, and Phoma exigua (440). Rishitin may not be the only product of induction in the host tissue under a given condition of infection; several, if not all, of the other related compounds (see Table V) may also be produced. The relative proportions of the compounds seem to depend on the incubation time, potato cultivar, and the fungal species (255,256,441).

The levels of the various phytoalexins and stress metabolites were measured in potato slices infected with either M. fructicola or G. cingulata (254,256,442,443) (Table VII), and analysis of the compounds after
TABLE VII

13C-Labeled Compounds from Potato Slices Infected with *Monilinia fructicola* and *Glomerella cingulata*.
Data recalculated from (254).

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>M. fructicola</em></th>
<th><em>G. cingulata</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rishitin</td>
<td>69</td>
<td>99</td>
</tr>
<tr>
<td>Lubimin</td>
<td>15</td>
<td>51</td>
</tr>
<tr>
<td>Epilubimin</td>
<td>3</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxylubimin</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Dihydrolubimin</td>
<td>-</td>
<td>148</td>
</tr>
<tr>
<td>Dihydroepilubimin</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Solavetivone</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td>Phytoberin</td>
<td>&lt;1</td>
<td>73</td>
</tr>
<tr>
<td>Phytoberol</td>
<td>-</td>
<td>21</td>
</tr>
</tbody>
</table>

Values in mg represent the amount obtained after purification from 54.5 kg of potatoes.

[13C]acetate feeding confirmed their synthesis via the acetate-mevalonate pathway. The analysis of the spectrum of rishitin (LXII) led to the suggestion of rapid, *de novo* synthesis of the compound, this being independent of pre-existing metabolic pools (254). In labeling experiments utilizing both [14C]acetate and [2-14C]mevalonate it was demonstrated that incorporation of mevalonate into rishitin occurred more rapidly than incorporation of acetate (444). It was postulated that farnesyl pyrophosphate, which originates from three molecules of mevalonate, cyclizes to 2,3-dihydroxygermacrene (LXXVIII), which is then transformed into a eudesmane derivative. This is followed by decarboxylation and double bond formation yielding rishitin (256,440,442,443) (Scheme 18). No details are available regarding the enzymes involved in the individual steps in this system and their possible induction/activation as a result of infection.

It was observed that when infected potato slices were fed with solavetivone (LXXIV), both lubimin (LXVII) and rishitin (LXII) were produced within 24 hr of incubation (270). Isolubimin (LXXIII) was also isolated from the slices. During the incubation there was a concomitant decrease in solavetivone levels and an increase in isolubimin. Sato et al. (445,446) similarly showed the formation of rishitin from acetate via 3-hydroxy-
Scheme 18. Postulated pathway for the biosynthesis of rishitin (440).
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A postulated sequence of reactions is presented in Scheme 19.

The list of $^{13}$C-labeled compounds in Table VII shows appreciable accumulation of solavetivone (LXXIV), lubimin (LXVII), and dihydrolubimin (LXIX) in the G. cingulata-infected tissue. The NMR spectra of these compounds showed similar $^{13}$C-coupling patterns (254). Dihydrolubimin, in this case, was possibly derived from lubimin, and the reaction may have been catalyzed by fungal enzymes (447). Solavetivone also accumulates in potato tissue after infection with P. infestans (266, 271). Fusarium avenaceum, Phoma exigua (266), and Erwinia carotovora (271, 448), and the fungitoxic lubimin (LXVII) also accumulates as a result of infection with P. infestans (288, 442, 449; cf. Table VII). A postulated pathway for the biosynthesis of lubimin and related compounds (Scheme 20) involves the formation of 2-hydroxygermacrene, which then undergoes oxidation of the methyl group followed by recyclization to produce lubimin and epilubimin (LXX) (256, 270, 443, 450, 451). Epilubimin may also be formed via enolization of lubimin (254). Reduction of the aldehydic function of lubimin and epilubimin may then lead to formation of the respective dihydro compounds (443, 452; cf. Ref. 270). It was suggested that the reverse reaction was also possible, and it is likely that the four compounds exist in equilibrium (Scheme 20).

Phytuberin (LXV) accumulates in potato tissues inoculated with either virulent or avirulent races of P. infestans, or with cell-free elicitor preparations obtained from these (264, 288, 453–455). Other fungal species also induce the formation of this phytoalexin (263, 264, 266, 448, 456–458). The NMR spectrum of the compound from $[^{13}$C]acetate-labeled tissues supported synthesis via a eudesmane precursor which undergoes cission of the bond between C-1 and C-2 and reattachment of C-2 to C-5 via oxygen. Analogous conclusions were drawn in the case of phytuberol (LXVI) (254). A postulated biosynthetic pathway is shown in Scheme 21; hydroxymethylation at C-1 and C-2 of the eudesmane derivative occurs followed by oxidative ring opening and then recyclization to produce the tricyclic structure of phytuberin. The elegant labeling experiments in potato have not yet been followed up by detailed enzymological investigations.

b. Phytoalexins of Sweet Pepper (Capsicum annuum) and Tobacco (Nicotiana spp.). The fungitoxic sesquiterpene, capsidiol (LXXVII) (288, 459), is formed in Capsicum annuum as a result of fungal infection and to a lesser extent by bacterial infection (280, 287, 460, 461). Capsidiol
Scheme 19. Formation of rhabitin from solavetivone (440).
Scheme 20. Postulated pathway for the biosynthesis of lubimin, epilubimin, and their dihydro derivatives (440).
Scheme 21: Postulated pathway for the biosynthesis of phytuberin and phytuberol (440).
accumulates in infection droplets on the leaves and fruits (460, 462-464). Following infection by *P. infestans*, capsidiol was shown to accumulate prior to the cytoplasmic disorganization of the infected cells; however, it was not clear whether the compound was also present in the adjacent uninfected cells (464).

Capsidiol was also formed in the tobacco species *Nicotiana tabacum* and *N. clevelandii* following viral infection (283). Glutinosone (LXXIX), another fungitoxic sesquiterpenoid, was formed in *N. glutinosa* after viral infection (284).

Capsidiol is synthesized via the acetate-mevalonate pathway (287, 465). Incorporation studies utilizing $[^{1,2-13}C]$acetate in *Monilinia fructicola*-inoculated *Capsicum annuum* supported the assumption that the angular methyl group of capsidiol at C-5 arises via migration from the C-10 of a eudesmane-type intermediate (465). Two biosynthetic pathways (Schemes 22a and b) have been suggested (256). One involves the formation of an intermediate, 1-keto-α-cyperone (a product of oxidation of the enone), which is then reduced to capsidiol (Scheme 22a) (466); the other pathway (Scheme 22b) is considered mechanistically more feasible. However, nothing is known regarding the enzymology of the pathways.

c. Phytoalexins of eggplant (*Solanum melongena*), Jimson Weed (*Datura stramonium*), and tomato (*Lycopersicon esculentum*). Lubimin (LXVII) and several other phytoalexins were shown to accumulate in *Solanum melongena* as a result of infection with fungi including *Monilinia fructicola*, *Penicillium frequentans*, *Aspergillus fumigatus*, *Botrytis cinerea*, and *Fusarium oxysporum* (256, 274, 275, 467). Detection of lubimin suggests a possible biogenetic relationship with the phytoalexins of potato (Scheme 20). Murai et al. (276, 468) revised the structure of one of the phytoalexins of *S. melongena*, auberginone (LXXVI), which was originally designated as an "enone sesquiterpenoid" (256, 274, 467), and proposed biogenetic pathways relating various stress metabolites of solanaceous plants. A possible biosynthetic pathway for the formation of auberginone is shown in Scheme 23.

The fruit capsule of *Datura stramonium* produced four phytoalexins—lubimin (LXVII), hydroxylubimin (LXVIII), capsidiol (LXXVII), and 2,3-dihydroxygermacrene (LXXVIII)—when inoculated with *Monilinia fructicola* or several other nonpathogenic fungi (278). The germacrene derivative is a possible precursor (276, 440) of the other compounds and has only moderate antifungal activity (278).

Phytoalexins common to other solanaceous plants may be associated
Scheme 22. Postulated pathways for the biosynthesis of capsidiol (256).
Scheme 23. Proposed pathway for the biosynthesis of auberginone (276).
with resistant responses of tomato plants (*Lycopersicon esculentum*) to vascular wilt caused by *Verticillium albo-atrum* (469,470), *Fusarium oxysporum* (471), and *V. dahliae* (472). Infection of *L. esculentum* with *V. albo-atrum* resulted in the formation of six phytoalexins, whereas only two were detected following infection with *F. oxysporum* (469,473,474). Of these, only one was characterized and identified as rishitin (LXII). The biosynthetic pathway leading to this compound in *L. esculentum* is presumably similar to that in *Solanum tuberosum* (Scheme 18). The occurrence of polyacetylenic phytoalexins in *L. esculentum* is discussed in Section IV.A.

d. Phytoalexins of Sweet Potato (*Ipomea batatas*). When *I. batatas* root tissue was inoculated with the fungus *Ceratocystis fimbriata*, the furanoterpene ipomeamarone (LXXXI) (290,291-294,475,476) first accumulated in the uninfected cells surrounding the infection site, and finally accumulated in the infected region itself (258,290). In addition, varying quantities of 4-ipomeanol (477), ipomeamaronol (LXXXII) (292,293,478), dehydroipomeamarone (LXXXIII) (295), ipomeamine (479), 4-hydroxyipomeamarone (480,481, Scheme 24), 4-hydroxydehydroipomeamarone (482,Scheme 24), and other unidentified furanoterpenoids (483,484) also accumulated. Ipomeamarone and some of the related induced terpenoid derivatives exhibited potent antifungal activity against *C. fimbriata* (483-487). Several other fungi and extracts prepared from them (488-491), abiatic elicitors (489,492,493), and even insect attack (494) induced accumulation of ipomeamarone in *I. batatas* roots.

In *in vivo* labeling experiments, [2-14C]acetate was incorporated into ipomeamarone (LXXXI) and into a lipid fraction (495). Increased extractable activity of acetyl-CoA synthetase (EC 6.2.1.1) and HMG-CoA reductase (EC 1.1.1.34) preceded the accumulation of terpenoids in the infected tissue (337,351,352,496). There was no significant increase in either terpenoid or enzyme levels in uninfected or wounded tissue. The abiatic elicitor HgCl2 induced similar increases in enzyme and terpenoid levels to those observed in the intact infection (338). The results of *in vivo* labeling experiments utilizing [14C]leucine and inhibitors of protein synthesis suggested that an inactive precursor of HMG-CoA reductase was synthesized in response to wounding; this was then converted to the active enzyme when the tissue was treated with HgCl2 (338).

An increase in the level of the entire enzyme system responsible for conversion of mevalonate to isopentenyl pyrophosphate was demonstrated in infected *I. batatas* tissue (408,497), the increase in pyrophos-
phomevalonate decarboxylase being particularly striking. Furthermore, 
$^{14}$C-labeled farnesol was shown to be readily incorporated into ipomea-
marone (498). However, the incorporation was low under strictly anaerobic
conditions. This suggested that some oxidative step might be involved in
the biosynthesis of ipomeamarone (498). Finally, dehydroipomeamarone
(LXXXIII) was shown to be the immediate precursor of ipomeamarone
(294). Attempts have been made to explain the biosynthetic relationships
between the various furanoterpenoids of *I. batatas* (252,499,500) and a
pathway has been proposed (Scheme 24).

3. Diterpenoid Phytoalexins

There are only a limited number of diterpenes that can be classified as
phytoalexins. These include casbene (LXXXIV) (297) and the momila-
tones (LXXXV, LXXXVI) (300).

Geranylgeranyl pyrophosphate (GGPP) is the initial precursor of the
diterpenoids. This compound is formed by the transfer of a prenyl residue
from isopentenyl pyrophosphate (IPP) to farnesyl pyrophosphate (FPP),
the reaction being catalyzed by a prenyl transferase or GGPP synthase.
Some prenyl transferases have been characterized (402,426,501) and their
mechanisms of action studied (331,502-509). In yeast, only one enzyme
was claimed to be responsible for the formation of GPP, FPP, and GGPP;
the enzyme was extensively purified and shown to have a molecular weight
of 84,000 and to consist of two subunits (510). However, in pumpkin
seeds two separate enzymes, GGPP synthase and FPP synthase, were
present. The former increased markedly during germination, whereas the
latter decreased (511).

Cyclization of GGPP can result in the formation of a wide variety of
diterpenoids in higher plants and fungi. The carbon skeleton of diterpenoids
is $C_{20}$; however, $C_{19}$ terpenoids are also found.

a. Phytoalexin of Castor Bean (*Ricinus communis*). The cyclic diter-
pene hydrocarbon casbene (LXXXIV) was formed when cell-free extracts
from young *R. communis* seedlings were incubated with $^{14}$Cmevalonic
acid (512,513). Several other diterpenes were also formed. Casbene was
synthesized at a higher rate if extracts from infected seedlings were used
(299). The fungal species utilized were *Rhizopus stolonifer*, *Aspergillus
niger*, and *Fusarium moniliforme*, and a proteinaceous elicitor (molecular
weight of approximately 30,000) of casbene synthesis was isolated from
the culture filtrate of *R. stolonifer* (297). This was later purified and
shown to be a polygalacturonase (514,515).
The cell-free extract from *R. communis* seedlings was able to convert GGPP directly to casbene (513), whereas other terpenes produced in the extract were synthesized via copalyl pyrophosphate (CPP), an intermediate in the biosynthesis of the gibberellins (512).

Casbene synthetase (reaction 8) has been isolated and characterized (296,516); it had a molecular weight of approximately 53,000 and exhibited a broad pH optimum of 7.5 to 9.0. The enzymic reaction was highly dependent on Mg$^{2+}$ concentration and showed maximal activity with concentrations above 5 mM. Manganese was less effective and was inhibitory at above 0.2 mM. The $K_m$ value for geranylgeranyl pyrophosphate was 1.9 $\mu$M. The above properties indicated that casbene synthetase was similar to other diterpenoid cyclization enzymes (296); however, the enzyme was only poorly inhibited by iodoacetamide and N-ethylmaleimide, suggesting the absence of highly reactive sulphydryl groups in this case. The synthetase was strongly inhibited by the plant growth retardant Phosphon D, although it was only weakly inhibited by the steroid synthesis inhibitor SKF-525A and the growth retardant Amo-1618. Maximum extractable activities of casbene synthetase were measured between 12 and 15 hr after the exposure of seedlings to *R. stolonifer* spores. The synthesis of casbene is one of the few cases where a single-step pathway catalyzed by a characterized enzyme leads to phytoalexin formation from a common branchpoint metabolite, and the suitability of this system for regulatory studies has been pointed out (296).

b. Phytoalexins of rice (*Oryza sativa*). Two diterpenoid phytoalexins have been isolated from rice leaves infected with the blast-fungus *Pyricularia oryzae* (300–302). These compounds were also produced when the leaves were irradiated with ultraviolet light (302). The phytoalexins were identified as momilactone A (LXXXV) and momilactone B (LXXXVI). The production of the compounds was enhanced by prior treatment of the
plants with 2,2'-dichloro-3,3'-dimethylcyclopropane carboxylic acid (WL28325), a systemic protectant (301). The diterpenes accumulated in the infection site and the neighboring tissue, and neither of the compounds occurred in uninfected leaves. A hypothetical pathway for the biosynthesis of the momilactones has been proposed (14).

4. Triterpenoid Phytoalexins

Theoretically, triterpenoids may be formed either by condensation of two molecules of FPP or through subsequent prenyl transfer from IPP to GPP. The triterpenoids that can be classified as phytoalexins are gossypol (LXXXVII), 6-methoxygossypol (LXXXVIII), and 6,6'-dimethoxygossypol (LXXXIX), which accumulate in cotton plants (Gossypium spp.) (303–305,517). Related compounds that also accumulate are hemigossypol (XC), 6-methoxyhemigossypol (XCI), desoxyhemigossypol (XCII), and desoxy-6-methoxyhemigossypol (XCIII) (252,303,305,517–521). Phytoalexin induction in Gossypium was elicited by fungal species including Aspergillus niger, Colletotrichum dematium, Meloidogyne spp., Rhizoctonia solani, and Verticillium spp. (517,522–524); fungal extracts were also active (522; also see Ref. 252).

Desoxyhemigossypol (XCII) and desoxy-6-methoxyhemigossypol (XCIII) can undergo autooxidation to form hemigossypol (XC) and 6-methoxyhemigossypol (XCI), respectively. Hemigossypol can then be enzymically transformed to gossypol (518,525; see also Ref. 252). From incorporation studies utilizing [14C]acetate and [14C]mevalonate in excised cotton roots, it was established that gossypol biosynthesis took place via the isoprenoid pathway (526,527). Stereospecific incorporation of mevalonate into gossypol was demonstrated using a cell-free enzyme system prepared from Gossypium roots (526). It was further shown that [2-14C]neryl pyrophosphate (NPP) was incorporated into gossypol more efficiently than GPP. The cis-cis and trans-cis isomers of FPP were better precursors than cis-trans and trans-trans FPP (528). This was consistent with the observation that the Δ6 double bond of farnesol had to be in the cis-configuration before incorporation into gossypol (526).

A high molecular weight enzyme was isolated, in a homogeneous state, from Gossypium root extracts. The enzyme catalyzed the formation of cis-cis FPP in addition to other isomers (529,530). The enzyme was eluted in a single protein peak when passed through a Sepharose 6B column. However, it was later shown that the protein was a multienzyme complex, and it was possible to split it into three protein fractions—I, II, and III (531,532). Analysis by SDS-gel electrophoresis revealed that fractions I
and III were monomeric forms with molecular weights of 51,500 and 98,000 respectively, whereas II was a trimeric form with molecular weights for the subunits of 32,400, 38,800, and 57,700. Fraction I showed no assayable enzymic activity, whereas II and III yielded acid labile products when incubated with IPP plus GPP, IPP plus NPP, or IPP alone; fraction II showed sigmoidal kinetics (531,532). Analysis of the reaction products indicated that fraction II was a prenyl transferase which could utilize GPP or NPP, together with IPP, for the synthesis of four isomers of FPP, whereas fraction III displayed IPP-DMAPP isomerase activity (531,532). Thus the complex could form FPP through a sequential reaction (Scheme 25).

The enzymic cyclization of cis-cis FPP possibly involves allylic rearrangement yielding an intermediate that was referred to by Heinstein et al. (531) as component 1 (see Scheme 25). The actual structure of the compound is not known; however, mass spectrometric analysis gave an apparent molecular weight of 218 or 220, and the fragment pattern was identical to that of a cadinene molecule (531,533). It was further demonstrated that the [14C]-labeled component 1 was incorporated into gossypol more efficiently than [2-14C]mevalonate (531). Component 1 accumulated if an iron- or copper-chelating agent was added to a reaction mixture containing [2-14C]mevalonate and a cell-free enzyme preparation from Gossypium roots, under anaerobic conditions (534). These conditions would clearly inhibit enzyme activities such as laccase or phenol oxidase. A peroxidase from Gossypium was shown to couple hemigossypol to form gossypol (535). Based upon these observations, a biosynthetic pathway for gossypol formation was postulated (531) (Scheme 25).

C. DEGRADATION OF TERPENOID PHYTOALEXINS

1. Host Metabolism

In tubers of Solanum tuberosum the level of sesquiterpenoids, in particular rishitin (LXII), reaches a maximum by 96-120 hr after infection; thereafter it decreases to negligible amounts by the tenth day. It was observed that exogenously applied [14C]rishitin was quickly metabolized by healthy, as opposed to infected, tissue of S. tuberosum (536). Two metabolites were identified as 13-hydroxyrishitin and 11,12-dihydorrishitin (537). Oxidation of exogenously added rishitin to 13-hydroxyrishitin was also demonstrated in S. tuberosum cell suspension cultures (538). Lubimin (LXVII) was similarly rapidly metabolized by the cultures, although the products were not identified in this case (538).
Scheme 25. Postulated pathway for the biosynthesis of gossypol (531).
The Capsicum phytoalexin, capsidiol (LXXVII), was metabolized when aseptically applied to healthy pepper tissue. Up to 65% of applied [14C]capsidiol was converted into a water-soluble product within 48 hr. This was later identified as 13-hydroxycapsidiol (538). Similar metabolism was obtained in tissue cultures of Capsicum. The early studies on metabolism of capsidiol led to the suggestion that induction of terpenoid phytoalexins might result from inhibition of their turnover (539; see comments in Section II.C.1).

Hydroxylation of terpenoid phytoalexins may in some cases be followed by glycosylation of the hydroxyl group. Glycosylated derivatives of solavetivone (LXXIV) have been detected in Nicotiana spp. (540).

2. Fungal Metabolism

Ward and Stoessel (541) demonstrated that capsidiol (LXXVII) was oxidized in vitro to its keto form, capsinone (LXXX), by Botrytis cinerea and Fusarium oxysporum f. vasicintum. These fungi belonged to a group of 10 species which were shown to induce capsidiol in sweet pepper. Capsinone displayed appreciably reduced fungitoxic activity. In vivo experiments were also carried out in which capsidiol was again shown to be oxidized. Using a number of Fusarium species it was demonstrated that there was no apparent relationship between detoxification of the phytoalexin and pathogenicity of the fungus (541).

Lubimin (LXVII) is converted to dihydrolubimin (LXIX) by Glomerella cingulata during infection of potato tissue (254). Whereas dihydrolubimin accumulated in potato-G. cingulata interactions, there were only traces of the compound detected in Monilinia fructicola-infected tissues (see Table VII). Several other fungi are also known to carry out enzymic reduction of lubimin to dihydrolubimin (447).

Ipomeamarone (XXXI), when incubated with Corticium rolfsii, disappeared over a period of six days (542). Similarly, some Fusarium species were able to transform 4-hydroxymyoporone (see Scheme 24) into a number of products (480,543,544). It is quite likely that formation of several of the terpenoid metabolites accumulating in infected plant tissues may be brought about by fungal enzymes.

IV. METABOLISM OF MISCELLANEOUS PHYTOALEXINS

The isoflavonoid phytoalexins of the Leguminosae and the terpenoids of the Solanaceae, Convolvulaceae, and Malvacae have been the subject of the majority of phytoalexin biosynthetic studies. However, a wide variety of
other compounds is also now accepted as phytoalexins, and many of these fall into clearly defined chemical classes (Table VIII). In addition to isoflavonoids, other shikimate-polymalonate-derived compounds may act as phytoalexins; these include vignafuran (CXVII) (545) from Vigna unguiculata (Leguminosae), the stilbene phytoalexins from Arachis (Leguminosae) and Vitis (Vitaceae), and the dihydrophenanthrene derivatives from Orchis and Loroglossum (Orchidaceae). Acetate-polymalonate-derived phytoalexins include the chromones, isocoumarins, and polyacetylenes. The occurrence of polyacetylenic phytoalexins in Vicia faba (Leguminosae) and Lycopersicon esculentum (Solanaceae), along with the usual isoflavonoid or terpenoid derivatives, represents one of the few cases where the taxonomic distribution of phytoalexins does not follow the expected pattern; polyacetylenic secondary products are generally characteristic of the Compositae. With the exception of the stilbenes, few reports exist concerning the cell-free formation of the phytoalexins, or their near precursors, described in this section.

A. ACETATE-POLYMALONATE DERIVATIVES

Biosynthetically, the acetate-polymalonate phytoalexins can be divided into two classes: those arising via polyketide intermediates and those synthesized from fatty acids (14). Few examples of the polyketide class have been reported so far, however, the best known example is the isocoumarin stress compound 6-methoxyxymellein (XCVII) from roots of Daucus carota (546,547). Early labeling experiments demonstrated the synthesis of this compound from $[^{14}C]$acetate (548), and its polyketide origin was confirmed by $^{13}$C biosynthetic studies (549). Both 6-methoxymellein and the closely related chromone eugenin (XCVI), as isolated from D. carota roots, may be synthesized via the same initial polyketide intermediate (549, Scheme 26). The labeling pattern for $^{13}$C incorporation from $[^{13}C]$acetate into eugenin suggested that the intermediate formed after ring closure may not be rigidly attached to an enzyme, thus allowing free rotation of the aryl ring (549). An alternative folding pattern for the polyketide during eugenin formation is also possible (549). Recently, two other chromone phytoalexins, lathodoratin (XCIV) and methyl-lathodoratin (XCV), have been isolated from Lathyrus odoratus (Leguminosae) (550). These compounds are unusual in their lack of a substituent at C$_2$ and their possession of an ethyl group at C$_3$. It was suggested that their biosynthetic origin may be via a polyketide, with the addition of a 1 carbon fragment at a later stage, or via the degradation of a 2',5'-dioxygenated isoflavone (550).
Following infection with *Botrytis* spp., *Vicia faba* produces a number of furanoacetylenic phytoalexins including wyerone (XCIX), wyerone acid (C), dihydrowyerone (CIII), dihydrowyerone acid (CIV), wyerone epoxide (CII), and wyerol (CI) (55,551–554). It has been proposed that wyerone acid, the most antifungal of the *V. faba* furanoacetylenes, is formed via the sequence wyerol → wyerone → wyerone acid, and a similar sequence of
<table>
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<th>Biosynthetic origin</th>
<th>Chemical class</th>
<th>Trivial name</th>
<th>Compound number</th>
<th>Structure</th>
<th>Species</th>
<th>References</th>
</tr>
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</table>
| Acetate-polymalonate| Chromone       | Lathodoratin | XCVI             | ![Structure 1](image1) | *Lathyrus odoratus*  
*Lathyrus hirsutus* | (550) |
|                     | Methyl-lathodoratin | XCV          | ![Structure 2](image2) | *Lathyrus odoratus*  
*Lathyrus hirsutus* | (550) |
|                     | Eugenin         | XCVI         | ![Structure 3](image3) | *Daucus carota* | (549) |
| Isocoumarin         | 6-Methoxymellein| XCVII        | ![Structure 4](image4) | *Daucus carota* | (546,547) |
| Acetophenone        | Xanthoxylin     | XCVIII       | ![Structure 5](image5) | *Citrus limon* | (589) |
| Furano-acetylene    | Wyerone         | XCVII        | ![Structure 6](image6) | *Vicia faba* | (55) |
|                     | Wyerone acid    | C            | As above, R = H | *Vicia faba* | (558) |
Acetate-poly-
malonate

Furano-
acetylene

Wyerol

Cl

\[
\text{CH}_2-\text{CH}-\text{CH}-\text{CCl}_3-\text{CH}-\text{CH}_2-\text{CO}_2\text{CH}_3
\]

\[\text{Vicia faba}\] (552)

Wyerone-4,5-
epoxide

ClII

\[
\text{CH}_2-\text{CH}-\text{CH}-\text{CCl}_3-\text{CH}-\text{CH}_2-\text{CO}_2\text{CH}_3
\]

\[\text{Vicia faba}\] (554)

Dihydro-
wyerone
(R = CH₃)

ClIII

\[
\text{CH}_2-\text{CH}_3-\text{CH}_2-\text{CH}-\text{CCl}_3-\text{CH}-\text{CH}_2-\text{CO}_2\text{CH}_3
\]

\[\text{Vicia faba}\] (553)

Dihydro-
wyerone acid

ClIV

As above, R = H

\[\text{Vicia faba}\] (553)

Polyacetylene

Safynol

CVIII

\[
\text{CH}_2-\text{CH}-\text{CH}-(\text{C}═\text{C})_3-\text{CH}-\text{CH}-(\text{CH}═\text{CH})\text{-CO}_2\text{CH}_3
\]

\[\text{Carthamus}
\text{tinctorius}\] (559,560)

Daucus carota

Lycopersicon
esculentum

(547)

(561)

Falcariol
(R = H)

CIX

\[
\text{CH}_2-(\text{CH})_2-\text{CH}-\text{CH}-(\text{C}═\text{C})_3-\text{CH}-\text{CH}_2
\]

\[\text{Vitis vinifera}
\text{(trans)}\]

\[\text{Arachis hypogaea}
\text{(African cultivars)}\]

(570)

(572)

Falcarindiol

CX

As above, R = OH

\[\text{Vitis vinifera}
\text{(trans)}\]

\[\text{Arachis hypogaea}
\text{(African cultivars)}\]

(570)

(572)

Shikimate-
poly-
malonate

Stilbene

Pinosylv

CXI

\[
\text{HO}
\]

\[\text{Vitis vinifera}
\text{(trans)}\]

\[\text{Arachis hypogaea}
\text{(African cultivars)}\]

(570)

(572)

Resveratrol
\text{(cis- and trans-)}

CXII

\[
\text{HO}
\]

\[\text{Vitis vinifera}
\text{(trans)}\]

\[\text{Arachis hypogaea}
\text{(African cultivars)}\]

(570)

(572)
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<th>Trivial name</th>
<th>Compound number</th>
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<td>Betagarin</td>
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<td>Hircinol (R = H)</td>
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<td>Loroglossum hircinum   (580)</td>
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</table>
reactions may occur for the dihydro derivatives (553, 555). Radiolabeling studies in HgCl₂-treated and *Botrytis cinerea*-infected cotyledons of *V. faba* have demonstrated the biosynthesis of wyerone from acetate, malonate, and oleate (556), thus confirming its origin via the fatty acid pathway. The enzymology of fatty acid biosynthesis in plants to the level of oleate is reasonably well understood (557). However, no information is available on the later stages of wyerone biosynthesis.

In addition to the host-catalyzed interconversions of the wyerone derivatives, both *Botrytis cinerea* and *B. fabae* can metabolize wyerone *in vitro* to the less antifungal wyerol (Cl) (552). Similarly, wyerone epoxide was converted to wyerol epoxide (CV) and dihydrodihydroxy-wyerol (CVI), these more polar metabolites again being less fungitoxic (554). Wyerone acid was converted by germinating conidia of *Botrytis fabae*, but not of *B. cinerea*, to a hexahydro derivative (CVII) (558).

The polyacetylenes safynol (CVIII) and falcarkinol and falcarindiol (CIX, CX) accumulate in infected tissues of *Carthamus tinctorius* and *Lycopersicon esculentum*, respectively, following infection by *Phytophthora drechsleri* (*C. tinctorius*) and *Cladosporium fulvum* (*L. esculentum*) (559–561). Falcarkinol also occurs in carrot root tissue infected with *Botrytis cinerea* (547). Like the furanoacetylenes, these polyacetylenes are derived from oleic acid.
B. SHIKIMATE-POLYMALONATE DERIVATIVES

In spite of the widespread occurrence of flavonoids in higher plants, the flavanone betagarin (CXIX) is the only member of this class to which phytoalexin properties have been ascribed so far (562). It accumulates in Beta vulgaris, along with the closely related isoflavone betavulgarin (CXX), following infection with Cercospora beticola (562). The 5-O-methyl, 2'-hydroxy, 4'-deoxy, 6,7-methylenedioxy substitution pattern of these two compounds is not found in the isoflavonoid phytoalexins of the Leguminosae. In particular, the B-ring hydroxylation pattern has interesting implications for both hydroxycinnamoyl-CoA ligase and chalcone synthase substrate specificity (see Section II.B.2).

The 2-arylbenzofuran vignafuran (CXVIII) accumulates along with the isoflavonoid phytoalexins kievitone (IV) and medicarpin (IX) in leaves of Vigna unguiculata infected with Colletotrichum lindemuthianum, and it is the most fungitoxic of the Vigna phytoalexins toward C. lindemuthianum (563). Results of labeling experiments utilizing L-phenylalanine[U-14C], DL-phenylalanine[1-14C], and DL-phenylalanine[2-14C] in ultraviolet irradiated seedlings of V. unguiculata led to the suggestion that vignafuran may be derived from an isoflavonoid precursor by a route involving loss of C-2 (isoflavonoid numbering). In this scheme, the phenylalanine-derived aromatic ring becomes the 2-aryl substituent and not a part of the benzofuran system (564). It was pointed out that alternative mechanisms could not be excluded, and attention was drawn to the possible formation of other arylbenzofurans such as the moracins (CXVII) [phytoalexins from Morus alba (565)] by oxidative cyclization of hydroxystilbenes (564).

Next to the isoflavonoids, the stilbenes are the most important class of phytoalexin derived from the shikimate-polymalonate pathway (14,566,
The antifungal activity of pinosylvin (CXI), a component of the heartwood of *Pinus* spp., has been recognized for some time (568), and its appearance in living sapwood in response to infection has resulted in its classification as a phytoalexin. *Trans*-resveratrol (CXII), its dimethyl derivative pterostilbene (CXIV), and the α-, β-, γ-, and ε-viniferins have all been isolated from infected or ultraviolet-irradiated leaves of *Vitis vinifera* (569-571). The antifungal activity of the viniferins is greater than that of the resveratrol monomer from which they are derived (569), although pterostilbene is the most antifungal of the *Vitis* phytoalexins (571). *Cis*- and *trans*-resveratrol are found in fungus-infected hypocotyls of African cultivars of peanut (*Arachis hypogaea*) (572), whereas in American cultivars of this plant these stilbenes (CXIII) are prenylated at position 4 (573) (see Table VIII).

Stilbene synthase was first detected in crude acetone powders from *Rheum rhaponticum* (574). The enzyme, which was not light-inducible, converted [2-14C]malonyl CoA and 4-coumaryl CoA (XXIa) to resveratrol, the incorporation of 14C into the A ring being shown by chemical degradation studies (574). The enzyme activity, of which a high percentage cosedimented with the 15,000 × g pellet of crude aqueous extracts, was dependent on dithiothreitol for activity and was inhibited by iodoacetamide. Dithiothreitol could not be replaced by 2-mercaptoethanol, and it was suggested that the enzyme might be regulated by oxidation reduction of a specific thiol group (574). A reaction sequence was proposed (Scheme 27) involving polyketide formation and intramolecular C-C bond formation in a manner analogous to the reaction catalyzed by 6-methylsalicylic acid synthase (574). It is interesting to compare the intramolecular aldol reaction (between carbon atoms a and b) in the stilbene synthase ring-closure step with the intramolecular acylation which results in chalcone formation (Scheme 2). Scheme 27 also summarizes a hypothetical reaction sequence for the formation of stilbene carboxylic acids, the reduction and dehydration of the polyketide being analogous to the 6-methylsalicylic acid synthase and putative 6'-deoxy chalcone synthase reactions.

Experiments to elucidate the B-ring substrate specificity of the *Rheum* stilbene synthase (575) have demonstrated that the enzyme is most active in the formation of resveratrol with 4-coumaryl CoA as substrate, although pinosylvin (CXI) was formed from cinnamoyl CoA at a lower rate (29%). In the context of the formation of rhaponticin (3,5,3'-tri hydroxy-4'-methoxy-stilbene 3-O-β-D-glucoside) by *Rheum*, the production of the corresponding B-ring-substituted stilbenes *in vitro* with feruloyl, isoferu-
Scheme 27. Proposed reaction sequence for stilbene synthase (574).
loyl, or caffeoyl CoA as substrates was always less than 10% of the rate observed with 4-coumaroyl CoA (575). These results therefore supported radiolabeling experiments which had suggested that the B-ring substituents of the stilbenes, other than the 4'-hydroxyl group, were determined after the stilbene synthetase reaction. Furthermore, pinosylvin (CXI) was not converted to rhapsonticin in Rheum, thus indicating that the stilbene 4'-hydroxyl was only inserted at the cinnamic acid level (575). The Rheum membrane-associated synthase obeyed normal Michaelis Menten kinetics, with a $K_m$ for 4-coumaroyl CoA of 13 $\mu M$ and for malonyl CoA of 370 $\mu M$ (575). The pH optimum was 7.5 for 4-coumaroyl CoA as substrate, although astringenin (3,5,3',5'-tetrahydroxy stilbene) formation from caffeoyl CoA had a pH optimum of around 6.5 (575); a similar variation of pH optimum for these substrates is observed with chalcone synthase (Section II.B.2).

A stilbene synthase has recently been isolated from the roots of six-week old Pinus seedlings (576). The enzyme formed pinosylvin from malonyl CoA and cinnamoyl CoA, but only poorly formed resveratrol from 4-coumaroyl CoA. The activity was induced in both needles and roots by exposure of the plants to ultraviolet light (576); induction occurred in the roots even if the needles were the only part of the plant exposed to irradiation. Similar ultraviolet-inducible stilbene synthases have been extracted from the leaves of five species of Vitaceae (577); these enzymes formed resveratrol and were coinducible with PAL and cinnamic acid 4-hydroxylase, maximum activities of all three enzymes occurring at 15 hr after irradiation. The stilbene synthase from Cissus antarctica was five times more active with 4-coumaroyl CoA than with cinnamoyl CoA, and it appeared particulate if extracted in aqueous buffers (577). The pH optimum of the particulate preparation was 5.0-6.0, whereas this increased to 7.0-8.0 if the enzyme was solubilized by detergent treatment. There is clearly enough information now available to help initiate detailed studies on the role and regulation of stilbene synthase during phytoalexin accumulation.

Indirect evidence for the intermediacy of resveratrol in the synthesis of the viniferins has come from studies of the time courses for phytoalexin accumulation in ultraviolet-irradiated leaf discs of Vitis vinifera (570); resveratrol accumulated rapidly, and its decline coincided with the appearance of $\varepsilon$-viniferin (CXV). Furthermore, resveratrol monomer, dimer ($\varepsilon$-viniferin), and trimer (a-viniferin) appeared sequentially during infection of intact plants by Plasmopara viticola (570). 4-Hydroxylated stilbenes may undergo oxidative dimerization in the presence of $\text{H}_2\text{O}_2$ and horseradish peroxidase (569). It was therefore suggested (569) that the
 viniferins may be formed by peroxidase attack in a manner similar to the formation of the dimeric phenylpropanoid lignans (578).

The dihydrophenanthrene phytoalexins of the Orchidaceae represent another class of shikimate-polymalonate-derived phytoalexins. A hypothetical scheme for the formation of orchinol (CXXI) has been given by Stoessl (14) (Scheme 28); this model is indirectly supported by the cooccurrence, in some plant species, of dihydrophenanthrenes, dihydrostilbenes, and/or spiro compounds (579). The latter may originate via the reduction of the intermediate CXXIb, and a biosynthetic origin for loroglossol (CXXIII) via cyclization of a suitably substituted dihydrostilbene (e.g., intermediate CXXIa) has been suggested elsewhere (580). No enzymic details are at present available.

C. OTHERS

The furanocoumarin xanthotoxin (CXXIV) has been implicated as a phytoalexin in roots of *Pastinaca sativa*, where its concentration increases in response to infection by a variety of nonpathogens (581). It is, however, also found in low quantities, along with at least five other coumarins, in uninfected plants. Radiolabeling experiments have demonstrated the formation of the linear furanocoumarins from the coumarin umbelliferone (CXXVII), although incorporation of [14C]mevalonate into the furan ring was very poor (582). However, the isolation, from cell suspension cultures of *Ruta graveolens*, of a dimethylallyl transferase catalyzing the formation of 6-dimethylallylumbelliferone (reaction 9) has lent acceptance to the mevalonate origin of the furan ring (213). The transferase, which was found in a readily sedimenting subcellular fraction assumed to be plastids, required Mn2+ and was specific for dimethylallylpyrophosphate and umbelliferone (213).

\[ \text{HO} \text{(CXXVII)} \xrightarrow{\text{DMAPP}} \text{HO} \text{(CXXIV)} \]

\[ (9) \]
Scheme 28. Proposed pathway for the biosynthesis of dihydronaphthrene phytoalexins (14).

R \rightarrow S
The simple phenylpropanoids coniferyl aldehyde (CXXV) and coniferyl alcohol (CXXVI) accumulate rapidly in leaf diffusates from incompatible interactions of near-isogenic flax lines with the rust fungus *Melampsora lini*, and their role as phytoalexins was proposed (583). Further work is necessary to establish whether these compounds originate de novo via the phenylpropanoid pathway from phenylalanine or are formed by the oxidation of preformed lignans. If the first case proves correct, these phytoalexins will be among the few for which each step in their formation has been demonstrated in plant cell-free systems. The enzymes responsible for the formation of coniferyl aldehyde and coniferyl alcohol (cinnamoyl CoA:NADP-reductase and cinnamyl alcohol:NADP-dehydrogenase) have been isolated from a number of plant species (584-586). The stereochemistry of the formation of coniferyl alcohol from feruloyl CoA has been elucidated (587,588), and a mechanism of action has been proposed for the cinnamyl alcohol:NADP-dehydrogenase from *Glycine max* (588).

V. Regulation of Phytoalexin Metabolism

A. BIOLOGICAL CONSIDERATIONS

Following a recent NATO Advanced Study Institute, a simple “Working Redefinition” of phytoalexins has emerged: “Phytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms” (590). This definition implies that phytoalexin accumulation is a highly regulated process, and the purpose of the remainder of the chapter is to review our present understanding of the control of phytoalexin accumulation.

As phytoalexin accumulation has now been reported in a large number of host-pathogen interactions (26), a number of general biological features have emerged which are relevant to an understanding of control:

1. There is a close correlation between phytoalexin accumulation and necrogenic (hypersensitive) resistance to fungal, bacterial, and viral infections (26). In many cases phytoalexins accumulate to levels as high as 10% of the dry weight of the infected tissue (591).

2. The accumulation of phytoalexins during the hypersensitive response is modulated by a number of factors including ontogenic changes in the host (546,592-596), temperature (597-601), light intensity and photoperiod (602,603), phytohormone concentrations (604,605), carbon (606) and nitrogen (607) status, ultraviolet irradiation (608), and fungi-
cide treatment (300,301). Clearly, therefore, the extent of phytoalexin accumulation by the host plant following attempted infection is highly dependent on the developmental and physiological status of the plant.

3. Induction of phytoalexin accumulation reflects specific changes in host metabolism. For example, in healthy hypocotyls of *Phaseolus vulgaris*, flavonol glycosides, leucoanthocyanins, and hydroxycinnamic acid derivatives account for all the phenylpropanoid derivatives. Induction of isoflavonoid-derived phytoalexins, such as kievitone (IV), coumestrol (XV), and phaseollin (XIII), is accompanied by negligible changes in the levels of the phenylpropanoids present in healthy tissue (118). Thus even within a group of products that are closely related biosynthetically, phytoalexin accumulation is a specific event. Such specificity has been observed in a number of cases (52,118,599,609,610).

4. Phytoalexin accumulation is highly localized spatially (111,611-615). Phytoalexins are synthesized in living cells (611,613,616); however, cell necrosis is closely related to phytoalexin accumulation, and phytoalexins accumulate in dead and dying cells (26,611). There is considerable evidence that phytoalexins are toxic to plant cells as well as to microorganisms (222,617-619), causing inhibition of mitochondrial respiration and disruption of cellular membranes. Phytotoxicity may result from vascular accumulation of phytoalexins above a critical concentration (617).

5. There is strong but indirect evidence that phytoalexin accumulation is a crucial component of necrogenic resistance in plants (26). The evidence is based on the following types of observation: (a) phytoalexins accumulate rapidly to fungitoxic levels in close association with expression of necrogenic resistance; (b) factors which affect phytoalexin accumulation have a corresponding effect on necrogenic resistance; (c) pathogens are less susceptible to phytoalexins than nonpathogens; and (d) pathogenic fungi can metabolize phytoalexins to less fungitoxic products. Exceptions to each of these four characteristics have been observed, and for many individual host-parasite interactions not all four features are exhibited. Nonetheless, the balance of evidence indicates that phytoalexins have a primary function in the protection of plants against attack by microorganisms, notably fungi.

It is clear that phytoalexin accumulation represents a major and specific metabolic commitment of plant cells that is highly controlled in both space and time. In recent years some progress has been made in the elucidation of the molecular mechanisms governing this defense response.
B. INDUCTION OF PHYTOALEXIN ACCUMULATION

The picture that emerges from ultrastructural, physiological, and genetic studies of the interaction between plants and fungal pathogens is that phytoalexin accumulation and necrogenic resistance are induced by a recognition process localized at the interface between host and pathogen (26). There is currently much interest in molecules from microorganisms that can elicit phytoalexin accumulation. The first elicitor to be identified was a polypeptide, monilicolin A, from the stone-fruit pathogen Monilinia fructicola, which stimulated production of phaseollin (XIII) in Phaseolus vulgaris (620). Subsequently the following types of molecules were identified as natural or biotic elicitors: (a) the lipids arachidonic acid and eicosapentaenoic acid (621); (b) protein-lipid-polysaccharide complexes (622,623); (c) lipopolysaccharides from bacterial outer cell wall membranes (624); (d) glycoproteins from the cell surfaces and culture filtrates of bacteria and fungi (72,297,514,515,625-631); and polysaccharides from the cell surfaces and culture filtrates of bacteria and fungi (21,72, 116,632-637).

The glycoproteins released from cell walls of Phytophthora megasperma f.sp.glycinea by alkali lose elicitor activity following periodate treatment, but not following heat or protease treatment, thus indicating that the carbohydrate moiety is the site or elicitor activity (630). An elicitor present in culture filtrates of Rhizopus stolonifer is a glycoprotein of molecular weight 30,000 ± 5000 which is active at concentrations as low as $2 \times 10^{-8} M$ (297). Activity is abolished by periodate, but in this case activity is also sensitive to protease treatment, suggesting that both carbohydrate and protein components are required for elicitor activity (297). Recently it has been shown that elicitor activity copurifies with the enzyme polygalacturonase (515), but it has not been established whether elicitor activity is dependent on expression of enzyme activity or merely on enzyme structure.

The relationship between elicitor structure and function is best characterized for the highly branched $\beta_1$$\rightarrow$3, $\beta_1$$\rightarrow$6-linked glucans present in culture filtrates and heat-released from cell walls of Phytophthora megasperma f.sp.glycinea (21). Chemical degradation studies have shown that the minimum structural entity required for elicitor activity is a branched nonasaccharide fragment, but active molecules up to a molecular weight of $10^6$ have been obtained. Similar elicitors have been obtained from a range of fungi (21) and even yeast extracts (638). The glucan elicitors from
Phytophthora megasperma f.sp.glycinea are capable of causing the formation of chemically diverse phytoalexins in a variety of host plants including glyceollin (XIV) in Glycine max, phaseollin (XIII) in Phaseolus vulgaris, and rishitin (LXII) in Solanum tuberosum (639). Furthermore, these glucan elicitors as isolated do not show race-specific features, and race-cultivar specificity in the interaction between Phytophthora megasperma f.sp.glycinea and G. max cannot apparently be accounted for in terms of specific induction of phytoalexin accumulation by these elicitors in resistant but not susceptible interactions (21). It was suggested that such elicitors trigger phytoalexin accumulation in a nonspecific defense response, and that race specificity was determined by extracellular glycoproteins that were only weak elicitors (640). These specificity factors were presumed to act by stimulation of a second, hitherto uncharacterized defense response. However, race specific glycoprotein elicitors have been isolated from cell walls of Phytophthora megasperma f.sp.glycinea (630) and the cell envelope of Pseudomonas glycinea (625).

There is some evidence that virulent pathogens may produce suppressors that inhibit or delay active resistance (26). Of special interest is the suggestion that in potato cultivars with R genes, specificity in resistance to physiological races of Phytophthora infestans is associated with compatibility, and a phytoalexin suppressor was proposed as the determinant of specificity (634,641–643). Mycelia and zoospores of a compatible race of P. infestans contain β1→3, β1→6-linked, low molecular weight glucans that can inhibit the rapid cell death, loss of electrolytes, tissue browning, and accumulation of terpenoid phytoalexins associated with the necrogenic resistance reaction of potato tissues to treatment either with elicitor from the same compatible race or infection by an incompatible race. Although the incompatible race contained similar glucans, they were much less active as suppressors of the hypersensitive reaction. The glucans consisted of nonanionic and anionic fractions, the latter containing a small proportion of glucose residues as phosphoryl monoesters. Furthermore, there was a correlation between suppressor inhibition of elicitor-induced protoplast lysis in a race-cultivar specific pattern that correlated with susceptibility.

The precise roles of elicitors, suppressors, and specificity factors remains to be established. First, elicitors are often obtained by relatively harsh treatments of cell walls, and it is not clear that they are released during plant: pathogen interaction. However, recently it has been shown that pea tissue contains enzymes with the potential to degrade the major com-
Pounds of *Fusarium* cell walls and release the elicitor chitosan (644). Similarly, extracts of soybean have been shown to release elicitors from cell walls of *Phytophthora megasperma* f.sp. *glycinea* (645) and degrade isolated glucan elicitor (646, 647). Exposure of insoluble fungal mycelial cell wall to soybean tissue for less than 2 min releases soluble elicitor (645). It has been suggested that host metabolism of fungal elicitors may be important for (a) cleavage of the elicitor to allow its passage through the host cell wall, and (b) subsequent destruction of elicitor activity to ensure a localized response (647). A further consequence of host enzyme-mediated release of elicitors is that race specificity may not reside in the structure of the released elicitor per se but in the linkage between the elicitor and fungal cell wall.

Second, elicitors, suppressors, and specificity factors present in culture filtrates are generally obtained from cultures grown axenically on relatively simple media. The nature of the extracellular molecules changes during the growth of such cultures (627, 648). Furthermore, it has become apparent that plant molecules, notably cell wall material, can cause selective induction of specific fungal extracellular enzymes and glycoproteins (648-650). A study of these molecules may be highly relevant to an understanding of host-pathogen interactions.

Third, fungal cell surface molecules may act in combination in host-pathogen interactions. Thus the sum of the effects on phytoalexin accumulation of eight fractions of the material heat-released from cell walls of *Colletotrichum lindemuthianum* was markedly different from the overall effect of the unfractionated material (116). Similarly, it was observed that insoluble cell wall material and the lipid fraction isolated from mycelia of *Phytophthora infestans* were separately ineffective but together induced rishitin accumulation and necrosis in *Solanum tuberosum* (651).

It is now well established that phytoalexin accumulation can also be induced by a wide range of unnatural or abiotic elicitors (223, 611), including (a) basic macromolecules, for example, poly-lysine, spermidine, histones, and autoclaved ribonuclease A (52, 104); (b) chloroform (652); (c) fungicides, for example, benomyl (653); (d) heavy metal salts, for example, CuCl₂ and HgCl₂ (111, 118, 225, 226, 338, 408, 606, 654-656); (e) localized freezing and thawing (227, 611, 657); (f) surfactants, for example, triton X-15, triton X-35, and sodium dodecyl sulphate (408, 658); (g) ultraviolet light (110, 111, 570, 576, 577, 659, 660); and (h) various DNA intercalating agents, for example, 5-bromo-deoxyuridine, 9- aminoacridine and photosensitive psoralen compounds (102, 106, 108, 660,
Detailed physiological studies by Bailey et al. (611) have shown a close association between plant cell death and accumulation of isoflavonoid phytoalexins in response to such abiotic elicitors. Subsequently it was shown that aqueous extracts of either dead or living French bean tissue stimulated the production of phytoalexins in *Phaseolus vulgaris* hypocotyls (227) or cultured cells (657), suggesting the presence of an endogenous elicitor in plant cells. It was postulated that cell death is the event that triggers isoflavonoid phytoalexin accumulation in *P. vulgaris* tissues treated with an abiotic elicitor (611). It is envisaged that the endogenous elicitor does not function in healthy cells because it exists in a bound, latent form or is compartmentalized. In dying or dead cells the elicitor is released or activated and then initiates phytoalexin accumulation in surrounding healthy cells. Subsequently, the phytoalexins diffuse from here into dead cells where they can accumulate to very high concentrations. Recently, an endogenous elicitor activity has been demonstrated in cell extracts from *Glycine max*, and it appears to be a pectic fragment of the cell wall (662). There is also evidence that a second pectic fragment is a proteinase inhibitor-inducing factor that transmits intercellularly the signal for wound-induced increases in proteinase inhibitor levels (663).

Furthermore, it has been shown that endogenous elicitor activity is released from cotyledons of *Pisum sativum* following treatment with HgCl₂, and pisatin (XI) subsequently was synthesized in living cells (656).

A major attraction of the endogenous elicitor hypothesis is that it might explain how diverse abiotic treatments lead to phytoalexin accumulation. It is also possible that a similar mechanism may operate in mediating phytoalexin accumulation in response to infection or treatment with biotic elicitors. The close association between infected plant cell death, phytoalexin accumulation, and necrogenic resistance has already been noted (26, 611). In *Phaseolus*, phytoalexins are not produced when infected cells remain alive during biotrophic fungal growth; they are produced after the death of infected cells and accumulate in dead cells (611). In this model, release of endogenous elicitor following injury by a pathogen would cause phytoalexin production in surrounding healthy cells and progressive cell death would provide a site for accumulation. Viruses cannot emit toxins or other chemical messages and must therefore be effective as elicitors in themselves; such a mechanism would explain viral induction of phytoalexin accumulation (35, 611).

Although there is little detailed information on the mechanism of action
of biotic elicitors, there is some evidence that such elicitors may be phyto-
toxic as predicted by this model. Thus a glycoprotein elicitor from Clado-
sporium fulvum which elicited rishitin formation in Lycopersicon escu-
ulentum also caused rapid leakage of ions and death of treated cells (626,
631,664). The same elicitor also induces isoflavonoid phytoalexins in
Pisum sativum and Glycine max (626). A glycoprotein elicitor from
Phytophthora infestans was toxic to the cells of many plants (665).
A relatively crude elicitor preparation from Phytophthora infestans causes
agglutination and death of S. tuberosum protoplasts (666). The glucan
elicitor from Phytophthora megasperma f.sp.glycinea inhibits growth of
cultured cells of G. max (113), although whether or not this is a direct
effect is unclear.

While the idea that phytoalexin accumulation is a consequence of cell
death and subsequent release of constitutive elicitors represents an attrac-
tive, unifying, and experimentally testable hypothesis, the evidence in
favor is circumstantial and indirect, and a causally related sequence of
events has not yet been established. It is possible to envisage biotic elicitors
acting by releasing constitutive elicitors independently of cell death. In
this context it is interesting to recall (a) that at least one potent elicitor is
a polygalacturonase (515), and (b) an endogenous elicitor in G. max is a
pectic fragment of the cell wall. Clearly it becomes important to establish
whether polygalacturonase is an elicitor by virtue of its ability enzymically
to release endogenous elicitor independently of cell death. However, it has
been demonstrated on a number of occasions that such enzymes also kill
plant cells (667).

The identification of an endogenous elicitor in extracts of plants cells
has focused attention on the transmission of the elicitation response. How-
ever, characterization of a pectic polysaccharide fragment as an endoge-
nous elicitor does not establish its role as a “second messenger” and other
candidates have been considered, notably the gaseous plant hormone
ethylene (668,669) and biogenic polyamines (670). Ethylene is induced
following infection or elicitor treatment in a number of systems (671-
674), and differences in the timing and extent of ethylene evolution in
susceptible and resistant interactions have been observed (672,673). In
G. max, elicitor treatment increased ethylene production after 1.5 hr
and glyceollin (XIV) accumulation after 6.0 hr (674). However, treatment
with the ethylene precursor aminocyclopropane carboxylic acid in the
absence of elicitor, while increasing ethylene production in a manner
similar to elicitor treatment, had no effect on glyceollin accumulation. Similarly, aminoethoxyvinylglycine, an inhibitor of ethylene biosynthesis, inhibited elicitor induction of ethylene production, but it had no effect on elicitor induction of glyceollin accumulation. It was concluded that ethylene production was an indicator of phytoalexin accumulation, but it did not function as a second messenger (674).

Putrescine, spermine, spermidine, and other biogenic amines accumulate in response to deficiencies of certain cations (675,676) and alter the stability of protoplasts (677). Biogenic amines induce pisatin accumulation in *Pisum sativum* (104), but inoculation with fungi causes no significant alteration in spermine and spermidine levels, suggesting that these molecules are not second messengers for phytoalexin accumulation (670).

Clearly an important step in elucidating the mechanism of action of biotic elicitors would be the characterization of a plant receptor. Agglutination of potato protoplasts by elicitor preparations indicates elicitor binding sites at the protoplast surface (666). Binding of radiolabeled culture filtrate molecules from *Cladosporium fulvum* to cell membranes of *Lycopersicon esculentum* has been reported (628,664). Race-specific oligosaccharide suppressors of the glucan elicitors of *Phytophthora infestans* appear to compete with elicitor for binding sites present in microsomal membrane preparations from *Solanum tuberosum* (641). Interestingly, *N,N*-diacetyl-D-chitobiose, the hapten for potato lectin, inhibits the hypersensitive reaction of potato tuber cells infected by incompatible and compatible races of *Phytophthora infestans* (678). The role of lectins in plant-microbe interactions has recently been comprehensively reviewed (679). However, in none of these cases has the biochemical specificity or biological significance of binding been established, and hence these reports cannot yet be taken as demonstrating elicitor receptor sites. Recently, antisera have been raised against purified cell walls of *Fusarium solani* f.sp. *pisi* (636). Using this probe, it was shown that within 15 min fungal wall components entered the plant cell and accumulated inside the cell wall as fungal growth was inhibited. Furthermore, *H*-labeled chitosan elicitor appeared to move into the plant cell and accumulate in the nucleus. It should be emphasized that a causal sequence of events between these early effects of elicitor and phytoalexin accumulation has not been established. Indeed, there are conflicting reports as to whether elicitor acts as a trigger (514) or is continually required for maximal enzyme or phytoalexin induction (116).
C. INDUCTION OF ENZYMES OF PHYTOALEXIN BIOSYNTHESIS

In recent years it has become generally accepted (64, 65) that the synthesis of plant natural products is controlled by appropriate changes in the levels of biosynthetic enzymes rather than by control of substrate supply (but see Ref. 119 for a dissenting view). Although measurement of the in vivo activity of an enzyme is of crucial importance in this context, the immense technical problems of a true in vivo assay have rarely been overcome, the method of Amrhein et al. (680) for measurement of phenylalanine ammonia-lyase being an elegant exception. However, this in vivo assay has not yet been applied in the study of PAL activity in relation to the accumulation of phenylpropanoid-derived phytoalexins. Hence, in general, it is necessary to extract enzymes and assay their activities in vitro. This procedure, in principle, provides no information on the in vivo activity or compartmentalization of the enzyme, and extraction will lead to changes in the concentration of physiological effectors of the enzyme. Furthermore, the enzyme in vitro is potentially susceptible to inhibition by factors which are in some way latent in vivo, but which are released into a crude cell extract. Important examples include (a) proteolytic enzymes, (b) inhibitors of proteolytic enzymes, and (c) phenolic substances and their oxidation products following exposure to phenolase enzymes. In the present context it is important to note that the levels of these interfering factors will themselves change following elicitor treatment or infection (681, 682). Thus analysis of the effects of mixing active and inactive extracts or coextraction of material from stimulated and non-stimulated tissue should be performed in each study (683). Inclusion of protease inhibitors prevents extensive proteolytic inactivation following extraction. Reductants such as 2-mercaptoethanol prevent phenolic oxidation, and phenolics can be removed by absorption on to resins (684). The presence of low molecular weight effectors can often be detected and corrected for by molecular sieving procedures.

If in the light of such considerations appropriate checks are made, precautions are taken, and the enzyme is assayed under defined, optimum conditions, then it is possible to obtain from extraction and in vitro assay procedures a useful measure of enzyme activity level which can be taken as a relative estimate of the amount of active enzyme molecules in the tissue. Using this approach, the relationship between changes in the activity levels of enzymes of phytoalexin biosynthesis in relation to phytoalexin accumulation following elicitor treatment or infection has now been
examined in a number of systems, notably those involving accumulation of phenylpropanoid-derived phytoalexins, where the enzymology is relatively well understood. The results of such studies have been discussed in Sections II.B, III.B, and IV. Additional relevant information is presented below.

In hypocotyls of Phaseolus vulgaris infected with a compatible race of Colletotrichum lindemuthianum, the causal agent of anthracnose disease, there is a marked increase in PAL activity 24 hr before the attainment of maximum phaseollin (XIII) and coumestrol (XV) levels which are spatially and temporally associated with necrotic lesions (685). In cell suspension cultures of the same plant, phaseollin accumulation can be induced by abiotic elicitors such as autoclaved ribonuclease A and by biotic elicitors present in culture filtrates and heat-released from a variety of fungi (52, 115,170,188). The phytohormone composition of the culture medium is important in determining the magnitude of phaseollin accumulation in response to exogenous elicitor. Furthermore, it is possible to increase phaseollin accumulation in the absence of added elicitors by manipulation of the hormone composition (604,605). At concentrations of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) greater than $2 \times 10^{-5}$ M, there is a marked inhibition of cell growth, and induction of phaseollin and PAL by exogenous biotic and abiotic elicitors is suppressed (604,605). There existed a positive correlation between PAL and phaseollin accumulation as a function of the concentration of 2,4-D. In contrast, manipulation of the concentration of 6-furfurylaminopurine (kinetin) can lead to PAL levels that are higher in control cultures than in induced cultures, and there is no positive correlation between enzyme activity and phaseollin accumulation (605). However, under hormonal conditions optimized for induction of phaseollin accumulation, there is a marked but transient increase in the activity of PAL concomitant with the onset of phaseollin accumulation in response to autoclaved RNase or an elicitor preparation heat-released from cell walls of Colletotrichum lindemuthianum (114,188). The induction of cinnamic acid 4-hydroxylase, 4-coumarate:CoA ligase, chalcone synthase, and chalcone isomerase in this and other systems has been discussed in Section II.B.

Although in P. vulgaris the kinetics of induction of PAL and chalcone synthase (CHS) are very similar, the dose responses for induction of these enzymes in cell cultures by elicitor heat-released from cell walls of Colletotrichum lindemuthianum show significant differences (115,116,170,686). Efficient induction of PAL is observed in two discrete ranges of elicitor.
concentration, with relatively weak induction at intermediate or supra-optimal elicitor concentrations (115, 116). Optimal elicitor concentrations in the two concentration ranges were 17.5 and 50 μg carbohydrate per ml culture. In contrast, CHS activity is only weakly induced at low elicitor concentrations that efficiently induce PAL activity, and optimal induction is observed at an elicitor concentration of 100 μg ml⁻¹ (116). The dose response for induction of phaseollin closely resembles that for induction of CHS.

Marked increases in PAL and 4-coumarate-CoA ligase activities occur in cell suspension cultures of *Petroselinum hortense* treated with elicitor preparations from the culture filtrate of *Phytophthora megasperma f.sp. glycinea* (177). The dose responses for induction of the two enzymes were very similar. In this system isoflavonoid-derived phytoalexins do not accumulate, and elicitor treatment does not cause an increase in CHS activity. Indeed, elicitor treatment completely inhibits the light-mediated increase in CHS activity associated with the accumulation of flavonoid pigments (177). Recent evidence suggests that furanocoumarin phytoalexins accumulate in response to elicitor treatment (687).

Inoculation of seedlings of *Ricinus communis* with *Rhizopus stolonifer*, or a glycoprotein elicitor from the fungus, causes accumulation of the diterpenoid phytoalexin casbene (LXXXIV) and induction of the enzyme system casbene synthase (Section III.B.3). Similarly, inoculation of the scutellum-embryonic axis of maize seedlings with *Rhizopus stolonifer* or other fungi causes a hundredfold increase in the extractable activity of an enzyme system capable of converting mevalonic acid into terpene hydrocarbons in the presence of Mn²⁺ and ATP (688). Geranylgeranylpyrophosphate and copalylpyrophosphate were also efficient precursors for five of the six terpene hydrocarbon products, indicating the accumulation of polycyclic diterpenoids (688), although the products have not yet been identified as phytoalexins.

Thus it is generally the case that induction of phytoalexin accumulation is correlated with increases in the activity levels of the appropriate biosynthetic enzymes. It should be noted that unlike fine control there is no theoretically rigorous criterion for proof that observed changes in enzyme levels regulate flux through a metabolic pathway. However, in those cases where there are marked increases in enzyme level from low basal levels in the unstimulated tissue, concomitant with the onset of phytoalexin accumulation, it is reasonable to conclude that production is regulated by enzyme induction. For example, in cell cultures of *Phaseolus vulgaris,*
PAL, CA4H, 4-coumarate:CoA ligase, and CHS are present at relatively low levels in control cultures and increase markedly and rapidly concomitant with the onset of phaseollin accumulation following elicitor treatment (114,170,188). On this basis it can be concluded that induction of these enzymes controls phytoalexin production. In contrast, chalcone isomerase is present at relatively high levels in control cultures and responds only sluggishly to elicitor treatment, and would therefore not be considered important in control of phaseollin accumulation in this system (114,188). Similarly, it can be concluded that in root discs of Ipomea batatas, HMG-CoA reductase and pyrophosphomevalonate decarboxylase, but not the enzymes of the cytosolic pathway of acetyl CoA formation, represent likely sites for control of sesquiterpenoid phytoalexin accumulation (338,408,689). The very low basal levels of PAL and CHS in cotyledons of Glycine max (138,151) provide further strong evidence, complementing the $^{14}$C$\text{O}_2$ in vivo labeling data of Moesta and Grisebach (225,226) (see Section II.C.1), that there is not a relatively high rate of phytoalexin biosynthesis in control tissue, and that abiotic elicitors do not act by inhibiting phytoalexin turnover against this constant rate of glyceollin synthesis. Hence glyceollin accumulation is regulated by changes in the levels of PAL, CHS (6'-deoxy?), and possibly other biosynthetic enzymes.

Major control sites within a group of enzymes that are under coordinate control and undergo broadly concomitant changes in activity levels can be identified by detailed comparison of the kinetics and dose responses for the induction of each of the enzymes with the kinetics and dose response for accumulation of product. On this basis, PAL and CHS, rather than CA4H and 4-coumarate:CoA ligase, would appear to be major control sites in phaseollin production in *P. vulgaris* cell cultures (114). Clear evidence for PAL as the primary control point in overall phenylpropanoid biosynthesis has been obtained with respect to light-induced flavonoid accumulation in *Petroselinum hortense* (64) and chlorogenic acid in *Solanum tuberosum* tuber discs (690). Analysis was facilitated by the fact that in both cases only one group of phenylpropanoid derivatives accumulated. However, it was recognized that secondary control may be exerted at the first enzymes of specific branch pathways: chalcone synthase in flavonoid biosynthesis (691) and hydroxycinnamoyl transferase in chlorogenic acid biosynthesis (692). In *P. vulgaris* cell cultures, in addition to phaseollin, wall-bound phenolic compounds also increase in response to elicitor (52), and it is likely that PAL controls overall flux in the phenylpropanoid pathway with CHS as a secondary control element regulating entry of material into the isoflavonoid/flavonoid specific branch pathway.
The importance of primary and secondary control sites is well illustrated by elicitor-mediated inhibition of light-induced CHS activity and synthesis in *Petroselinum hortense* (177). Similarly, in wounded hypocotyls of *Glycine max* the relatively weak additional induction of PAL activity concomitant with elicitor induction of glycineollin accumulation might be accounted for in terms of operation of a secondary control site channelling biosynthetic flux toward glycineollin and away from other phenylpropanoid products (112). Other apparent exceptions might reflect a lack of correlation between enzyme levels and phytoalexin accumulation arising from a comparison of responses in different physiological conditions (e.g., different hormone concentrations) rather than changes induced by an external signal against a constant internal physiological status.

Thus, it can be concluded that phytoalexin accumulation is largely controlled by changes in the levels of appropriate biosynthetic enzymes, hence a major task is to elucidate the molecular mechanisms governing these often marked, rapid and transient changes in enzyme level.

**D. MOLECULAR MECHANISMS REGULATING LEVELS OF PHYTOALEXIN BIOSYNTHETIC ENZYMES**

A change in enzyme activity level might reflect modulation of one or more of the following processes: *(a) de novo* synthesis, *(b) degradation*, *(c) activation*, and *(d) inactivation*.

In principle, the simplest way to demonstrate the involvement of *de novo* protein synthesis is by application of inhibitors such as cycloheximide, chloramphenicol, puromycin, or blasticidin S. Phytoalexin accumulation in response to infection (484,693) and treatment with biotic (694) and abiotic (102,106,660,661) elicitors is sensitive to inhibitors of protein synthesis. Necrogenic resistance can be destroyed by such inhibitors (484, 693,695-698), and in a number of cases, but not all (695), this correlates with inhibition of phytoalexin accumulation (484,693). With respect to enzymes of phytoalexin biosynthesis, Hadwiger et al. have noted that induction, by a range of biotic and abiotic elicitors, of both PAL activity and pisatin (XI) accumulation in tissue of *Pisum sativum* is sensitive to inhibitors of protein synthesis (102,110,660,661,694). These observations were interpreted in terms of elicitor induction of PAL synthesis as a link in the chain of events leading to phytoalexin accumulation. Similarly, in discs of *Ipomea batatas* root tissue, induction of HMG-CoA reductase activity, the enzyme system catalyzing the conversion of mevalonic acid to isopentenylpyrophosphate, and terpene production were inhibited by cycloheximide when applied to the cut surface together with the abiotic
elicitor HgCl₂, immediately after disc preparation (258,338). However, when HgCl₂ and cycloheximide were applied to discs that had been incubated for 24 hr, neither phytoalexin accumulation nor induction of enzyme activity was inhibited (258,338). It was suggested that inactive precursors of the enzymes are formed de novo in response to disc preparation, and are converted into the active forms only in response to fungal infection or elicitor treatment. Induction of the hypersensitive reaction in potato tuber tissue similarly appeared to be a two-stage process (699).

However, conclusions based on the use of protein synthesis inhibitors should be treated with caution, since it has become evident that such inhibitors have a number of side effects apparently unrelated to inhibition of protein synthesis (700). Furthermore, these inhibitors exert a blanket effect, and therefore, even if the effects observed are related to inhibition of protein synthesis, it may be that a second, regulatory protein is synthesized in response to elicitation rather than the biosynthetic enzyme itself. Even at best, inhibition of the response cannot be taken to indicate control over the rate of de novo synthesis, only that de novo synthesis of some entity is required. Thus the use of inhibitors of protein synthesis cannot provide rigorous proof of control over the synthesis of specific enzymes, and inhibition of the response should be considered only as a preliminary indication of the involvement of protein synthesis.

More rigorous evidence for the control of de novo synthesis of biosynthetic enzymes during phytoalexin accumulation has come from in vivo labeling with stable and radioactive isotopes. In vivo density labeling with ²H from ²H₂O followed by analysis of the equilibrium distribution of enzyme activity in CsCl density gradients has shown that both denatured RNase and elicitor heat-released from cell walls of Colletotrichum lindenmuthianum cause rapid, marked increases in the rate of labeling of PAL in cell cultures of Phaseolus vulgaris (188, 701). The activity level and rate of labeling of acid phosphatase, an internal control enzyme metabolically unrelated to PAL and phaseollin accumulation, were unaffected by elicitor treatment, thereby demonstrating that the specific activity of label in the amino acid pool is unaffected by elicitor (188). Therefore, the observed differences in the rate of labeling of PAL reflect differences in the turnover of the enzyme, and it was concluded that both elicitors cause an increase in the rate constant for de novo synthesis of PAL and that this was an early event in the phytoalexin defense response (188,701). The response was further investigated using the high-resolution density gradient solute KBr, which allows quantitative measurement of the
amounts of $^2$H-labeled and $^2$H-unlabeled enzyme independent of assumptions about, or measurement of, the specific activity of label in the amino acid pool from which the enzyme was synthesized (115). By this technique it has been shown that at low concentrations of the elicitor heat-released from cell walls of *Colletotrichum lindemuthianum*, the increase in PAL activity arises exclusively by stimulation of the rate of *de novo* synthesis against a constant rate of removal of active enzyme. In contrast, at higher elicitor concentrations, the increase in enzyme activity was accompanied by a marked, apparent stabilization of the enzyme *in vivo*, and the rapid but transient increase in enzyme activity was achieved by a program of reciprocal changes in the rate constant for *de novo* enzyme production and the rate constant for removal of enzyme activity (115).

Although density labeling uniquely provides simultaneous quantitative estimates of the amounts of labeled, newly synthesized PAL without assumptions about, or measurement of, the specific activity of label in the amino acid pools from which the enzyme was synthesized, the technique has a number of important limitations. First, quantitative data are only obtainable in favorable cases where the enzyme is stable in high concentrations of solute and is of high molecular weight so that labeled and unlabeled species are extensively resolved. Second, the technique measures the ratio of labeled to unlabeled enzyme and is therefore not applicable to studies requiring short pulses of label for detailed analysis of the early stages of rapid, regulatory responses. Third, the information obtained is essentially kinetic rather than molecular (702); putative inactive or processed forms of the enzyme are not monitored, and the technique cannot be extended to study protein synthesis *in vitro*.

Labeling with radioactive isotopes followed by specific immunoprecipitation offers a complementary approach free from these limitations. Therefore, the response has been further studied by measurement of changes in the rate of synthesis of PAL and CHS using *in vivo* pulse-labeling with $[^{35}\text{S}]$methionine coupled with specific, direct immunoprecipitation by antisera immunospecific for PAL and CHS, respective; this was followed by SDS-polyacrylamide gel electrophoresis of the immunoprecipitates and estimation of the incorporation of label into the enzyme subunits (170). Elicitor causes marked but transient increases in the rates of synthesis of both enzymes concomitant with the phase of rapid increase in enzyme activity at the onset of phaseollin accumulation. Increased rates of synthesis of both enzymes can be observed 20 min after elicitor treatment and the patterns of induction of synthesis of PAL and CHS are
broadly similar with respect to elicitor concentration and time, maximum rates of synthesis being obtained between 2.5 and 3.0 hr after elicitor treatment. Within this overall coordination, small but distinct differences between the enzymes were observed in (a) the precise timing of maximum enzyme synthesis, with that for CHS occurring 20–30 min earlier than that for PAL, and (b) the elicitor concentrations giving maximum enzyme synthesis. Synthesis of PAL and CHS each accounted for between 0.5 and 1% of the total protein synthesis in elicitor-treated cultures of *P. vulgaris* (170).

These observations highlight the importance of enzyme synthesis in the regulation of the activity levels of these enzymes. However, for a given rate of enzyme synthesis, induction of PAL and CHS activities is more efficient at high elicitor concentrations. This provides further evidence for the operation, under certain circumstances, of post-translational control of the activity level of PAL as implicated by *in vivo* density labeling with $^2$H (115), and it suggests that there may also be post-translational control of CHS.

Rapid, transient increases in the rate of synthesis of PAL correlated with rapid increases in enzyme activity have also been observed, by direct immunoprecipitation of *in vivo*-[35S]methionine-labeled subunits, in *Pisum sativum* tissue inoculated with *Fusarium solani* f.sp.pisi and *F. solani* f.sp.phaseoli (703), and in suspension cultures of *Petroselinum hortense* treated with the high molecular weight elicitor present in culture filtrates of *Phytophthora megasperma* f.sp.glycinea (177). As already discussed, in the latter case elicitor treatment completely inhibits light-stimulated CHS synthesis.

An increase in the overall rate of RNA synthesis occurs in a number of systems following infection (696,697,704–706), and inhibitors of RNA synthesis have been observed to inhibit necrogenic resistance (693,696), phytoalexin accumulation (102,104,106,108,660,661,693,694), and induction of appropriate biosynthetic enzymes (102,104,108,660,661). However, the interpretation of such observations is limited and qualified by the same kinds of considerations as those outlined above appertaining to inhibitors of protein synthesis. Indeed, although pisatin (XI) accumulation and induction of PAL activity is inhibited by actinomycin D at high concentrations, at low concentrations actinomycin D elicits these responses (102). There is evidence that certain abiotic elicitors alter the template activity and dye binding capacity of plant nuclear DNA, and a number of changes in nuclear properties have been noted (104,707). However, there
is no evidence that such changes are causally related to phytoalexin accumulation.

In vitro protein synthesis using a rabbit reticulocyte lysate translation system coupled with immunoprecipitation of PAL subunits has shown that elicitor treatment of Petroselinum hortense cell cultures causes a marked increase in the activity of the mRNA encoding for PAL (177). Similarly, it has recently been demonstrated, using a message-dependent rabbit reticulocyte lysate translation system, that elicitors cause an increase in activity level of the mRNAs encoding for PAL and CHS in Phaseolus vulgaris cell suspension cultures (686). Elicitor-induced changes in the activity levels of these mRNAs present in polysomes closely follow changes in the rate of enzyme synthesis as measured by in vivo labeling. Quantitative comparison of translation products using cellular RNA and polysomal RNA has shown that the increase in enzyme synthesis does not reflect selective recruitment of these mRNAs into polysomes following elicitor treatment (686).

The above changes in mRNA activity levels might reflect modulation of one or more of the following processes: (a) RNA synthesis, (b) processing of the primary transcript to yield mature mRNA, or (c) degradation of mature mRNA or precursors to mature mRNA. Availability of molecular clones of cDNA containing sequences that encode for enzymes of phytoalexin biosynthesis will allow measurement of elicitor-induced changes in mRNA amount by hybridization techniques. In principle, it should be possible, using recombinant DNA technology, to study the structure of the genes encoding for enzymes of phytoalexin biosynthesis, the molecular genetic environment of these genes, and the transcription and processing of the primary transcripts.

Little is known of the molecular mechanisms governing post-translational control of the enzymes of phytoalexin biosynthesis. High molecular weight inactivators and inhibitors of PAL have been reported (708–710), although their physiological roles in the control of enzyme activity have not been established. Following the light-induced increase in CHS activity in cell cultures of Petroselinum hortense, the subsequent exponential rate of decrease of enzyme activity was much greater than that of radioactivity in immunoprecipitable enzyme subunits, thus indicating a more rapid loss of catalytic activity than could be accounted for by actual degradation of the enzyme molecule (691). Comparison of the kinetics of light-induced changes in CHS catalytic activity and CHS protein as determined by a sensitive and specific radio-immunoassay revealed the accumulation of a
pool of inactive molecules during only the later stages of enzyme induction (711). However, the nature and molecular mechanisms governing the accumulation of inactive CHS have not been elucidated.

Recent evidence from in vivo labeling with $^2$H suggests that cinnamic acid might feedback-regulate PAL levels by a dual mechanism (712). Exogenous cinnamic acid not only inhibits de novo synthesis of the enzyme but also markedly stimulates the rate of removal of active enzyme. Although the molecular mechanisms underlying these effects remain to be elucidated, it has been established that this dual-feedback control mechanism operates in vivo following endogenous production of cinnamic acid. Changes in the levels and compartmentalization of cinnamic acid and biosynthetic derivatives during phytoalexin accumulation might then account for modulation of the apparent stability of the enzyme in vivo following elicitor treatment.

Following wounding, plant cells accumulate protease inhibitors (713). Wounding and elicitation exhibit many similarities, and it is possible to envisage accumulation of protease inhibitors leading to in vivo stabilization of certain enzymes by inhibition of plant cell proteolytic activity. Indeed, induction of protease inhibitors has been observed following infection of resistant tomato plants by Phytophthora infestans (714).

VI. Concluding Remarks

The first phase of research on phytoalexins over the last two decades has led to an understanding of the nature of these compounds and their possible role in plant disease resistance. In the next few years a more detailed understanding should emerge of the molecular mechanisms underlying their induction in plant cells. It is clear from the preceding discussions that we still only have limited knowledge of the enzymology of the reactions specific to phytoalexin formation. In terms of regulatory mechanisms, a general picture of the types of control involved can be presented, although further elucidation of the fine details will depend partly on an increased knowledge of the relevant enzymology and partly on the successful application of recombinant DNA technology and other molecular biological techniques. Furthermore, precise determination of the structures and sites of action of fungal and endogenous host elicitors is essential for a better understanding of the initial events determining the phytoalexin response.

An increased knowledge of the enzymic reactions involved in phyto-
alexin biosynthesis may also help our understanding of the biological role of these compounds. The use of inhibitors of PAL has already demonstrated the role of the induction of this enzyme in lesion limitation during the hypersensitive resistance of tobacco to tobacco necrosis virus (715). Very potent, specific inhibitors of the key enzymes PAL and HMG-CoA reductase are now available (716,350), and it should, in principle, be possible to use these compounds to produce artificial, phytoalexin-minus phenotypes. The behavior of such treated tissues, in the presence and absence of later intermediates capable of restoring phytoalexin production, may be investigated following fungal infection, and the results may allow conclusions to be drawn concerning the extent to which particular classes of phytoalexins themselves determine lesion limitation.

The induction of phytoalexin biosynthetic pathways by fungal elicitors is clearly an exciting new area for research in plant molecular biology. It is also interesting to consider the possible role of phytoalexins as "elicitors" of fungal enzymes involved in the degradation of these compounds. The structural requirements for induction of pisatin demethylase by phytoalexins have recently been demonstrated (reviewed in Ref. 717). Thus we may soon see attention being turned to the modulation of fungal gene expression by these plant products, a possibly important component of the host-parasite interaction which has so far received little attention.

Addendum

The above review covers the literature up to the latter half of 1981. Since then, important new work has been published in the area of fungal metabolism of phytoalexins (718–721), and several general reviews pertaining to the present Chapter have also recently appeared (722–726).

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