Phytochemistry, Vol. 25, No 5, pp. 979-995, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00

REVIEW ARTICLE NUMBER 16

BIOSYNTHESIS, ELICITATION AND BIOLOGICAL ACTIVITY OF ISOFLAVONOID PHYTOALEXINS

DAVID A. SMITH and STEPHEN W. BANKS†

Plant Pathology Department, University of Kentucky, Lexington, KY 40546-0091, U.S.A.

(*Received* 4 *October* 1985)

Key Word Index Leguminosae; phytoalexins; isoflavonoids; formation; toxicity; mode of action; tolerance; disease resistance.

Abstract –Isoflavonoid phytoalexins are a major class of low molecular weight, inhibitory compounds synthesized by certain plants, notably members of the Leguminosae. Accumulation of these isoflavonoids is often very marked following infection. This review summarizes their biosynthesis, elicitation and biological properties. Consideration is also given to their inferred function of limiting microbial invasion in plant tissues.

INTRODUCTION

Many flavonoids are antimicrobial [1-3]. These toxic compounds occur in plants constitutively [4], following stress [5], or in both circumstances [6, 7]. Their inhibitory nature has been taken, in part, as evidence that they help protect the producing plants from pathogens [3] and pests [8, 9]. In all of this work concerning the putative defensive capacities of flavonoids, great emphasis has been placed upon isoflavonoids formed in response to stress, primarily that caused by infection. These isoflavonoids have come to represent the predominant chemical class amongst the phytoalexins [10], a biologically-defined term encompassing toxic compounds which accumulate in plants after infection and which may represent a natural mechanism to resist microbial attack.

Post-infectional antimicrobial activity amongst the flavonoids, however, is not restricted to the isoflavonoid sub-class. The flavanone, betagarin, has been reported as a phytoalexin in sugar beet [11]. Broussonins A and B, which possess a 1,3-diphenylpropane skeleton, allowing them to be classified among the simplest flavonoids found naturally, are reputedly phytoalexins occurring in diseased shoots of paper mulberry [12]. Further studies led to the isolation of eight additional minor phytoalexins from infected cortical tissues: two flavans, four 1,3-diphenylpropanes and two chalcones [13]. The resistance of daffodil bulbs to infection by *Botrytis cinerea* involves the production of several antifungal compounds [14], of which three have been identified as hydroxyflavans [15].

The subject of those antimicrobial isoflavonoids that occur constitutively in plants also merits attention, albeit brief. A variety of isoflavonoids have been isolated from apparently healthy trees, where they may contribute to the resistance of woody tissues towards fungi and insects [16-18]. The antifungal activity of the prenylated isoflavone, luteone, found in immature fruits of *Lupinus luteus* [19] and on leaf surfaces of *L. albus* [4], was considered reason for it to be a hindrance to fungal development and, hence, infection. The distinction between the constitutive presence of antimicrobial isoflavonoids and their formation only after stress is, on occasion, blurred [6, 20]. Therefore, while it is often a division of convenience in orienting research programs, it may not always be of functional significance.

PHYTOALEXIN THEORY

Experimental observations consistent with the production of defensive chemicals by plants in response to microbial attack have been reported since at least the early years of this century, although the studies were few in number and limited in extent [21, 22]. Interest in the formation of post-infectional inhibitory compounds as one natural mechanism of disease resistance was spurred by publication of Müller and Börger's phytoalexin theory [23]. This proposed that restriction of fungal development in plant tissues was the result of the accumulation of some toxic chemical principle by living cells reacting to the invader. At that time, no chemicals were structurally characterized and the definition was of a biological nature and not of some precise chemical class.

By the late 1950s, the pioneering efforts of Müller [23, 24] inspired others to the chemical resolution of the first isoflavonoid phytoalexin (IP), pisatin (Fig. 1; **26**) [25, 26], from pea (*Pisum sativum*) tissues. Characterization of the related pterocarpanoid phytoalexin, phaseollin (Fig. 1; **27**), from bean (*Phaseolus vulgaris*) occurred soon thereafter [27, 28]. A few other chemicals which apparently satisfied the phytoalexin concept were identified about the same time [22] and a veritable flood would follow [29-31]. The notion was now well established of low molecular weight, toxic compounds, often absent from healthy tissues but formed after tissue damage which, accumulating at and around

† Present address: Biology Department, University of Toledo, Toledo, OH 43606, U.S.A.

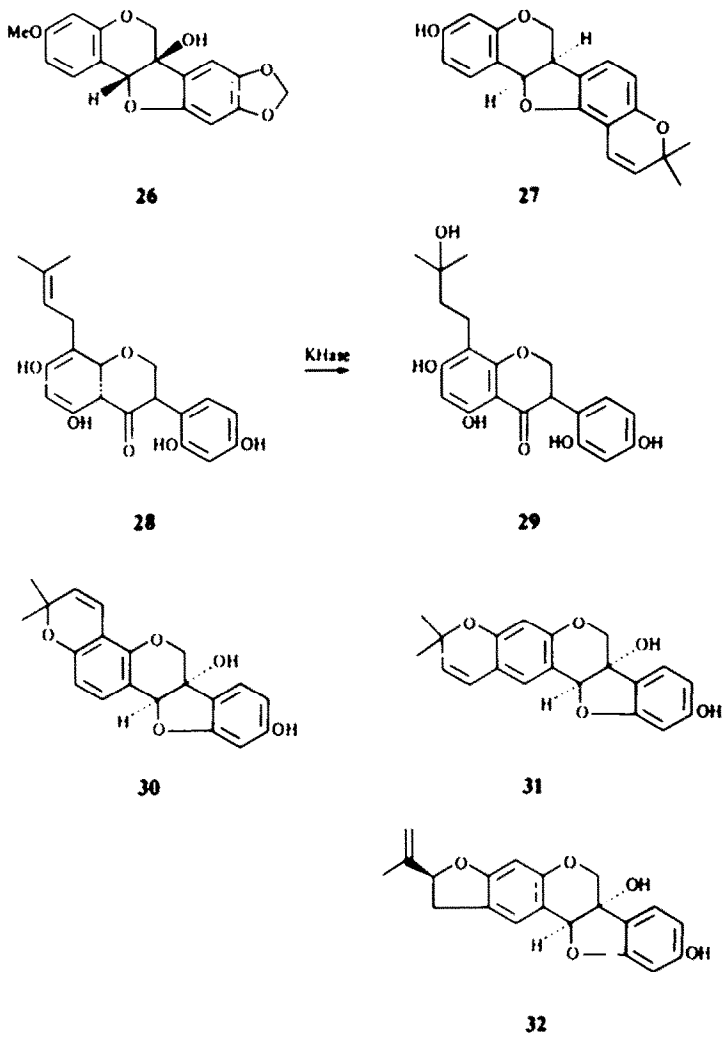


Fig. 1. Six isoflavonoid phytoalexins and one detoxification product. **26** (+)-(6a*R*,11a*R*)-Pisatin; **27** (−)-(6a*R*,11a*R*)-phaseollin; **28** kievitone; KHase = kievitone hydratase; **29** kievitone hydrate; **30** (−)-(6a*S*,11a*S*)-glyceollin I; **31** (−)-(6a*S*,11a*S*)-glyceollin II; **32** (−)-(6a*S*,11a*S*)-glyceollin III.

infection sites, were presumed to contribute to the limitation of microbial development in plants. Of these many chemicals—and several different classes are involved—the isoflavonoids predominate [10]. The many research efforts have also revealed an intriguing association, widespread though not restrictive, between IPs and the Leguminosae [30, 32].

FORMATION OF ISOFLAVONOID PHYTOALEXINS

Mention has been made of the possibility that phytoalexin accumulation might occur as a result of flavonoid glycoside hydrolysis [33]. Levels of the pterocarpanoid glycoside, trifolirhizin, decreased as symptom severity increased in infected red clover roots [34]. Although the subsequent appearance of the aglycone, maackiain—a phytoalexin of red clover foliage [35]—might suggest trifolirhizin hydrolysis as its source, the data were inconsistent and decreases in trifolirhizin levels were not always reflected by increases in maackiain. The general presumption has been, for some years now, that phytoalexins arise from remote precursors [36]. This assumption is based upon the comparative slowness with which IPs accumulate and the frequent absence of likely immediate precursors. Moreover, the lack of any change in flavonol concentration in beans during phaseollin accumulation suggested the stimulation, after infection, of a specific route leading to isoflavonoid synthesis [37]. The conviction is still strong that IP formation occurs from remote precursors following appropriate tissue irritation [38, 39].

Implicit in any consideration of phytoalexin production by plants are two related, yet distinct, issues. First, is the elucidation of pathways instrumental to the synthesis, in this particular instance, of isoflavonoids, with concomitant considerations of the regulatory mechanisms and enzymology involved. Second, is the nature of elicitation of phytoalexin biosynthesis, since the response is classically post-infectional [10].

Biosynthesis of isoflavonoid phytoalexins

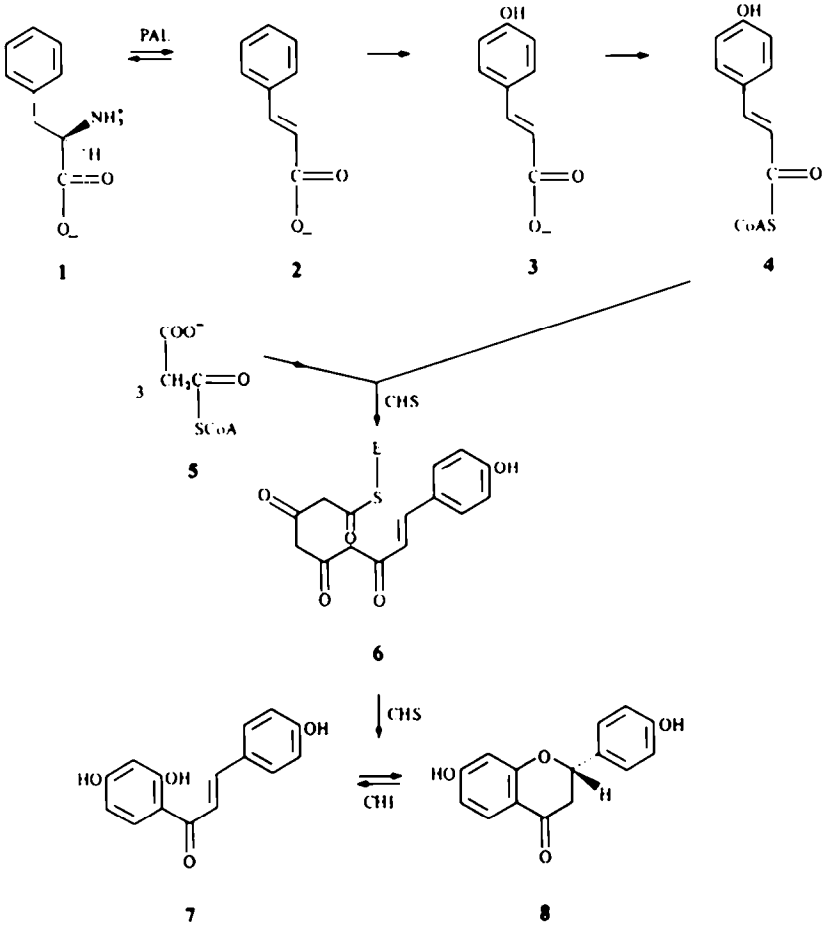
Pathway elucidation. Isoflavonoids differ from the flavonoids in having a rearranged 1,2-diphenylpropane

skeleton as opposed to a 1,3-diphenylpropane skeleton, and occur principally in the free state, rather than as glycosides. Like the flavonoids, the various classes of isoflavonoids are determined by changes in the oxidation level of the heterocyclic rings. Additional variation is conferred by the elaboration of the aryl groups by secondary reactions: hydroxylation, methylation and prenylation [40].

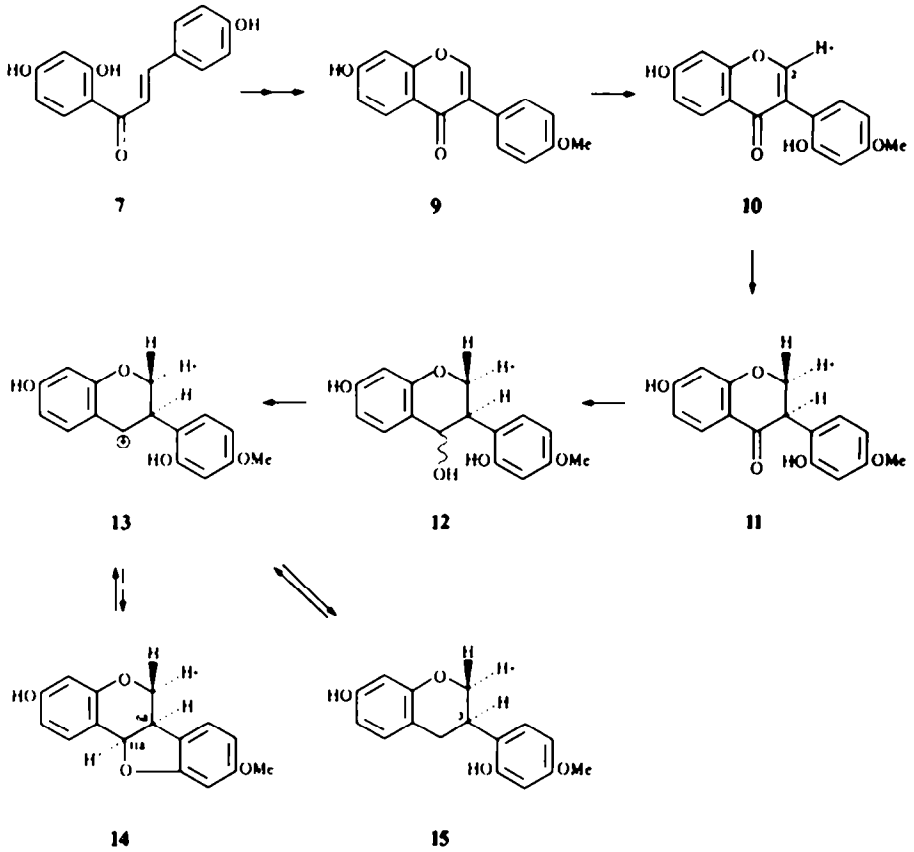
The biosynthetic pathways which culminate in the formation of IPs may be conveniently divided into three parts [3]: those early pathways shared with other secondary metabolites, those steps common to flavonoid and isoflavonoid biosynthesis and those reactions unique to the isoflavonoids. Summaries of the presumed routes determining the formation of certain IPs are presented in Schemes 1-3.

The essential C_{15} molecular framework of both flavonoids and isoflavonoids results from the convergence of the acetate-malonate and shikimic acid pathways (Scheme 1) [3]. Comprehensive studies by Grisebach and collaborators demonstrated that biosynthesis of the isoflavone skeleton from precursors of the general flavonoid pathway involved a 1,2-aryl shift of the B ring, and that this shift took place after the formation of the C_{15} chalcone intermediate [41–45].

One of the important problems to be investigated concerning the biosynthesis of isoflavonoids was the question of whether the chalcone or the isomeric flavanone acted as the substrate for aryl migration. An early experiment to resolve this question took the form of parallel competitive feeding experiments, in which either [^{14}C]-isoliquiritigenin (4,2',4'-trihydroxychalcone) (Scheme 1; 7), diluted with an equal amount of unlabelled (−)-liquiritigenin (7,4'-dihydroxyflavanone) (Scheme 1; 8), or [^{14}C]-(−)-liquiritigenin, diluted with an equal amount of unlabelled isoliquiritigenin, were fed to seedlings of *Trifolium subterraneum* [46]. Isolation of daidzein (Fig. 2; 33) and formononetin (Scheme 2; 9), followed by determination of the specific activity for each, showed that these were higher from the former mixture, thus indicating that the chalcone was the immediate substrate for aryl migration. Similar results were observed for flavone and flavanone metabolites in a subsequent experiment [47]. The relative efficiencies of chalcone and flavanone as precursors of daidzein and formononetin were studied by further competitive feeding experiments. The results strongly favoured the proposal [48] that chalcones were the more immediate precursors of isoflavones. Indeed, it is probable that only two chalcones, isoliquiritigenin (Scheme 1; 7) and 4,2',4',6'-tetrahydroxychalcone (Fig. 2;



Scheme 1. Early steps in phenylpropanoid biosynthesis. 1 L-Phenylalanine; PAL = phenylalanine ammonia-lyase; 2 *trans*-cinnamic acid; 3 4-hydroxycinnamic acid; 4 4-coumaroyl CoA; 5 malonyl CoA; CHS, E = chalcone synthase 6 $C_6C_3C_6$ intermediate; 7 isoliquiritigenin; CHI = chalcone/flavanone isomerase; 8 liquiritigenin.

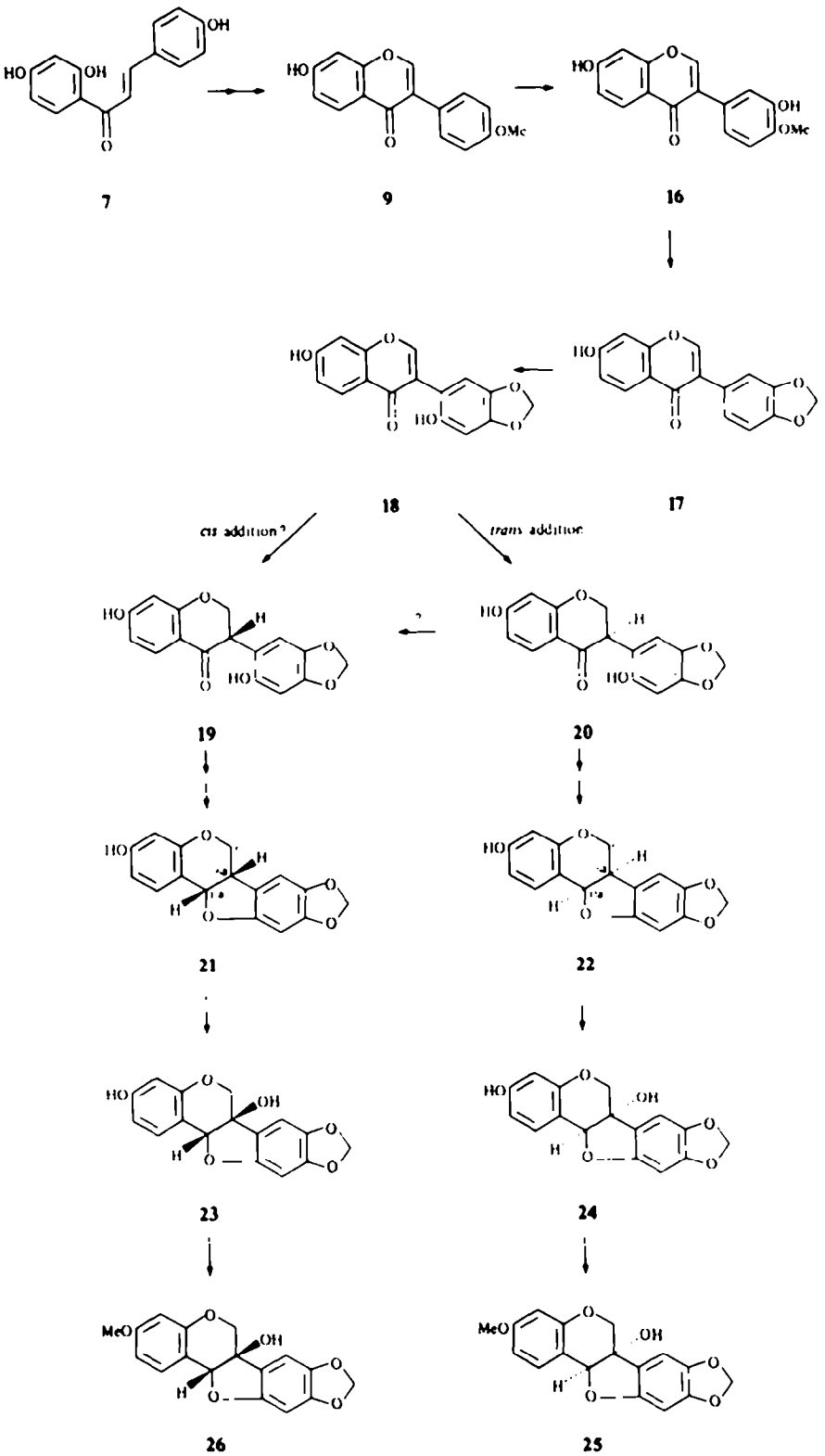


Scheme 2. Stereochemistry of isoflavone reduction during the biosynthesis of medicarpin and vestitol. 7 Isoliquiritigenin; 9 formononetin; 10 7,2'-dihydroxy-4'-methoxyisoflavone; 11 7,2'-dihydroxy-4'-methoxyisoflavanone; 12 7,2'-dihydroxy-4'-methoxyisoflavanol; 13 carbonium ion; 14 medicarpin; (−)-(6a*R*,11a*R*)-3-hydroxy-9-methoxypterocarpin; 15 vestitol; (−)-(3R)-7,2'-dihydroxy-4'-methoxyisoflavan.

42), normally act as substrates for aryl migration [49].

Possible mechanisms for the aryl migration have been examined by feeding [2-^{14}C-3-$^{3}H_2$]-7,4'-dihydroxyflavanone (Fig. 2; 35) to seedlings of *Cicer arietinum*. Grisebach and Zilg [50] found that on incorporation into formononetin, the ^{3}H:^{14}C ratio fell by 99% but, in contrast, [2-^{14}C-2-^{3}H]-5,7,4'-trihydroxyflavanone (Fig. 2; 36) was incorporated into biochanin A (Fig. 2; 34) with no change in the ^{3}H:^{14}C ratio. These findings demonstrated that no migration of heterocyclic protons accompanied the aryl migration. The aryl migration envisaged by Crombie, Dewick and Whiting [51], based on an earlier scheme [52], proceeds by a one electron transfer from the chalcone. A single electron loss forms the spirodienone, decomposition of which yields the isoflavone. Conversion of chalcones to isoflavones has also been accomplished by the protocols of organic chemistry [53–55], employing thallium III-induced oxidative rearrangements. This type of procedure has recently been utilized in the synthesis of phaseollin (Fig. 1; 27), though in its racemic form [56].

The origin of the acetate-derived ring in isoflavonoids has been investigated by feeding [1,2-^{13}C]-sodium acetate to $CuCl_2$-elicited pea pods [57] as well as to wounded bean cotyledons [58]. Tracer studies to resolve interrelationships between isoflavonoid classes have been applied in several areas. The first IPs to be investigated by these methods were maackiain (Scheme 3; 22) and medicarpin (Scheme 2; 14) which co-occur with formononetin in induced red clover (*Trifolium pratense*). Early feeding experiments with $CuCl_2$-treated seedlings established that ^{14}C-labelled isoliquiritigenin and formononetin were efficient precursors of both medicarpin and maackiain, whereas 2',4'-dihydroxy-4-methoxychalcone (Fig. 2; 37) and ^{3}H-labelled daidzein were poor precursors [59]. These results were interpreted as being consistent with the proposal that 4'-methylation accompanies the aryl migration step in isoflavonoid biosynthesis. It was observed during these experiments that there was a large pool of formononetin in red clover seedlings which appeared to be stable and was not immediately converted to the pterocarpans upon elicitation, indicating that the appearance of maackiain and medicarpin in treated tissue was the result of *de novo* synthesis. Later results [60] indicated that medicarpin was produced by a reductive sequence in which formononetin appeared to be 2'-hydroxylated (Scheme 2; 10), and was then reduced to the corresponding (3*R*)-isoflavanone (Scheme 2; 11), followed by further reduction to the isoflavanol (Scheme 2; 12). This could then lose water and cyclize to form the pterocarpan (Scheme 2; 14). The pathway to maackiain was envisaged [61] as proceeding by reduction of the isoflavone (Scheme 3; 18) to the corresponding isoflavanone



Scheme 3. Biosynthesis of the optical isomers of maackiain and pisatin. **7** Isoliquiritigenin; **9** formononetin; **16** calycosin; **17** ψ-baptigenin; **18** 6'-hydroxy-ψ-baptigenin; **19** (+)-(3S)-7,2'-dihydroxy-4',5'-methylenedioxyisoflavanone; **20** (−)-(3R)-7,2'-dihydroxy-4',5'-methylenedioxyisoflavanone; **21** (+)-(6a*S*,11a*S*)-maackiain; **22** (−)-(6a*R*,11a*R*)-maackiain; **23** (+)-(6a*R*,11a*R*)-6a-hydroxymaackiain; **24** (−)-(6a*S*,11a*S*)-6a-hydroxymaackiain; **25** (−)-(6a*S*,11a*S*)-pisatin; **26** (+)-(6a*R*,11a*R*)-pisatin.

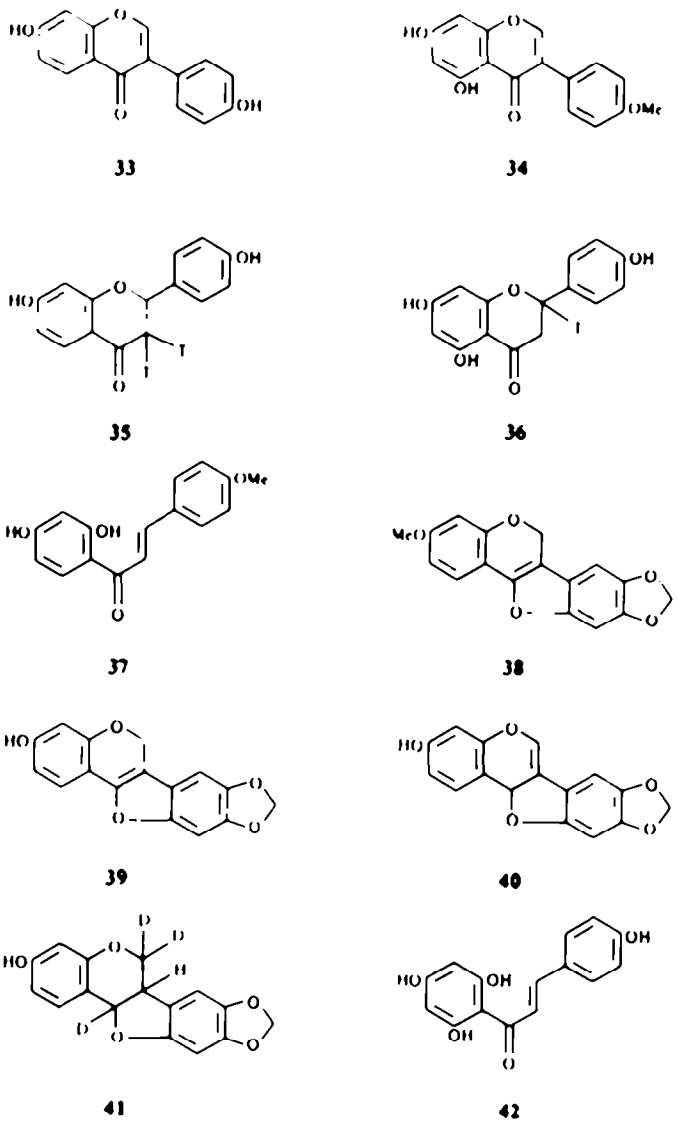


Fig. 2. Miscellaneous compounds tested experimentally in the elucidation of isoflavonoid biosynthetic pathways. **33** Daidzein; **34** biochanin A; **35** [2-^{14}C-3-^{3}H$_2$]-7,4'-dihydroxyflavanone; **36** [2-^{14}C-2-^{3}H]-5,7,4'-trihydroxyflavanone; **37** 2',4'-dihydroxy-4-methoxychalcone; **38** 3-methoxy-8,9-methylenedioxypterocarp-6a-ene; **39** 3-hydroxy-8,9-methylenedioxypterocarp-6a-ene; **40** 3-hydroxy-8,9-methylenedioxypterocarp-6-ene; **41** [6,11a-^{3}H$_2$]-maackiain; **42** 4,2',4',6'-tetrahydroxychalcone

(Scheme 3; **20**), further reduction to the isoflavanol and loss of water to form the pterocarpan in an analogous sequence to that described for medicarpin. A similar scheme, based on feeding experiments, has been proposed for the biogenesis of phaseollin (Fig. 1; **27**) [62].

The biosynthesis of pterocarpans is thought to involve a carbonium ion (Scheme 2; **13**). Strong support for this came from feeding experiments using induced seedlings of *Medicago sativa* in which vestitol (Scheme 2; **15**) and medicarpin (Scheme 2; **14**) were shown to be interconvertible [63]. Isoflavans and pterocarpans are believed to be biosynthesized simultaneously from a common intermediate which has been suggested as being the carbonium ion or its mesomeric counterpart.

The stereochemistry of isoflavone reduction during the biosynthesis of pterocarpans has also been studied. The feeding of [2-^{3}H]-2'-hydroxyformononetin (Scheme 2; **10**) to $CuCl_2$-treated seedlings of fenugreek revealed an overall *trans* addition of hydrogen to the double bond of the isoflavone in its transformation to (−)-(6a*R*,11a*R*)-medicarpin [64]. This is in contrast to the *cis* addition observed for the incorporation of [2-^{3}H]-formononetin into (+)-(6a*R*,11a*R*)-pisatin (Fig. 1; **26**) [65].

Pisatin represents an exception among pterocarpans because it is the only one acting as a phytoalexin that has a positive optical rotation. It is also interesting that in *P. sativum* a minor phytoalexin, (−)-(6a*R*,11a*R*)-maackiain (Scheme 3; **22**), is produced simultaneously with (+)-(6a*R*,11a*R*)-pisatin. By supplying exogenous (−)-(6a*R*,11a*R*)-maackiain to induced immature pea pods,

significant quantities of (−)-(6a*S*,11a*S*)-pisatin (Scheme 3; **25**) were synthesized. This suggested that the 6a-hydroxylation of maackiain during the biosynthesis of pisatin proceeded with retention of configuration at C-6a [66]. Confirmation of the direct hydroxylation of pterocarpans to form 6a-hydroxypterocarpans has come from the results of competitive feeding experiments in which the preferential incorporation of (+)-(6a*S*,11a*S*)-[^{3}H]maackiain (Scheme 3; **21**) over (−)-(6a*R*,11a*R*)-[^{14}C]maackiain (Scheme 3; **22**) into (+)-(6a*R*,11a*R*)-pisatin was observed. Similarly, (+)-(6a*R*,11a*R*)-6a-hydroxymaackiain (Scheme 3; **23**) was incorporated into (+)-pisatin to a much greater extent than (−)-(6a*S*,11a*S*)-6a-hydroxymaackiain (Scheme 3; **24**) [67]. The possible intermediacy of a pterocarp-6-ene or a pterocarp-6a-ene in the conversion of pterocarpans to 6a-hydroxypterocarpans has also been examined. The limited incorporation of [^{14}C]-3-hydroxy-8,9-methylenedioxy-pterocarp-6a-ene (Fig. 2; **39**) and [^{14}C]-3-methoxy-8,9-methylenedioxypterocarp-6a-ene (Fig. 2; **38**) into pisatin compared to the efficient incorporation of (+)-[^{14}C]-maackiain suggested that the pterocarp-6a-ene was not an intermediate [68]. ^{2}H-NMR Analysis of pisatin derived from [6,11a-$^2H_2$]-maackiain (Fig. 2; **41**) in $CuCl_2$-elicited pea pods clearly demonstrated the retention of all ^{2}H labels and thus confirmed that no pterocarp-6-ene (Fig. 2; **40**) or pterocarp-6a-ene was involved in the biosynthesis of pisatin [69].

The soybean phytoalexins, collectively known as the glyceollins (Fig. 1; **30**–**32**) are prenylated 6a-hydroxy-pterocarpans with negative optical rotations. Like pisatin, they have been shown to be derived from the appropriately substituted pterocarpan by 6a-hydroxylation with retention of configuration [70, 71], followed by prenylation and, finally, cyclization [72].

It is apparent that an extensive literature exists concerning isoflavonoid biosynthesis in general, as well as IP formation in particular, and the reader is referred to Dewick's review [1] for a comprehensive treatment of the subject. In addition, a recent list of all naturally occurring isoflavonoids can be found in the booklet by Ingham [32].

Enzymology and regulation. For many years it has been known that L-phenylalanine ammonia-lyase (PAL) (Scheme 1) catalysed the elimination of ammonia from L-phenylalanine (Scheme 1; **1**) to yield *trans*-cinnamic acid (Scheme 1; **2**)—the first committed step in the biosynthesis of all phenylpropanoids. Although increases in extractable PAL activity may precede IP accumulation [37, 73], the association is sometimes inconsistent and the importance of any regulatory role for this enzyme in IP formation remains moot. In both cotyledons and hypocotyls of *P. vulgaris*, phytoalexin elicitation occurred, on occasion, when PAL was suppressed below the wounded control level [74]. Furthermore, whereas kievitone (Fig. 1; **28**) accumulation occurred only in the upper half of treated *Vigna sinensis* hypocotyls, PAL activity increased throughout the hypocotyls [75]. Explanations for inconsistencies may lie in the presently insufficient appreciation of the different forms of PAL in plant tissues [76]. Certain isozymes, for example, with critical regulatory roles in isoflavonoid biosynthesis, might be enhanced upon particular tissue damage yet this precise response could be masked by other overall changes in PAL levels.

In a recent enzymological investigation [77] concerning glyceollin formation in soybean hypocotyls, stimulated by a glucan elicitor, phytoalexin accumulation was preceded by a transient rise in the extractable activity of PAL. Furthermore, there was an absolute correlation between increases in PAL activity and the eventual level of glyceollin accumulation in elicited seedlings. The results were felt to indicate a role for this 'early' enzyme in the regulation of the phytoalexin response in soybeans, by determining the amount of carbon from phenylalanine that is diverted into glyceollin biosynthesis. The possibility of a rate-limiting step later in the pathway was not, however, dismissed.

Although some studies have been undertaken of the appearance of cinnamic acid 4-hydroxylase, a mixed function monooxygenase which catalyses the conversion of *trans*-cinnamic acid to 4-hydroxycinnamic acid (Scheme 1; **3**), in wounded and/or illuminated tissues [78–80], few reports of its induction in systems producing phytoalexins exist [76]. Its activity, however, has been detected in association with phaseollin production in *P. vulgaris* [81] and glyceollin accumulation in soybean (*Glycine max*) [82]. Likewise, increases in hydroxycinnamoyl CoA ligase (= coumaryl CoA ligase) activity have also been reported to accompany phaseollin and glyceollin formation [77, 81–83].

Chalcone synthase (CHS) (Scheme 1), the first enzyme of phenylpropanoid metabolism specific to flavonoid/isoflavonoid biosynthesis, has been the focus of several different studies, although relatively few have directly concerned IP production [76]. This enzyme catalyses chalcone formation, despite the initial observation [84] that flavanone synthesis occurred—a result due to spontaneous cyclization of the first-formed product (Scheme 1; **6**). In *G. max* cotyledons, CHS activity peaked about 25 hr after phytoalexin elicitation, a time following PAL induction but prior to glyceollin accumulation [85]. Rapid, transient increases in CHS activity preceded phaseollin accumulation in *P. vulgaris* [81, 86]. Kievitone formation by elicitor-treated cell suspension cultures of *P. vulgaris* also followed transitory rises in PAL and CHS [87]. This research monitored 15 enzymes during kievitone accumulation, the results suggesting a highly selective induction of enzymes associated with IP synthesis. Furthermore, the findings implied that phytoalexin induction involved tight control mechanisms rather than general changes resulting from substantial metabolic disturbances following infection.

Chalcone isomerase (CHI) (Scheme 1) catalyses the stereospecific isomerization of a chalcone (Scheme 1; **7**) to its corresponding flavanone (Scheme 1; **8**) [76]. However, since the synthesis of isoflavonoids has been postulated to occur via direct formation of isoflavones from chalcones [76], any direct role for CHI in the synthesis of IPs seems unnecessary. Nonetheless, CHI increased in activity in infected soybeans [88] as well as in *P. vulgaris* following phytoalexin elicitation [81, 89].

Enzymic studies of the 2,3-aryl migration that generates isoflavones from their chalcone/flavanone precursors met with only limited success [90, 91] until, recently, Hagmann and Grisebach [92] reported that a microsomal preparation from soybean cell suspension cultures catalysed the rearrangement of (2*S*)-naringenin to genistein. The putative 'isoflavone synthase' involved was detected in cell cultures challenged with a phytoalexin elicitor prepared from *Phytophthora megasperma* f. sp. *glycinea* and appeared to be an NADPH- and dioxygen-dependent monooxygenase. The several-fold increase in isoflavone synthase activity following elicitation suggested that the

enzyme might play a role in regulating glyceollin accumulation.

Following isoflavone formation, various additional structural inter-conversions and elaborations occur in the generation of individual IPs. Only limited enzymic information is available concerning these steps. A 6a-hydroxylase, believed to be specifically involved in glyceollin biosynthesis, has been found in elicitor-challenged soybean seedlings and cell suspension cultures [71]. Cell suspension cultures of soybean and *Cicer arietinum* have also yielded *O*-methyltransferases [93, 94]. Prenylation is a necessary step in the biosynthesis of several IPs, such as glyceollins I, II and III [95] and kievitone [96]. Leube and Grisebach [95] reported that the prenylating enzyme, dimethylallylpyrophosphate: 3,6a,9-trihydroxypterocarpan dimethylallyltransferase, increased in soybean hypocotyls inoculated with *Phytophthora megasperma* f. sp. *glycinea* as well as in soybean cell cultures treated with a phytoalexin elicitor. The increase in 'prenyltransferase' activity had a longer lag phase than those for the increase of PAL or CHS activities, a finding consistent with the later action of the prenyltransferase in glyceollin biosynthesis.

Although enzymic studies, in combination with radiolabelling experimentation to elucidate biosynthetic sequences, allow relationships to be established between protein synthesis and phytoalexin formation, ultimate regulation of phytoalexin accumulation must lie at the level of gene expression. In a general sense, it has been known for some time that the overall rate of RNA synthesis increased in plants following infection and, moreover, that inhibitors of RNA synthesis diminished phytoalexin accumulation as well as the induction of appropriate, biosynthetic enzymes [76]. For example, actinomycin D inhibited the synthesis of poly (A)-containing mRNA in diseased soybeans [97]. Resistance and glyceollin production were also suppressed, suggesting that *de novo* mRNA synthesis was required for an effective defensive response and, furthermore, that genetic information governing eventual IP accumulation was expressed while the plant resisted infection.

An early, localized increase in CHS mRNA activity was evident in bean hypocotyls resisting *Colletotrichum lindemuthianum* [98]. This rise occurred prior to the onset of phaseollin accumulation. In contrast, in susceptible tissue, there was no induction of mRNA activity in the early stages of infection but, rather, a delayed and widespread increase as lesion development proceeded. Furthermore, rapid, transient increases in CHS mRNA were noted in cell cultures of *Phaseolus vulgaris* treated with a high molecular weight elicitor from *C. lindemuthianum* [99]. The rapid induction of mRNA from very low basal levels was felt to suggest that the elicitor initially caused a transitory increase in the transcription of the CHS gene(s), although the alternative possibilities of control over mRNA processing or degradation were not ruled out. Similar increases in mRNA activities related to phytoalexin synthesis were also observed after inoculation of soybean seedlings with *P. megasperma* f. sp. *glycinea* or after treatment of cultured soybean cells with a phytoalexin elicitor preparation [100]. The time course of changes in activity were alike for mRNAs coding for enzymes of general phenylpropanoid metabolism and for CHS mRNA. Cloned cDNA was used to demonstrate that the induced changes in CHS mRNA activity coincided with changes in the amount of mRNA. These results suggested that IP synthesis may be regulated by temporary gene activation. Therefore, the regulation of gene expression controlling phytoalexin build-up may be a key, early component in the natural defensive reactions of certain plant tissues.

Some cautionary notes must be expressed, however, as regards this type of work. First, the results of Bell *et al.* [98], discussed above, are presented in a manner that is inclined to overemphasize the apparent increase in CHS, as well as CHS mRNA, activity in the incompatible interaction. Second, employing a glucan elicitor from cell walls of *P. megasperma* f. sp. *glycinea*, Ebel and co-workers [10] noted increases in several enzyme activities, including those of PAL and CHS. The changes in PAL and CHS activities were correlated with corresponding alterations in mRNA activities encoding these enzymes. However, similar degrees of induction of PAL, CHS and the mRNA activities also occurred in response to an extracellular polysaccharide from *Xanthomonas campestris* which did not enhance glyceollin levels. This lack of coordination between PAL and CHS activities and the phytoalexin amount might imply that one or more later enzymes play the key regulatory role(s) in determining glyceollin accumulation. Certainly, as Ebel and colleagues [101] point out, great care must be exercized in interpreting induction of PAL and CHS as necessarily correlated with phytoalexin synthesis.

In the space of a comparatively short number of years, much has been learned about the chemical characterization of IPs, their precursors and biosynthetic sequences. These findings have, perhaps necessarily, led and outpaced understanding of the enzymes and regulatory mechanisms involved. Intensive use of the tools of molecular biology, however, should permit far more precise comprehension of the genetic and enzymic control of IP accumulation. Indeed, efforts in these directions represent some of the most intriguing phytoalexin research presently underway. The review by Dixon *et al.* [76] testifies to the accomplishments achieved and the challenges involved.

Elicitation of isoflavonoid phytoalexins

The obverse topic pertaining to phytoalexin formation, but distinct from biosynthesis as such, is resolution of what stimulates the accumulation after particular tissue damage. The expression commonly employed to encompass this phenomenon is 'elicitation', coming from Keen's proposal [102] that any factor which induced plants to accumulate phytoalexins be termed an 'elicitor'. Although the phytoalexin hypothesis was developed in the context of offering, at least, a partial explanation of how some plants might resist fungal colonization, it became apparent early on that elaboration of phytoalexins occurred in response to factors other than fungi and, indeed, even to abiotic elicitors [103]. Over the years, clarification of the mechanism(s) underlying elicitation has been a major focus for research, and not a little controversy [104–106]. Perhaps this fascination with resolving phytoalexin elicitation lies in the promise that it might lead to the ability to activate plant resistance via the application of exotic, but non-toxic, chemicals. Natural resistance in plants might, therefore, play a far greater role in practical disease control than is presently thought to be the case. Simultaneously, this should lessen the need for pesticide usage, moderating environmental impact prob-

lems that presently confront chemical control of plant diseases [107, 108].

In the particular case of IPs, definition of how elicitation occurs is not clearly resolved. One line of thought has been that, by mechanisms not yet understood, plant cells 'recognize' wall components of fungal invaders and, as a result, latent defenses —including IP formation—are activated. A recent series of papers [109–112] reported characterization of an hexa-(β-D-glucopyranosyl)-D-glucitol from mycelial walls of *P. megasperma* f. sp. *glycinea* that evoked phytoalexin accumulation in soybean. In spite of the precise chemical characterization of the active fraction isolated, its role as a natural elicitor remains uncertain for its release from the fungal walls followed hydrolysis with 2 N trifluoroacetic acid at 85° for 2.5 hr [112]. Furthermore, the isoflavonoid component(s) presumed to be produced in the elicitor assay were not individually identified but, rather, a composite absorbance value at 286 nm was used as a measure of their formation. Employing the same host–pathogen system, Keen and Legrand [113] had previously extracted glycoproteins from isolated preparations of the fungal walls with 0.1 N NaOH at 0° for 15 hr. Elicitor activity was associated with high molecular weight glycoproteins which appeared to contain only glucose and mannose as neutral sugars. Neither boiling nor pronase treatments destroyed elicitation, whereas periodate treatment did, suggesting that the carbohydrate components were important for activity. The significance of carbohydrate in the eliciting activity of *P. megasperma* f. sp. *glycinea* is underscored by the observation that an extracellular invertase, produced by the fungus and itself a mannan-glycoprotein, inhibited the activity of a glucan elicitor isolated from the cell wall of this pathogen [114].

Despite the discrepancies in the preparation protocols employed, as well as in the chemical composition of the elicitors, a common question emerges from these findings which bears upon their relevance to natural disease resistance. How might release –if release is required—of such eliciting materials, brought about by acid or base treatments in *in vitro* studies, be achieved in infected plant tissues? A possible explanation would rely upon appropriate enzymic activity. Working once more with soybean and *P. megasperma* f. sp. *glycinea*, Keen and Yoshikawa [115] determined that host tissues contained β-1,3-endoglucanase isozymes which released elicitor-active carbohydrates from the mycelial walls. The elicitors, in this instance, were glucomannans.

Opposed to the idea of the release of elicitor from a pathogen by a host enzyme, is the separation of host cell wall fragments resulting from the activity of a pathogen's enzyme(s). The plant wall component(s), in turn, elicit phytoalexin accumulation. Just such a situation has been reported and, again, with soybean, although in this instance the microorganism involved was a bacterium, *Erwinia carotovora* [116]. Heat-labile 'elicitor' activity in bacterial culture filtrates co-purified with α-1,4-endopolygalacturonic acid lyase activity. Elicitation was apparently indirect, a result of the enzymic release of active fragments from pectic polysaccharides in the plant cell walls.

Both pathogen and host, therefore, may serve as sources of IP elicitors. With regard to the pathogen, most work would indicate that a carbohydrate constituent of the outer layers triggers phytoalexin accumulation. An eliciting role for some pathogen component was, historically, the first situation reported [117]. The other circumstance is of a host fragment which is itself recognized as an elicitor by that same plant. This situation was first described by Hargreaves and Bailey [118] and subsequently reviewed by Bailey [119]. The many observations that diverse, and apparently unrelated, abiotic stimuli could result in IP production might, most easily, be rationalized if all were causing the release of some 'constitutive elicitor' in the plant which initiated phytoalexin formation. The accumulation of IPs may be variously evoked by heavy metal salts [73, 103, 120], DNA-intercalating chemicals [121], antibiotics [122], herbicides [123], chloroform [124], surfactants [125], ethylene [126], sulphur dioxide and ozone [123], freezing [118, 127], mechanical injury [128] and ultraviolet irradiation [129]. Bailey [119] speculated that healthy plants contain a constitutive elicitor, sequestered in some non-functional form, which is released or activated upon cell damage and stimulates phytoalexin formation. The chemical nature of Hargreves and Bailey's [118] putative constitutive elicitor is still unresolved.

Hahn and associates [130] reported an 'endogenous elicitor' from soybean hypocotyls that was shown to be a pectic fragment [131]. The adjective, endogenous, was employed because the elicitor was not active constitutively but, rather, had to be released from covalent attachment within the cell wall to be effective [104]. This distinction from a constitutive elicitor seems finely drawn and, while it might be valid on semantic grounds—although Hargreaves and Bailey did not suggest constitutive activity, merely constitutive presence—any implication of a different biological function or chemical nature may not be merited.

Although IP accumulation may be evoked by foreign biotic or abiotic factors, or by native biotic factors, and enzymic release of certain of these elicitors may be essential to their activity, the presence of carbohydrate is a nearly common denominator in active fractions. Additional, independent work supports this conclusion. Chitosans, constituted primarily of β-1,4-glucosamine, elicited pisatin accumulation in peas [132]. However, the eliciting activity of this polycation might have been the indirect result of it injuring plant cells [133] and so activating endogenous mechanisms [104]. Sucrose stimulated formation of the chalcone, pinostrobin, a phytoalexin in *Cajanus cajan* [134]. Plants may, then, respond to particular carbohydrate components, from external or internal sources, by activating IP formation. Details of the recognitional events involved remain unresolved, although evidence that a receptor site for a branched β-1,3-glucan elicitor of glyceollin exists on soybean membranes has been presented [135].

A not unexpected consequence of investigations of phytoalexin elicitation is the development of models encompassing the phenomena apparently involved in this event. Keen [136] proposed the presence of surface receptors—probably proteins or glycoproteins—on plant cells that recognized superficial molecules, the elicitors, of invading micro-organisms. 'Secondary messengers' were postulated to transmit this initial stimulus to the nucleus of the host cell contacted, as well as to neighboring cells via plasmodesmata. *De novo* transcription was presumed to result in the formation of new mRNA which, in turn, was translated, resulting in the production of enzymes catalysing phytoalexin formation. Accumulation of localized concentrations of the phytoalexins was assumed to

prevent, or at least delay, growth of any micro-organism sensitive to their inhibitory activities. Yoshikawa [137] elaborated slightly upon this scheme, emphasizing particularly a mechanism relevant to IP accumulation in soybean infected with *P. megasperma* f. sp. *glycinea*. In this case, elicitors were speculated to be released from the fungal cell wall surface as a result of glucanase activity constitutively present in the host cells.

Such models, while thought-provoking in developing theories for the role of phytoalexins in pathogenesis, must be viewed cautiously in light of the still uncertain status of natural elicitors, the limited information about receptor sites and the virtual dearth of data concerning secondary messengers. A possible candidate as a secondary messenger is the gaseous plant growth regulator, ethylene, which has been reported to elicit pisatin [126]. However, the evidence that it has a primary role in evoking phytoalexin accumulation is unconvincing. Although elicitors of glyceollin accumulation also increased ethylene formation and PAL activity, chemical manipulation of the ethylene response showed that it could be both stimulated and repressed without concomitant effects on glyceollin or PAL [138]. Others have suggested that these observations may have been artefactual [77], in the sense that ethylene may not be needed for mobilization of carbon within the detached cotyledonary tissues employed by Paradies *et al.* [138]. Kimpel and Kosuge [77] observed partial inhibition of glyceollin accumulation in intact soybean seedlings, when ethylene production was blocked, but no inhibition in seedlings lacking cotyledons. They speculate that, while ethylene may not control the initiation of the glyceollin response, it does mediate the flow of carbon and energy into glyceollin biosynthesis, such that it may be involved as a signal in the removal of reserves from the cotyledons to sites where phytoalexin formation is occurring. Cyclic AMP might represent another candidate as a secondary messenger mediating IP biogenesis, although Hahn and Grisebach [139] did not obtain results consistent with such a possibility.

One other issue pertinent to IP formation concerns the sites where biosynthesis and subsequent build-up occur. Excision of infected tissues and apparently healthy tissues adjacent to lesion areas has permitted determination of the IP levels accumulating and given a general picture of their localization [3, 38, 140, 141]. Phytoalexin concentrations were confined to lesion areas, or adjacent surrounding tissues, with generally no detectable amounts in healthy parts of the plant well removed from infection sites. Synthesis and accumulation, since they often occur in response to infection and consequent tissue damage, are frequently associated with necrotic reactions which involve tissue browning. Nonetheless, Rathmell and Bendall [142] indicated that phascollin formation may represent a specific stimulation of isoflavonoid metabolism separate from the general increase in phenolic compounds associated with necrosis. There seems little doubt that IPs are synthesized, *de novo*, from remote precursors soon after infection in the cells first contacted—prior to their death—and/or in living cells immediately surrounding the region of tissue damage. Some short-distance transport of the phytoalexins may occur to account, at least in part, for accumulation in dead cells at the infection site. The progression of cell death, as the lesion expands, may result in additional healthy cells activating phytoalexin synthesis until lesion size is limited and spread of the microorganism is restricted—unless, of course, the host response is overwhelmed by a highly adapted, virulent pathogen before an effective defense can be mounted, as may be the case with *Sclerotinia sclerotiorum* infection of bean leaves [143]. The supposed defensive capacity of IPs does not preclude the existence of other constitutive or activated resistance mechanisms functioning in series, or in concert, to contain microbial invasion [36, 144].

Although this overall picture may be quite accurate and is, in fact, supported by substantial data, relatively little information is available about the precise cellular location and concentration of phytoalexins. Techniques have not, generally, been developed to provide such exact information. Laser microprobe analysis has been employed to detect glyceollin at the cellular level in soybean [145]. However, the instrumentation is very expensive, elaborate tissue preparation is necessary and accurate quantification of glyceollin in tissue is not yet possible with this technique. More recently, Moesta and co-workers [146] reported a radioimmunoassay for glyceollin I that permitted quantitative determination of this phytoalexin in 15 μm microtome sections of infected soybean hypocotyl tissues. Together with the detection and quantitation of fungal hyphae by indirect immunofluorescence [147], this radioimmunoassay permitted quantitation of phytoalexin and hyphal colonization in alternate serial cryotome sections from *P. megasperma* f. sp. *glycinea*-infected soybean root tissue [148]. Techniques are now, therefore, close to the point of allowing very precise localization and measurement of IPs in diseased plant tissues. These abilities will help resolve controversies about the relevance of phytoalexin accumulation to natural disease resistance.

BIOLOGICAL ACTIVITIES OF ISOFLAVONOID PHYTOALEXINS

Another essential facet to be clarified before a thorough understanding of the natural role of IPs will emerge is whether they do generate a toxic milieu *in vivo* antagonistic to sensitive organisms. Müller and Börger [23] had stated that their conceptual phytoalexin would cause "... 'paralysis' or the premature death of the fungus" [149] and this has been the continuing presumption. The years have revealed, in addition, that phytoalexins are not uniquely toxic to fungi, but have inhibitory effects across much of the biological spectrum. While these research efforts have disclosed much about the biological properties of phytoalexins in general, and IPs in particular, the validity of relating laboratory findings to some natural inhibitory function in plant tissues remains a contentious issue [150].

Methodology

Many different types of bioassay have been employed to determine the effects of IPs [151]. These have involved studies on the germination of fungal spores, growth of germ-tubes, dry weight accumulation in liquid media and mycelial growth on agar surfaces. Antibacterial activity of IPs has been variously assessed by dispersing phytoalexin in agar subsequently streaked with inoculum, applying phytoalexin in organic solvent to bacteria-seeded agar, impregnating phytoalexin in antibiotic assay discs before placing these on seeded agar as well as introducing phytoalexin to liquid media and monitoring bacterial

growth by absorbance. More limited investigations have also been made of phytotoxic effects and of animal toxicity.

A general conclusion that has emerged from this considerable volume of work is that the particular assay conditions employed—the composition and pH of media, the growth stage of the assay organism—may have marked effects on the degree of inhibition observed. As examples, incorporation of rose bengal into agar modified the response of fungi to pisatin [152], alkaline conditions reduced kievitone's toxicity [153] and, whereas medicarpin was inhibitory towards spores and germ tubes of *Stemphylium botryosum* [154, 155], mycelium was relatively insensitive [156].

Another generality that can be drawn from these numerous bioassays is that IPs do not appear especially toxic, with effective doses falling within one order of magnitude, 10^{-5}–10^{-4} M. This conclusion, however, should be treated with considerable caution. Individuals interested in the relevance of phytoalexins to natural resistance employ bioassays designed to yield measures of effective concentrations that can be related to disease situations. Bioassay media, for example, have incorporated components of relevant plant tissues [141, 157]. More pertinent to considerations of IP efficacy, however, is the frequent use of plant pathogens in bioassays—organisms which may possess tolerance mechanisms towards IPs [158]. Therefore, an inadvertent bias may have been introduced into many studies whereby potential phytoalexin toxicity has been substantially underestimated. Few, if any, IP mode of action studies have been undertaken with a micro-organism deliberately selected for especial sensitivity to the individual phytoalexin being assessed.

Many procedural variables may affect *in vitro* estimates of phytoalexin toxicity and any one bioassay represents but a model system providing only limited information. Ideally, any IP should be bioassayed in several different systems and against a variety of organisms before definitive statements are made of its inhibitory nature.

Effects of isoflavonoid phytoalexins

The fungitoxicity of IPs is readily apparent from the inhibition they cause to germ-tube elongation, radial mycelial growth and dry weight accumulation [3, 154, 155, 159–161]. Such essentially superficial observations may be refined through examination of individually affected cells by both light and electron microscopy. A variety of cytological effects have been noted, including rapid cessation of cytoplasmic streaming, granulation of the cytoplasm, disorganization of cell contents and breakdown of the cell membrane [151, 153, 161–165]. Although damage may often prove fatal to individual fungal cells, cell or colony death is not an inevitable consequence of IP treatment. Skipp and Bailey [163] noted that phaseollin-treated sporelings often regrew from apparently unaffected hyphae, particularly from interstitial cells, and maackiain, medicarpin, or mixtures of these phytoalexins, caused only temporary cessation of germ-tube elongation [166].

Plasmalemma disruption, for which cytological evidence has already been cited, is supported by the substantial leakage of electrolytes and metabolites from fungal cells exposed to IPs. Phaseollin [165], maackiain [166] and kievitone [153], all induced loss of ^{14}C-labelled metabolites. The inevitable consequence of uncontrolled leakage of metabolites is loss of mycelial dry weight [151]. Influx, as well as efflux, however, may be affected. Phaseollin and kievitone inhibited the removal of ^{14}C-U-glucose from liquid media [153, 165] and, although maackiain caused no immediate marked effect on the rate of glucose assimilation by germinated spores, uptake was subsequently reduced [166].

Movement of molecules across the plasmalemma is not the solitary physiological process in fungi adversely affected by IPs. Phaseollin inhibited oxygen uptake by actively growing mycelium of *Rhizoctonia solani*, whereas the endogenous respiration of starved mycelium was stimulated [165]. Suppression of exogenous respiration may have been due to insufficient uptake of substrate as a consequence of membrane damage. The stimulation in starved mycelium might also have reflected membrane dysfunction, either because it allowed decompartmentalization of potential respiratory substrates within the mycelia or because it uncoupled oxidative phosphorylation. Certainly, since phaseollin failed to inhibit respiration by isolated mitochondria from *Neurospora crassa* [164], some indirect effect of the phytoalexin seemed likely.

Studies of the antibacterial activity of IPs have been more restricted in number and scope than investigations of fungitoxicity [151]. Effects may be either bacteriostatic or bactericidal. Whereas no inhibitory zones were evident when coumestrol was applied in antibiotic assay discs to nutrient agar seeded with *Pseudomonas* spp., this compound did prevent growth in liquid media for 36–48 hr [167]. Tests of eleven isoflavonoids, conducted in both soft agar and liquid media against eight *Rhizobium* strains [168], indicated that medicarpin and kievitone were most toxic, being bactericidal towards *R. japonicum* and *R. lupini*. Phaseollin and maackiain were moderately inhibitory, whereas pisatin and coumestrol, amongst others, showed little activity. An earlier survey [169] also indicated that kievitone was more inhibitory than either phaseollin or pisatin.

Gram-negative bacteria are usually less sensitive to antibiotics than Gram-positive genera [170]. This generalization appears to include isoflavonoid and flavonoid phytoalexins [171, 172]. Kievitone, phaseollin and 7-hydroxyflavan each inhibited six Gram-positive species at a concentration causing no apparent inhibition of six Gram-negative isolates [151, 171]. Govindarajan and Gnanamanickam [173] noted that kievitone was inhibitory towards three Gram-positive human pathogens, *Corynebacterium diptheriae* var. *mitis*, *Streptococcus haemolyticus* and *Staphylococcus aureus*, suggesting that certain plant isoflavonoids might have some utility in chemotherapy.

If the antifungal and antibacterial activities of IPs may be taken as circumstantial evidence that these compounds might contribute to curtailing microbial development in plant tissues, the argument may reasonably be expanded to encompass other pests and parasites. A survey of isoflavonoids as insect feeding deterrents revealed several with activity and raised the possibility that plants may utilize the same chemicals to deter insects as well as inhibit micro-organisms [20]. Microscopic nematodes are serious plant parasites and there is some evidence that certain of these, too, may be adversely affected by IPs. Glyceollin isomers limited the mobility of *Meloidogyne incognita* larvae, although the effect appeared to be

nematistatic, not nematicidal [174, 175]. Oxygen uptake by this sensitive nematode was inhibited when phytoalexin was added to larval suspensions.

Although most of the toxicological studies with IPs have focused on their antimicrobial activities, it is only sensible to question whether their accumulation to substantial concentrations in plant tissue might not also affect plants. In point of fact, phytotoxic effects of IPs are evidenced in several ways. Phaseollin caused rapid inhibition of respiration, less growth of suspension cultures and cell death [176]; affected cells of both bean and tobacco appeared granular. Glazener and VanEtten [177] noted cell death and intial reduced growth of suspension cultures of *Phaseolus aureus* and *P. vulgaris* following phaseollin treatment. Pisatin inhibited the growth of pea callus cultures [178] and retarded primary root growth in wheat [160]. Leakage of betacyanin and/or electrolytes occurred after exposure of beetroot discs to kievitone and phaseollin, but not pisatin or medicarpin [153, 179]. Root and hypocotyl growth, as well as seed germination in several clover species [180], was repressed by trifolirhizin.

A point worth bearing in mind, however, is that the toxicity shown by exogenous application of IPs to plant tissues capable of producing these compounds might not accurately reflect any effects occurring upon natural formation [177]. Nonetheless, even if individual plant cells are killed in local areas of high phytoalexin concentrations, there is no reason to suppose that such an outcome might not be of overall benefit to the plant. The localized death of plant cells occurring in the formation of a limited lesion and perhaps due, at least in part, to the accumulation of IPs to phytotoxic levels may represent a part of the plant's 'strategy' in containing invading microorganisms. Certainly, this notion is not inconsistent with phytoalexins playing a contributory role in hypersensitivity [181]. Disorganization of host cells with concomitant 'removal' of these as nutritional sources for pathogen sustenance, as a result of IP accumulation, could readily be envisaged as hindering extensive microbial colonization of plant tissues. This situation would seem likely to be particularly effective against obligate parasites, which require living host cells for successful growth and development. Accepting such an argument, IPs may conceivably be involved in curtailing viral replication [182] which, presumably, requires organized and functioning cellular machinery. It is worth bearing in mind, however, that isoflavonoids might not function solely as toxic agents in defensive responses. Suppression of plant growth raises the possibility that these compounds could act as endogenous growth regulators [1].

The paragraphs above represent a brief commentary on what is now a considerable literature on the physiological and cytological effects of IPs. Fungi, bacteria, nematodes—all may be susceptible to the inhibitory activities of IPs and may have their development in plant tissues curtailed as a consequence. Even viral replication may be limited indirectly as a result of the phytotoxic effects of isoflavonoids. Yet this does not quite complete the toxicological picture as regards isoflavonoids; a final point merits mention. Toxic consequences may result in animals, including humans, consuming plant tissues containing phytoalexins [183]. Although only a few investigations have addressed this topic, IPs, for example, may lyse red blood cells [151] and the effect may be rapid [165]. Coumestrol showed oestrogenic activity in mouse uterine bioassays and, as may be the case for other isoflavonoids [3], it may lead to infertility or other reproductive problems in mammals feeding on certain plants [3, 151, 184]. Pisatin repressed respiration in isolated rat liver mitochondria [185]. Other evidence of the toxicity of isoflavonoids to animal systems comes from observations that phaseollin, phaseollidin and phaseollinisoflavan killed water snails and brine shrimp [3, 186].

Any belief that IPs were uniquely fungitoxic chemicals long since disappeared. Nor is there evidence to suggest that they are even selectively active against fungi, for the concentrations required to express antifungal activity are comparable to those needed to inhibit members of other biological classes. Bacteria, higher plants and a range of animals, as well as isolated cells or organelles, are all vulnerable to individual IPs. Furthermore, cell death and non-lethal inhibition may occur, indicating that biocidal as well as biostatic activity may be expressed.

Mode of action

The sites of action of IPs in the spectrum of organisms affected seem likely to be similar, for no class seems particularly sensitive nor especially resistant. Clearly, the cytological and physiological consequences of IP treatment represent either direct or indirect manifestations of the toxicities of these chemicals. Ideally, in order to define a precise mode of action, the earliest effect on the target organism, employing the lowest amount of toxicant needed to accomplish inhibition, should be unambiguously established. It is probably fair to say that an exact mode of action has not been unequivocally determined for any IP.

Many of the observations mentioned above could most easily be rationalized by assuming that IPs are multi-site toxic chemicals, affecting many metabolic reactions and physiological processes, as opposed to site-selective compounds which primarily affect one particular reaction or process [151, 187]. The considerable range of organisms detrimentally affected, both prokaryotes and eukaryotes, is certainly suggestive of non-specific activity. Furthermore, the diverse physiological and cytological changes observed soon after treatment—especially in the fungi, which have been most thoroughly investigated—suggest that many 'secondary' effects may occur. Indiscriminate binding, apparent, as examples, in the substantial uptake of phaseollin by bean cells [176] as well as in the moderating effects of detergents, phospholipids and protein on kievitone's toxicity [153], likewise favors multi-site activity. Certainly polyphenolics, and perhaps even small phenols with multiple phenolic hydroxyls (a description befitting several IPs), may be rather indiscriminate in their binding, judged from the variety of interactions that may occur with proteins and polysaccharides [188]. There is also a teliological argument for IPs being multi-site inhibitors. These would be more difficult for a plant pathogen to counter, in an evolutionary sense, than site-selective compounds where an alteration in one pathogen gene might induce a change at the specific site of action and so confer resistance [189].

Generalization from the available data indicates dysfunction of membrane systems is, perhaps, particularly instrumental in the inhibitory action of IPs. Amongst treated fungi, substantial loss of dry weight, leakage, swelling and bursting of hyphae [151], are all consistent with membrane damage, particularly damage to the plasmalemma. Results from bacterial studies with glyci-

nol, glyceollin and coumestrol indicated that these acted as nonspecific membrane antagonists that altered the structural integrity of the membrane, thereby causing it to be a less efficient matrix for membrane-dependent processes [190]. The evidence also showed that the effects of these three IPs were of a general nature and not directed at a specific cellular process. Bacteriostatic concentrations of glycinol and glyceollin inhibited all cellular processes examined, regardless of whether these were coupled to respiration. Lysis of red blood cells, which obviously lack walls, as a consequence of exposure to IPs is also consistent with some action on the cell membrane. The apparent importance of a lipophilic nature for IPs is additional evidence making a membrane site of action probable. A lipophilic side chain is vital to the antifungal activities of wighteone [191] and kievitone [192]; this lipophilic nature likely permits effective penetration of fungal membranes [193]. The plasmalemma seems highly probable as a site of action, since it would likely be the first membrane encountered by any external toxic chemical. Hargreaves [179] suggested, however, from plant studies, that the tonoplast may be primarily affected by phaseollin. Of course, in those plants naturally producing IPs, their formation presumably would be intracellular and the tonoplast might, therefore, be encountered by the phytoalexins before the plasmalemma.

Although the balance of the evidence would appear to favor a case for IPs as multi-site toxicants with likely sites of action in cellular membrane systems, site-specific action against a particular metabolic process cannot be dismissed. Studies with *Meloidogyne incognita* indicated that glyceollin inhibited oxygen uptake by this nematode [175]. This report also revealed that glyceollin was a potent inhibitor of oxygen uptake by isolated mitochondria from soybeans and table beets and that it did not function as an uncoupler of oxidative phosphorylation but, rather, as an inhibitor of the electron transport system at some point after the succinate dehydrogenase site. Boydston and colleagues [194], who also addressed the issue of glyceollin inhibition of electron transport by isolated mitochondria, could not confirm this observation. Their data indicated that glyceollin specifically inhibited malate oxidation, acting as a site I inhibitor in a manner similar or identical to rotenone. Although these experiments indicated a glyceollin-specific site in the mitochondrial electron transport chain associated with the inner membrane, the possibility that glyceollin might also bind to, and influence, other membranes and/or enzymes was not ruled out. These investigations of respiratory effects, as Weinstein and Albersheim [190] point out, may not yet have been extensive enough to provide a convincing argument for a specific target for glyceollin's action and, furthermore, the observed inhibition of electron transport by glyceollin could be one of several secondary effects resulting from the interaction of glyceollin with cell membranes. Likewise, the action of pisatin as a respiratory uncoupler in rat liver mitochondria [185] might be mediated by the disruption of mitochondrial membranes.

The antifungal activity of pterocarpanoid phytoalexins was postulated by Perrin and Cruickshank [195] to be dependent on particular steric and compositional requirements. However, these conclusions were not supported by VanEtten's [196] subsequent investigations. An even more recent study [197] partially corroborates Perrin and Cruickshank's [195] earlier hypothesis. However, Kramer and colleagues [197] feel that the fungicidal property of the isoflavonoids is specific to individual fungi, substances and their concentrations, and that no absolute generalization is possible about structural requirements. It would appear that the structure–antifungal activity relationships of IPs, in general, require clarification [3, 198].

Bakker *et al.* [199] conducted an interesting investigation into the possible role of photodynamism in IP toxicity. They noted that, upon ultraviolet irradiation, phaseollin, 3,6a,9-trihydroxypterocarpan, glyceollin, tuberosin and pisatin, but not medicarpin, inactivated glucose-6-phosphate dehydrogenase in an *in vitro* assay, most likely as a result of free radical formation. Photodegradation of the phytoalexins occurred almost immediately upon the onset of illumination and, ultimately, the irradiation was highly destructive to all except medicarpin. The relevance of these observations to the *in vivo* activity of IPs is not, as yet, apparent, although the effects clearly merit further study.

Tolerance

There is no doubt that IPs exert toxicity towards many, varied organisms. However, not all are affected to the same extent. Differences in sensitivity occur between and within the different biological classes affected and, indeed, even within genera. Allusion has already been made to the apparently greater vulnerability of Gram-positive, as opposed to Gram-negative, bacteria [171, 172]. Phaseollin-treated bean cell suspension cultures, despite initial inhibition, eventually achieved substantial growth [176]. This long-term recovery was perhaps the result of individual cells escaping the phytotoxic effects or the consequence of phytoalexin conversion to non-toxic products. Furthermore, glyceollin isomers adversely affected the motility of the nematode, *M. incognita*, but no observed effect was noted on the related species, *Meloidogyne javanica* [174].

Although differential sensitivity and/or evidence of recovery from IP treatment, phenomena both indicative of tolerance mechanisms, have, therefore, been noted with bacteria, plants and animals, most is known concerning fungi [158]. Many different fungal types have been investigated in IP bioassays, not only phytopathogenic species, but also saprophytes and zoopathogens [151, 200]. Differences in phytoalexin vulnerability amongst fungi have been apparent for many years [160] and were at one time reputed to be associated with plant pathogenicity, pathogenic fungi being insensitive to their hosts' phytoalexin(s) while non-pathogens were susceptible. Such an overall generalization no longer seems warranted for, while instances in accord with this association occur, so, too, do several exceptions [151].

Tolerance towards IPs in fungi might have several mechanistic explanations, with parallels being drawn from fungicide research [201, 202]. Insensitive fungi might be less permeable to the phytoalexins or possess sites of action with weak phytoalexin affinities. In addition, less vulnerable cells might possess a metabolic bypass for the process inhibited by the phytoalexin or detoxification of the IP might occur before or after entry into the fungal cell. Other explanations may also exist and, furthermore, multiple tolerance mechanisms might be expressed by an individual organism or cell.

The relationship between plant pathogenicity and comparative insensitivity to host IPs is not always clear-

cut. Nonetheless, there are instances where fungal tolerance does seem critical for successful infection. Several papers published by VanEtten and co-workers [203–208] present a strong case that the ability to tolerate pisatin is essential for the virulence of *Fusarium solani* f. sp. *pisi* (*Nectria haematococca*) in pea. Although monooxygenase-catalysed degradation of pisatin by *F. solani* f. sp. *pisi* seems important here, this fungus also appears to possess an inducible, nondegradative tolerance to pisatin. This nondegradative component appears to be due to plasmalemma modification [204].

Detoxification by enzymic conversion of phytoalexins has been the most intensively studied tolerance mechanism [151, 158]. Isolation of a fungal enzyme catalysing detoxification of any IP was first accomplished for kievitone hydratase (KHase) (Fig. 1), which mediates transformation of kievitone (Fig. 1; **28**) to kievitone hydrate (Fig. 1; **29**) [209, 210]. The enzyme was first isolated from cell-free culture filtrates of *Fusarium solani* f. sp. *phaseoli* [209], but has since been shown to occur in *F. solani* f. sp. *phaseoli*-infected bean tissues, where it accumulates for several days following inoculation [211]. Highly-purified preparations of KHase, which is a predominantly extracellular enzyme [209], have been obtained [212]. The molecular weight of KHase was estimated to be 102 000; isoelectric focusing and affinity chromatography revealed that KHase was an acidic glycoprotein. Particularly relevant to present considerations, however, were studies undertaken to resolve whether any relationship existed in *Fusarium* between kievitone detoxification, mediated by KHase, and virulence to beans. Twenty-eight wild-type isolates of *Fusarium* and *Nectria* (*N. haematococca* is the perfect stage of *F. solani* [206]) were surveyed for their ability to produce kievitone hydrate and for their virulence towards *P. vulgaris* [213]. Only three of these isolates possessed demonstrable, extracellular KHase activity and these same three isolates were the only ones highly virulent on *P. vulgaris*. In addition to the wild-type analyses, several variants of a virulent strain of *F. solani* f. sp. *phaseoli* were tested for KHase activity, virulence to *P. vulgaris* and sensitivity to kievitone [211]. All variants with lowered KHase activity were more sensitive to the phytoalexin and less virulent to bean than the original strain from which they were derived.

These independent studies of two different plant–fungus interactions (pea—*F. solani* f. sp. *pisi* and bean—*F solani* f. sp. *phaseoli*) both indicate that greater tolerance of IPs is associated with more virulent behaviour by individual fungal isolates [203–213]. Another common feature of these investigations, but one that is perhaps more disquieting, is the occurrence of, at most, only very low levels of 3,6a-dihydroxy-8,9-methylendioxypterocarpan (DMDP) and kievitone hydrate, the primary metabolites of pisatin and kievitone, respectively, in infected plant tissues. Probably the most logical interpretation of these observations is that further conversion of the metabolites, catalysed either by the pathogen and/or its host, prevents their accumulation *in vivo*. Indeed, an intermediate role for DMDP in fungal detoxification of IPs has already been established [158, 214, 215], though this is not yet the case for kievitone hydrate and some other explanation may apply to its low recovery from *Fusarium*-infected beans.

Research into the metabolism of solitary IPs by fungi, while instructive of the mechanisms involved, may not represent the most appropriate models of natural situations. For example, *Phaseolus vulgaris* produces several antifungal stress metabolites following fungal colonization [30]. A successful pathogen, such as *Fusarium solani* f. sp. *phaseoli*, might need to render inactive several of these compounds while invading the plant tissues. Cultural studies indicated that this fungus detoxified different combinations of the IPs from bean [216, 217]. Within 24–30 hr, *F. solani* f. sp. *phaseoli* metabolized and detoxified a mixture of kievitone and phaseollidin as well as a mixture of kievitone, phaseollin and phaseollinisoflavan. Only kievitone, however, was subject to cell-free detoxification and, of the various enzymes presumably involved in these structural alterations of the phytoalexins, apparently only KHase was secreted extracellularly in an active form. The, as yet unbroken, association between tolerance of kievitone and virulence to bean in *F. solani* f. sp. *phaseoli*, together with the ability of this fungus to cope in culture with multiple treatments of IPs from bean, imply that the accumulation of post-infectional antimicrobial isoflavonoids might present a functional, inhibitory environment to any micro-organisms without the means to counter these compounds.

Studies of IP tolerance in fungi have, therefore, shed light on the role these compounds may play in diseased plants, though it is by no means established fact that they do generate effective toxic barriers against the development and spread of micro-organisms in infected tissues. A definitive statement on this matter will require refinement of bioassay systems, to establish the precise toxic potentials of IPs in infected plants, as well as ever more exact understandings of elicitation and the concentrations of phytoalexins occurring at micro-sties in damaged tissues. Research to elucidate the formation and biological properties of IPs, and the relevance of these phenomena to function, will need to continue to pursue several different avenues.

Acknowledgments—The authors wish to thank John Ingham for his constructive thoughts concerning the manuscript and Patricia Parrott, Denise Mattingly and Roger E. Deaton for preparing the typescript.

REFERENCES

1. Dewick, P. M. (1982) in *The Flavonoids: Advances in Research* (Harborne, J. B. and Mabry, T. J., eds) p. 535. Chapman & Hall, London.
2. O'Neill, T. M. and Mansfield, J. W. (1982) *Trans. Br. Mycol. Soc.* **79**, 229.
3. VanEtten, H. D. and Pueppke, S. G. (1976) in *Biochemical Aspects of Plant–Parasite Relationships* (Friend, J. and Threlfall, D. R., eds) p. 239. Academic Press, London.
4. Harborne, J. B., Ingham, J. L., King, L. and Payne, M. (1976) *Phytochemistry* **15**, 1485.
5. Smith, D. A. and Ingham, J. L. (1980) in *Advances in Legume Science* (Summerfield, R. J. and Bunting, A. H., eds) p. 207. HMSO, London.
6. Burden, R. S., Rogers, P. M. and Wain, R. L. (1974) *Ann. Appl. Biol.* **78**, 59.
7. Doherty, J. and Buescher, R. (1978) *J. Sci. Food Agric.* **29**, 853.
8. Hart, S. V., Kogan, M. and Paxton, J. D. (1983) *J. Chem. Ecol.* **9**, 657.

9. Russell, G. B., Sutherland, O. R. W., Lane, G. A. and Biggs, D. R. (1978) *Aust. Conf. Grassland Invert. Ecol.* 95.
10. Bailey, J. A. and Mansfield, J. W. (1982) *Phytoalexins*. Blackie, Glasgow.
11. Richardson, P. M. (1981) *Biochem. Syst. Ecol.* **9**, 105.
12. Takasugi, M., Anetai, M., Masamune, T., Shirata, A. and Takahashi, K. (1980) *Chem. Letters* 339.
13. Takasugi, M., Niino, N., Nagao, S., Anetai, M., Masamune, T., Shirata, A. and Takahashi, K. (1984) *Chem. Letters* 689.
14. O'Neill, T. M. (1981) Ph. D. Thesis. University of Stirling, U.K.
15. Coxon, D. T., O'Neill, T. M., Mansfield, J. W. and Porter, A. E. A. (1980) *Phytochemistry* **19**, 889.
16. Cocker, W., Dahl, T., Dempsey, C. and McMurry, T. B. H. (1962) *J. Chem. Soc.* 4906.
17. Donnelly, D. M. X. and Kavanagh, P. J. (1974) *Phytochemistry* **13**, 2587.
18. Harper, S. H., Kemp, A. D. and Underwood, W. G. E. (1969) *J. Chem. Soc.* (C) 1109.
19. Fukui, H., Egawa, H., Koshimizu, K. and Mitsui, T. (1973) *Agric. Biol. Chem.* **37**, 417.
20. Sutherland, O. R. W., Russell, G. B., Biggs, D. R. and Lane, G. A. (1980) *Biochem. Syst. Ecol.* **8**, 73.
21. Bernard, N. (1911) *Ann. Sci. Nat. (Bot.)* **14**, 221.
22. Deverall, B. J. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 1. Blackie, Glasgow.
23. Müller, K. O. and Börger, H. (1940) *Arb. Biol. Reichsanst. Land-u. Forstw. Berlin-Dahlem* **23**, 189.
24. Müller, K. O. (1958) *Nature* **182**, 167.
25. Cruickshank, I. A. M. and Perrin, D. R. (1960) *Nature* **187**, 799.
26. Perrin, D. R. and Bottomley, W. (1962) *J. Am. Chem. Soc.* **84**, 1919.
27. Cruickshank, I. A. M. and Perrin, D. R. (1963) *Life Sci.* **2**, 680.
28. Perrin, D. R. (1964) *Tetrahedron Letters* 29.
29. Coxon, D. T. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 106. Blackie, Glasgow.
30. Ingham, J. L. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 21. Blackie, Glasgow.
31. Kuć, J. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 81. Blackie, Glasgow.
32. Ingham, J. L. (1983) in *Fortschritte der Chemie organischer Naturstoffe* (Herz, W., Grisebach, H. and Kirby, G. W., eds) p. 1. Springer, Vienna.
33. Olah, A. F. and Sherwood, R. T. (1973) *Phytopathology* **63**, 739.
34. McMurchy, R. A. and Higgins, V. J. (1984) *Physiol. Plant Pathol.* **25**, 229.
35. Higgins, V. J. and Smith, D. G. (1972) *Phytopathology* **62**, 235.
36. Deverall, B. J. (1976) in *Biochemical Aspects of Plant–Parasite Relationships* (Freind, J. and Threlfall, D. R., eds) p. 208. Academic Press, London.
37. Rathmell, W. G. (1973) *Physiol. Plant Pathol.* **3**, 259.
38. Bailey, J. A. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 289. Blackie, Glasgow.
39. Stoessl, A. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 133. Blackie, Glasgow.
40. Al Ani, H. A. M. and Dewick, P. M. (1985) *Phytochemistry* **24**, 55.
41. Grisebach, H. (1959) *Z. Naturforsch.* **14b**, 802.
42. Grisebach, H. and Brandner, G. (1961) *Z. Naturforsch.* **16b**, 2.
43. Grisebach, H. and Brandner, G. (1962) *Experientia* **18**, 400.
44. Grisebach, H. and Patschke, L. (1965) *Z. Naturforsch.* **20b**, 399.
45. Patschke, L., Barz, W. and Grisebach, H. (1966) *Z. Naturforsch.* **21b**, 45.
46. Wong, E. (1968) *Phytochemistry* **7**, 1751.
47. Wong, E. and Grisebach, H. (1969) *Phytochemistry* **8**, 1419.
48. Grisebach, H. (1965) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.) p. 279. Academic Press, London.
49. Dewick, P. M. (1978) *Phytochemistry* **17**, 249.
50. Grisebach, H. and Zilg, H. (1968) *Z. Naturforsch.* **23b**, 494.
51. Crombie, L. Dewick, P. M. and Whiting, D. A. (1973) *J. Chem. Soc. Perkin Trans.* 1, 1285.
52. Pelter, A., Bradshaw, J. and Warren, R. F. (1971) *Phytochemistry* **10**, 835.
53. Farkas, L., Gottsegen, A., Nogradi, M. and Antus, S. (1974) *J. Chem. Soc. Perkin Trans.* 1, 305.
54. McKillop, A., Swann, B. P., Ford, M. E. and Taylor, E. C. (1973) *J. Am. Chem. Soc.* **95**, 3641.
55. Ollis, W. D., Ormand, K. L. and Sutherland, I. O. (1970) *J. Chem. Soc. C Pt.* 1, 119.
56. Thomas, P. and Whiting, D. A. (1984) *Tetrahedron Letters* **25**, 1099.
57. Stoessl, A. and Stothers, J. B. S. (1979) *Z. Naturforsch.* **34c**, 87.
58. Dewick, P. M., Steele, M. J., Dixon, R. A. and Whitehead, I. M. (1982) *Z. Naturforsch.* **37c**, 363.
59. Dewick, P. M. (1975) *Phytochemistry* **14**, 979.
60. Dewick, P. M. (1977) *Phytochemistry* **16**, 93.
61. Dewick, P. M. and Ward, D. (1978) *Phytochemistry* **17**, 1751.
62. Dewick, P. M. and Steele, M. J. (1982) *Phytochemistry* **21**, 1599.
63. Dewick, P. M. and Martin, M. (1979) *Phytochemistry* **18**, 591.
64. Dewick, P. M. and Ward, D. (1977) *J. Chem. Soc. Chem. Commun.* 338.
65. Banks, S. W., Steele, M. J., Ward, D. and Dewick, P. M. (1982) *J. Chem. Soc. Chem. Commun.* 157.
66. Banks, S. W. and Dewick, P. M. (1982) *Phytochemistry* **21**, 1605.
67. Banks, S. W. and Dewick, P. M. (1983) *Phytochemistry* **22**, 1591.
68. Banks, S. W. and Dewick, P. M. (1982) *Phytochemistry* **21**, 2235.
69. Banks, S. W. and Dewick, P. M. (1983) *Z. Naturforsch.* **38c**, 185.
70. Banks, S. W. and Dewick, P. M. (1983) *Phytochemistry* **22**, 2729.
71. Hagmann, M. L., Heller, W. and Grisebach, H. (1984) *Eur. J. Biochem.* **142**, 147.
72. Zahringer, U., Schaller, E. and Grisebach, H. (1981) *Z. Naturforsch.* **36c**, 234.
73. Hadwiger, L. A., Hess, S. L. and von Broembsen, S. (1970) *Phytopathology* **60**, 332.
74. Whitehead, I. M., Dey, P. M. and Dixon, R. A. (1982) *Planta* **154**, 156.
75. Munn, C. B. and Drysdale, R. B. (1975) *Phytochemistry* **14**, 1303.
76. Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983) in *Methods in Enzymology and Related Areas of Molecular Biology* (Meister, A., ed.) p. 1. Wiley, New York.
77. Kimpel, J. A. and Kosuge, T. (1985) *Plant Physiol.* **77**, 1.
78. Benveniste, I., Salaun, J.-P. and Durst, F. (1977) *Phytochemistry* **16**, 69.
79. Lamb, C. J. (1977) *Planta* **135**, 169.

80. Lamb, C. J. and Rubery, P. M. (1976) *Plant Sci. Letters* **7**, 33.
81. Dixon, R. A. and Bendall, D. S. (1978) *Physiol. Plant Pathol.* **13**, 295.
82. Ebel, J. (1979) in *Regulation of Secondary Product and Plant Hormone Metabolism* (Luckner, M. and Schreiber, K., eds) p. 155. Pergamon Press, Oxford.
83. Hahlbrock, K., Schroder, J. and Viregge, J. (1980) *Adv. Biochem. Eng.* **18**, 40.
84. Kreuzler, G. and Hahlbrock, K. (1975) *Eur. J. Biochem.* **56**, 205.
85. Zahringer, U., Ebel, J. and Grisebach, H. (1978) *Arch. Biochem. Biophys.* **188**, 450.
86. Dixon, R. A., Dey, P. M. Murphy, D. L. and Whitehead, I. M. (1981) *Planta* **151**, 272.
87. Robbins, M. P., Bolwell, G. P. amd Dixon, R. A. (1985) *Eur. J. Biochem.* **148**, 563.
88. Partridge, J. E. and Keen, N. T. (1977) *Phytopathology* **67**, 50.
89. Dixon, R. A. and Lamb, C. J. (1979) *Biochim. Biophys. Acta* **586**, 453.
90. Grisebach, H. and Brandner, G. (1962) *Biochim. Biophys. Acta* **60**, 51.
91. Wong, E. (1965) *Biochim. Biophys. Acta* **111**, 358.
92. Hagmann, M. and Grisebach, H. (1984) *FEBS Letters* **175**, 199.
93. Poulton, J. E., Grisebach, H., Ebel, J., Schaller-Hekeler, B. and Hahlbrock, K. (1976) *Arch. Biochem. Biophys.* **173**, 301.
94. Wengenmayer, H., Ebel, J. and Grisebach, H. (1974) *Eur. J. Biochem.* **50**, 135.
95. Leube, J. and Grisebach, H. (1983) *Z. Naturforsch.* **38c**, 730.
96. Burden, R. S., Bailey, J. A. and Dawson, G. W. (1972) *Tetrahedron Letters* 4175.
97. Yoshikawa, M., Yamauchi, K. and Masago, H. (1978) *Plant Physiol.* **61**, 314.
98. Bell, J. N., Dixon, R. A., Bailey, J. A., Rowell, P. M. and Lamb, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3384.
99. Ryder, T. B., Cramer, C. L., Bell, J. N., Robbins, M. P., Dixon, R. A. and Lamb, C. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5724.
100. Schmelzer, E., Borner, H., Grisebach, H., Ebel, J. and Hahlbrock, K. (1984) *FEBS Letters* **172**, 59.
101. Ebel, J., Schmidt, W. E. and Loyal, R. (1984) *Arch. Biochem. Biophys.* **232**, 240.
102. Keen, N. T. (1975) *Science* **187**, 74.
103. Cruickshank, I. A. M. and Perrin, D. R. (1965) *Aust. J. Biol. Sci.* **18**, 803.
104. Darvill, A. G. and Albersheim, P. (1984) *Ann. Rev. Plant Physiol.* **35**, 243.
105. Keen, N. T. and Bruegger, B. (1977) in *Host Plant Resistance to Pests* (Hedin, P. A., ed.) p. 1. Am. Chem. Soc., Washington.
106. West, C. A. (1981) *Naturwissenschaften* **68**, 447.
107. Cartwright, D. W., Langcake, P. and Ride, J. P. (1980) *Physiol. Plant Pathol.* **17**, 259.
108. Kuć, J. and Caruso, F. L. (1977) in *Host Plant Resistance to Pests* (Hedin, P. A., ed.) p. 78. Am. Chem. Soc., Washington.
109. Ossowski, P., Pilotti, A., Garegg, P. and Linberg, B. (1984) *J. Biol. Chem.* **259**, 11337.
110. Sharp, J. K., Albersheim, P., Ossowski, P., Pilotti, A., Garegg, P. and Lindberg, B. (1984) *J. Biol. Chem.* **259**, 11341.
111. Sharp, J. K., McNeil, M. and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11321.
112. Sharp, J. K., Valent, B. and Albersheim, P. (1984) *J. Biol. Chem.* **259**, 11312.
113. Keen, N. T. and Legrand, M. (1980) *Physiol. Plant Pathol.* **17**, 175.
114. Ziegler, E. and Pontzen, R. (1982) *Physiol. Plant Pathol.* **20**, 321.
115. Keen, N. T. and Yoshikawa, M. (1983) *Plant Physiol.* **71**, 460.
116. Davis, K. R., Lyon, G. D., Darvill, A. G. and Albersheim, P. (1984) *Plant Physiol.* **74**, 52.
117. Cruickshank, I. A. M. and Perrin, D. R. (1968) *Life Sci.* **7**, 449.
118. Hargreaves, J. A. and Bailey, J. A. (1978) *Physiol. Plant Pathol.* **13**, 89.
119. Bailey, J. A. (1980) *Ann. Phytopathol.* **12**, 395.
120. Stössel, P. and Magnaloto, D. (1983) *Experientia* **39**, 153.
121. Hadwiger, L. A. and Schwochau, M. E. (1971) *Plant Physiol.* **47**, 346.
122. Kobayashi, M. and Ohta, Y. (1983) *Phytochemistry* **22**, 1257.
123. Rubin, B., Penner, D. and Saettler, A. W. (1983) *Environ. Toxicol. Chem.* **2**, 295.
124. Bailey, J. A. and Berthier, M. (1981) *Phytochemistry* **20**, 187.
125. Hargreaves, J. A. (1981) *New Phytol.* **87**, 733.
126. Chalutz, E. and Stahmann, M. A. (1969) *Phytopathology* **59**, 1972.
127. Rahe, J. E. and Arnold, R. M. (1975) *Can. J. Botany* **53**, 921.
128. Takahashi, H. and Jaffe, M. J. (1984) *Physiol. Plant.* **61**, 405.
129. Bridge, M. A. and Klarman, W. L. (1973) *Phytopathology* **63**, 606.
130. Hahn, M. G., Darvill, A. G. and Albersheim, P. (1981) *Plant Physiol.* **68**, 1161.
131. Nothnagel, E. A., McNeil, M. and Albersheim, P. (1983) *Plant Physiol.* **71**, 916.
132. Hadwiger, L. A. and Beckman, J. M. (1980) *Plant Physiol.* **66**, 205.
133. Young, D. H., Kohle, H. and Kauss, H. (1982) *Plant Physiol.* **70**, 1449.
134. Cooksey, C. J., Garratt, P. J., Dahiya, J. S. and Strange, R. N. (1983) *Science* **220**, 1398.
135. Yoshikawa, M., Keen, N. T. and Wang, M.-C. (1983) *Plant Physiol.* **73**, 497.
136. Keen, N. T. (1982) in *Advances in Plant Pathology* (Ingram, D. S. and Williams, P. H., eds) Vol. 1, p. 35. Academic Press, London.
137. Yoshikawa, M. (1983) in *Biochemical Plant Pathology* (Callow, J. A., ed.) p. 267. Wiley, Chichester.
138. Paradies, I., Konze, J. R., Elstner, E. and Paxton, J. (1980) *Plant Physiol.* **66**, 1106.
139. Hahn, M. G. and Grisebach, H. (1983) *Z. Naturforsch.* **38c**, 578.
140. Rahe, J. E. (1973) *Can. J. Botany* **51**, 2423.
141. Smith, D. A., VanEtten, H. D. and Bateman, D. F. (1975) *Physiol. Plant Pathol.* **5**, 51.
142. Rathmell, W. G. and Bendall, D. S. (1971) *Physiol. Plant Pathol.* **1**, 351.
143. Sutton, D. C. and Deverall, B. J. (1984) *Plant Pathol.* **33**, 377.
144. Ride, J. P. (1983) in *Biochemical Plant Pathology* (Callow, J. A., ed.) p. 215. Wiley, Chichester.
145. Moesta, P., Seydel, U., Linder, B. and Grisebach, H. (1982) *Z. Naturforsch.* **37c**, 748.
146. Moesta, P., Grisebach, H. and Ziegler, E. (1983) *Plant Physiol.* **73**, 233.
147. Moesta, P., Grisebach, H. and Ziegler, E. (1983) *Eur. J. Cell Biol.* **31**, 167.
148. Hahn, M. G., Bonhoff, A. and Grisebach, H. (1985) *Plant Physiol.* **77**, 591.
149. Paxton, J. D. (1980) *Plant Disease* **64**, 734.
150. Pueppke, S. G. (1978) *Mycopathologia* **65**, 115.
151. Smith, D. A. (1982) in *Phytoalexins* (Bailey, J. A. and

Mansfield, J. W., eds) p. 218. Blackie, Glasgow.
152. VanEtten, H. D. (1973) *Phytopathology* **63**, 1477.
153. Bull, C. A. (1981) Ph.D. Thesis, University of Hull, U.K.
154. Higgins, V. J. (1969) Ph.D. Thesis, Cornell University, Ithaca, NY, U.S.A.
155. Higgins, V. J. and Millar, R. L. (1968) *Phytopathology* **58**, 1377.
156. Higgins, V. J. (1972) *Physiol. Plant Pathol.* **2**, 289.
157. Pueppke, S. G. and VanEtten, H. D. (1976) *Phytopathology* **66**, 1174.
158. VanEtten, H. D., Matthews, D. E. and Smith, D. A. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 181. Blackie, Glasgow.
159. Cruickshank, I. A. M. (1962) *Aust. J. Biol. Sci.* **15**, 147.
160. Cruickshank, I. A. M. and Perrin, D. R. (1961) *Aust. J. Biol. Sci.* **14**, 336.
161. Smith, D. A. (1976) *Physiol. Plant Pathol.* **9**, 45.
162. Grisebach, H. and Ebel, J. (1978) *Angew. Chem. Int. Ed. Engl.* **17**, 635.
163. Skipp, R. A. and Bailey, J. A. (1976) *Physiol. Plant Pathol.* **9**, 253.
164. Slayman, C. L. and VanEtten, H. D. (1974) *Plant Physiol. Suppl.* **54**, 24.
165. VanEtten, H. D. and Bateman, D. F. (1971) *Phytopathology* **61**, 1363.
166. Higgins, V. J. (1978) *Phytopathology* **68**, 338.
167. Lyon, F. M. and Wood, R. K. S. (1975) *Physiol. Plant Pathol.* **6**, 117.
168. Pankhurst, C. E. and Biggs, D. R. (1980) *Can. J. Microbiol.* **26**, 542.
169. Wyman, J. G. and VanEtten, H. D. (1978) *Phytopathology* **68**, 583.
170. Glasby, J. S. (1976) *Encyclopaedia of Antibiotics*. Wiley, London.
171. Gnanamanickam, S. S. and Mansfield, J. W. (1981) *Phytochemistry* **20**, 997.
172. Gnanamanickam, S. S. and Smith, D. A. (1980) *Phytopathology* **70**, 894.
173. Govindarajan, G. and Gnanamanickam, S. S. (1981) *Curr. Sci.* **50**, 1077.
174. Kaplan, D. T., Keen, N. T. and Thomason, I. J. (1980) *Physiol. Plant Pathol.* **16**, 309.
175. Kaplan, D. T., Keen, N. T. and Thomason, I. J. (1980) *Physiol. Plant Pathol.* **16**, 319.
176. Skipp, R. A., Selby, C. and Bailey, J. A. (1977) *Physiol. Plant Pathol.* **10**, 221.
177. Glazener, J. A. and VanEtten, H. D. (1978) *Phytopathology* **68**, 111.
178. Bailey, J. A. (1970) *J. Gen. Microbiol.* **61**, 409.
179. Hargreaves, J. A. (1980) *Physiol. Plant Pathol.* **16**, 351.
180. Chang, C., Suzuki, A., Kumai, S. and Tamura, S. (1969) *Agric. Biol. Chem.* **33**, 398.
181. Mansfield, J. W. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 253. Blackie, Glasgow.
182. Bailey, J. A., Vincent, G. G. and Burden, R. S. (1976) *Physiol. Plant Pathol.* **8**, 35.
183. Kuć, J. and Currier, W. (1976) in *Mycotoxins and Other Fungal Related Food Problems* (Rodricks, J. R., ed.) p. 356. Am. Chem. Soc., Washington.
184. Loper, G. M., Hanson, C. H. and Graham, J. H. (1967) *Crop Sci.* **7**, 189.
185. Oku, H., Ouchi, S., Shiraishi, T., Utsumi, K. and Seno, S. (1976) *Proc. J. Acad.* **52**, 33.
186. Bailey, J. A. and Skipp, R. A. (1978) *Ann. Appl. Biol.* **89**, 354.
187. Siegel, M. R. (1975) in *Pesticide Selectivity* (Street, J. C., ed.) p. 21. Dekker, New York.
188. Haslam, E. and Lilley, T. H. (1986) in *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure–Activity Relationships* (Middleton, E., Jr., Cody, V. and Harborne, J. B., eds). Alan Liss, New York (in press).
189. Delp, C. J. (1980) *Plant Disease* **64**, 652.
190. Weinstein, L. I. and Albersheim, P. (1983) *Plant Physiol.* **72**, 557.
191. Ingham, J. L., Keen, N. T. and Hymowitz, T. (1977) *Phytochemistry* **16**, 1943.
192. Smith, D. A. (1978) *Phytopathology* **68**, 81.
193. Harborne, J. B. and Ingham, J. L. (1978) in *Biochemical Aspects of Plant and Animal Coevolution* (Harborne, J. B., ed.) p. 343. Academic Press, London.
194. Boydston, R., Paxton, J. D. and Koeppe, D. E. (1983) *Plant Physiol.* **72**, 151.
195. Perrin, D. R. and Cruickshank, I. A. M. (1969) *Phytochemistry* **8**, 971.
196. VanEtten, H. D. (1976) *Phytochemistry* **15**, 655.
197. Kramer, R. P., Hindorf, H., Jha, H. C., Kallage, J. and Zilliken, F. (1984) *Phytochemistry* **23**, 2203.
198. Johnson, G., Maag, D. D., Johnson, D. K. and Thomas, R. D. (1976) *Physiol. Plant Pathol.* **8**, 225.
199. Bakker, J., Gommers, F. J., Smits, L., Fuchs, A. and de Vries, F. W. (1983) *Photochem. Photobiol.* **38**, 323.
200. Gordon, M. A., Lapa, E. W., Fitter, M. S. and Lindsay, M. (1980) *Antimicrob. Agric. Chemother.* **17**, 120.
201. Dekker, J. (1977) in *Systemic Fungicides, 2nd Edition* (Marsh, R. W., ed.) p. 176. Longman, London.
202. Dekker, J. (1977) in *Proceedings of the British Crop Protection Conference*, p. 689. British Crop Protection Council.
203. Denny, T. P. and VanEtten, H. D. (1983) *J. Gen. Microbiol.* **129**, 2893.
204. Denny, T. P. and VanEtten H. D. (1982) *J. Gen. Microbiol.* **129**, 2903.
205. Matthews, D. E. and VanEtten, H. D. (1983) *Arch. Biochem. Biophys.* **224**, 494.
206. Tegtmeier, K. J. and VanEtten, H. D. (1982) *Phytopathology* **72**, 604.
207. Tegtmeier, K. J. and VanEtten, H. D. (1982) *Phytopathology* **72**, 608.
208. VanEtten, H. D. (1979) in *Recognition and Specificity in Plant Host–Parasite Interactions* (Daly, J. M. and Uritani, I., eds) p. 301. Jpn. Scientific Societies Press, Tokyo, and University Park Press, Baltimore.
209. Kuhn, P. J. and Smith, D. A. (1979) *Physiol. Plant Pathol.* **14**, 179.
210. Kuhn, P. J., Smith, D. A. and Ewing, D. F. (1977) *Phytochemistry* **16**, 296.
211. Smith, D. A., Wheeler, H. E., Banks, S. W. and Cleveland, T. E. (1984) *Physiol. Plant Pathol.* **25**, 135.
212. Cleveland, T. E. and Smith, D. A. (1983) *Physiol. Plant Pathol.* **22**, 129.
213. Smith, D. A., Harrer, J. M. and Cleveland, T. E. (1982) *Phytopathology* **72**, 1319.
214. Fuchs, A., de Vries, F. W. and Platero Sanz, M. (1980) *Physiol. Plant Pathol.* **16**, 119.
215. MacFoy, C. A. and Smith, I. M. (1978) *Physiol. Plant Pathol.* **14**, 99.
216. Smith, D. A., Harrer, J. M. and Cleveland, T. E. (1981) *Phytopathology* **71**, 1212.
217. Zhang, Y. and Smith, D. A. (1983) *Physiol. Plant Pathol.* **23**, 89.