Phytochemistry, Vol. 27, No. 1, pp. 133–150, 1988.
Printed in Great Britain.

0031–9422/88 $3.00 + 0.00

BIOSYNTHESIS AND METABOLISM OF SESQUITERPENOID PHYTOALEXINS AND TRITERPENOIDS IN POTATO CELL SUSPENSION CULTURES

PHILIP A. BRINDLE*, PAUL J. KUHN† and DAVID R. THRELFALL

Department of Plant Biology and Genetics, University of Hull, Hull HU6 7RX, U.K.; †Shell Biosciences Laboratory, Sittingbourne Research Centre, Sittingbourne, ME9 8AG, U.K.

(*Revised received* 15 *October* 1986)

Key Word Index—*Solanum tuberosum*; Solanaceae; potato; tuber discs; cell suspension cultures; *Phytophthora infestans*; biosynthesis; metabolism; sesquiterpenoids; triterpenoids; phytoalexins; sterols.

Abstract—The accumulation and turnover of sesquiterpenoid phytoalexins and the effects of the induction of phytoalexin biosynthesis on triterpenoid synthesis have been studied in potato tuber tissue discs and cell suspension cultures inoculated with sporangia of either an incompatible (Race 4) or compatible (Complex) race of *Phytophthora infestans*. A comparative study of the incompatible and compatible interaction using Kennebec (R_1) tuber tissue showed the accumulation of rishitin (major component) and lubimin in both interations, though the patterns of accumulation and the observed fungal development were different. Rishitin turnover in both interactions was demonstrated by administration early in the time course of a small dose of [2-^{14}C]mevalonate though no accumulation of a rishitin metabolite was apparent. The assay of the incorporation over a series of short time periods (2 hr) of either [2-^{14}C]MVA or [2-^{14}C]acetate into squalene and sterols suggested that triterpenoid synthesis by tuber discs (*P. infestans* is unable to synthesize sterols) was inhibited during the period of phytoalexin accumulation.

A similar study with Kennebec-cell suspension cultures showed the accumulation of rishitin, mostly in the growth medium, when the cultures were inoculated with sporangia of *P. infestans*. Unlike the tuber discs, however, the patterns of accumulation of rishitin were identical with both races of fungus and the zoospores liberated from the sporangia did not germinate and infect the potato cells. A marked loss of rishitin from the inoculated culture occurred after 24 hr, but, as with tuber discs, this loss took place without the accumulation of any rishitin metabolite. As judged by the incorporation over short time periods (2 hr) of [2-^{14}C]MVA into squalene, sterols and phytoalexins, triterpenoid synthesis was markedly reduced just prior to the onset of phytoalexin accumulation. Potato cv. Majestic (r)-cell suspension cultures inoculated with sporangia of *P. infestans* Complex race accumulated lubimin, 3-hydroxylubimin, rishitin, phytuberol and phytuberin in the growth medium. The patterns of accumulation of phytoalexins in the presence or absence of a saturating concentration for sterol synthesis of either [2-^{14}C]MVA (3.3 mM) or [2-^{14}C]acetate (1 mM) were in agreement with the partial biosynthetic sequence lubimin → 3-hydroxylubimin → rishitin. Triterpenoid synthesis, as measured by the incorporation of the 3.3 mM [2-^{14}C]MVA and 1 mM [2-^{14}C]acetate, ceased very abruptly just prior to the start of phytoalexin accumulation. The reason for this appeared to be due to the inhibition or loss of squalene synthetase activity. The presence of 3.3 mM [2-^{14}C]MVA in the cultures resulted in a large increase in the levels of squalene, squalene 2,3-oxide and cycloartenol in the cells of healthy cultures and of phytoalexins accumulated in the growth medium of the inoculated cultures. This apparent lack of any regulatory control of the incorporation of MVA into the three triterpenoids and into phytoalexins is presumably the principal reason for the need to bring about the rapid and complete inhibition of squalene synthetase activity in the cells of inoculated cultures and, by implication, in the cells responsible for the synthesis of phytoalexins in infected tuber discs.

INTRODUCTION

The carbocyclic sesquiterpenoid phytoalexins rishitin (**1**) and lubimin (**2**) are synthesized *de novo* and accumulate in the upper cell layers of tissue discs of *Solanum tuberosum* L. (potato) cv. Kennebec tubers which have been aged and the upper cell surfaces inoculated with zoospores of either an incompatible (Race 4) or an appropriate compatible (e.g. Complex race) race of the fungus *Phytophthora infestans* (Mont.) de Bary [1–5] or with biotic elicitors (e.g. cell-free sonicate, arachidonic acid etc.) prepared from the fungus [see 6].

The phytoalexin content of a treated tuber disc at any given time is probably determined by the availability of farnesyl diphosphate (FPP) in the cells responsible for phytoalexin biosynthesis and the balance between the activities of the enzymes involved in the biosynthesis of phytoalexins from FPP, and those involved in their catabolism. The finding that concomitant with the induction of phytoalexin accumulation steroid glycoalkaloid (solanine and chaconine) accumulation by the discs is inhibited [2, 7–9], led to the suggestion that steroid glycoalkaloid accumulation, which along with an increase in the sterol content of aged potato tuber discs [10] is part

*Present address: Zoecon Corporation, 975 California Avenue, Palo Alto, CA 94304, U.S.A.

Abbreviations: FPP, *trans,trans*-farnesyl diphosphate; MVA, mevalonic acid; SDW, sterile distilled water; dimethyl-, monomethyl and demethyl-sterols, 4,4-dimethyl-, 4α-monomethyl- and 4-demethyl-sterols respectively.

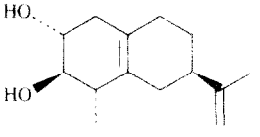
of the ageing process (wounding reaction), is inhibited due to the diversion of FPP away from steroid glycoalkaloid (and by implication sterol and steryl glycoside) biosynthesis and into phytoalexin biosynthesis [7–9], possibly as a result of loss of squalene synthetase activity [9]. This quantitatively attractive [see data in refs 7, 9], although somewhat simplistic interpretation, is open to criticism on the grounds that both the common and divergent parts of the biosynthetic pathways for the synthesis of sesquiterpenoid terpenoids and steroid glycoalkaloids may be under separate and independent controls, i.e. alkaloid accumulation is a function of wound periderm formation and is unrelated to phytoalexin formation [11]. In support of this criticism a recent study has shown that the HMG-CoA reductase activity of potato discs is increased markedly on ageing and undergoes another marked increase on infection with *P. infestans* [12]. Commensurate with synthesis *de novo* of the reductase both increases are inhibited by blasticidin S.

There is little known about the regulation and properties of the enzymes involved in the biosynthesis and metabolism of phytoalexins in potato tissues apart from the changes in HMG-CoA reductase activity in infected discs [12]. There is some evidence, albeit indirect, that the carbocyclase responsible for the formation from FPP of the first intermediate (germacrene A) on the post-FPP of the biosynthetic pathway is synthesized *de novo* in response to infection, to inoculation with biotic elicitors or to the presence of later intermediates on the pathway. First, only very small amounts of rishitin are synthesized from ^{14}C-labelled acetate in untreated tissues [13]. Second, the capacity of cell-free extracts of potato tuber tissue inoculated with sporangia of *P. infestans* or biotic elicitors to synthesize lubimin from isopentenyl diphosphate is not observed until some three hours after inoculation and, in parallel with the accumulation of phytoalexins in tuber tissue, rises to a maximum some 48–96 hr after treatment, declining thereafter [5, 14]. Third, the accumulation of the vetaspiranes (**2**–**4**) implicated in the biosynthesis of rishitin is induced following incubation of tuber tissues or cell-suspension cultures with solavetivone (**3**) or rishitin (**1**) [15–17]. Fourth, the enzyme (casbene synthetase) responsible for the conversion of geranylgeranyl diphosphate to the diterpenoid phytoalexin casbene in castor bean seedlings (*Ricinus communis* L.) is synthesized *de novo* in response to infection of the seedlings with *Rhizopus stolonifer* [18].

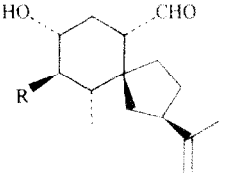
It is not known if the conversions of exogenous solavetivone, (**3**), lubimin (**2**) and 3-hydroxylubimin (**4**) (three putative precursors of rishitin) into rishitin (**1**) and the metabolism of rishitin to 13-hydroxyrishitin and 11, 12-dihydro-13-hydroxyrishitin in untreated tuber tissue and healthy potato cell-suspension cultures [15, 17, 19–25] are due to the synthesis *de novo* of the appropriate enzymes, enzyme activation, the presence of active enzymes or a combination of these three possibilities.

In order to study the biosynthesis and metabolism of potato phytoalexins and the interrelationship of phytoalexin biosynthesis with triterpenoid (sterols, steryl glycosides and pentacyclic triterpenes) biosynthesis in the absence of the wounding reaction, we have turned our attention to the use of potato-cell suspension cultures. The suitability of suspension cultures for such studies was provided by the demonstration that potato-cell suspension cultures [ex Kennebec (R_1) tuber tissue] accumulate the sesquiterpenoid phytoalexins lubimin, rishitin and solavetivone after inoculation with sporangia of either a compatible (Complex race) or incompatible (Race 4) race of *P. infestans* [4].

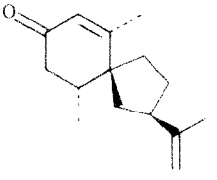
In this paper, we report on (i) the turnover of rishitin and the apparent inhibition of triterpenoid synthesis in potato [cv Kennebec (R_1)] tuber discs infected with *P. infestans*, (ii) the time courses and patterns of phytoalexin accumulation of potato-cell suspension cultures [ex Kennebec (R_1) and Majestic (r)] inoculated with sporangia of *P. infestans* and (iii) the inhibition of triterpenoid biosynthesis in fungal-inoculated suspension cultures.



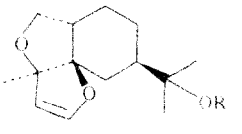
1



2 R = H
4 R = OH



3



5 R = Ac
6 R = H

RESULTS

The experiments reported in this paper were not replicated since (i) they were performed in such a way as to minimize biological variation within an experiment and (ii) it was expected that the magnitudes of the more important changes would be so much greater than those due to biological and analytical variations that replication was unwarranted.

The single-TLC/X-ray autoradiographic procedure used to determine the radioactive contents of the phytoalexins in the various extracts is an obvious target for criticism. It has, however, been shown to give accurate and reproducible values because the radioactive areas on the plate are located with great precision and the radioactive phytoalexins are well separated from each other and from other radioactive materials in the extracts.

Experiments with potato tuber discs

The phytoalexins rishitin (**1**) and lubimin (**2**) were accumulated in the top mm of tissue of aged (24 hr), tuber discs the upper surfaces of which had been inoculated with sporangial suspensions of either *P. infestans* Race 4 (incompatible race) or *P. infestans* Complex race (compatible race) and then 30 hr later administered a single, small dose of high specific activity (*R*, *S*)-[2-^{14}C] MVA (Fig. 1). In both interactions rishitin was accumulated as the major phytoalexin. No other phytoalexins were detected in the interactions and no phytoalexins were detectable in the SDW-controls either by GC or, in the radiochemical parts of the experiment, by TLC/X-ray autoradiography.

The amount of radioactivity associated with the rishitin following the administration of (*R*, *S*)-[2-^{14}C]MVA rose rapidly to a maximum and then declined, even though the accumulation of rishitin continued unabated throughout most of the incubation period in the incompatible reaction (Fig. 1). The maximum amount of radioactivity incorporated into rishitin represented 13.4% of the biologically active isomer (3*R*) of the [2-^{14}C]MVA in the case of the incompatible reaction and 8.2% in the compatible reaction. The patterns of incorporation of radioactivity into lubimin were less dramatic, with only up to 0.9% of the utilisable isomer of the [2-^{14}C]MVA being incorporated into this phytoalexin.

The incorporation of radioactivity from either [2-^{14}C]acetate or (*R*, *S*)-[2-^{14}C]MVA into sterols plus squalene as measured over a two hr incubation period was increased markedly on ageing of freshly cut tuber discs (Fig. 2). In aged, infected discs, however, it was very much reduced. As expected, there was a significant incorporation of radioactivity into the phytoalexins rishitin and lubimin in the infected discs. There was also some radiochemical evidence for the synthesis of small amounts of phytoalexins in the uninfected discs.

The radioactivity in the unsaponifiable fractions from which the squalene and sterols were isolated was distributed between squalene (2–13%, MVA; 1–7%, acetate), dimethyl sterols (55–78%, MVA, 40–81%, acetate), monomethyl sterols (11–22%, MVA; 14–36%, acetate) and demethyl sterols (3–20%, MVA; 3–22%, acetate), the dimethyl sterols accounting for the greatest proportion of the radioactivity.

The proportion of the radioactivity associated with the monomethyl sterols and demethyl sterols rose from 10 and 3% respectively in the case of fresh discs to some 20 and

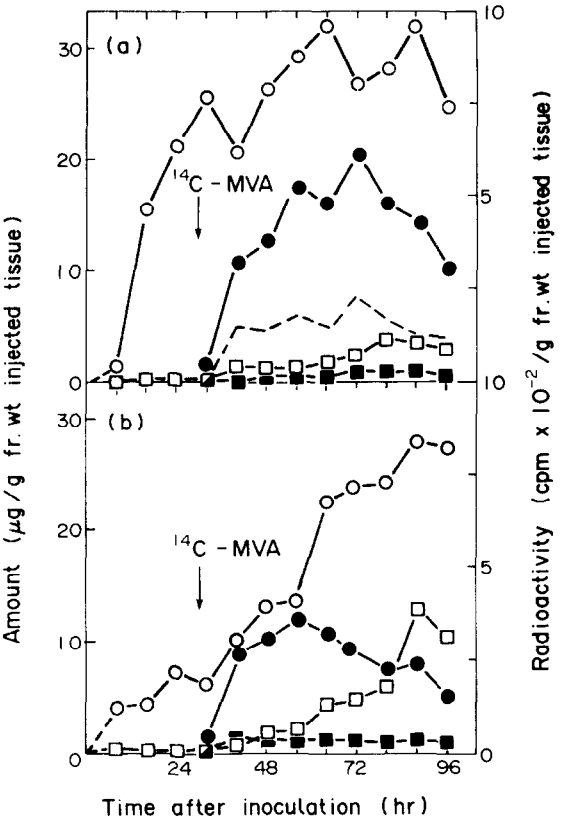


Fig. 1. Accumulation (open symbols) of, and incorporation of radioactivity (closed symbols) from a single dose of (*R*, *S*)-[2-^{14}C]MVA (53 mCi/mmol; 0.11 μCi/5 discs) into, rishitin (○, ●) and lubimin (□, ■) in aged tuber (cv. Kennebec) discs inoculated with SDW, *P. infestans* Race 4 (Upper graph) or *P. infestans* Complex race (Lower graph) [---, cpm × 10^{-1}/μg rishitin]. The top mm from five discs (*ca* 2.2 g fr. wt) was taken for analysis. No Phytoalexins were detectable by GC or TLC/X-ray autoradiography in the SDW-inoculated control.

30% in discs which had been aged for 72 hr. The incorporation of radioactivity into squalene in aged, infected tissue was always 50–90% lower than that observed in freshly cut discs, in discs aged for 24 hr and in aged discs treated with SDW. The amounts of individual sterols were not measured in these experiments, although the sterol composition of Kennebec tuber tissues was known to be as shown in Table 1.

Examination by light microscope of sectioned and stained tuber tissue six days after infection revealed that in the compatible interaction the Complex race of the fungus had grown through the 5 mm thick disc whereas in the incompatible reaction the intercellular hyphae of Race 4 had not penetrated beyond the first mm.

In the final experiment with tuber discs, the effect of a pulse of unlabelled MVA on the incorporation of radioactivity from (*R*, *S*)-[2-^{14}C]MVA into phytoalexins by tuber tissue which had been aged for 24 hr prior to inoculation with a mycelial sonicate was examined (Table 2).

Experiments with cell suspension cultures

These were performed with dark-grown cell suspension cultures. However, although the cultures were maintained

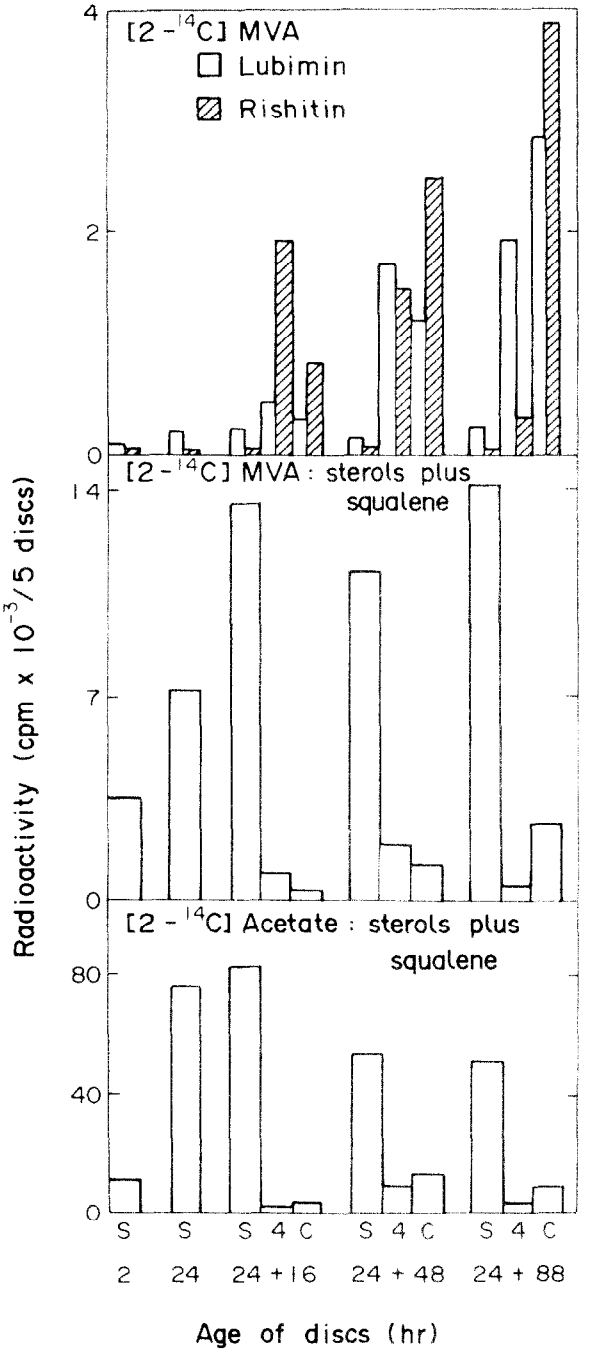


Fig. 2. Short-term (2 hr) incorporation of radioactivity from either (*R, S*)-[2-^{14}C]MVA (53 mCi/mmol; 0.22 μCi/5 discs) or [2-^{14}C]acetate (58 mCi/mmol; 1.5 μCi/5 discs) into phytoalexins and squalene plus sterols in freshly cut tuber (cv. Kennebec) discs, discs aged for 22 hr prior to administration of the radiosubstrate and discs aged for 24 hr inoculated with SDW, *P. infestans* Race 4 or *P. infestans* Complex race and then incubated for a further 14, 46 and 86 hr prior to administration of the radiosubstrate. The times shown are those at which the discs were taken for analysis i.e. they include the two hr incubation period in the presence of the radiosubstrate. S, SDW-treated discs, C, discs inoculated with *P. infestans* Complex race; 4, discs inoculated with *P. infestans* Race 4. The top mm from five discs (*ca* 2.2 g fr. wt) was taken for each analysis. The amounts of lubimin and rishitin in the tissues infected for 16, 48 and 88 hr were similar to those shown in Fig. 1.

Table 1. Major sterol composition of Kennebec tuber tissue

	Sterols	Amount (mg kg fr. wt)
4-Demethyl		
	Cholesterol	0.12
	Campesterol	0.05
	Stigmasterol	0.47
	Sitosterol	0.43
	Isofucosterol	2.67
4α-Methyl		
	Obtusifoliol	0.08
4,4-Dimethyl		
	Cycloartenol	0.26

in the dark and the experimental systems were incubated in the dark, no special precautions were taken to exclude light from the cultures when the experiments were set up.

The cultures were white in appearance and were composed mainly of aggregates each made up of some 15–20 cells. In the case of the Kennebec (R_1) cultures, the lag phase of growth was *ca* three days and the logarithmic period was *ca* six days. The corresponding periods for the Majestic cultures were much shorter.

On inoculation of the white cultures with sporangia of *P. infestans* a noticeable browning of the suspension became visible at two hr. Thereafter browning became more intense and was maximal by 8–12 hr. At this time, the harvested cells had an orange-brown colouration. Examination of the cells with a light microscope showed that even after 24 hr only a small number of zoospores had germinated and there was no apparent infection of potato cells by zoospores of either race of *P. infestans* (race 4 or Complex race). There was also no evidence of lysis of the potato cells, an observation which was borne out by the fresh weight yields of cells from infected cultures.

The phytoalexins were accumulated mostly (*ca* 90% of the total amounts present in the culture) in the culture medium, facilitating their extraction in a pure form; indeed, they accounted for most of the weight and, in radiochemical experiments, all of the radioactivity in the ether extracts of the medium.

Experiments with Kennebec cultures

These were similar in design to those parts of the first two experiments carried out with aged tuber discs (Figs 1 and 2). The most important modification made to the experimental procedure was that the short-term (two hr) incorporations of radioactivity from (*R, S*)-[2-^{14}C]MVA into phytoalexins and into sterols plus squalene were measured throughout the phases of phytoalexin induction and accumulation.

Rishitin (**1**) was the only phytoalexin accumulated in the culture filtrates of the cultures inoculated with sporangia of *P. infestans* (Fig. 3) though the presence of lubimin (**2**) and 3-hydroxylubimin (**4**) in the ether extracts of the culture filtrates was indicated by TLC/X-ray autoradiography (Fig. 4). No phytoalexins were detectable by GC in

Table 2. Accumulation of, and incorporation of radioactivity into, rishitin in aged tuber (cv Kennebec) tissue inoculated with an autoclaved mycelial sonicate of *P. infestans* followed by a pulse of (*R*, *S*)-[2-^{14}C]MVA (55 mCi/mmol; 0.44 μCi/5 discs) and a chase of (*R*, *S*)-MVA (1000-fold excess)

Time after inoculation (hr)	Rishitin (μg/g fr. wt)		Rishitin (10^{-3} cpm/g fr. wt)	
	Unchased	Chased	Unchased	Chased
6*	0	—	0	—
12†	11	—	37	—
18	10	18	61	50
24	13	30	95	83
36	15	27	120	87

The top mm from five discs was taken for each analysis.
*Pulse added.
†Chase added.

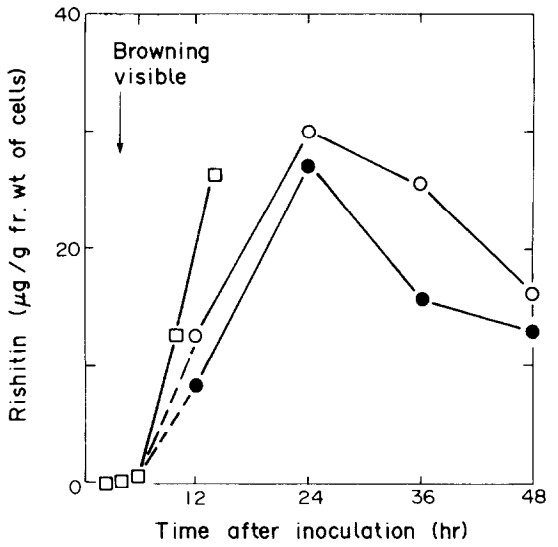


Fig. 3. Accumulation of rishitin in the culture medium of Kennebec-cell suspension cultures inoculated with sporangia of either *P. infestans* race 4 (●) or *P. infestans* Complex race (○, □). The cultures used for the long term experiment (○, ●) were from the same transfer and were each inoculated with (*R*, *S*)-[2-^{14}C]MVA (55 mCi/mmol; 1.2 μCi/60 ml) eight hr after inoculation with sporangia. The cultures used for the short term experiment (□) (Fig. 4) were each inoculated with (*R*, *S*)-[2-^{14}C]MVA (55 mCi/mmol; 1.1 μCi/45 ml) two hr prior to analysis. The cultures used in the long term experiment contained *ca* 25 g fresh weight of cells/100 ml of culture. Those used in the short term contained *ca* 22 g fresh weight of cells/100 ml of culture.

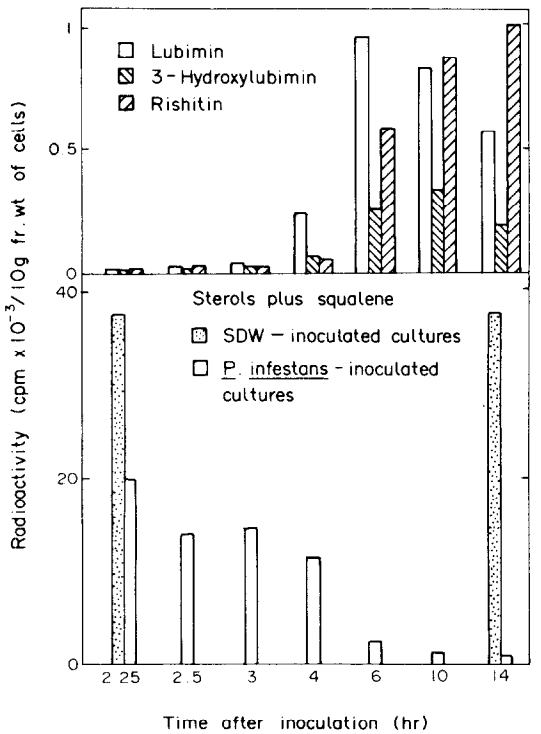


Fig. 4. Short-term (two hr) incorporation of radioactivity from (*R*, *S*)-[2-^{14}C]MVA (55 mCi/mmol; 1.1 μCi/45 ml) into phytoalexins in the medium and squalene plus sterols in the cells of Kennebec-cell suspension cultures inoculated with *P. infestans* Complex race. The radiosubstrate was administered two hours prior to harvest of the cultures at the times shown. The radioactivity associated with the three phytoalexins in the 2.25 and 14 hr SDW-inoculated cultures was similar to that in the 2.25 hr *P. infestans*-inoculated culture. The amount of rishitin present at each time point is shown in Fig. 3.

the ether extracts of the culture filtrates of the SDW-inoculated cultures. However, small amounts of radioactivity in the extracts co-chromatographed on TLC with standards of rishitin, lubimin and 3-hydroxylubimin. The cells from the fungal-inoculated cultures contained small amounts of ^{14}C-labelled rishitin and negligible amounts of ^{14}C-activity which co-chromatographed on TLC with standards of lubimin and 3-hydroxylubimin.

The specific activities of the the rishitin samples isolated from cultures inoculated with sporangia of *P. infestans*

and eight hr later administered a single dose of (*R*, *S*)-[2-^{14}C]MVA (Fig. 3) were effectively doubled between 12 and 24 hr and then remained essentially constant (average 188 dpm/μg rishitin) for the remainder of the experiment. Despite the marked decline in the amounts of rishitin in the culture filtrates in the second half of the experiment no radiochemical or GC evidence was found for the presence of either ether-soluble metabolites of rishitin or glycosides with ether-soluble aglycones derived from rishitin in the culture filtrates.

The incorporation of radioactivity from (*R*, *S*)-[2-^{14}C]MVA into phytoalexins in the culture filtrate as measured over a two hour period rose rapidly between three and six hours in the fungal-inoculated cultures whereas that measured for the sterols plus squalene in the unsaponifiable lipids of the cells fell rapidly over the first 2.5 hr of the experiment (Fig. 4).

In the control incubations, the percent distribution of radioactivity between squalene, dimethyl sterols, monomethyl sterols and demethyl sterols respectively was 25, 58, 12 and 5% at the end of the first incubation period and 27, 40, 13 and 20% at the end of the second. The percentage of the radioactivity associated with squalene in the cells of the fungal-infected cultures fell within the range 27–38%.

Experiments with Majestic cultures

In these experiments the time courses of the accumulation of, and incorporation of radioactivity into, phytoal-

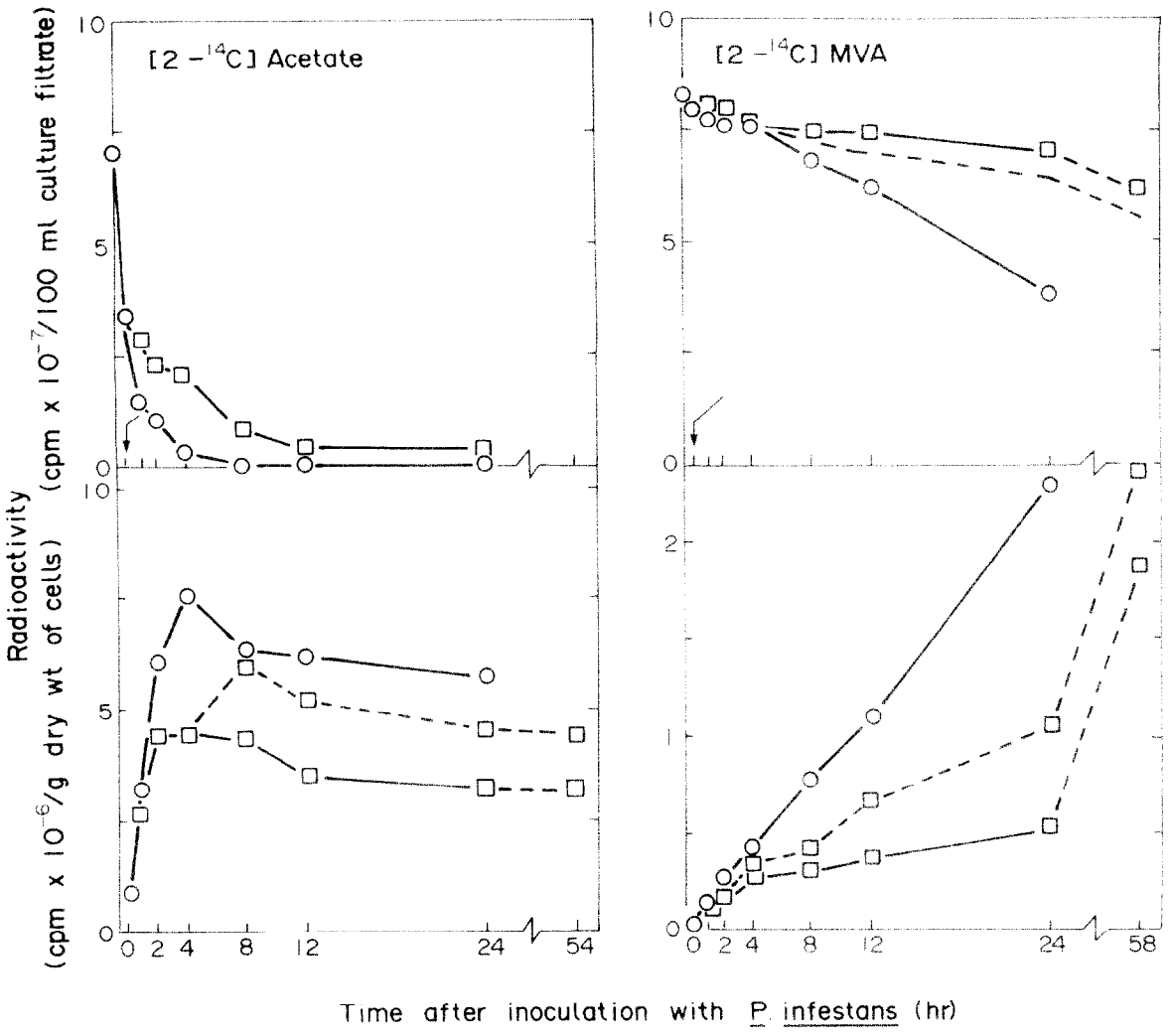


Fig. 5. Disappearance of radioactivity from the culture medium and its incorporation into phytoalexins in the medium and unsaponifiable lipids in the cells of Majestic-cell suspension cultures administered a single dose of either (*R*)-[2-^{14}C]MVA (13 μC/mol; 3.3 mM) or [2-^{14}C]acetate (0.75 mCi/mol; 1 mM) at a saturating concentration for sterol synthesis and then inoculated with sporangia of *P. infestans* Complex race or SDW. Top graphs: ○—○, ^{14}C in the medium in SDW-inoculated cultures; □—□, ^{14}C in the medium in *P. infestans*-inoculated cultures; ---, corrected for the presence of phytoalexins in the medium. Bottom graphs: ○—○, ^{14}C in unsaponifiable lipids of cells in SDW-inoculated cultures; □—□, ^{14}C in unsaponifiable lipids of cells in *P. infestans*-inoculated cultures; □---□, ^{14}C in unsaponifiable lipids (cells) and phytoalexins (medium) in *P. infestans*-inoculated cultures. At harvest the suspensions contained *ca* 25 g fresh weight (*ca* 1 g dry wt) of cells/100 ml of culture.

exins and triterpenoids were determined for suspension cultures which had been administered a single dose of either (*R*)-[2-^{14}C]MVA (plus unlabelled (*S*)-MVA) or [2-^{14}C]acetate at a saturating concentration for sterol synthesis and then, after 30 min, inoculated with SDW or a sporangial suspension of the Complex race of *P. infestans* (Figs 5–10). The substrate concentrations used were based upon data for the maximal rates of sterol synthesis in cell suspensions of sycamore (*Acer pseudoplatinus* L) in the presence of different substrates [26].

The radioactivity supplied as [2-^{14}C]acetate (1 mM) was rapidly lost from the culture medium of the cell suspension culture inoculated with SDW (Fig. 5). A similar, though less rapid, loss of radioactivity occurred in the case of the fungal-inoculated culture. In the incubations containing (*R*)-[2-^{14}C]MVA (3.3 mM), the radioactivity disappeared from the medium very gradually (Fig. 5).

The synthesis from [2-^{14}C]MVA of the constitutive terpenoids in the cells of the control cultures (as measured by the incorporation of radioactivity into the unsaponifiable lipids) occurred at a constant rate throughout the period of the experiment whereas, coincident with the onset of phytoalexin biosynthesis, the rate fell rapidly after about two hours in the fungal-inoculated cultures (Fig. 5). It is noteworthy that the fungal-inoculated cultures, however, recovered the ability to synthesize constitutive terpenoids between 24 and 54 hr. The results for the synthesis of the constitutive terpenoids from [2-^{14}C] acetate were not as clear cut due to the depletion of the pool of added acetate soon after the start of the experiment. Nevertheless, inhibition of constitutive terpenoid synthesis was discernible at the onset of phytoalexin biosynthesis (Fig. 5).

Lubimin (**2**), 3-hydroxylubimin (**4**) and rishitin (**1**) accumulated in the culture filtrates of the fungal-inoculated suspensions in both experiments (Fig. 6). In the presence of [2-^{14}C]MVA, however, phytuberin (**5**) and phytuberol (**6**) were also accumulated in the culture filtrates.

The administration of either [2-^{14}C]MVA or [2-^{14}C]acetate brought about a large increase in the final amounts of phytoalexins accumulated in the culture filtrates of the fungal-inoculated cultures (Fig. 6). In the presence of [2-^{14}C]MVA, the greater part of the increase had occurred by 12 hr and as shown by the specific activity data the added [2-^{14}C]MVA was acting as the major/sole source of carbon for the synthesis of phytoalexins (Table 3). In the presence of [2-^{14}C]acetate, the increase occurred much later when the [2-^{14}C]acetate, due to its depletion (Fig. 5), was not acting as a source of carbon for phytoalexin synthesis (c.f. Fig. 6 and Fig. 7).

The cells from the fungal-inoculated cultures contained relatively low levels of lubimin and rishitin, the patterns of accumulation of which corresponded to those observed in the culture filtrates (Fig. 6). The cells were not analysed for the presence of either 3-hydroxylubimin or phytuberin.

The sterol, steryl glycoside and pentacyclic triterpene composition of the unsaponifiable lipids of the potato cells after 30 min incubation with [2-^{14}C]MVA is given in Table 4. No evidence could be found for the presence of steroid glycoalkaloids in either the cells or the culture medium. The sterol composition was very similar to that determined for Kennebec tubers (Table 1). It is noteworthy that the percentage compositions of the monomethyl sterol and demethyl sterol and steryl glycoside fractions were unaffected by any of the four treatments, despite the very large changes in the amounts of each of the first two classes of sterols.

In the presence of [2-^{14}C]MVA, the amounts of squalene plus squalene 2,3-oxide (based on the radioactivity data) and the cycloartenol showed a 53- and 20-fold increase, respectively, over the 24 hr time period while the 24-methylene cycloartanol and monomethyl sterols each showed a more modest nine–ten-fold increase (Fig. 8). It was calculated that the squalene plus squalene 2,3-oxide content of the cells at 24 hr [9.5 (sp. act. = theoretical) − 11 (sp. act = cycloartenol) μmol/g dry wt] exceeded even that of the cycloartenol (8 μmol/g dry wt). The levels of demethyl sterols (Fig. 8), apart from a small increase over the first few hr, remained relatively unaffected as did those of the demethyl steryl glycosides and the pentacyclic triterpene lupeol (Fig. 8), apart from a small sustained increase throughout the course of the experiment. The specific radioactivities of the sterols reflected the availability of the substrate throughout the experiment, their positions on the sterol biosynthetic pathway and the observed increases in their pool sizes. The highest specific radioactivity values of the cycloartenol (66 μCi/mmol), uncorrected for the amount of cycloartenol present at the start of the experiment, were some 85% of the theoretical value (78 μCi/mmol) which would have been observed if the radiosubstrate (13 μCi/mmol) had acted as the sole carbon source. Although the specific radioactivities of the 24-methylene cycloartenol and lupeol were not measured, radio-GC showed that their radioactivity content increased with time in the expected manner (Fig. 9).

The synthesis of triterpenoids from [2-^{14}C]MVA was more or less completely inhibited at the level at squalene formation soon after inoculation of the cultures with sporangia (Fig. 8). The inhibition was strikingly obvious at four hr from the radioactivity content of the squalene plus squalene 2,3-oxide and from the mass and specific radioactivity data of the cycloartenol. Indicative of a lack of inhibition of sterol synthesis from squalene the radioactivity content of the squalene declined rapidly after four hr and the specific radioactivities of the four classes of sterols increased throughout the experiment. The lower levels of monomethyl sterols, dimethyl sterols and steryl glycosides accumulated in the inoculated cells were taken to be a reflection of the decreased pool size of the cycloartenol rather than to inhibition of the enzymes on the post-squalene part of the pathway. Radio-GC suggested that lupeol formation also was diminished in response to infection. As judged from the final time point, the fungal-inoculated cultures recovered some of their ability to synthesize squalene towards the end of the experiment.

In the presence of [2-^{14}C]acetate, the changes in the levels of sterols were not as large as those with [2-^{14}C]MVA due to the very rapid uptake and utilization of most of the radiosubstrate by the cells within the first two hours of the experiment (Fig. 8). Nevertheless, in the control culture there were slight increases in the levels of cycloartenol (*ca* 50%) and demethylsterols over the initial eight hr period, after which the former compound levelled off at *ca* 200 μg/g dry wt cells (Fig. 10); a level comparable to that observed at the first sampling point of the [2-^{14}C]MVA incubation. No such changes took place in the infected cultures. The specific radioactivity of the cycloartenol from the control cultures increased up to four hours and then, in keeping with the rapid loss of substrate

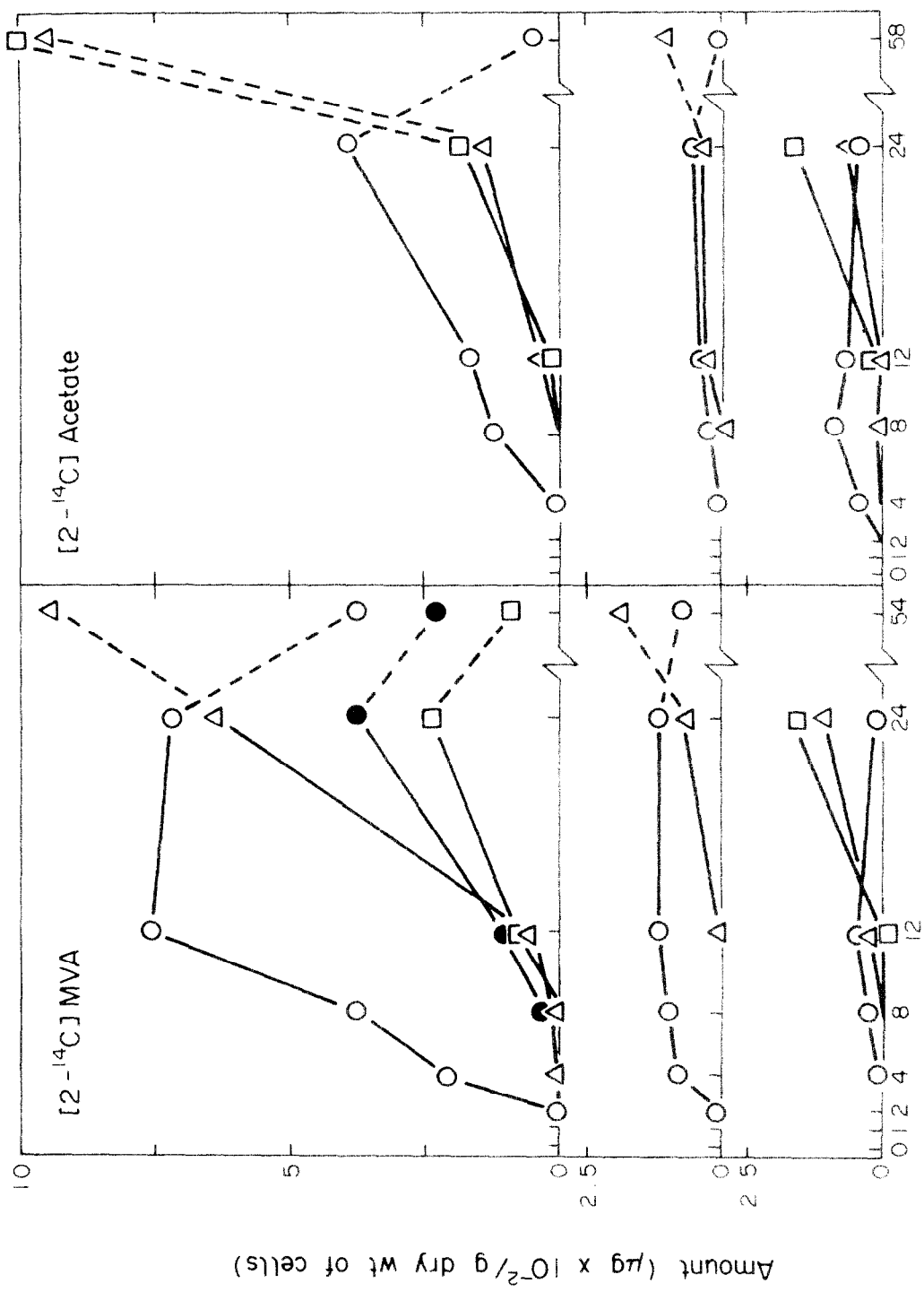


Fig. 6. Accumulation of lubimin (□), 3-hydroxylubimin (△), rishitin (○) and phytuberin (●) in Majestic-cell suspension cultures in the presence or absence of either 3.3 mM (*R*)-[2-^{14}C]MVA or 1 mM [2-^{14}C]acetate. Top and middle graphs: phytoalexins in the culture medium and cells respectively of *P. infestans*, ^{14}C-substrate-inoculated cultures. Bottom graphs: culture medium of *P. infestans*-inoculated controls. At harvest the suspensions contained *ca* 1 g dry weight/100 ml of culture.

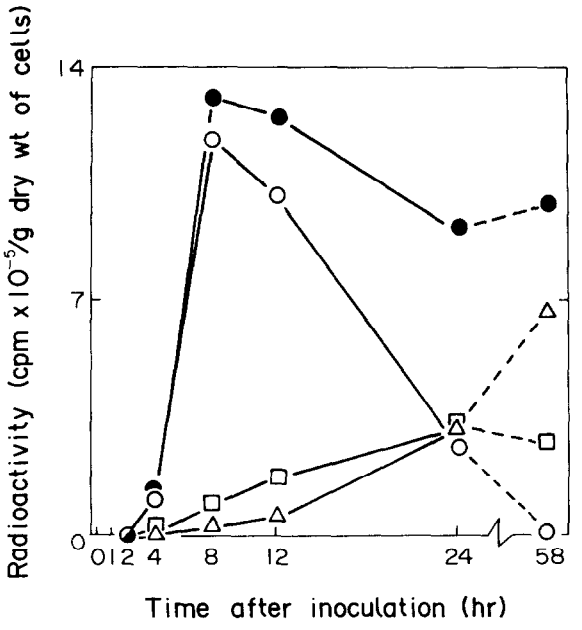


Fig. 7. Incorporation of radioactivity from 1 mM [2-^{14}C]acetate into phytoalexins accumulated in the culture medium of Kennebec-cell suspension cultures inoculated with *P. infestans*. ○—○, lubimin; □—□, 3-hydroxylubimin; △—△, rishitin; ●-●, sum of ○, □ and △.

from the culture medium, declined. The corresponding values from the fungal-inoculated cultures showed that the inhibition of the cycloartenol synthesis was detectable as early as two hr after inoculation and that there was some synthesis *de novo* of cycloartenol throughout the experiment.

DISCUSSION

It is confirmed that the sesquiterpenoids rishitin (**1**) (major phytoalexin) and lubimin (**2**) are accumulated in the top mm of aged potato tuber cv. Kennebec (R_1) discs when the upper surfaces of the discs are inoculated with sporangia of either an incompatible race (Race 4) or an appropriate compatible race (complex race) of the fungus *P. infestans* (Fig. 1), and that rishitin is accumulated in potato cv. Kennebec cell suspension cultures inoculated with sporangia of either one of the aforementioned strains of *P. infestans* (Fig. 3). In addition it is shown for the first time that lubimin, 3-hydroxylubimin (**4**), rishitin, and in the presence of a saturating concentration for sterol biosynthesis of (*R*)-MVA, phytuberin (**5**) and small amounts of phytuberol (**6**) are accumulated in potato cv. Majestic (r) cell suspension cultures inoculated with sporangia of the complex race of *P. infestans* (Race 4 not tested) (Fig. 6). The failure of the Kennebec cultures to accumulate solavetivone (**3**) and lubimin in response to inoculation with sporangia after the fifth transfer of the cells (c.f. [4] and this paper) and the gradual loss of the ability of both cell cultures to produce any phytoalexins in response to inoculation with sporangia show that potato cell suspension cultures are unstable with regard to phytoalexin production. Indeed, it is due to the loss of the ability of the Kennebec cultures to produce phytoalexins that the key experiments in this study are performed with the Majestic cultures.

The faster rate of rishitin accumulation in tuber discs inoculated with the incompatible race of the fungus compared to that in the discs inoculated with the compatible race (Fig. 1) is in keeping with the proposal that the rapid localized accumulation of rishitin by the host tissue is involved in the containment of the fungus [27]. The absence of a differential response in the cell suspension cultures inoculated with the different strains of the fungus (Fig. 3) is not too surprising since, unlike the situation in the fungus/tuber interaction, the fungal zoospores do not germinate and invade the potato cells.

The greater part (*ca* > 90%) of the phytoalexins in the suspension cultures is recovered from the culture filtrate. This use of the culture medium as a sink for phytoalexins is consistent with the view that in infected tuber discs phytoalexins are able to move out of the host cell in which they are synthesized and, in the case of the incompatible reaction, diffuse into necrotic cells [28].

It is clear from studies with non-terpenoid phytoalexin-producing systems that the amounts of phytoalexins present in infected tissues is the net product of their biosynthesis and catabolism, and that the rates of catabolism can be high even during the phase of active accumulation of phytoalexins [29]. The incorporation of radioactivity into, and its subsequent loss from, rishitin in tuber discs inoculated with *P. infestans* and then administered a single dose of high specific [2-^{14}C]MVA shows that there is turnover of rishitin in both interactions and that if the biosynthetic pools are in rapid equilibrium with the accumulated pools it is extremely high in the compatible reaction (Fig. 1). Somewhat surprisingly, the pulse chase experiment (Table 1) suggests that there is no turnover of rishitin in potato discs elicited with a fungal sonicate. The single dose [2-^{14}C]MVA experiment performed with fungal-inoculated Kennebec cell suspension cultures provides no evidence for turnover of rishitin during the accumulation phase, possibly because the supply of label never becomes exhausted (Fig. 4). If the loss of rishitin from fungal-elicited tuber discs (Fig. 1) and cell suspension cultures (Fig. 4) is due to enzymic rather than non-enzymic reactions then the appropriate activities are almost certainly associated with the potato cells since there is no evidence that *P. infestans* is able to metabolise rishitin [31]. Aged, healthy Kennebec tuber tissue and Kennebec cell suspension cultures are able to metabolise exogenous rishitin to 13-hydroxy-rishitin, 11,12-dihydro-13-hydroxyrishitin and glutinisone [22,23,32]. These compounds, however, are not detectable in fungal-inoculated discs or fungal-inoculated cultures despite the fact that there is a rapid loss of rishitin from the Kennebec cell suspension cultures (Fig. 4). The possibility that glycosylated forms of rishitin and/or ether-soluble metabolites of rishitin are accumulated in the culture medium is also eliminated because acidic and enzymic (emulsin) hydrolyses of the ether extracted medium fail to release any ^{14}C-labelled ether-soluble compounds.

The results of the short-term (2 hr) radiochemical incubations indicate that some biosynthesis *de novo* of very small amounts of phytoalexins takes place in freshly cut tubers, in aged tubers and in cell suspension cultures (Figs 2, 4). This is in keeping with the findings of a previous study [7], in which the capacity for rishitin synthesis was demonstrated in various parts of potato

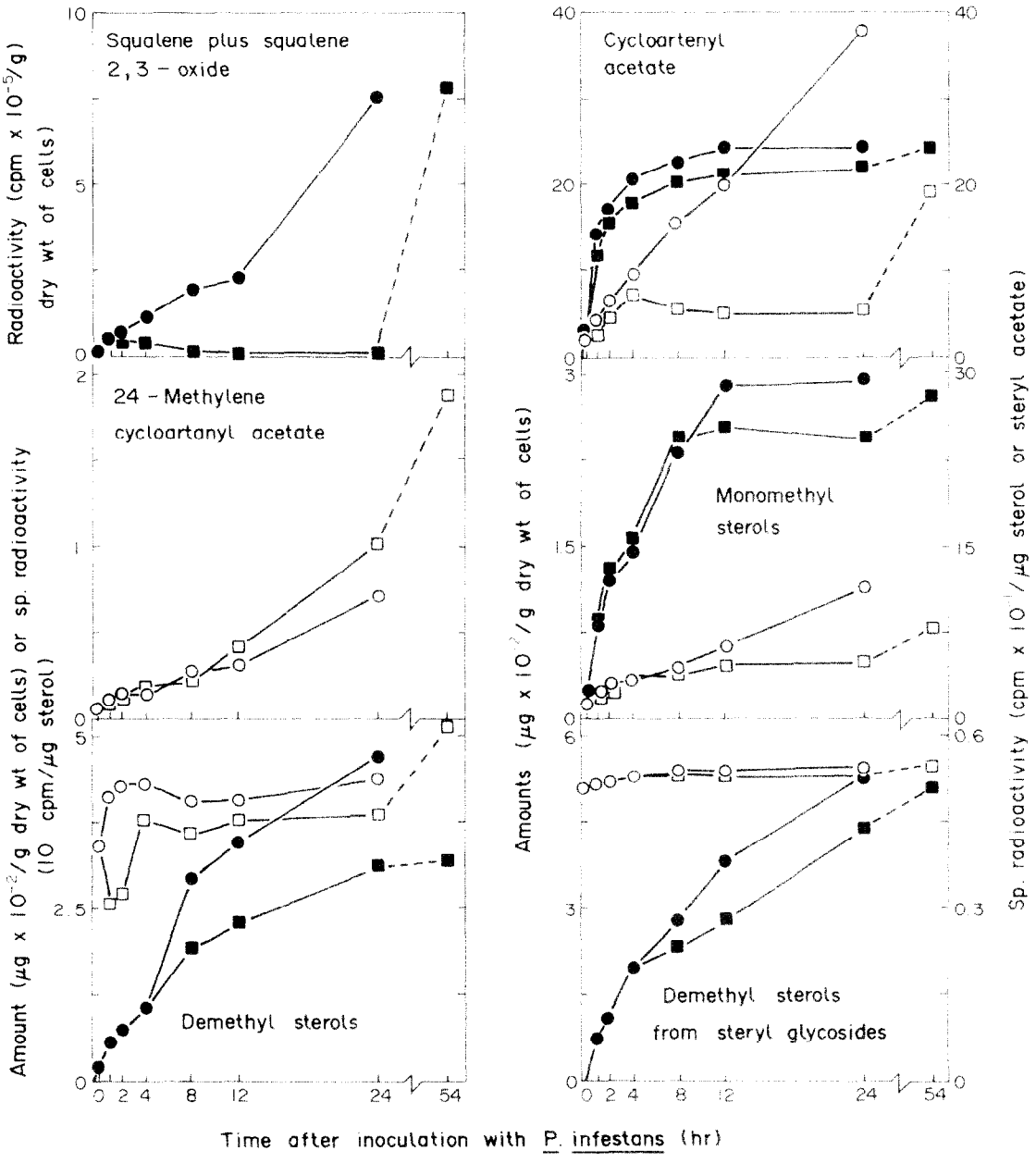


Fig. 8. Amounts (open symbols) and specific radioactivities (closed symbols) of triterpenoids isolated from the unsaponifiable lipid of the cells of Majestic-cell suspension cultures administered 3.3 mM (*R*)-[2-^{14}C]MVA and then inoculated with *P. infestans* (□, ■) or SDW (○, ●). Some symbols are omitted for the sake of clarity.

plants in response to slicing. In this context it is of interest that lubimin has been considered to be a normal metabolite which is subject to a high rate of turnover in healthy tissue [29]. The uninfected discs and uninoculated cell suspension cultures clearly have some capacity for phytoalexin turnover because no phytoalexins are detectable by GC in any of the SDW controls.

The sequential changes with time of the rates of incorporation of radioactivity from [2-^{14}C]MVA into lubimin, 3-hydroxylubimin and rishitin in fungal-inoculated Kennebec cell suspension cultures (Fig. 3) and of the masses of the same three phytoalexins in Majestic cell suspension cultures which have been co-inoculated with sporangia of *P. infestans* and a saturating concentration for sterol synthesis of either (*R*)-[2-^{14}C]MVA (3.3 mM) or [2-^{14}C]acetate (1 mM) (Fig. 6) are consistent with: (i) the operation of the biosynthetic sequence: (acetate→MVA→FPP→) lubimin (**2**)→3-hydroxylubimin (**4**)→rishitin (**1**) [15, 19, 20] and (ii) the sequential induction and/or activation of the post-FPP enzymes of this sequence. The most compelling evidence for the sequence **2**→**4**→**1** is provided by the quantitative transfer of ^{14}C from [2-^{14}C]acetate-labelled **2** to **4** and **1** in the Majestic cultures co-inoculated with *P. infestans* and 1 mM [2-^{14}C]acetate

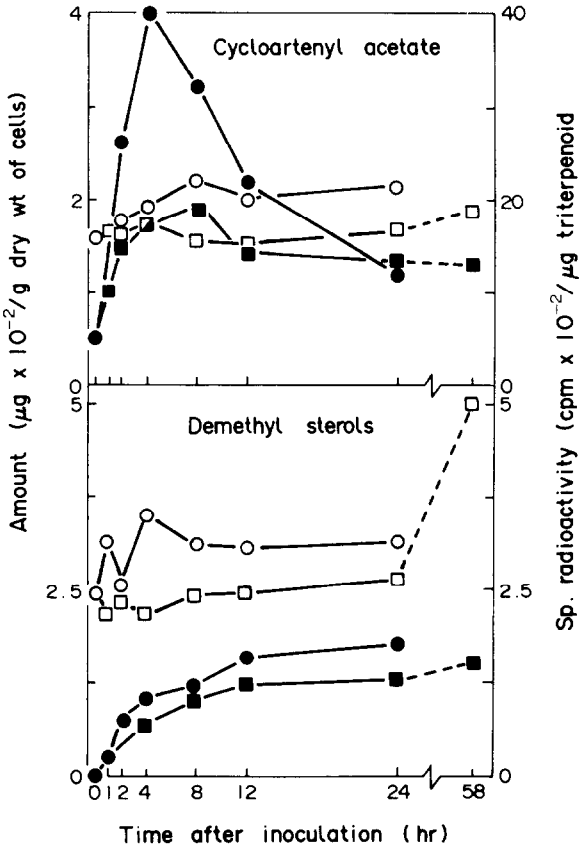


Fig. 9. Amounts (open symbols) and specific radioactivities (closed symbols) of cycloartenol and demethyl sterols isolated from the unsaponifiable lipid of the cells of Majestic-cell suspension cultures administered 1 mM [2-^{14}C] acetate and then inoculated with *P. infestans* (□, ■) or SDW (○, ●).

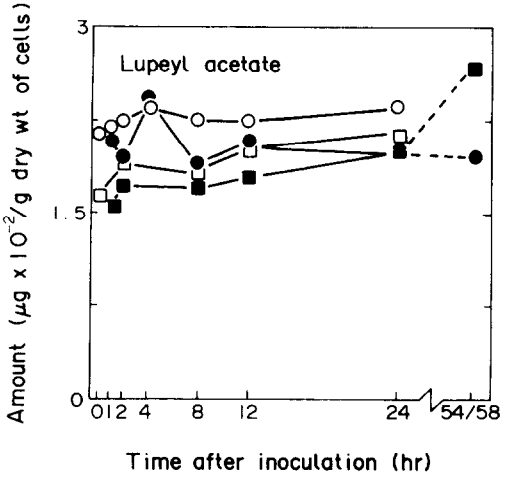


Fig. 10. Amounts of lupeol isolated from the unsaponifiable lipid of the cells of Majestic-cell suspension cultures administered either 3.3 mM (*R*)[2-^{14}C]MVA (○, ●) or 1 mM [2-^{14}C]acetate (□, ■) and then inoculated with *P. infestans* (●, ■) or SDW (○, □).

(Fig. 7). This somewhat unexpected effect is due to the very rapid utilization of the [2-^{14}C]acetate by the potato cells (see Fig. 5).

The marked increases in phytoalexin accumulation when elicitor-treated tuber discs are exposed to a pulse of [2-^{14}]MVA followed by a chase of unlabelled MVA (Table 2) and when cell suspension cultures are co-inoculated with sporangia of *P. infestans* and a saturating concentration for sterol synthesis of (*R*)-[2-^{14}C]MVA (Fig. 6) show that the synthesis of phytoalexins from MVA is not rate limiting in either of these systems and that the rate of phytoalexin synthesis is regulated either

Table 3. Specific radioactivities of phytoalexins isolated from the culture filtrate of the Majestic potato-cell suspension supplied with a saturating concentration for sterol synthesis of [2-^{14}C]MVA and inoculated with *P. infestans*

Time after inoculation (hr)	Specific radioactivity [cpm/μg (relative to Me arachidate)]			
	Lubimin	3-Hydroxylubimin	Rishitin	Phytuberin
2	250	—	—	—
4	230	—	330	—
8	205	—	310	375
12	250	470	310	365
24	220(280)*†	430(510)	290(350)	355(440)
54	270	450	300	390
Mean	238†	443	308	371

*( ) determined by radio-GLC.

†The absolute specific activities of the rishitin and lubimin calculated from the radio-TLC/GLC (mean values) and radio-GLC values were 35.2 and 35 μCi/mmol and 34.5 and 34.1 μCi/mmol, respectively, cf theoretical value of 39 μCi/mmol if [2-^{14}C]MVA was acting as the sole source of isoprene units. The corresponding values for phytuberin and 3-hydroxylubimin could not be calculated as sufficient quantities of these compounds were not available for the determination of the response factors.

Table 4. Triterpenoid composition of unsaponifiable lipids from the cells of Majestic-cell suspension cultures

Sterols	Amount (μg/g dry wt cells)*
4-Demethyl	
Cholesterol	18
Campesterol	5
Stigmasterol	26
Sitosterol	169
Isofucosterol	122
As glycosides	
Stigmasterol	10
Sitosterol	430
Isofucosterol	69
4α-Monomethyl	
31-Norlanosterol	1
Obtusifoliol	3
Cycloeucalenol (tentatively identified)	2
24-Methylene lophenol (tentatively identified)	7
4,4-Dimethyl	
Cycloartenol	182†
24-Methylene cycloartanol	6†
Pentacyclic triterpene	
Lupeol	215†

*Levels present after 30 min incubation with 3.3 mM [2-^{14}C]MVA.

†Calculated as acetate.

somewhere between acetyl CoA and MVA or by the availability of simple substrates (eg. acetyl CoA) in the transformed cell. The indirect stimulation of phytoalexin synthesis in cell suspension cultures co-inoculated with sporangia of *P. infestans* and a saturating concentration for sterol synthesis of acetate is difficult to explain.

The results from the short-term (two hr) incubation of fresh, aged and fungal-inoculated Kennebec tuber discs with small amounts of high specific activity [2-^{14}C]MVA or [2-^{14}C]acetate (Fig. 2) indicate that sterol (free sterols and steryl ester) synthesis is markedly enhanced in discs which have been aged (Fig. 2). In agreement with an earlier study carried out with Bintje tuber tissue, the ageing process is found to increase particularly the incorporation of radioactivity into demethyl sterols although most of the radioactivity is associated with dimethyl sterols [10]. In Bintje this enhanced incorporation of radioactivity into demethyl sterols is related directly to large increases in the levels of isofucosterol until it becomes the predominant sterol. This sterol is the most predominant sterol (70% of total demethyl sterols) present in Kennebec tuber tissue (Table 1) and the observed enhancement of demethyl sterol synthesis in tuber discs may also be in its favour. In aged discs inoculated with either race of the fungus, the incorporation rates are very much reduced. These findings are, therefore, complementary to those which have shown that the accumulation of, and incorporation of radioactivity from [2-^{14}C]MVA or [2-^{14}C]acetate into steroid glycoalkaloids is inhibited in aged tuber discs infected with *P. infestans* [2, 7, 8] and suggest that sterol synthesis in general is inhibited in infected discs. The observed reduction in the incorporation of radioactivity into squalene in the infected discs indicates that the inhibition is at the level of squalene formation. These interpretations must, however, be treated with caution because (i) the number, sizes and variations in the sizes of the endogenous pools of acetate and MVA are unknown and (ii) the comparisons are made between one set of tissues undergoing the dramatic metabolic changes associated with the wounding reaction and another set of tissues in which the equally dramatic metabolic changes brought about as a result of the infection process are superimposed on those of the wounding reaction. Indeed it could be argued that the reduction in the rates of sterol synthesis and the inhibition of steroid glycoalkaloid accumulation are simply a reflection of a large number of dead host cells in the infected tissues taken for analysis.

The results from a similar series of experiments performed with Kennebec-cell suspension cultures indicate that on inoculation of the cultures with sporangia of *P. infestans* squalene and sterol (free and esterified) synthesis is inhibited by as much as 41% within 2.25 hr of inoculation and progressively becomes more inhibited up to and throughout the period of phytoalexin accumulation (Fig. 4). They are, however, still subject to the criticism that the changes observed may simply reflect an increase in the pool size of endogenous MVA in the potato cells following inoculation of the cultures, despite the rapidity with which the inhibition occurs.

Unequivocal proof that triterpenoid synthesis in general is inhibited in potato cell suspension cultures inoculated with *P. infestans* is provided by the changes in the rates of synthesis and accumulation of squalene, sterols (free and esterified), steryl glycosides (free and esterified), lupeol and phytoalexins which take place in Majestic cell suspension cultures administered a single dose of ether (*R*)-[2-^{14}C]MVA (3.3 mM) or [1-^{14}C]acetate (1 mM) at a saturating concentration for sterol synthesis and then inoculated with SDW or a compatible race of *P. infestans* (Figs 5–11 and Tables 3, 4).

The [2-^{14}C]MVA, as expected, acts as the major/sole substrate for terpenoid biosynthesis throughout the experimental period. The [2-^{14}C]acetate, however, is taken up rapidly by the cells and metabolized in such a way that very little of it is incorporated into terpenoids. The presence of [2-^{14}]MVA in the medium causes a rapid, sustained and large increase in the terpenoid content of the SDW- and fungal-inoculated cultures (Fig. 11). In the former the increase is mainly due to an increase in the triterpenoid content of the cells; in the latter it is made up of a short, rapid increase in the triterpenoid content of the cells followed by a rapid but sustained increase in the phytoalexin content of the medium. This last increase is maintained for some 24 hr after which time phytoalexin accumulation ceases and triterpenoid accumulation in the cells is resumed (c.f. Figs 5, 8, 11). The slow continued increase in the terpenoid content of the MVA/fungal-inoculated cells between 4 and 24 hr is in part attributable to the accumulation of phytoalexins in the cells. The greater part, however, is on the basis of the analyses performed in this study (c.f. Figs 5, 8, 11) due to the accumulation of terpenoids other than sesquiterpenoids and triterpenoids. In the acetate experiments evidence for some continued synthesis of sterols in the fungal-inoculated cultures is provided by the fall in the specific activity of the cycloartenol following induction of phyto-

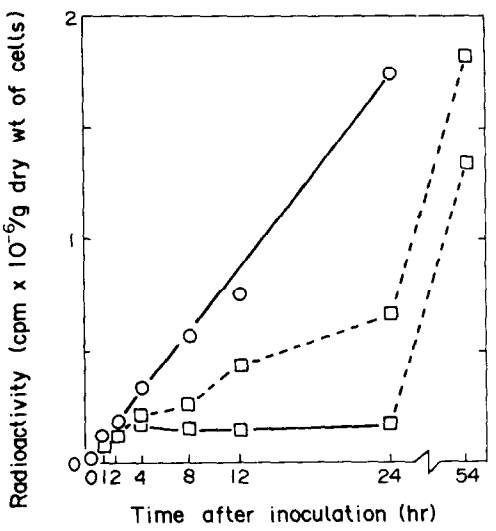


Fig. 11. Incorporation of radioactivity from 3.3 mM (*R*)-[2-^{14}C]MVA into triterpenoids (○) in Majestic-cell suspension cultures and into triterpenoids (□) and triterpenoids plus phytoalexins (■) in cultures inoculated with *P. infestans*. The values are calculated from the data contained in Figs 6 and 8 and contain estimates of the small amounts of radioactivity incorporated into 24-methylene cycloartanol and lupeol.

alexin synthesis. In view of the MVA results, this synthesis probably takes place in untransformed cells.

The increase in the triterpenoid content of the cells from the cultures administered MVA and then inoculated with SDW is almost entirely attributable to a very large increase in the squalene, squalene 2,3-oxide and cycloartenol (Fig. 8) contents of the cells. Similar but quantitatively less significant increases are also shown by 24-methylene cycloartanol and the monomethyl sterols (Fig. 8). The demethyl sterol content of the cells rises over the first two hours after which it shows no measureable change (Fig. 8). The sterol glycosides and the lupeol contents of the cells undergo a very small but sustained increase (Fig. 8). The percentage composition of the monomethyl sterol, demethyl sterol and steryl glycoside pools remain unchanged throughout the experiment.

The triterpenoid constituents of the cells from the cells from the cultures administered MVA and then inoculated with fungus show a similar behaviour to those from MVA/SDW-inoculated cultures for the first four hr, thereafter the squalene, squalene 2,3-oxide and cycloartenol contents of the cells decline whilst the 24-methylene cycloartanol, monomethyl sterol, demethyl sterol and steryl glycoside rise slowly throughout the course of the experiment. A plot of the total radioactivity incorporated into the triterpenoid fractions against the radioactivity incorporated into phytoalexins reveals that *de novo* triterpenoid synthesis in the inoculated cells stops abruptly just prior to the onset of phytoalexin accumulation (Fig. 11).

A complementary set of results to those obtained for the [2-^{14}C]MVA experiment is provided by the results of the [2-^{14}C]acetate experiment after allowance is made for the rapid utilization of the acetate by the cells at the start of the experiment.

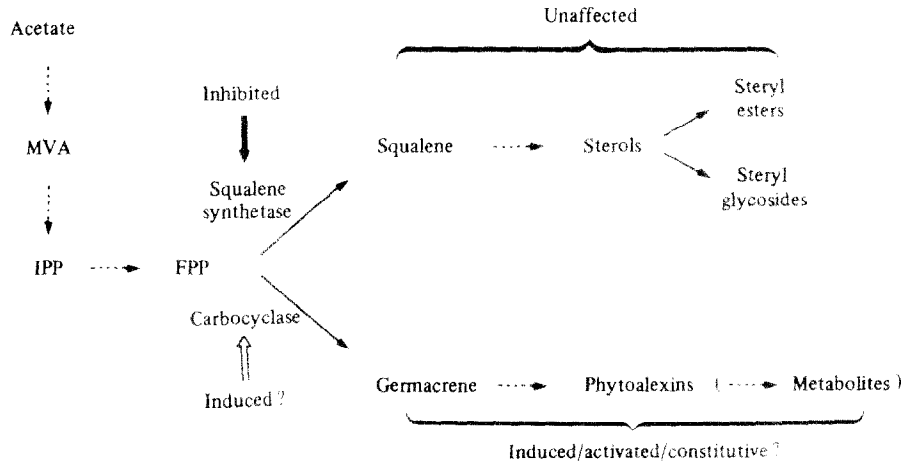
The time courses for the increases and decreases in the amounts and specific activities of triterpenoids labelled from either [2-^{14}C]MVA or [2-^{14}C]acetate are consistent with, the now well-established [33], biosynthetic sequences: acetate → MVA → FPP → squalene (→ lupeol) → squalene 2,3-oxide → cycloartenol → 24-methylene-cycloartanol → monomethyl sterols → demethyl sterols → steryl glycosides. In addition they show that the synthesis of cycloartenol from MVA proceeds in an unregulated manner, that the methylation of cycloartenol to form 24-methylene cycloartanol is probably a rate limiting step and that the cells' ability to accumulate lupeol, demethyl sterols and steryl glycosides is strictly limited. It is noteworthy that, despite the very large pool of cycloartenol in the cells administered [2-^{14}C]MVA only the glycosylation reaction is restricted to stigmasterol, sitosterol and isofucosterol.

The results of these experiments show quite clearly that in most, if not all, of the cells in the fungal-treated cultures the incorporation of FPP into sterols is inhibited rapidly and completely due to the inhibition of squalene formation. The fact that, as shown by the mass data and the specific radioactivity data, the operation of the biosynthetic pathways beyond squalene are unaffected shows that the inhibition is confined to squalene formation. The recovery of the ability to synthesize sterols from MVA by fungal-treated cultures could be due to the removal of the inhibition of squalene formation at the end of the phase of phytoalexin accumulation or to the growth of untransformed cells in the population.

The inhibition of squalene formation is probably brought about as a direct result of the inhibition of squalene synthetase. The possibility that it is due to the FPP-carbocyclase involved in phytoalexin synthesis having a lower K_m and a higher V_{max} that squalene synthetase is unlikely, since the inhibition occurs before appreciable synthesis of any phytoalexins has taken place and saturating amounts for sterol synthesis of exogenous MVA fail to relieve the inhibition. The need to inhibit squalene synthetase is presumably related to the finding that the syntheses of squalene and cycloartenol from MVA and of phytoalexins from MVA are unregulated. Thus by the simple expedient of inhibiting the enzyme which catalyses the first committed reaction on the triterpenoid pathway all the available FPP in the cells of fungal-inoculated cell suspension cultures and, by implication, in the cells responsible for the synthesis of phytoalexins in infected tuber discs is channelled into phytoalexin formation and is not 'wasted' in the unnecessary synthesis of triterpenoids (Scheme 1). It is noteworthy that the synthesis of the other terpenoids in the cell appear to be unaffected in fungal-treated cells. This may reflect the fact that they are synthesized in other compartments of the cell (i.e. chloroplasts and mitochondria) to those involved in the biosynthesis of sesquiterpenoids and triterpenoids (i.e. cytosol and microsomes).

EXPERIMENTAL

Radiochemicals. Na[2-^{14}C]acetate (58 mCi/mmol), (*R, S*)-[2-^{14}C]MVA lactone (53 mCi/mmol) and (*R*)-[2-^{14}C]MVA lactone (53 mCi/mmol) were from Amersham International plc. Each lactone, after the careful evaporation of the C_6H_6 in which it was supplied, was converted into Na [2-^{14}C]MVA by the addition of an equivalent amount of aq. $NaHCO_3$. The low sp. act. species of Na [2-^{14}C]acetate (0.74 mCi/mmol) and Na (*R*)-[2-^{14}C]MVA (13 μCi/mmol) used in the experiments with Majestic-cell suspension cultures were prepared by mixing Na [2-^{14}C]acetate



Scheme 1. Biosynthesis and metabolism of triterpenoids in fungal-inoculated potato-cell suspension cultures and, by implication, phytoalexin-synthesizing cells in infected tuber discs.

(58 mCi/mmol) and Na (*R*)-[2-^{14}C]MVA (53 mCi/mmol) with the appropriate quantities of NaOAc and Na (*R, S*)-MVA (prepared from the lactone supplied by Sigma) respectively. In The latter case allowance was made for the biologically inactive isomer.

Chemicals. These were of the best grade available from commercial sources. All solvents were redistilled before use, the Et_2O from over reduced Fe.

Biological material. Phytophthora infestans, Race 4 (Hull University culture collection) and a Complex race known to be able to match the R_1 major gene (a generous gift from Dr D. D. Clarke, Glasgow University), was maintained on bean agar at 18 ± 3° and subcultured monthly [34]. The agar was supplemented with cholesterol and sitosterol to 50 μg/ml to promote sporangia formation [35]. Sporangial suspensions were prepared by flooding 9–12 day old cultures with sterile distilled H_2O (SDW). Inocula were quantified by estimating the concentration of sporangia with a haemocytometer.

Tubers of *Solanum tuberosum* cv. Kennebec (R_1) were obtained from a commercial grower in Aberdeen, Scotland. Those of cv. Majestic (r) were grown at the University Botanic Gardens, Hull. After harvest in Autumn the tubers were stored at 4°, in darkness, until required.

The Kennebec-cell suspension cultures (ex. tuber tissue) were the later subcultures of the one used in a previous study [4]. The Majestic-cell suspension cultures (ex. tuber tissue) were established by the same procedure as that used to establish the Kennebec cultures [4]. The cultures were grown at 27°, in the dark, with constant agitation (150 rpm; Gallenkamp Orbital Incubator) in Murashiges minimal organic medium (MS[36]-glycine), with kinetin (0.1 mg/l) and NAA (1.0 mg/l) as growth hormones. They were subcultured every 11 (Majestic) or 21 (Kennebec) days by transferring 30 ml of the suspension culture to 70 ml of fresh medium contained in a 250 ml conical flask. The characteristics of the cultures are described in Results.

Incubation conditions. All the manipulations described in this section were carried out under aseptic conditions.

(i) Tuber discs. Discs (15 mm dia × 5 mm) of Kennebec tuber tissue were prepared from cylindrical cores taken from 8-month-old tubers that had been equilibrated to room temp. and surface sterilized with 15% Everechlor for 15 min, followed by rinsing in EtOH. The discs were rinsed (× 3) in SDW and aged in Petri dishes for 24 hr on 1% water-agar at 18 ± 3°, in darkness, prior to inoculation.

All the experiments with tuber discs, except the pulse chase experiment, were part of one large experiment which was executed with a single batch of potato tubers and a single inoculum of each race of *P. infestans*. In this experiment the upper surface of each of the aged discs was inoculated with 0.05 ml of sporangial suspension (1.2×10^5 sporangia/ml) or SDW and the incubation continued until harvest or administration of radiochemicals. The effects of wounding, ageing and infection of potato tuber tissue on the incorporation of ^{14}C-acetate and ^{14}C-MVA into phytoalexins and sterols were measured in freshly cut discs, discs which had been aged for 22 hr and discs which had been both aged for 24 hr and inoculated with sporangia or SDW and then incubated for 14, 46 and 86 hr. Two batches of five potato discs each were used for each set of determinations. One batch was treated with 0.22 μCi (*R, S*)-[2-^{14}C]MVA (53 mCi/mmol) whilst the other was treated with 1.5 μCi [2-^{14}C]acetate (58 mCi/mmol). To ensure good penetration of the radioisotopes, an aq. solution (50 μl/disc) of the isotope was gently vacuum-infiltrated for 10 min into the upper cell layers of the discs by placing the Petri dishes containing the treated discs in a sterile vacuum desiccator attached to a water pump. The time courses of the accumulation and turnover of phytoalexins were determined using discs which had been infected for 8, 16 and 24 hr and infected discs which had been incubated for 30 hr, vacuum-infiltrated (conditions as above) with (*R, S*)-[2-^{14}]MVA (53 μCi/μmol; 0.11 μCi/5 discs) and then incubated for the times shown in Fig. 1. Five discs were used for each analysis.

In the pulse-chase experiment, the pulse consisted of a single dose of (*R*)-[2-^{14}C]MVA (53 mCi/mmol) administered by mild vacuum-infiltration to aged potato discs (0.44 μCi/5 discs) 6 hr after inoculation with a mycelial sonicate (50 μl/disc; 100 mg mycelia/ml) prepared as described in [5]. The chase consisted of a 1000-fold excess of MVA [66 mM (*R, S*)-MVA; 50 μl/disc] administered six hr later by mild vacuum infiltration. The control discs were infiltrated with SDW.

(ii) Kennebec-cell suspension cultures. Two experiments were carried out with this cell line. In the first, 21-day-old suspension cultures from the ninth transfer were pooled, thoroughly mixed and then divided into two batches of 240 ml each and one batch of 120 ml. The 2 × 240 ml batches were each inoculated with 3 ml sporangial suspension (6.5×10^5 sporangia/ml) of either race 4 or the Complex race of *P. infestans* and the 1 × 120 ml batch of culture was inoculated with 1.5 ml SDW. The cultures from each treatment were apportioned into 60 ml samples contained in

sterile 250 ml conical flasks and returned to the orbital incubator. After 8 hr in the incubator, 1.2 μCi aq. (*R,S*)-[2-^{14}C]MVA (53 mCi/mmol) was supplied to each flask. The contents from one flask of the cell cultures inoculated with sporangia were taken for analysis of phytoalexins at 12, 24, 36 and 48 hr after inoculation. The SDW controls were analysed at 12 and 48 hr. At harvest the suspensions contained *ca* 25 g fr. wt of cells/100 ml medium.

In the second experiment, 21-day-old cultures from the tenth transfer were pooled. A 315 ml batch of the pooled culture was inoculated with 3 ml sporangial suspension (8.6×10^5 sporangia/ml) of the Complex race of *P. infestans* and the dispensed into 7×45 ml volumes contained in 250 ml flasks. The remainder (90 ml) of the pooled culture was inoculated with 0.8 ml SDW and divided into the equal vols. The cultures were then returned to the orbital shaker. At each of a series of preselected times (inoculated cultures, 0.25, 0.5, 1, 2, 4, 8 and 12 hr; control (SDW) cultures, 0.25 and 12 hr) 1.1 μCi aq. (*R,S*)-[2-^{14}C]MVA (53 mCi/mmol) was added to the flask from each treatment. The culture plus radiochemical was then incubated for a further two hours after which the levels of radioactivity associated with the phytoalexins in the Et_2O extracts of the culture filtrates and the sterol fractions from the lipid extracts of the cells were measured. At harvest the suspensions contained *ca* 22 g fr. wt cells/100 ml medium.

(iii) Majestic-cell suspension cultures. two experiments were performed with this cell line. In the first, the effect of a single dose of a saturating concn for sterol synthesis of [2-^{14}C]acetate on phytoalexin and sterol biosynthesis was investigated. 11-day-old cultures from the fourth transfer were pooled (1550 ml) and thoroughly mixed. A 200 ml aliquot was removed and set aside, and the remainder of the culture was then supplemented with [2-^{14}C]acetate (0.74 mCi/mmol; 1.35 mmol in 1 ml SDW) to a final molarity of 1 mM. Addition of the ^{14}C-acetate raised the pH of the culture medium from 4.8 to 5.1. After thorough mixing, the suspension was divided into a 700 and a 651 ml batch which, after incubation for 30 min at RT with periodic shaking, were inoculated with 7 ml SDW and 6.5 ml sporangial suspension of *P. infestans* (Complex race; 8×10^5 sporangia/ml) respectively. The 200 ml aliquot removed earlier was also inoculated with 2 ml of the same fungal inoculum. Immediately after inoculation a 101 ml aliquot of SDW/^{14}C-acetate-inoculated cell suspension was taken for analysis. The remainder of the SDW/^{14}C-acetate-inoculated suspension (606 ml), the fungal/^{14}C-acetate-inoculated suspension (657 ml) and the fungal-inoculated suspension (202 ml) were apportioned into 101 ml batches (plus one of 51 ml in the case of the fungal/^{14}C-acetate-inoculated suspension) contained in 250 ml conical flasks. The flasks were then returned to the orbital shaker. The contents from one flask of each of the treatments involving [2-^{14}C]acetate were taken for analysis at 1, 2, 4, 8, 12 and 24 and 58 (51 ml fungal/^{14}C-acetate culture) hr from the time of inoculation with either SDW or sporangia. The two ^{14}C-acetate-free cultures were harvested as four aliquots (*ca* 50 ml) at 4, 8, 12 and 24 hr after Inoculation. At harvest the suspensions contained 26–30 g fr wt of cells/100 ml (0.93–1.06 g dry wt of cells/100 ml).

In the second experiment, the effect of a single dose of a saturating concentration for sterol synthesis of [2-^{14}C]MVA on phytoalexins and sterol biosynthesis was investigated. Apart from differences in the number of transfers undergone by the subculture, the volumes of suspensions used and the final molarity of the radiosubstrate, the experimental protocol was the same as the one described above. In summary, the important features were as follows: next (fifth) transfer of the culture used in the previous experiment; total volume of suspension 1335 ml; a mixture of (*R*)-[2-^{14}C]MVA (13 μCi/mol) and (*S*)-MVA added to give a final concentration of 3.3 mM with respect to both isomers; pH rose from 4.9 to 5.3 on addition of the MVA; 86 ml SDW/^{14}C-MVA-inoculated suspension; 6×86 ml plus 1 $\times$ 61 ml (54 hr time point) of fungal/^{14}C-MVA inoculated suspension; 7×86 ml of SDW/^{14}C-MVA-inoculated suspension and 2×86 ml of fungal inoculated suspension; 22–26 g fr wt of cells/100 ml medium at harvest (0.9–1.2 g dry wt for cells/100 ml medium; incubation times as for ^{14}C-acetate experiment.

Extraction, purification and estimation of phytoalexins and sterols. The extraction and purification of these compounds were carried out without exposure of the samples to excess heat, except when an essential part of the procedure. All tissues and extracts (dissolved in cyclohexane) were stored under N_2 at $-20°$.
(i) Tuber discs. The first mm of tissue was excised from the top surfaces of treated tuber discs (5 discs/treatment), weighed (2.03–2.41 g) and then homogenized (Ultraturrax) in $CHCl_3$–MeOH (2:1, 5 ml/g fr. wt) for 1 min. The homogenate was vacuum-filtered through a sintered glass funnel and the tissue residue rinsed with more $CHCl_3$–MeOH (2.5 ml/g starting wt). The Filtrate plus rinsing was washed ($\times$ 2) with either H_2O (1 vol.) (non-radioactive extracts) or aq. 1 mM $NaHCO_3$ (1 vol.) (radioactive extracts), to facilitate removal of any unmetabolized ^{14}C-substrate, and the $CHCl_3$ phase reduced to dryness under vacuum, using a small addition of EtOH to remove traces of H_2O. The residue ($CHCl_3$-soluble lipids) was taken up in a small, known vol. of cyclohexane.

The non-radioactive extracts were subjected to prep. TLC on silica gel G (0.5 mm) developed with EtOAc–cyclohexane (1:1). After development, the marker spots and authentic phytoalexin standards were visualized by spraying with vanillin–H_2SO_4 reagent (for R_fs and colours, see below) and the area of gel containing the bands of lubimin and rishitin eluted with Me_2CO. The extract was taken to dryness under a stream of N_2 then redissolved in a known vol. of a standard soln. of Me arachidate (0.4 mg/ml) in cyclohexane for estimation of the phytoalexins by GC (see below).

In the radiochemical experiments, the radioactivity associated with lubimin and rishitin was determined as follows. A one-third aliquot of the extract was subjected to prep. TLC and an X-ray autoradiograph prepared of the developed plate. The autoradiograph was then used to locate the bands of ^{14}C-lubimin and ^{14}C-rishitin (clearly defined and coincident with phytoalexin markers) which were removed and the radioactivity quantified using a Scintillation Counter, transferring the areas of gel directly into 10 ml vols of scintillant. The quantification of the amount of each phytoalexin was determined from the remainder (two thirds) of the extract by using TLC and GC as described above. In the analyses where the ^{14}C associated with sterols and squalene was determined, the demethyl-, monomethyl- and dimethyl-sterols, steryl esters and squalene were then eluted from the upper part of the plate (R_f 0.67–1.00) with Et_2O (10 ml) and subjected to cold saponification (10 ml Et_2O extract, 2 ml 60% KOH) overnight at room temp. The saponification mixture was diluted with H_2O (12 ml) and extracted ($\times$ 3) with Et_2O (1 vol). The Et_2O extracts were combined, washed with H_2O until free of alkali (no colour with alcoholic phenolphthalein) and taken to dryness under vacuum, using small additions of EtOH to remove H_2O. The unsaponifiable lipid was dissolved in a minimum vol of cyclohexane and subjected to prep. TLC on silica gel G (0.5 mm) developed ($\times$ 2) with EtOAc–C_6H_6 (3:22) along with authentic samples of a demethyl sterol (cholesterol), a monomethyl sterol, (obtusifoliol), a dimethyl sterol (cycloartenol) and squalene. The three groups of ^{14}C-labelled sterols and ^{14}C-squalene were located by X-ray autoradiography and were shown to have the same R_f values as the marker compounds [located under UV (350 nm) after spraying the marker lanes with Rhoamine 6G in Me_2CO. For R_f values see below]. The radioactivity associated

with each of the groups of sterol and the squalene was determined in a Scintillation Counter, transferring the areas of gel directly into 10 ml vols of scintillant.

(ii) Kennebec cell suspension cultures. The culture was filtered through Whatman no. 541 hardened, ashless filter paper under vacuum and the retained cells rinsed (× 2) with H_2O (0.5 vol of culture filtrate), after which the cells were weighed and, if not analysed immediately, stored at −20°.

The culture filtrate plus rinsings were extracted (× 3) with Et_2O (1 vol). The Et_2O extracts were then bulked and, after the addition of a small vol. of EtOH, evapd to dryness under vacuum. If the sample was radioactive then the Et_2O extracts were washed with 1 mM $NaHCO_3$ (1 vol.) to remove unmetabolized ^{14}C-substrate prior to removal of the solvent. The Et_2O extract was then redissolved in a small, known vol. of cyclohexane and the phytoalexins estimated by GC. The radioactivity associated with the phytoalexins was measured by the same procedure as that described under (i). In all cases, greater than 90% of the radioactivity in the Et_2O extracts was associated with phytoalexins. The phytoalexin content of the whole cells was determined by the same procedure as that described under (i).

The extraction and separation of sterols was performed as follows. A sample of cells was refluxed for 1 hr in a mixture of MeOH (1 ml/g fr. wt) and 60% KOH (0.5 ml/g fr. wt). The saponification mixture was then cooled, diluted with H_2O (3 vols) and filtered through glass wool. The residue was rinsed with H_2O (1 vol.) and the total filtrate extracted (× 4) with petrol (40–60°). The petrol extracts were bulked, washed (× 3) with H_2O (1 vol.) to remove alkali and taken to dryness under vacuum. The unsaponifiable lipid was taken up in a small vol. of cyclohexane containing cholesterol, obtusifoliol, cycloartenol and squalene (ea. at 5 μg/g fr. wt of cells) and subjected to prep. TLC on Rhodamine 6G-impregnated silica gel G developed (× 2) with EtOAc–C_6H_6 (3:22). The areas of gel containing sterols and squalene were located under UV (350 nm) and assayed for radioactivity by scintillation counting, transferring the gel directly into 10 ml scintillant. (iii) Majestic cell suspension cultures. These were analysed by similar procedures to those described under (ii), except that: an aliquot (5 μl) of the filtered culture medium was assayed for radioactivity prior to the rinsing of the cells with H_2O; a 2 g sample of the harvested cells was taken for dry wt determination by lyophilization; the ^{14}C-labelled phytoalexins were recovered from TLC immediately after development, the sterols (free plus esterified) were analysed quantitatively as were the steryl glycosides (free plus esterified).

A sample (16 g) of frozen potato cells was homogenized (Ultraturrax) for 2 × 1 min in 85 ml $CHCl_3$–MeOH (2:1) and then vacuum-filtered through glass wool. The residue was re-extracted with a further 85 ml $CHCl_3$–MeOH, filtered and the bulked filtrates taken to dryness under vacuum. The extract was then taken up in EtOH (30 ml) and refluxed for 2 hr with 65% KOH (10 ml). The saponification mixture was cooled, diluted with H_2O (40 ml) and extracted (× 3) with Et_2O (60 ml). The Et_2O extracts were combined, washed (× 3) with H_2O (100 ml) until free of alkali and, after the addition of a small vol. of EtOH, taken to dryness under vacuum. The unsaponifiable lipid was then taken up in a small vol of $CHCl_3$ and together with authentic markers subjected to prep. TLC on Rhodamine 6G-impregnated silica gel G (0.75 mm) developed with EtOAc–C_6H_6 (3:22). After development, the plate was examined under UV (350 nm) and the bands of gel corresponding to authentic markers of cycloartenol, obtusifoliol and cholesterol removed and eluted individually with Et_2O (3 × 5 ml). The area of gel containing the squalene and squalene 2,3-oxide was transferred directly into 10 ml of scintillation fluid and assayed for radioactivity, except in the case of the 24 hr control sample. The squalene and squalene 2,3-oxide in the 24 hr [2-^{14}C]MVA control sample were eluted separately with Et_2O (3 × 5 ml) (^{14}C-squalene: ^{14}C-squalene 2,3-oxide :: 1:2) and characterized (see below). The gel containing the steryl glycosides was mixed EtOH (10 ml) and 6 M H_2SO_4 (5 ml) and refluxed for 2 hr. The mixture was then cooled, diluted with H_2O (2 vols) and extracted (× 3) with Et_2O (1 vol.). The Et_2O extracts were bulked, washed (× 3) with H_2O (1 vol.), taken to dryness and the demethyl sterols recovered by prep. TLC under the conditions just described above. After removal of Et_2O, the sterol fractions representing the dimethyl (plus lupeol)-, monomethyl- and demethyl-sterols and the aglycones of the steryl glycosides were redissolved in cyclohexane and appropriate aliquots assayed for radioactivity and subjected to GC. To separate and characterize the dimethyl sterols from each other and from lupeol acetylation and a further purification step were needed.

The dimethyl sterol fraction, after removal of solvent under a stream of N_2, was acetylated (0.5 ml Ac_2O plus 4 drops C_5H_5N) overnight at room temp. The acetylation mixture was then diluted with 2 M HCl (40 ml) and the acetates extracted into Et_2O (3 × 40 ml). The Et_2O extracts were bulked, washed, taken to dryness under vacuum and subjected to prep. TLC on 10% $AgNO_3$-impregnated silica gel G (0.5 mm) developed with petrol (60–80°)–C_6H_6 (1:1). The plate was then sprayed with Rhodamine 6G and bands of gel corresponding to authentic cycloartenyl acetate (R_f 0.53) and 24-methylene cycloartanyl acetate plus lupeyl acetate (R_f 0.42) located under UV (350 nm) and eluted individually with Et_2O (3 × 5 ml) and the recovered acetates assayed for radioactivity and subjected to GC, radio-GC and GC-MS (EI).

The squalene and squalene 2,3-oxide from the 24 hr [2-^{14}C]MVA control sample were assayed for radioactivity and then characterized by TLC (EtOAc–C_6H_6, 0.5:99.5. Squalene, R_f 0.91; squalene 2,3-oxide, R_f 0.54) and GC-MS. The radiochemical purity of each sample was confirmed by purification of a mixture of the ^{14}C-sample plus unlabelled carrier material via its squalene-thiourea adduct [37]. The authentic sample of squalene 2,3-oxide used for the characterization studies was prepared from squalene by the procedure described in [38].

In a study undertaken prior to the one detailed above, it was established that steroid alkaloids were not present in Majestic cells and culture filtrates from either healthy or browned, fungal-inoculated cultures. The method of analysis was based on that described in [39], in which the aglycone solanidine and its dehydration product solanthrene are detected by GC following acid hydrolysis of any solanine and chaconine in the sample. As a check on the method, it was shown that Kennebec tuber tissue taken directly from under the skin of a tuber i.e. a known source of solanidine glycosides, yielded solanidine and solanthrene on acid hydrolysis.

(iv) Extractions of sterols from Kennebec tuber tissue. In outline the procedure followed was as follows. Peeled potato tubers (1 kg) were chopped into small pieces and homogenized in EtOH (2.5 l). The homogenate was filtered, concentrated under vacuum to 200 ml and refluxed with H_2O (30 ml) and KOH (32 g) for 2 hr. After dilution with ice-cold H_2O (3 vols) the saponification mixture was extracted (× 3) with petrol (500 ml), the bulked petrol extracts washed (× 3) with H_2O (500 ml) and then taken to dryness under vacuum. The unsaponifiable lipid was dissolved in 95% EtOH (15 ml) and the sterols pptd as digitonides [40]. The sterols were recovered from the digitonides [41] and separated into demethyl-, monomethyl- and dimethyl-sterols by prep. TLC on Rhodamine 6G-impregnated plates developed with EtOAc–C_6H_6 (3:22). The three groups of sterols were eluted individually from the gel (Et_2O), taken up in cyclohexane and identified and quantitated by GC and GC-MS.

Characterizations of phytoalexins and sterols and confirmation of radiochemical purities. Phytoalexins were identified either by co-TLC and colour reactions on TLC, co-GC, MS and GC-MS (rishitin and lubimin) or by TLC (R_f and colour reactions), GC, MS and GC-MS (3-hydroxylubimin, phytuberin, phytuberol). The TLC, GC and MS data were in good agreement with those reported in the literature (eg. [4, 15, 42–44]). Sterols, steryl acetates, squalene, squalene 2,3-oxide, lupeol and lupeyl acetate were identified by co-TLC, co-GC and GC-MS. The GC-MS identifications of the sterols and the tentative identification of lupeol were aided greatly by computer matching of the acquired spectra with those of an extensive sterol library. Lupeol and 3-hydroxylubimin were also characterized by ^{1}H NMR (360 MHz, $CDCl_3$, TMS as int. standard). The spectra obtained were in good agreement with those published in the literature [15, 45].

The radiochemical purities of the compounds recovered from the TLC procedures were checked, when they contained sufficient radioactivity, by subjecting representative samples to radio-GC (see below), and collecting the splitter effluent as a series of 1 min fractions between mass peaks and as a series of 10 (short R_t peaks) −30 (long Rt peaks) sec fractions for each mass peak. In each sample tested greater than 90% of the radioactivity injected onto the column was co-eluted with same profile(s) as the mass peak(s) [or marker mass peak(s)] of the appropriate phytoalexin(s), sterol(s) or steryl acetate(s).

TLC. Analytical and prep. TLC of $CHCl_3$- and Et_2O-soluble lipids, unsaponifiable lipids and sterol fractions were carried out on silica gel G or Rhodamine 6G-impregnated silica gel G (unsaponifiable lipids) developed with either EtOAc–cyclohexane (1:1) or EtOAc–C_6H_6 (3:22) (unsaponifiable lipids and sterols). Dimethyl steryl acetates and lupeyl acetate were separated on 10% $AgNO_3$-impregnated silica gel G developed with petrol (60–80°)–C_6H_6 (1:1). Phytoalexin markers were located by spraying the plates with vanillin–H_2SO_4 reagent [31]. Sterols and squalene were located by examination of Rhodamine 6G-impregnated or Rhodamine 6G-sprayed plates under UV (350 nm). As an additional aid to the location and identification of sterols, sterol esters and sterol glycosides the marker spots were sometimes sprayed with Liebermann–Burchard reagent. The R_fs, and in the cases of phytoalexins, colours given by vanillin–H_2SO_4 reagent are as follows: EtOAc– cyclohexane (1:1): 3-hydroxylubimin, 0.10 (purplish blue), rishitin, 0.22 (blue), lubimin, 0.30 (turquose), solavetivone, 0.44 (buff), phytuberin, 0.58 (reddish pink); EtOAc–C_6H_6 (3:2) (× 2): steryl glycosides, 0.05, demethyl sterols, 0.40, monomethyl sterols, 0.48, dimethyl sterols, 0.55, squalene 2,3-oxide, 0.85, squalene, 0.9.

GC, GC-MS and radio-GC. Estimation and preliminary identification of phytoalexins, sterols and steryl acetate was by FID-GC. The conditions used were as follows: phytoalexins: 3% OV 225 on Gas Chrom Q (100–200 mesh) in a glass column (2.1 mm × 2 mm i.d.), col. temp. 180°, detector 300°, injection port 225°, N_2 35 ml/min. RR_t (Me arachidate): phytuberin, 0.36, solavetivone, 0.56, rishitin, 0.72, lubimin, 1.20, 3-hydroxylubimin, 4.75. Sterols and steryl acetates: 3% OV 17 on Gas Chrom Q (80–100 mesh) in a glass column (2 m × 2 mm i.d.), col. temp. 240° (250° for steryl acetates), detector 300°, injection port 360°, N_2 40 ml/min, RR_t (cholestane): cholesterol, 2.6, campesterol, 3.5, stigmasterol, 3.8, sitosterol, 4.3, isofucosterol, 4.9, 31-norlanosterol, 3.8, obtusifoliol, 4.3, cycloeucalenol, 5.1, 24-methylene lophenol, 7.1. Acetates RR_t (cycloartenyl acetate), lupeyl acetate, 1.0, 24-methylene cycloartanyl acetate, 1.1.

The peak areas of the phytoalexins, demethyl- and monomethyl-sterols and steryl acetates were computed by an electronic integrator relative to Me arachidate, sitosterol and cycloartenyl acetate respectively. The rishitin and lubimin values were corrected by the appropriate response factors. The factors used for phytuberin and 3-hydroxylubimin were calculated from the cpm/μg phytoalexin (relative to Me arachidate) data obtained in the Majestic experiment (Table 3) by making the assumption that the absolute sp. activities (μCi/mmol) of these phytoalexins were the same as those of rishitin and lubimin.

Radio-GC analyses were performed under the same conditions to those just described, except that a glass lined stream splitter was incorporated into the apparatus and set at 15% flow to the FID. Effluent fractions from the splitter were condensed onto cooled (0°) small bore glass tubes then eluted with 10 ml scintillation fluid directly into counting vials.

GC-MS of the phytoalexins and sterols were performed at Shell Research Ltd. Kent and the Department of Biochemistry, University of Liverpool, respectively.

Radioactivity measurements. The radioactivity contents of the samples were assayed by liquid scintillation spectrophotometry. An aliquot of sample (usually not more than 10 μl) was added to 10 ml scintillation fluid [6 g PPO, 0.3 g POPOP, 100 g naphthalene in 1.3 l toluene–ethoxyethanol (1:3)] and counted to an accuracy of at least 5%. All radioassays were corrected for background radioactivity and where a result was calculated as disintegrations per minute (dpm) corrections were made for the counting efficiency (77.3%) of the instrument. Only on a few occasions was it necessary to make any correction for quenching.

Autoradiographs were obtained from thin layer radiochromatograms using Industrex CV film (Kodak). At the end of the exposure time the film was developed in D19 Developer (Kodak) as recommended by the manufacturer.

Acknowledgements—We thank Dr F. Cottee, Shell Research Ltd., and Dr L. J. Goad, University of Liverpool, for GC-MS data, Dr P. D. Regan, Shell Research Ltd, for ^{1}H NMR data, and Mrs Susan Swetez for expert technical assistance. One of us (P. A. B.) was in receipt of a SERC CASE award.

REFERENCES

1. Varns, J. L., Kúc, J. and Williams, E. B. (1971) *Phytopathology* **61**, 174.
2. Currier, W. W. and Kúc, J. (1975) *Phytopathology* **65**, 1194.
3. Bostock, R. M., Nuckles, E., Henfling, J. W. D. M. and Kúc, J. (1983) *Phytopathology* **73**, 435.
4. Brindle, P. A., Kuhn, P. J. and Threlfall, D. R. (1983) *Phytochemistry* **22**, 2719.
5. Coolbear, T. and Threlfall, D. R. (1985) *Phytochemistry* **24**, 2219.
6. Kúc, J. and Rush, J. S. (1985) *Arch. Biochem. Biophys.* **236**, 455.
7. Shih, M., Kúc, J. and Williams, E. B. (1973) *Phytopathology* **63**, 821.
8. Shih, M. and Kúc, J. (1973) *Phytopathology* **63**, 826.
9. Tjamos, E. C. and Kúc, J. (1982) *Science* **217**, 542.
10. Hartman, M. A. and Benveniste, P. (1974) *Phytochemistry* **13**, 2667.
11. Ishizaka, N. and Tomiyama, K. (1972) *Plant Cell Physiol.* **13**, 1053.
12. Ôba, K., Kondo, K., Doke, N. and Uritani, I. (1985) *Plant Cell Physiol.* **26**, 873.
13. Sakai, S., Tomiyama, K. and Doke, N. (1979) *Phytopath. Soc. Jpn* **45**, 705.
14. Coolbear, T. and Threlfall, D. R. (1985) *Phytochemistry* **24**, 2219.
15. Murai, A., Sato, K. Osada, A., Katsui, N. and Masamune, T. (1982) *J. Chem. Soc. Chem. Commun.* **1**, 32.
16. Alves, L. M., Heisler, E. G., Kissinger, J. C., Patterson, J. M.

and Kalan, E. B. (1979) *Plant Physiol.* **63**, 359.
17. Zacharius, R. M. and Kalan, E. B. (1984) *Plant Cell Reports* **3**, 189.
18. Moesta, P. and West, C. A. (1985) *Arch. Biochem. Biophys.* **238**, 325.
19. Kalan, E. B. and Osman, S. F. (1976) *Phytochemistry* **15**, 775.
20. Sato, K., Ishiguri, Y., Doke, Y., Tomiyama, T., Yagohashi, F., Murai, A., Katsui, N. and Masamune, T. (1978) *Phytochemistry* **17**, 1901.
21. Stoessl, A. and Stothers, J. B. (1981) *Can. J. Botany* **59**, 637.
22. Ishiguri, Y., Tomiyama, K., Murai, A., Katsui, N. and Masamune, T. (1978) *Ann. Phytopath. Soc. Jpn* **44**, 775.
23. Murai, A., Katsui, N., Yagihashi, F., Masamune, T., Ishiguri, Y. and Tomiyama, K. (1977) *J. Chem. Soc. Chem. Commun.* 670.
24. Brindle, P. A., Coolbear, T., Kuhn, P. J. and Threlfall, D. R. (1985) *Phytochemistry* **24**, 1219.
25. Ward, E. W. B., Stoessl, A. and Stothers, J. B. (1977) *Phytochemistry* **16**, 2024.
26. Goad, L. J. (1983) *Biochem. Soc. Trans.* **11**, 548.
27. Doke, N. (1982) *Physiol. Pl. Path.* **21**, 85.
28. Nakajima, T., Tomiyama, K. and Kinukawa, M. (1975) *Ann. Phytopath. Soc. Jpn* **41**, 49.
29. Stoessl, A., Robinson, J. R., Roch, G. L. and Ward, E. W. B. (1977) *Phytopathology* **67**, 64.
30. Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983) *Adv. Enzymol. Related Areas Molec. Biol.* **55**, 1.
31. VanEtten, H. D., Matthews, D. W. and Smith, D. A. (1982) in *Phytoalexins* (Bailey, J. A. and Mansfield, J. W., eds) p. 181. Blackie, Glasgow.
32. Zacharius, R. M., Kalan, E. B. and Kimoto, W. I. (1985) *Plant Cell Reports* **4**, 1.
33. Goodwin, T. W. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W. and Spurgeon, S. L., eds) Vol. 1, p. 350. Wiley, New York.
34. Rogerson, F. A. (1978) Ph.D. Thesis, University of Hull.
35. Langcake, P. (1974) *Trans. Br. Mycol. Soc.* **63**, 573.
36. Murashige, T. and Skoog, F. (1962) *Physiol. Plant.* **15**, 473.
37. Nicolaides, N. and Laves, F. (1954) *J. Am. Chem. Soc.* **76**, 2596.
38. Nadeau, R. G. and Hanzlik, R. P. (1969) *Meth. Enzymology* **15**, 346.
39. Coxon, D. T., Price, K. R. and Jones, P. G. (1979) *J. Sci. Food Agric.* **30**, 1043.
40. Windaus, A. (1909) *Ber. Chem. Dtsch. Ges.* **42**, 238.
41. Schoenheimer, R. and Dam, H. (1933) *Hoppe-Seylers Z. Physiol. Chem.* **215**, 59.
42. Stoessl, A., Stothers, J. B. and Ward, E. W. B. (1976) *Phytochemistry* **15**, 855.
43. Coxon, D. T., Curtis, R. F., Price, K. P. and Howard, B. (1974) *Tetrahedron Letters* 2363.
44. Katsui, N., Matsunaga, A., Kithara, H., Yagihashi, F., Murai, A., Masamune, T. and Sato, M. (1977) *Bull. Chem. Soc. Jpn* **50**, 1217.
45. Schmidt, J., Lien, N. T. and Adam, G. (1983) *Phytochemistry* **22**, 1032.