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MEVALONATE-ACTIVATING ENZYMES IN GREENING TISSUE CULTURES

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Abstract—Isopentenyl pyrophosphate (IPP)* was identified as one of the products in assays of mevalonate-activating enzymes in acetone powders prepared from callus culture of *Kalanchoë crenata*. Mevalonate-5-phosphate (MVAP) and mevalonate-5-pyrophosphate (MVAPP) were the main products isolated from assay mixture. There was an increase in mevalonate-activating enzyme activity in illuminated tissue cultures in various stages of greening and this was associated with the development of chloroplasts.

INTRODUCTION

WHEN dark-grown callus cultures of *Kalanchoë crenata* were transferred to 16-hr days there was an increase in photosynthetic terpenoids such as carotenoids[1] and phytol.[2] This was correlated with the development of chloroplasts in the cultured cells, a process which occurred more slowly[3] than in etiolated leaves.[4] The photosynthetic ability of the green cultures will be dependent, in part, on the presence in the plastids of various terpenoids which, in turn, will be dependent on the state and activity of enzymes concerned with their synthesis. The present paper describes investigations on changes in mevalonate-activating enzyme activity in dark-grown callus cultures exposed to light.

RESULTS

When ^{14}C—MVA was incubated with all types of callus, two major radioactive products, apart from unchanged starting material, were always present. These products were tentatively identified as MVAP and MVAPP from their R_f values which compared closely with published data[5] (Table 1). A fourth compound was observed in small amounts on chromatograms developed in the isobutyric acid–NH_3–H_2O solvent (R_f 0·26) from every assay mixture.

To finally establish that the products were MVAP and MVAPP, the radioactive areas on the chromatograms were cut out, the compounds eluted in distilled water and the eluates freeze-dried. The residues were dissolved in 1 ml H_2O and divided into two 0·5-ml portions. 0·5 ml 2 N HCl was added to one portion and hydrolysis carried out at 100° for 7 min. To

* Abbreviations: IPP, isopentenyl pyrophosphate; MVAP, mevalonate-5-phosphate; MVAPP, mevalonate-5-pyrophosphate; MVA, mevalonic acid.

[1] A. K. STOBART, I. MCLAREN and D. R. THOMAS, *Phytochem.* **6**, 1467 (1967).
[2] A. K. STOBART, N. R. WEIR and D. R. THOMAS, *Phytochem.* **8**, 1089 (1969).
[3] A. K. STOBART and D. R. THOMAS, *Phytochem.* **7**, 1963 (1968).
[4] D. VON WETTSTEIN, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. 1, p. 19, Academic Press, New York (1966).
[5] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **99**, 381 (1966).

the other portion was added 0·5 ml of 0·1 M Tris-HCl buffer, pH 7·6, containing 4 mg calf intestinal alkaline phosphatase. The enzyme mixtures were incubated at 36° for 4 hr. Chromatography of the hydrolysed solutions was carried out using the two solvent systems shown in Table 1. Acid hydrolysis of the compound corresponding to MVAPP converted it almost totally into a compound with chromatographic properties identical to MVAP whereas similar treatment of the MVAP-like substance produced no change. Alkaline phosphatase converted both compounds into free mevalonic acid. It thus seems clear that MVAP and MVAPP are formed from MVA in reaction mixtures containing soluble protein from all tissue-culture sources.

TABLE 1. R_f VALUES OF LABELLED PRODUCTS PRODUCED FROM MVA BY CALLUS CULTURES

Solvent	R_f value		
	MVAPP	MVAP	MVA
Butanol–formic acid–H_2O (77:10:13, v/v)	0·02	0·14	0·70
Isobutyric acid–NH_3 (sp. gr. 0·88)–H_2O (22:1:10 v/v)	0·21–0·24	0·32–0·42	0·6–0·82

TABLE 2. ACTIVITY OF THE ISOPENTENOL-3,5-DINITROBENZOATE PREPARED FROM UNKNOWN PRODUCTS OF ^{14}C—MVA

Crystallization	Radioactivity in isopentenol-3,5-dinitrobenzoate* (counts/min per mg)	
	(*a*)	(*b*)
1st	68	36
2nd	31	43
3rd	23	41
4th	16	45
5th	8	44

* (*a*) From the unknown radioactive compound eluted from paper chromatograms developed in solvent 2 and (*b*) from the unknown radioactive compound eluted from Dowex-1 formate resin.

The fourth product was at first thought to be IPP. It became increasingly radioactive with time, suggesting that it was, in fact, a member of the terpenoid biosynthetic pathway. When ^{14}C—MVA, ^{14}C—MVAP and ^{14}C—MVAPP were fed to enzyme extracts of dark-grown callus, more activity was incorporated into the unknown from MVAPP than from either MVAP or MVA which suggested that the unknown compound was synthesized at a stage in the pathway after MVAPP. The labelled unknown compound was isolated chromatographically and subjected to hydrolysis with snake venom phosphatase to convert any IPP present to isopentenol.[6] Carrier isopentenol was added and the mixture extracted with benzene. The benzene extracts were bulked, dried over anhydrous $MgSO_4$, and the 3,5-dinitrobenzoate prepared. The 3,5-dinitrobenzoate was repeatedly recrystallized from ethanol and the specific activity determined after each crystallization. From the results (Table 2) it is evident

[6] K. BLOCH, S. CHAYKIN, A. H. PHILLIPS and A. DEWAARD, *J. Biol. Chem.* **234**, 2595 (1959).

that the specific activity rapidly diminished after each crystallization, indicating that no radioactive isopentenol was present. These results prove that the unknown fourth compound obtained by paper chromatography was not IPP and probably may be a later metabolite in terpenoid biosynthesis.

Reaction mixtures from all the tissue cultures under investigation were also subjected to column chromatography on Dowex-1 formate resins and eluted with formic acid–ammonium formate mixtures. The positions of the eluted compounds were determined by their radioactive peaks. Unchanged ^{14}C—MVA was eluted almost immediately with ^{14}C—MVAP and ^{14}C—MVAPP following. A fourth radioactive peak was eluted by the 4 N—HCOOH + 0·8 M—$HCOONH_4$ solvent and its position, relative to the other eluted compounds, was again suggestive of IPP. This compound was subjected to the same procedures used above for investigating the unknown compound found by chromatography in the isobutyric acid solvent. The 3,5-dinitrobenzoate, prepared from the cobra venum hydrolysate plus carrier isopentenol, was repeatedly recrystallized and assayed for radioactivity. The results (Table 2) showed that the activity remained constant after the first crystallization. This was interpreted as indicating that this product of ^{14}C—MVA metabolism was indeed IPP. The radioactive component was therefore co-chromatographed on paper with synthetically prepared IPP. After the radioactive area had been located by autoradiography, the paper was sprayed with the Hanes and Isherwood reagent[7] to locate organic phosphates. The radioactive compound ran coincidental with the synthetic IPP in both solvents (Table 1, Solvent 1, R_f 0·11; Solvent 2, R_f 0·42). The radioactive and the synthetic IPP could not however be separated from MVAP in either of the solvent systems used. The MVAP spot, produced on all chromatograms of reaction mixtures containing soluble protein from acetone powders of all sources of callus, was therefore a composite spot containing IPP. However, the presence of composite MVAP and IPP spots on chromatograms was not considered to alter substantially the interpretation of results since the amount of activity in IPP was about twenty times less than that in MVAP.

The unknown radioactive product found using paper chromatography was not evident using column chromatography with Dowex-1 formate resin, indicating that it was either eluted with one of the other radioactive products, remained on the column or was destroyed. No further attempts were made to determine the nature of this unknown compound.

Hence, the products of ^{14}C—MVA utilization, by enzymes extracted from dark-grown callus and illuminated cultures, were ^{14}C—MVAP and ^{14}C—MVAPP as the two major products, with relatively smaller amounts of IPP. The activities of the MVA and MVAP kinases extracted from dark-grown and greening cultures were next investigated, as differences between the various calluses might occur.

Buffered protein extracts of acetone powders from dark-grown, first-generation and third-generation callus and from green callus were prepared, and their ability to incorporate activity from ^{14}C—MVA into MVAP and MVAPP investigated. The protein concentrations of each preparation were adjusted to within the same range and equal amounts of ^{14}C—MVA (8×10^6 counts/min in a total reaction mixture volume of 0·4 ml) used in each assay. After an appropriate time interval, the reactions were stopped, and the mixtures separated by paper chromatography using the butanol–formic acid–H_2O solvent. Results were expressed as a percentage of the total radioactivity incorporated into MVAP and MVAPP, and also as counts incorporated per milligram protein, after 5, 10, 20, 30 and 45 min incubation time.

The results (Table 3) indicated that mevalonate-activating enzyme activity increased in

[7] C. S. Hanes and F. A. Isherwood, *Nature* **164**, 1107 (1949).

callus tissues on exposure to light. The percentage incorporation from ^{14}C—MVA into MVAP after 5 min reaction time was 3·6 per cent for dark-grown callus and 6·1 per cent for green callus extracts. Radioactivity detected in MVAPP also increased; 1·1 per cent was recovered in MVAPP in dark-grown callus extracts but 4·1 per cent and 3·8 per cent were recovered in the third-generation callus and green callus, respectively.

TABLE 3. MEVALONATE-ACTIVATING ENZYME ACTIVITY OF BUFFERED EXTRACTS PREPARED FROM ACETONE POWDERS OF CALLUS

Incubation (min)	Protein content per 0·4 ml reaction mixture (μg)	% Incorporation into MVAP	% Incorporation into MVAPP	Counts/min $\times 10^{-3}$ per mg protein into MVAP	Counts/min $\times 10^{-3}$ per mg protein into MVAPP
		Dark-grown callus (no chlorophyll)			
5	798	3·6	1·1	9·1	2·8
10		7·9	2·8	19·7	7·0
20		12·3	6·4	30·6	15·9
30		16·8	9·9	41·7	24·6
45		18·9	13·3	47·6	33·5
		1st-generation callus (no chlorophyll)			
5	837	4·9	2·6	11·8	6·3
10		8·8	4·8	21·2	11·6
20		14·3	8·3	33·9	19·6
30		19·7	12·5	47·1	29·9
45		21·5	16·9	51·6	40·6
		3rd-generation callus (0·19 mg chlorophyll/g acetone powder)			
5	793	5·3	4·1	15·3	10·3
10		11·0	8·9	28·4	23·0
20		18·3	13·6	46·5	34·5
30		20·8	18·7	50·9	45·8
45		18·9	18·8	46·2	45·9
		Green callus (0·32 mg chlorophyll/g acetone powder)			
5	815	6·1	3·8	14·3	8·9
10		12·7	9·2	30·6	22·2
20		17·9	18·6	44·4	46·1
30		15·3	21·6	37·6	53·1
45		13·1	23·6	32·5	57·7

Each assay mixture contained 8×10^6 counts/min ^{14}C—MVA.

0·02 ml incubation mixture was applied to the chromatograms in each case. Percentage incorporation of radioactivity into MVAP and MVAPP was calculated as a percentage the total counts recovered on chromatograms in MVAP, MVAPP and the biologically active isomer of D, L-MVA remaining.

In the dark-grown callus and first-generation callus extracts the percentage incorporation of radioactivity in both MVAP and MVAPP increased steadily over 40 min, the activity in MVAPP always being markedly lower than that found for MVAP. A different incorporation pattern was found for the third-generation callus and green callus extracts (both calluses contained detectable amounts of chlorophyll—see Table 3).

The activity incorporated into MVAP by extracts of third-generation callus increased for 30 min to 20·8 per cent, but after 45 min had fallen to 18·9 per cent. The activity in MVAPP increased steadily and after 45 min equalled the activity recovered in MVAP. In the green

callus extracts the incorporation into MVAP reached a maximum after 20 min (17·9 per cent and then declined finally to 13·1 per cent after 45 min. However, activity recovered in MVAPP increased rapidly and MVAPP contained more activity than MVAP after 20 min. When incorporation of activity was expressed as counts/min per mg protein a similar pattern of results emerged.

Since the protein concentrations of buffered extracts of all types of callus were similar in all the assays, the data presented is interpreted as demonstrating an enhancement of mevalonate kinase and mevalonate phosphate kinase activity which is correlated with chloroplast production in illuminated cultures of *Kalanchoë* callus. However, this assay with ^{14}C—MVA involved two enzyme steps and any increase in activity of the first enzyme catalysing these reactions may produce more substrate available for the second enzyme, and so the results obtained for MVAP kinase might not be due to any enhancement of its activity, but simply a consequence of more MVA kinase.

To investigate this possibility, MVAP kinase was assayed using ^{14}C—MVAP supplied to enzyme extracts of acetone powders of dark-grown callus and green callus. The substrate,

TABLE 4. MVAP KINASE ACTIVITY IN DARK-GROWN AND GREEN CALLUS

Callus	Incubation (min)	Protein content per 0·4 ml reaction mixture (μg)	Counts/min × 10^{-3} recovered in MVAP	Counts/min × 10^{-3} recovered in MVAPP	% Incorporation in MVAPP	Counts/min × 10^{-3} in MVAPP per mg protein
Dark-grown	5	695	21·1	3·0	12·4	4·3
	30		20·4	5·0	19·7	7·2
Green	5	790	18·2	5·9	24·8	7·6
	30		15·6	9·9	38·8	12·5

Each assay mixture contained 1×10^6 counts/min ^{14}C—MVAP. 0·01 ml incubation mixture was applied to the chromatogram in each case.

^{14}C—MVAP, was obtained from assay reaction mixtures when ^{14}C—MVA was supplied to extracts of dark-grown callus. The ^{14}C—MVAP was purified on Dowex-1 formate resin columns. The MVAP fed to each reaction mixture had an activity of 1×10^6 counts/min. The results (Table 4) are presented in the same manner as the previous experiments; the counts incorporated into MVAPP being expressed as a percentage of the total counts applied to the chromatograms. The results show that more activity was incorporated from ^{14}C—MVAP into MVAPP by green callus extracts than by dark-grown callus extracts. These results were interpreted as showing that green callus contained a greater MVAP kinase activity than dark-grown callus.

Acetone powders of 3000 *g* and 20,000 *g* fractions from dark-grown, first generation, third generation and green callus were prepared. Acetone precipitates of the final supernatants were obtained by the addition of 0·9 vol. cold acetone, followed by centrifugation. Chlorophyll determinations were made on the acetone extracts obtained in the preparation of acetone powders from third-generation and green callus fractions. Buffered extracts of these powders were prepared and assays of mevalonate enzyme activity carried out as described previously. The protein concentrations were adjusted to within the same range in each assay. The radioactivity incorporated from ^{14}C—MVA into MVAP plus MVAPP was determined after 35 min incubation. Results were expressed as the percentage radioactivity

of the total radioactivity on the chromatogram, incorporated into MVAP and MVAPP and also as radioactivity incorporated per milligram protein.

Most activity was incorporated by the supernatant fraction of each callus (Table 5). The percentage incorporation into this fraction by each callus was similar. The amount of activity incorporated into MVAP plus MVAPP by the 3000 *g* and 20,000 *g* fractions varied; it was highest for the fractions from the chlorophyll-containing cultures (third generation and green callus) and was low in those fractions from dark-grown callus. The percentage incorporation by the 3000 *g* and 20,000 *g* fractions increased for calluses grown in the light for the longer times. Presumably, the increase in activity of the 3000 *g* fraction was associated with the development of mevalonate-activating enzymes in the maturing chloroplasts of illuminated calluses. The 20,000 *g* fractions of third generation and green callus contained chlorophyll

TABLE 5. INTRACELLULAR DISTRIBUTION OF MEVALONATE-ACTIVATING ENZYMES ACTIVITY IN CALLUS CULTURES

Callus	Fraction	Protein content per 0·4 ml reaction mixture (μg)	Chlorophyll content mg/g acetone powder	Counts/min × 10^{-3} in MVA + MVAP + MVAPP	Counts/min × 10^{-3} in MVAP + MVAP	% Incorporation into MVAP + MVAPP per mg protein
Dark-grown	3000 *g*	786	Absent	194·4	1·2	0·7
	20,000 *g*	916	Absent	184·9	6·5	3·8
	Supernatant	864	Absent	167·8	44·0	30·3
1st generation	3000 *g*	904	Absent	187·6	4·9	2·9
	20,000 *g*	916	Absent	197·5	6·1	3·4
	Supernatant	923	Absent	194·9	54·8	30·4
3rd generation	3000 *g*	897	6·1	199·4	12·6	7·0
	20,000 *g*	894	3·8	203·5	8·5	4·7
	Supernatant	918	0·8	196·6	50·9	28·2
Green	3000 *g*	914	8·9	196·8	13·6	7·5
	20,000 *g*	909	2·7	201·0	12·9	7·0
	Supernatant	928	0·9	201·5	54·6	29·2

$4·0 \times 10^5$ counts/min ^{14}C—MVA in a total reaction mixture volume of 0·4 ml used in each assay. 0·2 ml incubation mixture was applied to the chromatograms in each case.

and presumably chloroplast membranes must be present in this fraction. Some small chloroplasts and developing plastids might also be expected to be present and the increase in activity incorporated into MVAP and MVAPP by these fractions from progressively greener callus is again most likely correlated with the light-stimulated development of plastids and an increase in the mevalonate-activating enzymes. The high enzyme activity of the supernatants, in part, may be attributed to chloroplast fragments and leached-out plastid protein, but it is considered that extraplastid enzymes were primarily responsible for the high enzyme activities in the supernatant. The last suggestion is supported by the similar enzyme activity noted for supernatants from all types of callus.

DISCUSSION

In all the soluble protein extracts from acetone-dried powders of *Kalanchoë* callus, MVA kinase, MVAP kinase and pyrophosphomevalonate decarboxylase, were demonstrated, as ^{14}C—MVAP, ^{14}C—MVAPP and ^{14}C—IPP were shown to be products of ^{14}C—MVA. Rogers

et al.[5] only detected MVAPP from MVA in their assays of French bean leaves, tissue cultures of Paul's Scarlet Rose, the green tissue of variegated *Pelargonium kewensis* leaves and leaves of *Hibiscus*. The only extracts that gave a radioactive spot identifiable as MVAP were obtained from the white portion of the variegated *Pelargonium* leaves. These workers also detected small amounts of MVAP in one experiment with a weakly active chloroplast preparation. Other workers[6] found, using yeast autolysates, that providing sufficient ATP was present, the reaction catalysed by MVAP kinase proceeded rapidly and led to the formation of MVAPP. Rogers *et al.*[5] used incubation times of 3–15 hr in their assays, whereas with *Kalanchoë* callus extracts, the maximum incubation was only 45 min. It would seem possible, therefore, that shorter incubation periods might account for the finding of MVAP in the experiments reported here.

The increase in mevalonate-activating enzyme activity in illuminated tissue cultures in various stages of "greening" and hence in various stages of chloroplast maturity, was associated with the development of chloroplasts. The fractionation experiments confirmed this interpretation, since when chloroplasts developed, enzymic activity of the 3000 *g* fractions increased, whereas the enzyme activity in the supernatants (cytoplasm) remained similar. Thus, in the greening cultures, two mevalonate-activating systems were present, with the cytoplasmic complement remaining static and the increases in enzyme activity associated with the development of chloroplasts. It is possible that as chloroplasts develop and more substrate became available (i.e. from CO_2 fixation) for entrance into the terpenoid pathway via MVA, an induced enzyme synthesis occurs or pre-existing protein is activated to produce enzyme activity.

Rogers *et al.*[8] demonstrated the presence of isoenzymes of mevalonic kinase in French bean seedlings. The mevalonic kinase of chloroplasts had a pH optimum of 7·5. while the cytoplasmic kinase had a pH optimum of 5·5. Rogers *et al.* obtained very little overlap of activity for their two enzymes since in extracts of etiolated French bean cotyledons there was demonstrable activity at pH 5·5 and virtually none at pH 7·5. Extracts of isolated French bean chloroplasts, on the other hand, were active at pH 7·5 but not at pH 5·5. Thus, as the assay procedures of the present work were carried out at pH 7·4, it was possible that mevalonate kinase, extracted from dark-grown callus, would not have been detected. However, etiolated French bean leaves contained both mevalonate kinase enzymes[8] and these etiolated leaves presumably contain etioplasts. Callus cultures of Paul's Scarlet Rose[5] contained a mevalonate kinase, active at pH 7·4, and this callus, if similar to *Kalanchoë* callus, would contain proplastids.[3,9] It therefore remains possible that mevalonate kinase with an optimum at pH 7·4 was associated with the proplastids of the callus cultures. However, most activity was recovered in the supernatant fraction of dark-grown callus and, unless the proplastids were extremely fragile and broke up on fractionation, or the enzyme was leached out of the proplastid, it seems likely that the mevalonate kinase recovered in this fraction was cytoplasmic in origin. The enzyme was active at pH 7·4 but, until detailed enzyme studies are carried out, it is not possible to interpret the present results further.

MATERIALS AND METHODS

Plant Material

The growth medium and culture conditions for the *Kalanchoë* callus has been reported elsewhere.[1,10] A summary of the types of callus used in the experiments is given in Table 6. Plant material was fractionated in 0·35 M NaCl buffer, pH 7·4, by standard procedures.[3]

[8] L. J. ROGERS, S. P. J. SHAH and T. W. GOODWIN, *Biochem. J.* **100**, 14 c (1966).

[9] ANNE T. PICKERING and D. R. THOMAS, *New Phytol.*, in preparation.

[10] I. MCLAREN and D. R. THOMAS, *New Phytol.* **66**, 683 (1967).

TABLE 6. TERMINOLOGY USED TO DESCRIBE *Kalanchoë* CALLUS GROWN FOR VARIOUS PERIODS IN 16-hr days

Terminology	Description
Dark-grown callus	Callus cultures derived from stem cultures and which have been grown for 3 yr in the dark with monthly subculturing
1st-generation callus	Callus subcultured from dark-grown callus and then grown in 16-hr days for 4 weeks
2nd–3rd-generation callus	Callus subcultured from previous generation of callus and then grown in 16-hr days for 4 weeks
Green callus	Callus grown in 16-hr days for 3 yr with subculturing at monthly intervals

Radioisotope Techniques

D L-Mevalonic acid—2^{14}-C lactone was purchased from the Radiochemical Centre, Amersham. D L-Mevalonic acid—2^{14}-C was prepared from the lactone by the method of Rogers *et al.*[5] Strips of chromatography paper containing the separated compounds were scanned on a Scanogram 2, chromatogram scanner (Atomic Accessories Inc.). Radioactive areas on the chromatograms were cut out, immersed in scintillation fluid (5 g 2,5-diphenyloxazole and 0·3 g 1,4-bis-(4-methyl-5-phenyloxazol-2-yl) benzene per litre toluene), and counted in a Packard Tri-Carb scintillation counter. Autoradiographs were prepared with Ilford X-ray film.

Mevalonate-Activating Enzyme Assay

Acetone-dried powders were prepared from callus tissues by the method of Loomis.[11] Buffered extracts (0·04 M-potassium phosphate, pH 7·4, containing 0·04 M-$MgSO_4$) were used in each assay. All procedures employed in the preparation of such extracts, were carried out at 0°. The protein content was determined with Folin's reagent.[12] Chlorophyll, extracted from the callus during the preparation of acetone-dried powders, was determined in diethyl ether.[13]

The reaction mixture[5] for each assay contained KH_2PO_4, 14·0 μM; $MgSO_4$, 4·0 μM; sucrose, 120 μM; L-cysteine, 600 μM; ATP, 2·4 μM; and D, L-2-^{14}C MVA. The activity, per reaction mixture, of the MVA, is given in the text. The final volume of the reaction mixture, including extracted protein, was 0·4 ml. The assay was carried out at 36° with shaking. The reaction was stopped by boiling for 2–3 min and the precipitated protein removed by centrifugation.

Chromatography

Known volumes (0·01 or 0·02 ml) of reaction mixture from the assays were chromatographed on Whatma No. 1 in the following solvent systems[5,14] given in Table 1. Columns of Dowex-1 formate resin were als used to separate products of ^{14}C—MVA metabolism.[6] The resin was prepared by the method of Harlbe *et al.*[15] and washed with 3 M sodium formate until the eluate gave a negative reaction with $AgNO_3$ and the washed thoroughly with H_2O. The column (14 × 1·5 cm) was eluted with 300 ml 4 N—HCOOH, 300 n 4 N—HCOOH + 0·4 M $HCOONH_4$ and 300 ml 4 N—HCOOH + 0·8 M $HCOONH_4$. Fractions containin separated radioactive compounds were bulked, freeze-dried and the ammonium formate removed by sublima tion.

Authentication of the Products from ^{14}C—MVA Feeding

MVAP and MVAPP, eluted from paper chromatograms or from the ion exchange columns, were authenticated from their products of hydrolysis.[5] IPP was subjected to cobra snake-venom (*Naja, naja*, Sigma) hydrolysis,[6] and the product, after the addition of carrier isopentenol, extracted in benzene. The 3,5-dinitrobenzoates were prepared[16] using freshly prepared 3,5-dinitrobenzoyl chloride, and repeatedly recrystallized from ethanol. Isopentenol and IPP were chemically synthesized from 3-chloro-2-methyl-1-propene.[17,18]

[11] W. D. LOOMIS, *Plant Physiol.* **34**, 541 (1959).
[12] O. H. LOWRY, N. J. ROSEBROUGH, A. LEWIS-FARR and R. J. RANDALL, *J. Biol. Chem.* **234**, 2595 (1959).
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Expression of Results

The activity in the separated products of the enzyme reactions was expressed as counts/min per total amount of compound. Activities presented for ^{14}C—MVA represent the biologically active isomer of the supplied DL-2-^{14}C—MVA. The percentage incorporation into MVAP and MVAPP was calculated from the total counts/min in MVAP, MVAPP and in the unchanged biologically active isomer of DL-2-^{14}C—MVA.