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INCORPORATION OF [^{14}C]PHENYLALANINE AND [^{14}C]CINNAMIC ACID INTO CAPSAICIN IN CULTURED CELLS OF *CAPSICUM FRUTESCENS*

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Key Word Index—*Capsicum frutescens*; Solanaceae; cell suspension cultures; immobilized cells; secondary metabolism; capsaicin.

Abstract—Cells of *Capsicum frutescens* were supplied with either [^{14}C]phenylalanine or [^{14}C]cinnamic acid, when cultured as either free suspensions or when immobilized in reticulate polyurethane, under growth-promoting or growth-inhibiting conditions. Under both culture conditions, immobilized cells generally incorporated higher levels of radioactivity from the precursors into capsaicin throughout a 27-day culture period, although under growth-promoting conditions the relative amount of radioactive capsaicin accumulated was related to the phase of the growth cycle. The accumulation of radioactive and total capsaicin is reduced in culture conditions promoting cell division, and it is suggested that both protein and cell-wall metabolisms are potential sinks for capsaicin precursors and that the size of these sinks can be manipulated experimentally.

INTRODUCTION

The secondary metabolic activity of cultured cells of the chilli pepper, *Capsicum frutescens*, can be manipulated readily. The accumulation of the pungent principle capsaicin can be enhanced significantly by altering the physical organization of liquid-suspended cells as occurs when they are immobilized in reticulate polyurethane foam [1], by reducing the rate of cell division by omitting growth regulators [1] or nitrate [2] from the growth medium, and by exogenously supplying precursors to capsaicin [1, 3]. It can be hypothesized that all three forms of manipulation of the cells have biochemically common features; namely, they may each be effected according to the concept of an antagonistic relationship between primary and secondary metabolic pathways which may compete for common intermediates. Evidence in favour of such a hypothesis has been discussed for a number of secondary metabolites [2, 4–8].

Phenylalanine is a precursor to capsaicin and protein; cinnamic acid, the product of the reaction catalysed by phenylalanine ammonia-lyase (PAL, EC 4.3.1.5.) would also be expected to be incorporated into capsaicin, although this has not been demonstrated previously in cultured cells. Cinnamic acid has been reported to be involved in cell-wall metabolism [9] and this may proceed in competition with capsaicin synthesis. The aim of this paper, therefore, is to study and compare the patterns of incorporation of [^{14}C]phenylalanine and [^{14}C]cinnamic acid into capsaicin under growth (SHO medium) and non-growth (SHind medium) conditions in immobilized and suspended cultured cells of *C. frutescens*; and so to identify some of the limitations to capsaicin synthesis and accumulation.

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RESULTS

Growth characteristics of immobilized and freely suspended cells cultured in complete medium (SHO) and medium lacking nitrate and growth regulators (SHind)

Suspended cells (approximately 1.5 g fr. wt per flask) and immobilized cells (three polyurethane blocks, approximately 1.5 g fr. wt per flask) were grown on either complete modified medium (SHO), containing 2.5 $\times 10^{-6}$ M 2,4-D, 5×10^{-6} M kinetin and 24.72 mM nitrate, or with a medium lacking 2,4-D, kinetin and nitrate (SHind). Both immobilized and suspended cells cultured on SHind medium failed to show any increase in dry weight over a 27-day period (Fig. 1).On SHO medium, however, both types of culture exhibited sigmoidal growth curves: suspended cells had a lag phase of dry weight increase of between 6 and 9 days, and the lag phase of the immobilized cells was extended to between days 9 and 15; there was a rapid increase in dry weight between days 12 and 15 (Fig. 1). By days 21–24 the suspended cells had stopped increasing in dry weight, and the immobilized cells had stopped increasing by days 24–27. The suspended cells showed a greater dry weight increase than the suspended cells throughout the growth cycle, a pattern also reflected by a greater increase in fresh weight (data not shown).

The incorporation of [^{14}C]*cinnamic acid into capsaicin*

On incubation of cultures of immobilized and suspended cells (as described above) with 74 kBq of [3-side chain-^{14}C]cinnamic acid, approximately 80–90% of the initial radioactivity was removed from the medium within 2 hr, and the curve takes the form of a rectangular hyperbola.

Since capsaicin is predominantly released into the medium [10] and is not catabolized (Aitchison, P. A. and Yeoman, M. M., unpublished observations), it is possible

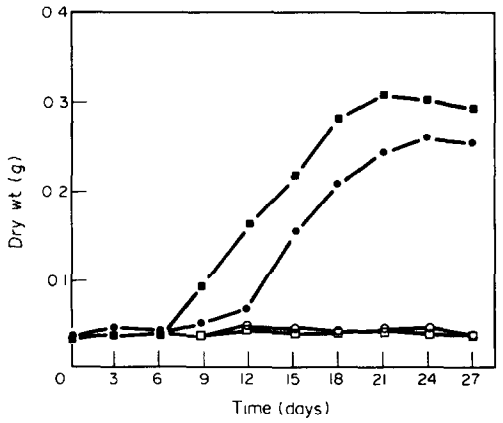

Fig. 1. Increase in dry weight of immobilized (circles) and freely suspended (squares) cell cultures in SHO (closed symbols) and SHind (open symbols) media.

that [^{14}C]capsaicin, newly synthesized from the [^{14}C]cinnamic acid, could accumulate rapidly in the medium and so give a reduced measurement of uptake of the precursor. In order, therefore, to investigate the short-term synthesis of capsaicin from exogenous cinnamic acid, cultures of immobilized cells were incubated for 48 hr in SHind medium and then supplied with 37 kBq of [^{14}C]cinnamic acid for 5, 15, 30 and 60 min. After each pulse, the cells were removed from the medium and both cells and medium were immediately assayed for [^{14}C]capsaicin. The results of this experiment are presented in Table 1, and it can be seen that even after a 5 min pulse of [^{14}C]cinnamic acid, radioactivity is found in capsaicin, representing 0.22% of the radioactivity in the medium extract and 0.28% of the radioactivity in the soluble cell extract. After a radioactive pulse of 60 min, a larger number of counts and a greater proportion of total radioactivity in the chloroform extract were found in capsaicin in the medium; but the amount of radioactivity in capsaicin in the cell extract was smaller after a 60 min pulse of precursor, suggesting its release from the cells into the medium. The radioactivity in the methanol-soluble cell extracts similarly declined with increasing pulse time. This decline could not be correlated with increasing radioactivity in the chloroform-soluble fraction of the medium, but there was a concomitant increase in the accumulation of radioactivity in the methanol-insoluble fraction of the cells. This result suggests some binding of cinnamic acid or its derivatives to the cell wall.

To measure more directly the release of radioactive capsaicin from immobilized cells, cultures were incubated for 48 hr in SHind medium, and then incubated for a further 24 hr in the same medium supplemented with 74 kBq of [^{14}C]cinnamic acid to label the capsaicin. After the labelling period the immobilized cells were washed with 10 ml of fresh SHind medium (lacking any precursor) and transferred at intervals to fresh SHind medium (again lacking any precursor) and the radioactive capsaicin released by the cells was determined by assaying extracts of the medium of each flask. The results of two such experiments are presented in Figs 2a and 2b. In the first experiment the short-term release of capsaicin was followed. Cells immediately after the labelling period (time 0)

Table 1. The fate of [^{14}C]cinnamic acid in immobilized cell cultures in SHind medium*

Duration of pulse (min)	Radioactivity in medium extract (a) (dpm × 10^{-3}/culture)			Radioactivity in soluble cell extract (b) (dpm × 10^{-3}/culture)			Radioactivity in insoluble (c) cell extract		% Recovery of radioactivity
	Capsaicin	Remainder	% Capsaicin	Capsaicin	Remainder	% Capsaicin	(dpm × 10^{-3}/culture)	(% total)	
5	3.1 ± 0.3	1398.1 ± 7.4	0.22	0.7 ± 0.07	244.9 ± 2.7	0.28	672.0 ± 28.4	30.3	97.6
15	3.7 ± 0.2	1033.5 ± 5.2	0.36	0.7 ± 0.05	155.9 ± 8.4	0.46	781.9 ± 22.4	35.2	97.3
30	5.0 ± 0.4	964.7 ± 12.4	0.51	0.2 ± 0.01	95.2 ± 10.2	0.24	813.1 ± 40.4	36.6	97.2
60	5.7 ± 0.4	665.8 ± 14.3	0.86	0.2 ± 0.01	60.5 ± 10.0	0.28	1841.1 ± 47.3	83.5	99.4

*Cultures were supplied with 37 kBq of precursor for pulses of either 5, 15, 30 or 60 min, after which time the following extractions were performed: chloroform extract of the medium (a) and methanol extract of the cells (b). Radioactivity was assayed in capsaicin and the remainder of each extract, including the methanol-insoluble cell fraction (c).

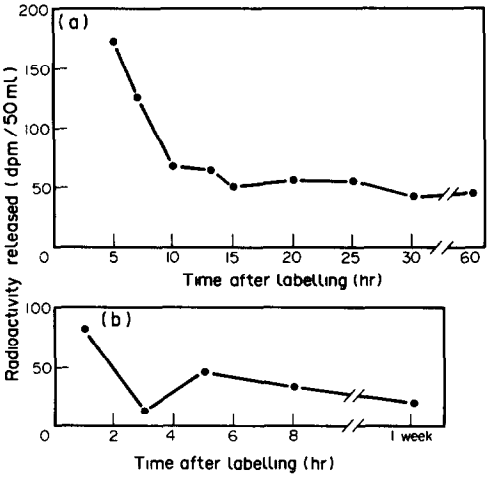

Fig. 2. Release of [^{14}C]capsaicin from immobilized cells cultured in SHind medium over a period of (a) 60 min and (b) 8 hr and 1 week.

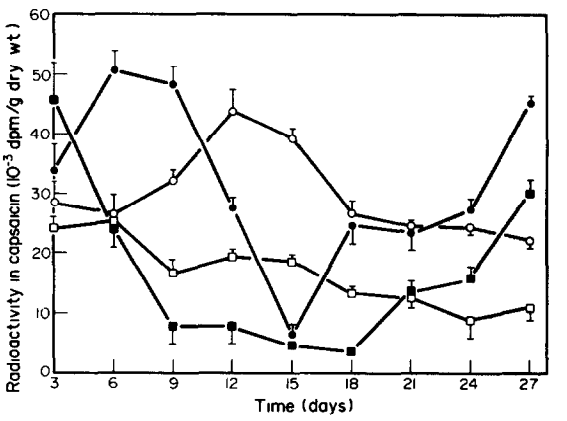

Fig. 3. Incorporation of [3-side chain-^{14}C]cinnamic acid into capsaicin in immobilized cells (circles) and freely suspended cells (squares) cultured in SHO (closed symbols) and SHind (open symbols), over a 27-day culture period. Bars represent half standard errors.

contained 729 dpm capsaicin, and on transfer to fresh medium there was observed a pattern of release of capsaicin which approximated to a hyperbolic curve, approaching an asymptote (Fig. 2a). Analysis of the immobilized cells themselves at 60 min indicated that significant levels of radioactive capsaicin were retained, more than five times the amount that was released in the previous 30 min, suggesting some intracellular binding of capsaicin, or its inaccessibility to the medium by compartmentalization. In the second experiment (Fig. 2b), the long-term release of capsaicin was followed, over a week's incubation. After the first hour in fresh medium, only very low levels of capsaicin accumulated in the medium. When the cells were assayed for their contents of radioactive capsaicin at 1 week, it was found that they contained a small amount of radioactivity (91 dpm), perhaps due either to binding or compartmentalization of capsaicin which was synthesized during the labelling period, or which was synthesized during the subsequent precursor-free incubation period; it is possible that [^{14}C]cinnamic acid and its derivatives were compartmentalized or bound (perhaps to the cell wall) and gradually released for capsaicin synthesis.

In order to investigate the accumulation of [^{14}C]capsaicin from [^{14}C]cinnamic acid over a longer time course, and to compare the pattern of incorporation in immobilized and suspended cells under growth and non-growth conditions, triple replicate cultures were set up as described above, supplied with 50 ml of either SHO (growth) or SHind (non-growth) media. The pattern of incorporation of [^{14}C]cinnamic acid into capsaicin was determined over a 27-day culture period, by supplying the cultures with 74 kBq of [3-side chain-^{14}C]cinnamic acid in 24 hr pulses at 3-day intervals. After each labelling period the uptake of radioactive precursor was estimated by measuring the uptake of radioactivity from the medium, and in all cases it was found to be not less than 80% of that added. The results of the incorporation of radioactivity into capsaicin are presented in Fig. 3.

(a) *In SHO (growth) medium.* Between days 3 and 9 (the lag and exponential phase) the level of radioactivity in capsaicin in suspension cultures declined dramatically to a steady level of 4000–8000 dpm/g dry wt between days 9 and 18 (linear phase). Between day 18 and 27 (stationary phase) the incorporation increased to approximately 30 000 dpm/g dry wt (day 27). The incorporation of radioactivity into capsaicin was greater in immobilized than in suspended cell cultures throughout the culture period after day 3; the level declined dramatically between days 9 and 15, to just over 6300 dpm/g dry wt and increased thereafter.

(b) *In SHind (non-growth) medium.* The radioactivity in capsaicin in suspended cell cultures in SHind medium was higher than that in suspended cell cultures in SHO medium between days 6 and 18, after which time it declined to a lower level. For the immobilized cell cultures, incorporation into capsaicin in SHind medium was greater than that in SHO medium between days 12 and 15, and also to a smaller extent between days 18 and 21, after which time it declined to a lower level. At all times in the experiment, immobilized cell cultures incorporated [^{14}C]cinnamic acid into capsaicin to a greater extent than did suspended cell cultures, in SHind medium.

The incorporation of [^{14}C]*phenylalanine into capsaicin*

Immobilized cells of *C. frutescens* have previously been shown to be able to synthesize capsaicin from radioactive phenylalanine [1, 2]. In order to investigate further this activity in immobilized cells, a comparison of the pattern of incorporation was made into freely suspended cells on growth and non-growth media over a 27-day culture period; the methodology was as for the previous experiment, except that the precursor supplied was 74 kBq L-[U-^{14}C]phenylalanine. The results are presented in Fig. 4.

(a) *In SHO (growth) medium.* The suspended cell cultures incorporated relatively low levels of radioactivity into capsaicin throughout the culture period, the levels being at their lowest between days 12 and 18 (approximately 3000 dpm/g dry wt), and increasing towards the end of the growth cycle. The level of incorporation in immobilized cell cultures was much greater, especially at

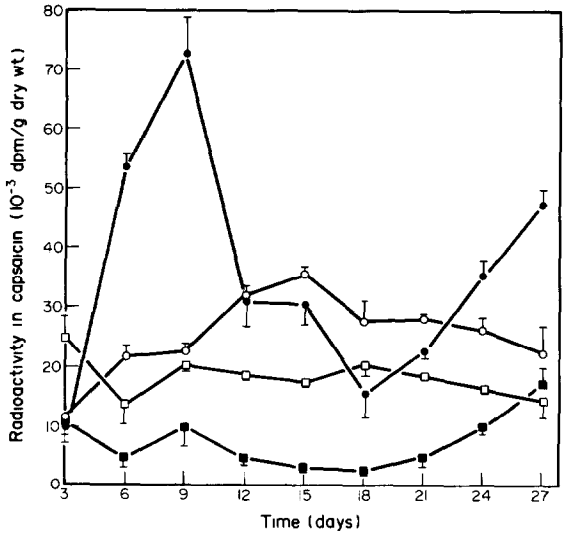

Fig. 4. Incorporation of L-[U-^{14}C]phenylalanine into capsaicin in immobilized cells (circles) and freely suspended cells (squares) cultured in SHO (closed symbols) and SHind (open symbols), over a 27-day culture period. Bars represent half standard errors.

days 6–9, when there was a more than seven-fold difference in the yield of [^{14}C]capsaicin between the two types of culture, a ratio which was approximately maintained to day 18, although the yield of radioactive capsaicin at this time was smaller. At day 27, there was a four-fold greater yield of radioactive capsaicin in the immobilized cell cultures.

(b) *In SHind (non-growth) medium.* The suspended cells incorporated higher levels of [^{14}C]phenylalanine into capsaicin than when on SHO medium (a greater than seven-fold difference at day 18) until day 18, when the yields declined in SHind medium and increased in SHO medium. The immobilized cell cultures on SHind medium produced higher yields of [^{14}C]capsaicin than those on SHO medium only between days 15 and 21, when the cells on SHO were dividing.

The accumulation of total capsaicin

The results of the production of total non-radioactive capsaicin by immobilized cells are presented in Fig. 5.

(a) *In SHO (growth) medium.* An increase in the rate of capsaicin accumulation was delayed until between days 15 and 18, with a maximum rate of accumulation between days 18 and 21. Thereafter the rate of accumulation increased only gradually to reach a level of approximately 0.3 mg/g dry wt per culture (50 ml) at day 27. This is significantly higher than the yield which was accumulated by cultures of freely suspended cells at day 27 (0.5–1.5 μg/g dry wt per 50 ml culture).

(b) *In SHind (non-growth) medium.* There was a generally higher yield of capsaicin throughout the culture period than in cells on SHO medium (e.g. a 4.7-fold greater yield at day 15), with an increase in accumulation between days 6 and 15 but no further production thereafter.

The synthesis and accumulation of soluble protein in immobilized cells

Previous results [1, 2] have suggested an inverse correlation between the incorporation of [^{14}C]phenylalanine into capsaicin and soluble protein. In order to investigate this relationship further, the incorporation of [^{14}C]phenylalanine into, and total accumulation of, soluble protein was determined at intervals in immobilized cells cultured on growth and non-growth media.

The incorporation of L-[U-^{14}C]phenylalanine (37 kBq) into soluble protein (TCA-insoluble fraction) after 30 min pulses was determined in triple-replicate cultures supplied with 50 ml of SHO or SHind, set up as described above, at days 0, 3, 9, 15 and 24. The results are presented in Fig. 6 and show that at each point in the culture cycle the cells cultured in SHO (growth) medium incorporated significantly higher levels of radioactivity into soluble protein—there was a linear increase in incorporation from ap-

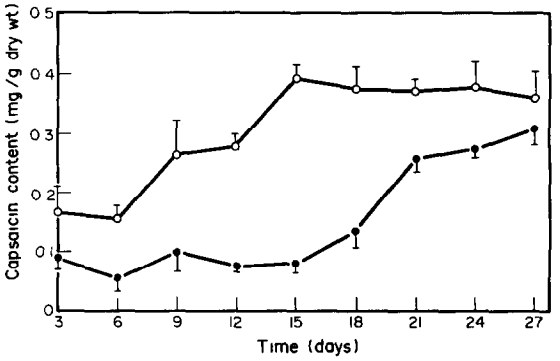

Fig. 5. Capsaicin accumulation by immobilized cells cultured for 27 days in SHO (closed circles) and SHind (open circles). Bars represent half standard errors.

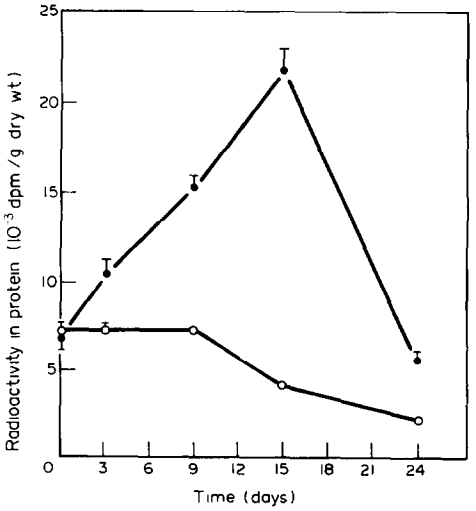

Fig. 6. Incorporation of L-[U-^{14}C]phenylalanine into soluble protein (TCA-insoluble fraction) by immobilized cells cultured for 24 days in SHO (closed circles) and SHind (open circles). Bars represent half standard errors.

proximately 7000 dpm/g dry wt up to day 15, to a value of approximately 22 000 dpm/g dry wt followed by a 75% drop in incorporation at day 24. Cells on SHind medium incorporated approximately the same amount of radioactivity (approximately 7000 dpm/g dry wt) up to day 9, after which time the level gradually declined. The greatest difference in incorporation between the cultures was observed at day 15.

Total alkali-soluble protein accumulation was measured, similarly, at days 0, 3, 9, 15 and 24, and the results are presented in Fig. 7. There was a general correlation between the pattern of total protein content and of incorporation of radioactivity into protein over the culture period, and there were similarly marked differences between cells cultured in SHO and SHind media. On SHO medium, total protein accumulated throughout the culture period, but the rate of accumulation declined slightly after day 15, reflected by a decline in incorporation of [^{14}C]Phe. Protein content declined after day 9 in SHind cultures, and protein content at day 24 was 58% that of SHO cultures at the same point in the culture period; there seemed to be degradation but no net synthesis of protein between days 9 and 24 on the non-growth medium.

The synthesis and accumulation of other soluble phenolics

On examination of chloroform and methanol extracts of the medium and cells, respectively, by TLC and HPLC, it was observed that the numbers and patterns of the major components, presumed to be soluble phenolics, were different. Up to five major TLC spots, visualized by the $FeCl_3$–$K_3Fe(CN)_6$ spray for phenolics and of R_f values 0.50, 0.29, 0.26, 0.22 and 0.09 in the chloroform–methanol system [1], were eluted and attempts made to identify them by (1) their retention times on HPLC, and (2) the UV spectral absorbance pattern and maxima of the HPLC peaks as measured by an on-line diode array spectrophotometer. There was no difference in their colour reaction to the spray. The five TLC spots had absorbance spectra with λ_{max} of either 240 and 292 nm (R_f 0.50) or 253, 266 and 285 nm (R_f 0.29, 0.26, 0.22 and 0.09), suggesting that the latter spots were compounds of closely related structures; but it was not possible to identify the compounds. Comparisons with the retention times and spectra of known intermediates of the capsaicin pathway (as listed in the Experimental) indicated that the compounds were none of these. Figures 8 and 9 show the pattern of incorporation of label from either [^{14}C]cinnamic acid or [^{14}C]phenylalanine, respectively, into total extracted phenolic compounds, after labelling with 74 kBq of precursor for 24 hr and extraction of the cultures at 3-day intervals, as described above.

(1) [^{14}C]*cinnamate into soluble phenolics* (*Fig.* 8). In SHO (growth) medium, cultures of immobilized cells

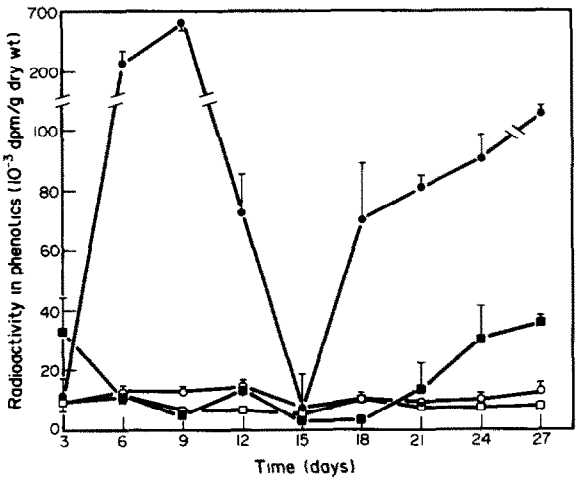

Fig. 8. Incorporation of [3-side chain-^{14}C]cinnamic acid into soluble phenolics in immobilized cells (circles) and freely suspended cells (squares) cultured in SHO (closed symbols) and SHind (open symbols), over a 27-day culture period. Bars represent half standard errors.

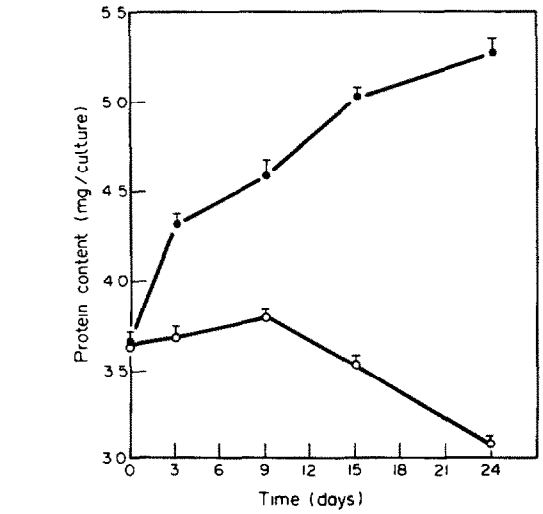

Fig. 7. Protein accumulation by immobilized cells cultured for 24 days in SHO (closed circles) and SHind (open circles). Bars represent half standard errors.

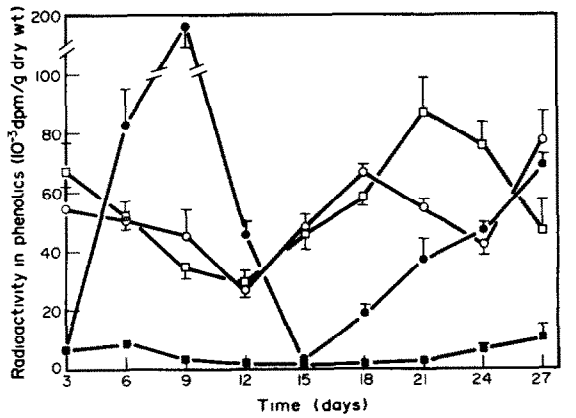

Fig. 9. Incorporation of L-[U-^{14}C]phenylalanine into soluble phenolics in immobilized cells (circles) and freely suspended cells (squares) cultured in SHO (closed symbols) and SHind (open symbols), over a 27-day culture period. Bars represent half standard errors.

incorporated increasingly high levels of radioactivity into phenolics up to approximately day 9. Between days 9 and 15, the levels of incorporation declined, but increased thereafter to day 28. Suspended cells incorporated relatively low levels into the soluble phenolics throughout the culture period, with greatest incorporation at the beginning (day 3) and end (days 24 and 28) of the period. In SHind (non-growth) medium, incorporation from [^{14}C]cinnamate was relatively low throughout the entire culture period in both immobilized and suspended cell cultures, with the immobilized cell cultures incorporating only slightly more radioactivity than the suspended ones.

(2) [^{14}C]*Phenylalanine into soluble phenolics* (*Fig.* 9). In SHO (growth) medium, a very similar pattern of incorporation to that from [^{14}C]cinnamic acid was observed for both immobilized and suspended cultures; the levels of incorporation from [^{14}C]Phe were, however, generally lower, e.g. 3.4-fold lower for the day 9 immobilized cells and 3.3-fold lower for the day 27 suspended cells.

In SHind (non-growth) medium, the incorporation of radioactivity from [^{14}C]phenylalanine was generally greater than that from [^{14}C]cinnamic acid in both immobilized and suspended cell cultures. For suspended cells, incorporation was greater in SHind than in SHO (greater than 37-fold at day 21), and in immobilized cells incorporation was greater than that in SHO only between days 15–21, i.e. during the time of the greatest rate of increase in dry weight (3.5 times greater incorporation in SHind at day 18). On non-growth medium, there was little difference in the extent of incorporation of [^{14}C]phenylalanine into soluble phenolics between immobilized and suspended cell cultures.

The effect of capsaicin on its own synthesis

In order to determine whether capsaicin accumulation in the medium has any influence on its own synthesis, the effect of a range of concentrations of exogenous capsaicin on the incorporation of [^{14}C]phenylalanine and [^{14}C]cinnamic acid into capsaicin was examined. Triple-replicate cultures of immobilized pepper cells were set up as described above in 50 ml of SHind medium and incubated for 24 hr. After 24 hr, 1 ml of solutions of capsaicin in 2% (v/v) methanol was added to the flasks to give a final concentration of 0.01, 0.05, 0.10 or 0.20 mM. Control flasks were supplied with 1 ml of 2% (v/v) methanol lacking capsaicin, and all were incubated for a further 24 hr. After this time, all cultures were supplied with 37 kBq of either L-[U-^{14}C]phenylalanine or [3-side chain-^{14}C]cinnamic acid and incubated for 24 hr, when determinations were made of the increases in cell fresh weight and incorporation of radioactivity into capsaicin. There was no detectable change in the fresh weight of any of the cultures over the total 3-day experimental period. Results of the analysis of radioactivity in capsaicin are presented in Table 2, and indicate that in cultures supplied with either radioactive precursor, incorporation into capsaicin decreased in a way which was directly correlated with the initial concentration of exogenously applied capsaicin. A concentration of 0.2 mM resulted in a 50.9% decrease in [^{14}C]phenylalanine incorporation and a 66.2% decrease in [^{14}C]cinnamic acid incorporation, compared with controls. Since there was no observable difference in the growth rate (fresh weight increase or decrease) of the cells under the experimental regime, it would seem likely that the effect is unrelated to any effect of capsaicin on the growth rate of the cultures.

DISCUSSION

Immobilization techniques have been applied to plant cells only relatively recently (see ref. [11] for review), and little work has been done to characterize, in biochemical terms, the effects of such techniques on the entrapped cells. Some observations have been made which suggest that immobilization alters the expression of secondary metabolic activity, resulting in the enhanced accumulation of specific secondary metabolites synthesized in multi-enzyme processes [11, 12] as opposed to one- or two-step biotransformations where the enhancement effects are less apparent [13]. It has been proposed [1, 2] that this enhancement of secondary metabolic activity in immobilized cells is associated with the reduced rate of cell division exhibited by cells cultured under such conditions, resulting in a divergence of metabolic intermediates from primary (essentially growth-associated) to secondary pathways. It has been argued that such a switch in emphasis from primary to secondary metabolism may not require the induction of *de novo* enzyme synthesis [7] and that

Table 2. Incorporation of radioactivity into capsaicin in cultures of immobilized cells cultured in the presence of capsaicin*

Concn of initial exogenous capsaicin (mM)	Radioactivity in capsaicin from [^{14}C]-Phe (% control)	Radioactivity in capsaicin from [^{14}C]-cinn. (% control)
0 (controls)	100†	100‡
0.01	89.7	84.8
0.05	68.7	65.3
0.10	54.9	41.5
0.20	49.1	33.8

*Cells were supplied with 37 Bq of either L-[U-^{14}C]phenylalanine or [3-side chain-^{14}C]cinnamic acid. Standard errors of three determinations are given.

†1772 ± 122 dpm/g fr. wt.

‡3432 ± 194 dpm/g fr. wt.

capsaicin accumulation is largely limited by precursor availability [2].

The results presented in this article support this concept. It should be noted that, since the specific activity of the [3-side chain-^{14}C]cinnamic acid added to the culture was approximately 10% that of the L-[U-^{14}C]phenylalanine, a similar level of radioactivity in, say, capsaicin from each precursor might indicate an approximately ten-fold greater flux of intermediates from [^{14}C]cinnamic acid, but, of course, this is dependent on dilution by endogenous metabolites. Chilli pepper cells which were entrapped in reticulate polyurethane foam and cultured on a growth medium incorporated significantly higher levels of radioactivity in both capsaicin and unidentified phenolics than did freely suspended cells throughout a 27-day culture period, when supplied with either [^{14}C]phenylalanine or [^{14}C]cinnamic acid. The level of incorporation of both these precursors was related to the phase of the growth cycle of the cells cultured on a growth medium, being reduced during the period of greatest increase in dry weight of the cultures; it was at this time that the difference in the extent of incorporation was least between the immobilized and suspended cells (Figs 3 and 4). In general, the greatest difference between the growth and non-growth media in their effect on incorporation into capsaicin in like cultures, that is, in either immobilized or suspended cell cultures, was observed when the growing cultures exhibited their greatest rate of increase in dry weight. Immobilized cells similarly accumulated higher total yields of capsaicin when cultured on a non-growth, compared with a growth-promoting, medium throughout the 27-day culture period. The pattern of incorporation of [^{14}C]phenylalanine into soluble protein in immobilized cells on growth medium increased to a maximum at day 15 (Fig. 6) when incorporation into capsaicin was declining rapidly and incorporation into the unidentified soluble phenolics was at a minimum (Fig 9), and this is consistent with the view that capsaicin synthesis is in part limited by a high rate of protein synthesis, which would limit the phenylalanine supply [2]. Since, however, it would not be expected that [^{14}C]cinnamic acid would be incorporated into protein, this argument does not explain the pattern of incorporation of this precursor into capsaicin. The results in Table 1 suggest that cinnamic acid is readily bound to the methanol-insoluble (mainly cell wall) fraction, an observation also recorded by Fry [9] in exponentially growing cultured spinach cells. It is therefore suggested that the inverse relationship between dry weight increase and incorporation of [^{14}C]cinnamic acid into capsaicin and soluble phenolics is due to the preferential esterification of cinnamic acid and its derivatives to the cell wall in dividing cells. If this is the case, then the cell wall can be considered to be, like protein, a sink for intermediates in capsaicin biosynthesis. Moreover, since only a small proportion (10–30%) of the total radioactivity from [^{14}C]phenylalanine accumulates in TCA-insoluble protein, the cell wall might be expected to be a larger sink for potential precursors of capsaicin than is protein, and in terms of the final accumulation capsaicin, a more important limitation than the activity of phenylalanine ammonia-lyase (PAL), which catalyses the first step of hydroxycinnamic acid metabolism and thence capsaicin synthesis from phenylalanine.

In relation to this is the apparent inconsistency in the pattern of labelling of capsaicin by both precursors, but particularly by [^{14}C]phenylalanine, and the total accumulation of capsaicin in growing immobilized cells. There was found to be a sharp increase in incorporation of [^{14}C]phenylalanine into capsaicin during the lag phase (Fig. 4), but there was no observable increase in the accumulation of capsaicin in cultures not supplied with precursor (Fig. 5). It is suggested that these observations show that although lag phase cells do not normally accumulate high yields of capsaicin (compared with stationary phase cells, on a w/w basis), they are nevertheless capable of doing so if supplied with a precursor, such as phenylalanine or cinnamic acid, indicating that in the lag phase the limitation to capsaicin production is at the level of precursor supply. There was significant protein synthesis during this period of the growth cycle (Figs 6 and 7), although an increase in the dry weight of the culture was not indicated; and it is possible that by addition of the precursor during the lag phase the demand for phenylalanine for protein synthesis was saturated. Addition of [^{14}C]cinnamic acid may similarly have been sufficient to saturate the binding sites in the cell wall. Addition of the same amount of precursor at a later stage of the growth cycle (day 15), at which time protein and cell wall synthesis was proceeding at a greater rate, was apparently insufficient to saturate these 'sinks' and incorporation into capsaicin was, it is suggested, thereby limited. The 'induction' of enzyme activity by the exogenous supply of phenylalanine or cinnamic acid cannot be excluded, since there is evidence that *trans*-cinnamic acid, at least, may induce the activities of enzymes in phenylpropanoid metabolism [14, 15].

The differences in the extent of incorporation of both [^{14}C]phenylalanine and [^{14}C]cinnamic acid between immobilized and suspended cells cultured in a growth-limiting medium are of some interest. Since neither the immobilized nor the suspended cultures exhibited an increase in either fresh or dry weight over the 27-day incubation period, the lower incorporation of the precursors into capsaicin in the suspended cells is unlikely to have been due to differences in the activities of growth-related processes. It is possible that, in the suspended cells, which were much more dispersed than the immobilized cells, the intermediates were more readily washed out of the cells into the liquid medium, so limiting incorporation into capsaicin. A second possibility is that immobilized cells possess higher activities of one or more enzymes of the capsaicin biosynthetic pathway, due, for example, to a higher amount of active enzyme(s), a reduced physical compartmentalization of enzyme(s) and substrate(s), or an enzyme or enzymes with lower K_m values for substrate(s), which could be dependent on the degree of structural organization or intercelluar communication between cultured cells. This possibility was first suggested by Lindsey and Yeoman [1] to explain the difference in response of immobilized and suspended cells to feeding with a precursor to capsaicin, isocapric acid, which had no known sink in growth-related metabolic processes. Both possibilities are now being examined. It is interesting to note that, in non-growing cultures of immobilized and suspended pepper cells (i.e. in SHind medium) there was relatively little or no difference in the extent of incorporation of either [^{14}C]phenylalanine or [^{14}C]cinnamic acid into soluble phenolics, although there was a difference in incorporation into capsaicin. This could be evidence for a rapid metabolism of soluble phenolics in non-dividing cells such that they do not accumulate to high levels in either immobilized or suspended cells. Preliminary results

(Hall, R. D., Holden, M. and Yeoman, M. M., unpublished observations) indicate that intermediates of the capsaicin pathway do not accumulate to significant levels in either cultured cells or in the intact chilli pepper fruit.

One further potential limitation to capsaicin production by cultured chilli pepper cells is the effect that accumulated capsaicin has on its own synthesis. The results in Tabl 2 show that exogenous concentrations of capsaicin as low as 10 μM caused a reduced incorporation of radioactivity from either [^{14}C]phenylalanine or [^{14}C]cinnamic acid. This could be due to a negative feedback inhibition effect of capsaicin on one or more of the enzymes in the aromatic part of the biosynthetic pathway, presumably closer to the product than PAL, since the incorporation of [^{14}C]cinnamic acid is adversely affected. It is also possible that the observed effect is due to an increase in the pool sizes of intermediates in the biosynthetic pathway due to the relatively high concentrations of end-product—this would result in a dilution of radioactivity and a low final specific activity of accumulated capsaicin and further work must be performed to determine this. In this context it is known that the activity of PAL is inhibited by the accumulation of cinnamic acid and other phenylpropanoids [16, 17]. Since capsaicin is rapidly released from the cells into the surrounding medium (Fig. 2a), it is unlikely that intracellular levels, which are of the order of < 10 nmol/g dry wt [10], are high enough to inhibit capsaicin synthesis significantly. Nevertheless, from the biotechnological point of view, it would seem that in order to maximize the yield of capsaicin or any other secondary metabolite which inhibits its own synthesis, the product should be continuously removed from the cells and medium. It is becoming apparent that for the large-scale production of specific secondary metabolities which are the products of multistep biosyntheses, the source–sink relationships of both precursor and product may be critical in determining the viability of the process.

EXPERIMENTAL

Cell cultures. Cells of *Capsicum frutescens* Mill. cv. annuum were cultured as free suspensions or immobilized in 1 cm^3 blocks of reticulate polyurethane foam (Declon, Corby, Northants, U.K.) according to the method of Lindsey *et al.* [11]. Cultures were maintained in 250 ml Erlenmeyer flasks containing 50 ml of either a growth medium (SHO), namely Schenk and Hildebrandt medium [18] supplemented with 2.5×10^{-6} M 2,4-dichlorophenoxyacetic acid (2,4-D), 5×10^{-6} M kinetin and 30 g/l. sucrose (pH 5.8), or a non-growth medium (SHind), namely Schenk and Hildebrandt medium modified by the omission of nitrate (as KNO_3) and of 2,4-D and kinetin. All flasks were agitated on a rotary shaker of orbital diameter 1.5 cm at 96 rpm, at $24 \pm 1°$ in continuous illumination (Warmwhite fluorescent lamps) of photon flux density 20 μmol/m^2/s^1.

Cell fr. wt was determined after filtering on a Buchner vacuum filter, and dry wt was determined after drying the cells at 90° in a hot air oven for 24 hr.

Experiments were performed in triple replicate.

Analytical methods. Radioactive capsaicin was determined by scintillation counting of TLC spots of MeOH extracts of cells and $CHCl_3$ extracts of medium, after separation in $CHCl_3$–MeOH (49:1), according to ref. [1].

The determination of the incorporation of radioactivity into soluble protein was performed by scintillation counting of the TCA-insoluble fraction, according to ref. [8]. Total protein was determined spectrophotometrically on KOH-soluble extracts of cells according to ref. [19]. The determination of the total capsaicin content of cells and medium was performed by isocratic HPLC according to ref. [1].

HPLC of soluble phenolics and the determination of their UV spectral characteristics were performed using a Hewlett Packard HP 1090 system with an on-line diode-array spectrophotometer. The solvent system was MeOH–HOAc–H_2O (95:3.75:1.25) at a flow rate of 1 ml/min. The column was a Hypersil MOS (15 cm × 4.6 mm) (Hichrom, Reading, U.K.), maintained at a temp. of 40°. The detector measured the signal at 280 nm against a background of 550 nm, and the spectral range of the peaks determined was 240–352 nm.

Chemicals. All chemicals were analytical reagent grade. Authentic capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), and intermediates of the capsaicin biosynthetic pathway (*trans*-cinnamic acid, *p*-coumaric acid, caffeic acid, ferulic acid, vanillin and vanillic acid) were obtained from the London Sigma Chemical Co. (Poole, Dorset, U.K.). L-[U-^{14}]Phenylalanine (18.4 GBq/mmol) and [3-side chain-^{14}C]cinnamic acid (2.07 GBq/mmol) were obtained from Amersham International (Amersham, Bucks., U.K.) and HPLC-grade solvents were obtained from Fisons (Loughborough, Leics., U.K.).

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REFERENCES

1. Lindsey, K. and Yeoman, M. M. (1984) *Planta* **162**, 495.
2. Lindsey, K. (1985) *Planta* **165**, 126.
3. Lindsey, K. and Yeoman, M. M. (1984) *J. Exp. Botany* **35**, 1684.
4. Davies, M. E. (1972) *Planta* **104**, 50.
5. Phillips, R. and Henshaw, G. G. (1977) *J. Exp. Botany* **28**, 785.
6. Yeoman, M. M., Miedzybrodzka, M. B., Lindsey, K. and McLauchlan, W. R. (1980) in *Plant Cell Cultures: Results and Perspectives* (Sala, F., Parisi, B., Cella, R. and Cifferi, O., eds) pp. 327–343 Elsevier-North Holland, Amsterdam.
7. Yeoman, M. M., Lindsey, K., Miedzybrodzka, M. B. and McLauchlan, W. R. (1982) in *British Society of Cell Biology, Symposium Vol. 4: Differentiation* in Vitro (Yeoman, M. M. and Truman, D. E. S., eds) pp. 65–82. Cambridge University Press, Cambridge.
8. Lindsey, K. and Yeoman, M. M. (1983) *J. Exp. Botany* **34**, 1055.
9. Fry, S. C. (1984) *Phytochemistry* **23**, 59.
10. Lindsey, K., Yeoman, M. M., Black, G. M. and Mavituna, F. (1983) *FEBS Letters* **155**, 143.
11. Lindsey, K. and Yeoman, M. M. (1986) in *Plant Cell Culture Technology* (Yeoman, M. M., ed.) Botanical Monograph Series, pp. 226–265. Blackwell Scientific Publications, Oxford.
12. Brodelius, P., Deus, B., Mosbach, K. and Zenk, M. H. (1979) *FEBS Letters* **103**, 93.
13. Alfermann, A. W., Schuller, I. and Reinhard, E. (1980) *Planta Med.* **40**, 218.
14. Lamb, C. J. (1977) *FEBS Letters* **75**, 37.
15. Gerrish, C., Robbins, M. P. and Dixon, R. A. (1985) *Plant Sci.* **38**, 23.

16. Dixon, R. A., Browne, T. and Ward, M. (1980) *Planta* **150**, 279.
17. Sato, T., Kiuchi, F. and Saukawa, U. (1982) *Phytochemistry* **21**, 845.
18. Schenk, R. U. and Hildebrandt, A. C. (1972) *Can. J. Botany* **50**, 199.
19. Bearden, J. C. (1978) *Biochim. Biophys. Acta* **533**, 525.