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# THE BIOSYNTHESIS OF LUBIMIN FROM [1-$^{14}$C]ISOPENTENYL PYROPHOSPHATE BY CELL-FREE EXTRACTS OF POTATO TUBER TISSUE INOCULATED WITH AN ELICITOR PREPARATION FROM *PHYTOPHTHORA INFESTANS*

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**Key Word Index**—*Solanum tuberosum*; Solanaceae; *Phytophthora infestans*; tubers; cell-free system; biosynthesis; sesquiterpenoids; phytoalexins; lubimin.

**Abstract**—A cell-free enzyme system, which catalyses the incorporation of radiolabel from [1-$^{14}$C]isopentenyl pyrophosphate into the sesquiterpenoid phytoalexin lubimin, has been prepared from tuber tissue of *Solanum tuberosum* inoculated with an elicitor preparation from *Phytophthora infestans*. Biosynthesis of lubimin is optimum at pH 7.3–7.5 and is dependent upon $Mg^{2+}$ and NADPH. Lubimin labelling by cell-free enzyme system prepared from tissue 48 hr after treatment with elicitor rises rapidly to a maximum over the first 30 min of incubation and does not decline for a further 150 min. The biosynthetic capacity for lubimin in cell free extracts can be observed as early as 3 hr after inoculation of tuber tissue, and rises to a maximum at about 48 hr after treatment, declining thereafter. Lubimin labelling is inhibited by iodoacetamide, the effect of which is reversed by 3,3-dimethylallylpyrophosphate. Preliminary observations on the cell-free system show that it will also catalyse the incorporation of [2-$^{14}$C]mevalonic acid into lubimin in the presence of an ATP-generating system.

## INTRODUCTION

Terpenoids constitute one of the three main types of phytoalexins produced by higher plants in response to infection, the other two being phenolic or acetylenic in nature. Monoterpenes are, somewhat paradoxically, not often referred to as phytoalexins, even though certain members of this class of compound have been shown to have antimicrobial activity (e.g. carene [1]) and accumulate as a result of infection (see ref. [2]). The biosynthesis of monoterpenes has been studied extensively [3] both in *in vivo* studies, e.g. [2-$^{14}$C]MVA* feeding to needles of *Pinus* [4], and in cell free systems with regard to relevant intermediates (e.g. [5]).

Casbene was one of five diterpene hydrocarbons synthesized by preparations derived from seedlings of *Ricinus communis* [6, 7]. When the seedlings were stressed with various fungi, casbene synthesis in the derived cell-free preparations was markedly stimulated [8]. The cell free system, utilizing the phenolic adsorbent Polyclar AT (insoluble polyvinylpyrrolidone), catalysed the incorporation of both [2-$^{14}$C]MVA and [2-$^{14}$C]GGPP into the bicyclic phytoalexin. The purification of casbene synthetase from *Ricinus communis* treated with *Rhizopus stolonifer* spores [9] marked the first report of the isolation of an enzyme responsible for the production of a terpenoid phytoalexin. The enzyme was not present in uninfected tissue.

Of the sesquiterpenoid phytoalexins, the biosynthesis of the acarbocyclic compound ipomeamarone has been the subject of a great deal of attention. This furanosesquiterpene is synthesized in *Ipomoea batatas* in response to infection by *Ceratocystic fimbriata* (see ref. [10]) and recently a particulate fraction from root tissue has been isolated which catalyses the reduction of dehydroipomeamarone to ipomeamarone [11]. The biosynthesis of gossypol (a dimeric sesquiterpenoid with phytoalexin properties) from [2-$^{14}$C]MVA has been demonstrated and a comparison made of the relative efficiency of GPP, neryl-PP and the isomers of FPP as precursors using a high speed supernatant fraction from seedlings of *Gossypium* [12].

The three major eudesmanoid phytoalexins from potato systems are rishitin (**3**) [13], lubimin (**2**) [14] and solavetivone (**1**) [15]. The biosynthesis of these compounds is undoubtedly from acetate via a pathway common to that of sterols up to the level of FPP (see [16]). The incorporation of radiolabel from $^{14}$C-acetate and $^{14}$C-MVA into rishitin in slices of *Solanum tuberosum* was demonstrated over a decade ago [17]. Since then $^{13}$C NMR studies have provided much information on the biosynthesis of these compounds (see ref. [16]) but the *in vivo* relationships remain to be unequivocally demonstrated. The synthesis of FPP has been demonstrated in a number of cell free systems from higher plants and certain of the participating enzymes have been isolated (see ref. [18]). There is only one account, to date, of the cell-free biosynthesis of eudesmanoid phytoalexins, this preliminary work comprising part of a Ph.D. thesis from this laboratory [19]. The system used was derived from *Solanum tuberosum* infected with *Phytophthora infestans*.

This paper presents details of a cell-free system derived from tuber tissue of *S. tuberosum* cv. Kennebec inoculated

*Abbreviations: MVA, mevalonic acid; DMAPP, IPP, GPP, FPP and GGPP, 3,3-dimethylallyl-, isopentenyl-, geranyl-, farnesyl- and geranylgeranyl-pyrophosphates.

with an elicitor preparation from *Phytophthora infestans* (Mont) de Bary, race 4 which catalyses the incorporation of radiolabel from [1-$^{14}$C]IPP into the phytoalexin lubimin. Preliminary results regarding the successful use of [2-$^{14}$C]MVA as a precursor in the same system are also given.

## RESULTS

### *Biosynthetic activity of discs and slices*

[2-$^{14}$C]MVA (0.5 $\mu$Ci) was applied in 20 $\mu$l amounts to the surface of tuber discs (prepared as described in Experimental) 48 hr after inoculation with elicitor. After periods of 0.5, 1 and 2 hr the whole discs were extracted (see Experimental) and radioactive products analysed. Labelling was found in rishitin and lubimin and also in a number of compounds which were incompletely resolved in the TLC system used (even when double developed) and had $R_f$ values of between 0.5 and 0.7. These compounds were eluted from the TLC plates together and analysed as a single compound, designated fraction H.

The rate of incorporation of MVA into rishitin, lubimin and fraction H was greatly increased at 2 hr compared to that at 30 min (Fig. 1A), probably reflecting the physical barriers to equilibration of the applied label with the biosynthetic pool. Discs which had been inoculated with sterile distilled water catalysed only low levels of incorporation of [2-$^{14}$C]MVA into compounds unresolved on TLC which migrated with an average $R_f$ within the limits found for fraction H. No labelling of rishitin or lubimin was found (results not shown).

Slices prepared from discs inoculated with elicitor were found to incorporate [2-$^{14}$C]MVA and [1-$^{14}$C]IPP (Figs 1B and 1C respectively) into rishitin and lubimin, albeit at very low levels under the conditions used. There was also some labelling of fraction H. Incorporation of MVA into rishitin was highest in the second layer slices (i.e. 0.5–1.0 mm), being double that observed in the other slices. The levels of rishitin labelling from IPP was similar in the first and second layer slices, but was greatly reduced in the third. Labelling of lubimin from both precursors was greatest in the first layer slices but maximum levels were only approximately 15% of that for rishitin. Incorporation of MVA into fraction H increased through the layers, but that of IPP was greater in the first and second layers. Incorporation of MVA and IPP into phytoalexins was not observed in slices cut from discs treated with sterile distilled water. Compounds chromatographing on TLC within the range of fraction H were labelled with both MVA and IPP in these slices, but levels were low and little difference was seen between the slices from the three layers (results not shown).

The results demonstrated that potato tuber tissue under these conditions actively biosynthesized phytoalexins and was therefore used to make cell-free enzyme preparations.

### *Preparation of the cell-free enzyme system*

The top 0.5 mm of tissue was removed from discs which had been aged for 24 hr and inoculated with either elicitor or sterile distilled water for a period of 48 hr (see Experimental). This tissue was weighed and, using a mortar and pestle with acid washed sand, homogenized in potassium phosphate buffer (0.1 M, 1.25 ml. g fr. wt tissue$^{-1}$), pH 8.0, containing sucrose (0.5 M), polyvinylpyrrolidone (8%), EDTA and 2-mercaptoethanol (each at $1 \times 10^{-2}$ M). The homogenate was centrifuged at 600 *g* for 15 minutes and a 2.0 ml aliquot applied to the top of a PD10 column (Pharmacia) equilibrated in running buffer which comprised potassium phosphate ($5 \times 10^{-2}$ M), pH 7.5, containing sucrose (0.5 M), EDTA and 2-mercaptoethanol (each at $1 \times 10^{-3}$ M). The sample was allowed to run into the top of the gel, as was 0.5 ml running buffer. Further running buffer was applied to the top of the column and the first 2.5 ml effluent was discarded. The

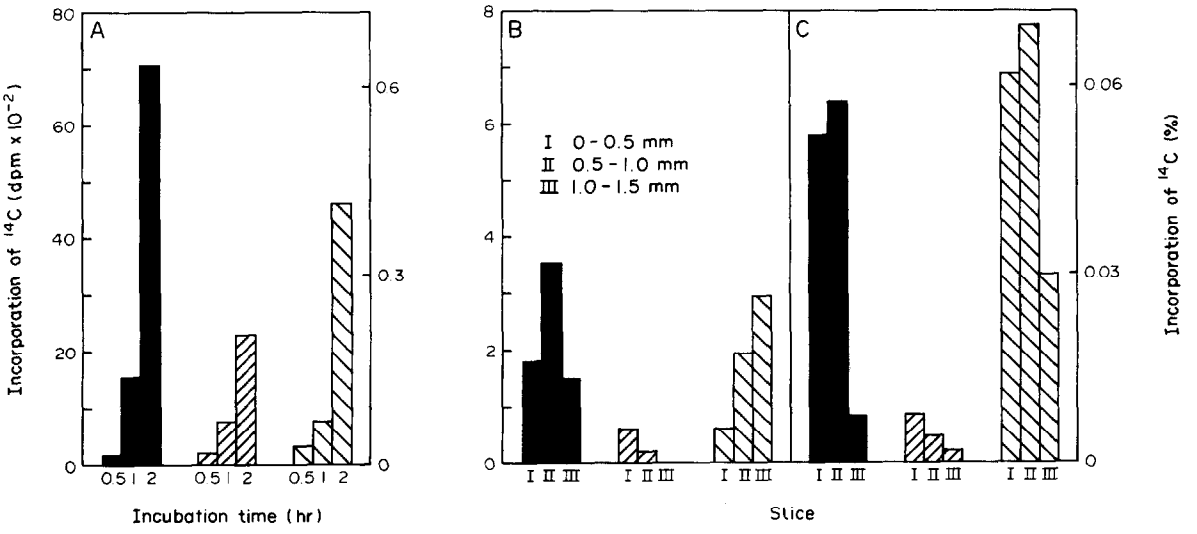



Fig. 1. Incorporation of label from DL-[2-$^{14}$C]MVA and [1-$^{14}$C]IPP into rishitin (■), lubimin (▨) and fraction H (▩) by potato tuber discs and slices: (A) incorporation of DL-[2-$^{14}$C]MVA by discs 48 hr after inoculation with elicitor, incubation times as shown; (B) and (C) incorporation of DL-[2-$^{14}$C]MVA and [1-$^{14}$C]IPP respectively by three successive (I–III) 0.5 mm thick slices cut from discs 48 hr after inoculation with elicitor. Incubations were for 2 hr.

next 3.5 ml of effluent comprised the cell-free enzyme preparation (0.6 mg protein $ml^{-1}$) and was used in incubations as described in the Experimental.

*Incorporation of [1-$^{14}$C]IPP into lubimin and other metabolites over a period of 2 hr catalysed by cell-free enzyme preparation*

Cell-free enzyme preparations were incubated for 3–120 min with [1-$^{14}$C]IPP, the results being presented in Fig. 2. Incorporation of label into lubimin rose almost linearly over the first 30 min after which no large increase overall was observed. Labelling of fraction H was almost linear over the first 12 min, the rate being greater than that for lubimin. The rate of increase in labelling then slowed presenting a maximum rate at 25 min followed by a rapid fall over the subsequent 10 min. From 35 to 120 min there was, overall, a steady decrease in the amount of labelling seen in fraction H.

In a parallel experiment a cell-free enzyme preparation was made from control discs. Labelling of compounds which were incompletely resolved in the TLC system used and having an average $R_f$ similar to fraction H (designated fraction H') was seen to increase steadily over 60 min to a maximum level which was unchanged for a further 1 hr (results not shown).

Incorporation of [1-$^{14}$C]IPP into lubimin and fraction H was found to be dependent upon $Mg^{2+}$. In incubations in which $MgCl_2$ was omitted, no ether extractable metabolites of IPP were observed. The use of NADPH (rather than NADPH generating system) was ineffective in the cell-free system, as were NADP, NAD and NADH. In certain experiments cell-free incubations were performed for an initial 30 min at which point further additions were made of [1-$^{14}$C]IPP, or the NADPH generating system or its individual components, and the incubations continued for a further 30 min. The levels of incorporation of [1-$^{14}$C]IPP into lubimin and fraction H were compared to unsupplemented 60 min incubations. No increases in labelling were observed in any of the supplemented incubations (over that present in 60 min incubations) except in the case of fraction H labelling in the presence of additional glucose-6-phosphate.

It should be noted that preformed rishitin and lubimin in the discs (from which cell-free enzyme preparations were made) were removed from the extracts during passage through the PD10 columns. Lubimin, at the level of 0.37 nmoles, was totally inhibitory as regards [1-$^{14}$C]IPP incorporation into ether extractable material in the cell-free enzyme incubations.

*Changes in the level of lubimin biosynthesis by cell-free enzyme preparations derived from discs 0–72 hr after inoculation with elicitor*

Cell-free enzyme preparations were made from tuber discs 0–72 hr after inoculation with elicitor and incubated with [1-$^{14}$C]IPP for 5, 15 and 60 min, the patterns of lubimin labelling being presented in Fig. 3. Incorporation of label from [1-$^{14}$C]IPP into lubimin was clearly seen in 15 min incubations of cell-free enzyme preparations made only 3 hr after inoculation of discs with elicitor. The biosynthetic capacity of the derived enzyme preparations increased up to 48 hr after inoculation and thereafter declined, as evidenced by the 15 min incubations. That the 60 min incubations do not fully reflect this pattern is probably due to [1-$^{14}$C]IPP becoming limiting in the longer incubations with the most active preparations. In parallel experiments with enzyme preparations from sterile distilled water inoculated discs, some lubimin synthesis was noted in 60 min incubations of enzyme preparations made 60 hr after inoculation. The level of lubimin synthesis rose slightly 72 hr after inoculation, but the maximum level of activity seen was less than 1% of that in the corresponding incubation of cell-free enzyme preparations from discs inoculated with elicitor.

An estimation of the mass of phytoalexin in these discs was not possible, but parallel sets of discs were used to obtain information regarding the accumulation of phytoalexins over the first 72 hr of treatment. It was observed that after a short lag period, the rate of accumulation of rishitin and lubimin rapidly increased and was fairly linear from 12 hr. Between 48 and 72 hr the rate of accumulation slowed but the levels present at 72 hr were still some 25% higher than at 48 hr.

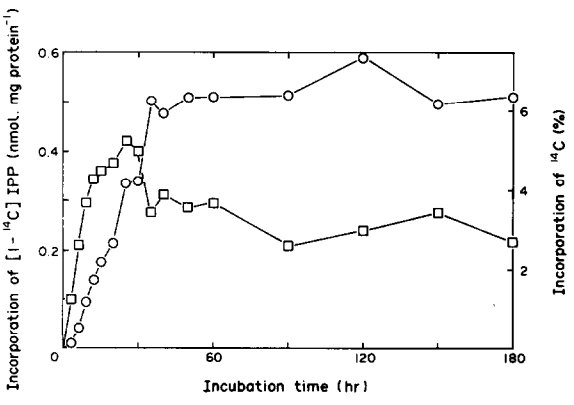



Fig. 2. Incorporation of label from [1-$^{14}$C]IPP into lubimin (○) and fraction H (□) over 180 min by cell free enzyme preparations made from potato tuber discs 48 hr after treatment with elicitor.

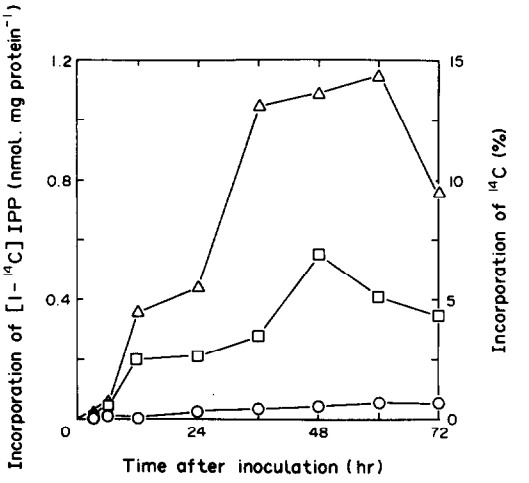



Fig. 3. Incorporation of label from [1-$^{14}$C]IPP into lubimin by cell free enzyme preparations made from potato tuber discs 0–72 hr after treatment with elicitor. Incubations were for 5 (○), 15 (□) and 60 (△) min.

Incorporation of label into fractions H and H' was seen as early as 3 hr after inoculation of the discs. Labelling of fraction H exhibited an overall increase over the 72 hr inoculation period, although fluctuations were quite marked. Generally, labelling in 15 min incubations was higher than that in 60 min incubations, correlating with the findings in the previous section. In the control experiment the rise in labelling of fraction H was more gradual over the 72 hr period and appeared to plateau between 48 and 60 hr after inoculation. Incorporation of label from [1-$^{14}$C]IPP into fraction H' in 60 min incubations was approximately double that in 15 min incubations at all time points.

*pH dependence of incorporation of [1-$^{14}$C]IPP into lubimin and other metabolites catalysed by cell-free enzyme preparations*

Incubations of cell-free enzyme preparations made 48 hr after inoculation of discs with elicitor were modified with regard to the phosphate buffer so that the final pH of incubations were in a series between 6.5 and 8.2 without alteration of ionic strength. Incubations with [1-$^{14}$C]IPP were performed for 5 and 60 min, the results being presented in Fig. 5. Incorporation of [1-$^{14}$C]IPP into lubimin (Fig. 5A) exhibits a pH optimum of between 7.3 and 7.5. At pH 7.1 and 7.7 incorporation was 40% and 55% of the maximum seen in 60 min incubations and 60% and 55% of that in 5 min incubations respectively. At pH 6.6 and 8.2 the levels of incorporation were almost negligible in both 5 and 60 min incubations.

Maximum incorporation of [1-$^{14}$C]IPP into fraction H (Fig. 5B) was also observed to be between pH 7.3 and 7.5 in 5 min incubations, but appeared to be shifted towards a more alkaline pH in 60 min incubations. In the 5 min incubations the levels of incorporation into fraction H were 68% and 46% of the maximum at pH 7.1 and 7.7 respectively, and was very low at pH 6.6 and 8.2. In 60 min

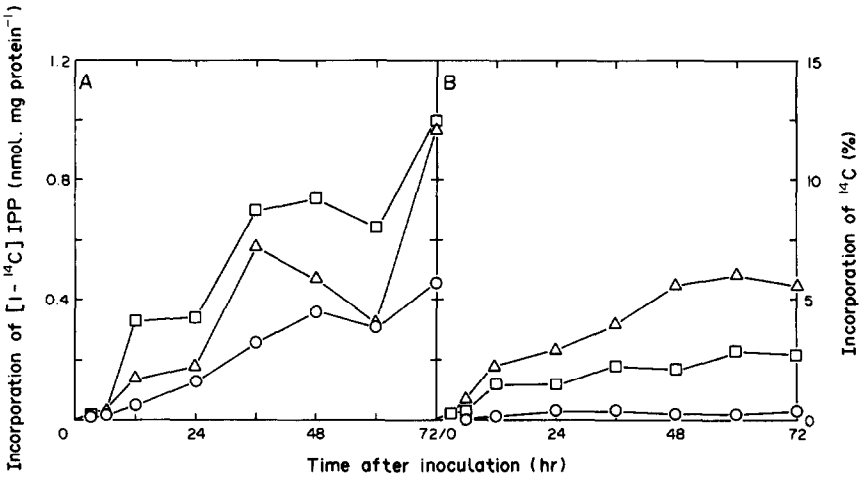



Fig. 4. Incorporation of label from [1-$^{14}$C]IPP into (A) fraction H and (B) fraction H' by cell-free enzyme preparations made from potato tuber discs 0–72 hr after treatment with elicitor or sterile distilled water respectively. Incubations were for 5 (○), 15 (□) and 60 (△) minutes.

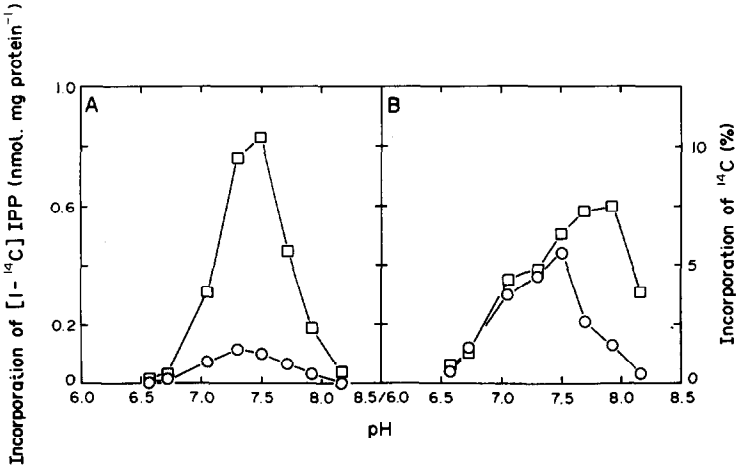



*Fig. 5.* Dependence upon pH of the incorporation of label from [1-$^{14}$C]IPP into (A) lubimin and (B) fraction H by cell-free enzyme preparations made from potato tuber discs 48 hr after treatment with elicitor. Incubations were for 5 (○) and 60 (□) min.

incubations the incorporation was 96% of the maximum at pH 7.7 and remained at 52% at pH 8.2.

*NADPH dependence of the biosynthesis of lubimin by cell-free enzyme preparations made from discs 48 hr after inoculation with elicitor*

Incubations of the cell-free enzyme preparations with various amounts of NADPH-generating system were performed for 30 min (Table 1). In the absence of the NADPH-generating system, the levels of incorporation of [1-$^{14}$C]IPP into lubimin were in the order of 5% of that seen in normal incubations containing 0.25 μmol NADP. Fraction H labelling was found to be three times higher in these incubations compared to normal. With the inclusion of NADPH-generating system containing 0.05 μmol NADP levels of incorporation similar to those seen in normal incubations were observed, and labelling of fraction H had fallen by nearly 50%. Slightly reduced levels of lubimin labelling compared to normal were observed with higher levels of NADPH-generating system, but no large increase was seen until the inclusion of four times the normal amount. In these incubations the pH was found to have become more acid, even though the additional NADPH generating systems had been introduced in buffered solution. Fraction H labelling was also observed to decrease, compared to that in incubations with 0.5 μmol NADP, but not to such a great extent.

*Inhibition of lubimin biosynthesis in cell-free enzyme preparations, by iodoacetamide and its reversal by DMAPP*

Iodoacetamide (20 nmol) and DMAPP (4 nmol) were added separately and in combination to incubations of [1-$^{14}$C]IPP with cell-free enzyme preparation made 48 hr after inoculation of discs with elicitor. Incubations were performed in the presence and absence of NADPH generating system for 5, 15 and 60 min, the results being presented in Fig. 6.

Incorporation of IPP into lubimin in normal incubations (Fig. 6A) was almost completely inhibited by the addition of iodoacetamide (Fig. 6B). The addition of DMAPP to iodoacetamide inhibited incubations (Fig. 6C) resulted in the restoration of labelling of lubimin to a level over and above that seen in normal incubations and also in 60 min incubations containing DMAPP but not iodoacetamide (Fig. 6D). In these latter incubations, incorporation of [1-$^{14}$C]IPP into lubimin was greatly increased over that normally seen at all time points, and also almost double that observed in 5 and 15 min incubations containing iodoacetamide and DMAPP together. As expected, negligible levels of lubimin labelling were seen in incubations where NADPH-generating system was omitted (Fig. 6E–H).

Labelling of fraction H in incubations in which NADPH-generating system was present exhibited similar responses to iodoacetamide and DMAPP as seen for lubimin. Marked differences were apparent, however, in incubations in which there was no addition of NADPH-generating system. When DMAPP was added to iodoacetamide inhibited incubations (Fig. 6G) incorporation of [1-$^{14}$C]IPP into fraction H was greatly increased over that seen not only in normal incubations (Fig. 6E), but also in incubations in which DMAPP was included but iodoacetamide was not (Fig. 6H).

## DISCUSSION

A series of experiments [T. Coolbear, unpublished work] on Kennebec tuber discs and slices inoculated with an elicitor preparation from *Phytophthora infestans* confirmed that the stressed tissue incorporated label from DL-[2-$^{14}$C]MVA and from [1-$^{14}$C]IPP into rishitin and lubimin. Incubations using the elicitor preparation as the only source of enzyme protein did not present any activity with regard to IPP metabolism. The levels of incorporation were optimum 48 hr after treatment and so this protocol was used as a basis for the preparation of the cell-free enzyme system.

The use of polyvinylpyrrolidone (PVP) to adsorb phenolic compounds (which, through hydrogen bonding or covalent condensation after oxidation, combine with and thus inhibit enzymes) is well documented [20–22]. The inhibition of phenol oxidases by chelating agents such as EDTA and by thiols has also been reviewed [22]. With regard to PVP, both soluble and insoluble forms have been used in the isolation of plant enzymes and organelles (reviewed by ref. [21]). Relevant to the present study is the use of the insoluble Polyclar AT in the preparation of extracts of *Ricinus communis* seedlings (e.g. [9]),

Table 1. Incorporation of label from [1-$^{14}$C]IPP into lubimin and fraction H: dependence upon NADPH-generating system

| Amount of generating system (μmol NADP*) | Incorporation of [1-$^{14}$C] (μmol mg·protein$^{-1}$) | |
|---|---|---|
| | Lubimin | Fraction H |
| 0.0 | 0.029 (0.36)† | 0.529 (6.61) |
| 0.05 | 0.714 (8.93) | 0.293 (3.66) |
| 0.25‡ | 0.704 (8.80) | 0.171 (2.14) |
| 0.40 | 0.593 (7.41) | 0.139 (1.74) |
| 0.50 | 0.489 (6.11) | 0.136 (1.70) |
| 1.00 | 0.129 (1.61) | 0.064 (0.80) |

Cell-free enzyme preparations were made from discs 48 hr after inoculation with elicitor. Incubations were for 15 min.

*Initial amount added as part of NADPH-generating system.

†Figures in parentheses give percentage incorporation.

‡Normal level used in incubations.

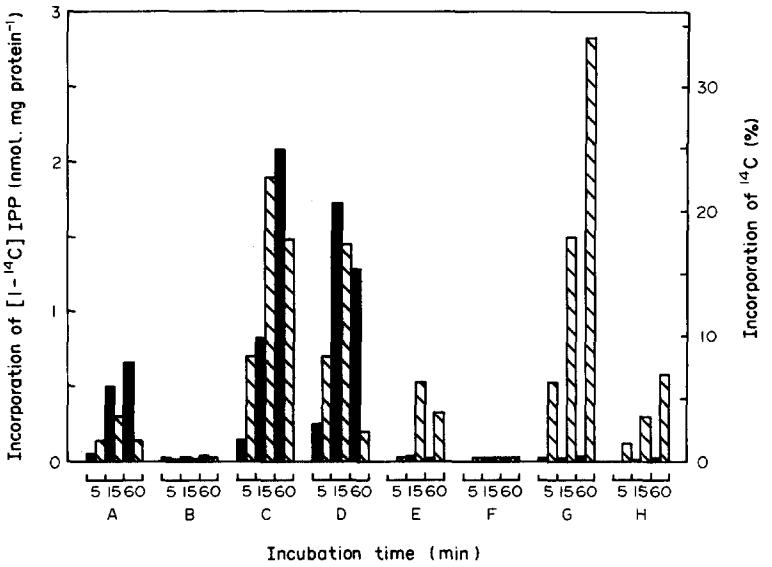



Fig. 6. Effect of the addition of iodoacetamide and DMAPP and the exclusion of NADPH generating system (GS) on the incorporation of label from [1-$^{14}$C]IPP into lubimin (■) and fraction H (▨) by cell free enzyme preparations made from potato tuber discs 48 hr after treatment with elicitor: (A) normal conditions; (B) + iodoacetamide; (C) + iodoacetamide + DMAPP; (D) + DMAPP; (E) − GS; (F) − GS, + iodoacetamide; (G) − GS, + iodoacetamide, + DMAPP; (H) − GS, + DMAPP. Incubations were for 5, 15 and 60 min.

*Phaseolus vulgaris* and *Zea mays* leaves [23], *Pisum sativum* fruits [24] and *Ipomoea batatas* roots [25]. These preparations have been used to investigate the biosynthesis of various terpenoids. Banthorpe *et al.* [5] have reported the use of both insoluble and soluble PVP to enhance prenyltransferase activity in *Tanacetum vulgare* leaf extracts. In the present study soluble PVP was used to protect the cell-free enzyme preparation from phenolic disruption during homogenization and the presence of mercaptoethanol was essential for the maintenance of enzyme activity through the chromatographic procedure.

The cell-free enzyme system was found to synthesize not only lubimin from [1-$^{14}$C]IPP, but also a number of other metabolites (fraction H) which remained unidentified during the course of the work described here. It was noticeable that rishitin was not generally produced by the system, this activity being observed on one unique occasion only. In this instance the cell-free enzyme system had been derived from tissue which had been initially prepared from Kennebec tubers harvested the same day from the University of Hull Botanical Gardens. Lack of tissue prevented further investigation of this finding. Accumulation of rishitin was apparent in inoculated discs prepared from potato tubers at any stage of storage, although the actual amounts of lubimin and rishitin relative to each other varied.

The nature of the components of fraction H are still under investigation. The differences in the labelling patterns observed in incubations of cell-free extracts prepared from elicitor inoculated discs compared to those from sterile distilled water inoculated discs indicate that fraction H contains at least one component that is induced by the elicitor treatment. This component would appear to be labelled rapidly and then further metabolized, thus presenting the peak of labelling of fraction H seen at 25–30 min incubation time (Fig. 2). A second component of fraction H appears to have a similar time course of labelling as that for fraction H′ from control tissue, i.e. a gradual increase to a maximum at 60 min, and may prove to have the same identity. Whether the rapidly labelled component of fraction H is a precursor of lubimin is being investigated.

The different pH optima observed in 5 and 60 min incubations with regard to fraction H labelling supports the contention of two distinct components. In 5 min incubations the rapidly labelled component would predominate and its coincident pH optimum with that for lubimin biosynthesis may support a speculative precursor relationship.

When cell-free enzyme preparations were made at various times after inoculation of discs with elicitor, lubimin biosynthesis was observed in preparations made only 3 hr after inoculation. A point of further interest was that in preparations made from discs inoculated with sterile distilled water, labelling of fraction H′ was also dependent upon the time at which the discs were processed, up to 48 hr after inoculation. This may be the result of increased biosynthetic activity in the discs due to an ageing process. It is known that during ageing of discs respiration rate and protein and RNA synthesis increases. Carbohydrate and phospholipid metabolism is also stimulated as in the *de novo* synthesis of enzymes from storage tissue (see refs [26–28]). Sodium [1-$^{14}$C]acetate labelling of certain sterols and their precursors is also stimulated during ageing [29].

The dependence of lubimin biosynthesis upon NADPH, and the observations that the cytochrome P450 inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) inhibits the labelling of lubimin from DL-[2-$^{14}$C]MVA when applied to the surface of metabolizing potato discs [T. Coolbear, unpublished work], indicate the involvement of cytochrome P450 monooxygenases in

the oxidation of precursors of lubimin. Furthermore, it has now been demonstrated that the hydroxyl groups of rishitin and lubimin are derived from molecular oxygen [30]. The presence of low levels of lubimin biosynthesis in the absence of exogenous NADPH would tend to indicate the existence of an endogenous pool of reducing power. The passage of the cell-free extract through the PD10 columns would have been expected to remove any free NADPH from the extract. This may indicate a pool of protein bound NADP or NADPH in the extracts, which do contain NADP reducing activity. The increased level of labelling of fraction H in the absence of exogenous NADPH can be explained in terms of the accumulation of a lubimin precursor, the formation of which is not NADPH dependent.

It was conceivable that the labelling of lubimin from [1-$^{14}$C]IPP was the result of the incorporation of IPP at the level of FPP synthetase rather than at IPP isomerase. Iodoacetamide, an inhibitor of the latter enzyme, was used to distinguish between these possibilities. The marked inhibition of labelling of lubimin in incubations with iodoacetamide clearly supports the contention that the majority of the IPP is entering the pathway at the earlier point and therefore accounts for all the carbon atoms of lubimin. The low level of lubimin labelling in the presence of iodoacetamide may be the result of incomplete inhibition or the presence of an endogenous pool of either preformed DMAPP or GPP. Again, free molecules of either compound would have been removed during the preparation of the cell-free extracts, but protein bound pools of DMAPP have been postulated in earlier work [5].

The reversal of iodoacetamide inhibition by DMAPP leads to some problems in interpretation of the results. The level of labelling of lubimin in incubations containing both DMAPP and iodoacetamide was higher than in the unmodified incubations. The actual amount of lubimin produced may well be higher than that indicated by the levels of labelling since the specific activity of the lubimin would be decreased by virtue of the DMAPP being unlabelled. Since no isomerization of DMAPP should occur one of the three isoprene residues constituting the lubimin skeleton would be unlabelled and the specific activity reduced by one third. The observed increases in labelling may either reflect the fact that IPP does not need to be isomerized to give DMAPP, therefore omitting one step in the pathway, or be a result of the high concentration of DMAPP causing the rate of reaction to increase to a level that more than compensates for label dilution effects. This rationale can be extended to explain the apparently anomalous levels of lubimin labelling in incubations in which DMAPP was included without iodoacetamide. In these incubations the level of labelling at 60 min was markedly less than at 15 min and also less than that seen in 60 min incubations with DMAPP and iodoacetamide together. In the absence of iodoacetamide, IPP isomerase would be active and therefore the exogenous DMAPP could contribute to the biosynthetic pool of IPP, thus diluting out the labelled substrate. The incorporation of [1-$^{14}$C]IPP into lubimin would therefore be reduced. A decrease in the level of labelling at 60 min compared to 15 min would imply further metabolism of lubimin. This was not observed in unmodified incubations, therefore either metabolism occurs with the higher concentrations of lubimin, or other factors are operative. Fraction H labelling would be subject to similar effects of dilution of label and the results interpreted accordingly.

A series of preliminary experiments (results not shown) were undertaken on the incorporation of label from DL-[2-$^{14}$C]MVA into metabolites by the cell-free enzyme preparation. Using the standard incubation negligible activity was observed and was not significantly different in the presence of various concentrations of ATP. When an ATP-generating system (comprising ADP, creatine phosphate and creatine kinase EC 2.7.3.2, with additional $MgCl_2$) was employed, however, labelling of lubimin and fraction H was greatly increased. The ATP-generating system was also found to slightly enhance the incorporation of label from [1-$^{14}$C]IPP into lubimin whereas ADP and ATP alone inhibited synthesis, as did additions of bovine serum albumen and lubimin (0.37 $\mu$mol).

The biosynthesis of lubimin and rishitin from IPP may well follow the pathway shown in Scheme 1 although other routes have been postulated (see ref. [16]). The intermediacy of solavetivone and lubimin in the formation of rishitin was proposed on the basis of the feeding of unlabelled solavetivone [31] and isolubimin [32] to healthy potato tuber tissue. $^{13}$C NMR studies did not support the postulated conversion of dihydrolubimin to lubimin [33], but experiments with [8,8-$^{2}H_2$]solavetivone confirmed a precursor relationship via lubimin and hydroxylubimin in aged tissue [34]. The conversion of [2-$^{14}$C]acetate to rishitin via [$^{14}$C]hydroxylubimin has also been shown in potato slices inoculated with *P. infestans* zoospores [35].

The cell-free enzyme system described here contains all the necessary enzymes for the production of lubimin from IPP (and from MVA to IPP). If the pathway in Scheme 1 applies, this would include IPP isomerase, FPP synthetase, FPP cyclase and various oxidative enzymes. Whether the enzymes for conversion of lubimin to rishitin are absent or inactive remains to be determined.

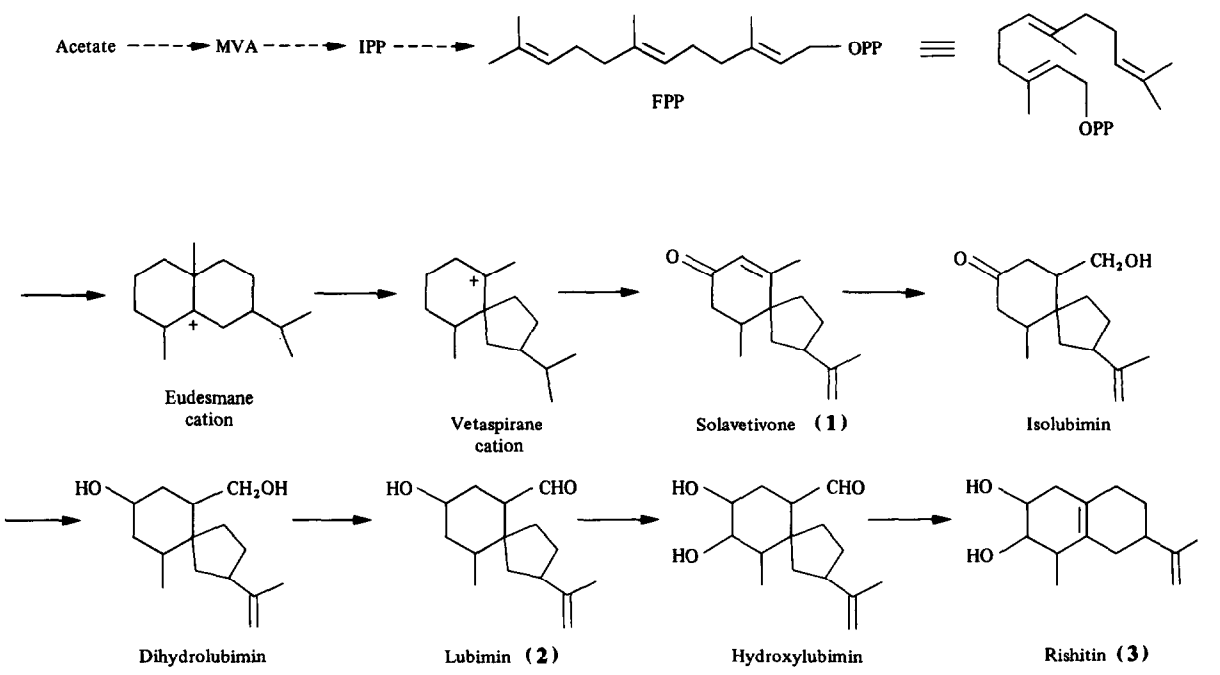
## EXPERIMENTAL

*Media and chemicals.* Pea agar was prepared by homogenizing 125 g peas in 500 ml distilled water using a Waring Blender. The homogenate was strained through two layers of muslin, poured into 500 ml 4% Oxoid agar No. 3 and the total volume made to 1 l. The pea agar was then autoclaved at 121° for 15 min.

Toluene based scintillation fluid for counting radioactive samples was prepared by dissolving 6 g 2,5-diphenyloxazole (PPO), 0.3 g 1,4 di[2-(5-phenyloxazolile)]-benzene and 100 g naphthalene in 1 l. toluene to which was added 300 ml ethoxyethanol. DL-[2-$^{14}$C]MVA (53 mCi·mmol$^{-1}$) and [1-$^{14}$C]IPP triammonium salt (53 mCi·mmol$^{-1}$) were from Amersham plc. Glucose-6-phosphate, G-6-P dehydrogenase (EC 1.1.1.49, 190–220 U·mg solid$^{-1}$), NADP and iodoacetamide were from Sigma. Polyvinylpyrrolidone (soluble form) was from BDH. Solvents were redistilled before use.

*Plant and fungal materials.* Tubers of *Solanum tuberosum* L. cv. Kennebec ($R_1$) were from a commercial grower (Roy, Huntley, Aberdeenshire, Scotland) and stored in the dark at 4°. *Phytophthora infestans* (Mont) de Bary, race 4 was obtained from the culture collection of the Department of Plant Biology, University of Hull and maintained on pea agar petri plates. Subculturing was undertaken after 10–14 days growth at 21° in the dark.

*Preparation of elicitor from* P. infestans. Petri plates of *P. infestans* on which the mycelial mat covered the surface, were held at −20° overnight. The mycelia were scraped off the frozen agar,




Scheme 1. Biosynthesis of lubimin and rishitin.

weighed and homogenized in 0.1 M KPi buffer, pH 7.0 (12.5 ml · g fr. wt mycelia$^{-1}$). The homogenate was sonicated for a total of 3 min at 75 W at 0° in 30 sec periods, then centrifuged at 30000 *g* (MSE 8 × 35 ml rotor) for 30 min. The supernatant was decanted, divided into 1 ml aliquots and stored at −20° until used as the elicitor preparation.

*Preparation of potato tuber discs.* When required, unwounded, non-photosynthetic tubers were washed, allowed to equilibrate overnight to room temp and then surface sterilized in 1.5% NaClO (15% aq. Everchlor: ICI), rinsed in absolute EtOH and allowed to dry in a laminar flow cabinet. Discs (1.0 mm diameter, 0.5 mm thick) were cut aseptically from the tubers using a cork borer and razor blades, avoiding the use of material within 1 cm of the surface of the tuber. The discs were rinsed three times in sterile distilled water, placed on wet filter papers in petri dishes and aged for 24 hr in the dark at 21°. After ageing the discs were inoculated with 20 μl of either elicitor or sterile distilled water (controls) and replaced in the dark at 21° for periods specified in the text. The discs were either used to make cell-free enzyme preparations (see text) and slices or extracted for phytoalexins as described below. Modifications to the above procedure necessitated by particular experiments are specified in the text.

*Preparation and incubation of potato tuber slices.* Three successive 0.5 mm thick slices were cut aseptically from tuber discs (prepared as above and treated for 48 hr) starting from the inoculated surface and bulked according to the layer from which they were taken. The slices were suspended in 5 ml 50 mM KPi buffer, pH 7.5, containing sucrose (0.5 M), EDTA ($1 \times 10^{-3}$ M) and mercaptoethanol ($1 \times 10^{-3}$ M) and incubated in 25 ml flasks for 2 hr at 30° with 0.5 μCi of either [2-$^{14}$C]MVA or [1-$^{14}$C]IPP. The discs were then removed and extracted as described below.

*Incubation of cell-free enzyme preparation.* The basic incubation of cell-free enzyme preparations (see text) comprised 0.75 ml enzyme (0.45 mg protein) in a total vol. of 1.375 ml containing $MgCl_2$ ($6 \times 10^{-3}$ M) and an NADPH-generating system equivalent to 0.18 mM NADPH consisting of NADP ($1.8 \times 10^{-4}$ M), glucose-6-phosphate ($2.4 \times 10^{-3}$ M) and glucose-6-phosphate dehydrogenase (0.73 U). After a 5 min pre-incubation of these components at 30°, 4 μl [1-$^{14}$C]IPP (0.2 μCi) was added and the incubations continued for periods specified in the text. The incubations were terminated by the addition of $Et_2O$ (5 ml) and the labelled products extracted (see below). Modifications of the basic incubation procedure are specified in the relevant sections of the Results.

*Extraction of discs and slices.* Finely diced discs or slices were homogenized in $CHCl_3$–MeOH (2:1) using 2 ml per disc. The homogenate was passed through a glass sinter (No. 3) under vacuum and the retained tuber tissue rehomogenized in a further vol. of $CHCl_3$–MeOH (2:1). This was again filtered, the residue on the sinter washed with $CHCl_3$–MeOH (2:1) and the two lots of filtrate and washings bulked. The aq. phase was removed and shaken with an approximately equal vol. of $CHCl_3$–MeOH (2:1). The resulting $CHCl_3$ layer was removed and bulked with the original $CHCl_3$ extract which was then washed (× 2) with $H_2O$. The $CHCl_3$ phase was carefully reduced to dryness under vacuum, using EtOH to remove the last traces of $H_2O$. The residue was taken up in a small, known vol. of $CHCl_3$–MeOH (2:1).

*Extraction of cell-free enzyme incubations.* The contents of flasks containing terminated cell-free enzyme incubations were decanted into tubes. The flasks were then rinsed with 5 ml $Et_2O$ and 5 ml $H_2O$ which were also decanted into the tubes. The $Et_2O$ layers in the tubes were removed and retained. The remaining aq. layers were extracted with further 5 ml lots of $Et_2O$ which had been used to rinse the flasks for a second time. The $Et_2O$ layers were removed and bulked with the initial extracts which were then reduced in vol. to 5 ml and washed (× 2) with 5 ml lots of $H_2O$. The washed $Et_2O$ extracts were carefully evaporated to dryness, using EtOH to remove the last traces of $H_2O$, and the residues taken up in a small, known vol. of $CHCl_3$–MeOH (2:1).

*Identification, purification and estimation of compounds extracted from discs and cell-free enzyme incubations.* Samples were subjected to TLC on silica gel G in EtOAc–cyclohexane (1:1). Visualisation of marker spots and authentic standards was

achieved by spraying with vanillin reagent (1 g vanillin, 0.2 ml $H_2SO_4$, 30 ml MeOH) and heating to 120°. 0.5 mm plates were used for $^{14}C$-labelled samples, 1.0 mm plates for the preparation of samples for GLC. Photographic records of the chromatographed radioactive samples were obtained using a Radiochromatogram Spark Chamber fitted with a Polaroid CU5 camera (Birchover Instruments Ltd.). These were used to locate the radioactive areas on the TLC plates which could then be removed and quantified using an Intertechnique Scintillation Counter, transferring the gel directly into 10 ml scintillant.

Samples to be analysed by GLC were eluted from the gel with $Me_2CO$. The extracts were taken to dryness under $N_2$, after having removed the gel by filtration through a glass fibre disc, and then redissolved in up to 20 $\mu$l $Me_2CO$—the actual vol. depending on the concn of extracted compounds. Estimation of phytoalexins in the extracts was by FID-GC (Pye Unicam 104 and GCD) using 3% OV 225 on Gas Chrom Q packed into a glass column (2.4 m × 2.0 mm). The conditions were: column temperature 190°, injection temp. 250°, detector temp. 300°, $N_2$ flow 40 ml. $min^{-1}$. Methyl stearate (1 $\mu g \cdot \mu l^{-1}$) was used as internal standard. In analyses where GLC was used to confirm the identities of radio-labelled compounds a glass lined stream splitter (S.G.E. Ltd.) was incorporated into the apparatus and set at 15% flow to the FID. Samples of undetectable mass content (i.e. those from cell-free incubations) were analysed before and after the addition of carrier phytoalexins. Effluent from the GC coincident with compounds detected by the FID were condensed onto cooled small bore glass tubes then eluted either with $Me_2CO$, if further TLC was to be undertaken, or with 10 ml of scintillation fluid directly into counting vials for radioactivity determinations. Fractions from around the detected compounds were also collected and were found to be free of radioactivity.

*Characterization of $^{14}C$-labelled lubimin from cell-free enzyme incubations.* Authentic samples of lubimin were converted to the alcohol 15-dihydrolubimin by reduction with methanolic $NaBH_4$. Monoacetyl-lubimin and diacetyl-15-dihydrolubimin were made from lubimin and 15-dihydrolubimin respectively using $Ac_2O$–$C_5H_5N$. The compounds were separated by TLC on silica gel G in *iso*-PrOH–EtOAc (1:9), giving the following $R_f$ values: lubimin, 0.59; 15-dihydrolubimin, 0.41; monoacetyl-lubimin, 0.71; diacetyl-15-dihydrolubimin, 0.75. On GLC (conditions as described earlier) the $RR_t$s (Me stearate = 1) of these compounds were lubimin, 2.18; 15-dihydrolubimin, 3.58; monoacetyl-lubimin, 2.18 (i.e. co-chromatographed with lubimin); diacetyl-15-dihydrolubimin, 3.08). Lubimin was also subjected to HPLC using a Spherisorb S5 ODS column (12.5 cm × 4.9 mm), eluting with MeOH–$H_2O$ (7:3) at a flow rate of 1.0 ml $min^{-1}$. An $R_t$ of 5.32 min was assigned to the authentic compound.

Samples of the $^{14}C$-labelled product co-chromatographing with lubimin on TLC in EtOAc–cyclohexane (1:1) were either mixed with authentic lubimin and derivatized as above or injected directly on to the HPLC column. The chromatographic (TLC, GLC and HPLC) properties of the derivatized and non-derivatized $^{14}C$-labelled compounds showed them to be identical to the authentic samples and confirmed the identity of the starting compound as $^{14}C$-lubimin.

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