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FORMATION OF ISOPRENOID PYROPHOSPHATES FROM MEVALONATE BY ORANGE ENZYMES

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Abstract—Enzyme preparations from orange juice vesicles decarboxylated mevalonate-1-^{14}C in presence of ATP and Mg^{++}. These enzyme preparations also converted isopentenyl pyrophosphate-1-^{14}C (IPP) to dimethylallyl pyrophosphate (DMAPP). The products of mevalonate-2-^{14}C metabolism include mevalonate phosphate, mevalonate pyrophosphate, IPP and DMAPP. Linalool was the major 10-carbon product detected in enzymatically hydrolyzed reaction mixture from mevalonate-2-^{14}C. Juice vesicle preparations also phosphorylated linalool-3-^{14}C in presence of ATP and Mg^{++}. Evidence of enzymes that convert mevalonate to linaloyl pyrophosphate suggests that citrus fruits synthesize terpenoids from mevalonate via linaloyl pyrophosphate.

INTRODUCTION

PARTICIPATION of isoprenoid pyrophosphates in terpene biosynthesis has been implicated by past studies with a number of biological systems. Mevalonate-5-phosphate[1] (MVAP), mevalonate-5-pyrophosphate[2,3] (MVAPP), isopentenyl pyrophosphate[4,5] (IPP) and dimethylallyl pyrophosphate[6] (DMAPP) have been recognized as successive intermediates in terpene synthesis from mevalonate (MVA). Geranyl pyrophosphate (GPP) is generally assumed to be the immediate precursor of all monoterpenes.[7] Recently, however, neryl pyrophosphate (NPP) was reported as the 10-carbon product of mevalonate metabolism in a pine seedlings system.[8] In addition, search for the origin of citrus flavor led Attaway *et al.*[9] to speculate that linaloyl pyrophosphate (LPP) was the most likely precursor of monoterpenes in citrus tissues.

Studies were initiated in this laboratory to elucidate the pathway of terpenoids in citrus. In a previous communication[10] we reported the presence in orange juice extracts, of enzymes for mevalonate activation. Other products such as IPP were not detected among the reaction products from MVA probably because low concentrations of enzymes and substrate were

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TABLE 1. DECARBOXYLATION OF MVA-1-^{14}C BY ORANGE ENZYME PREPARATION

	$^{14}CO_2$ liberated (cpm × 10^3)	
	MVA-2-^{14}C	MVA-1-^{14}C
Enzyme	0·14	111·73
Heated enzyme	0·15	0·18
No enzyme	0·14	0·19

The $^{14}CO_2$ liberated from MVA-2-^{14}C and MVA-1-^{14}C was trapped in hyamine hydroxide and counted by liquid scintillation.

used in those experiments. Since then, we have demonstrated that orange juice vesicles contain the full complement of enzymes for the step-wise transformation of MVA through IPP and DMAPP to LPP. The results of these studies are reported in this paper.

RESULTS

Mevalonate Phosphorylated and Decarboxylated to IPP

The enzyme that decarboxylates MVAPP to IPP (ATP-5-pyrophosphomevalonate carboxy-lyase (dehydrating, E.C. 4.1.1.33) was assayed in orange juice vesicles by two methods, measurement of CO_2 liberated in the reaction as described by Henrikson and Smith[11] and measurement of IPP. The results in Table 1 show that C-1 and not C-2 of MVA is liberated as CO_2 and that the reaction is enzyme dependent. The enzymic nature of this reaction is further evident from data in Fig. 1. Evolution of $^{14}CO_2$ increased with increase in protein concentration in the system. Maximal decarboxylation occurred at pH 7·0. The pH curve shows that the alkaline range was more favorable for decarboxylation than the acidic.

IPP formation from mevalonate-2-^{14}C was measured after separating the pyrophosphate from the reaction mixture on the Dowex-formate column and purifying it by paper chromatography. In addition to ^{14}C-labeled IPP, ^{14}C-labeled MVAP, MVAPP and IP were also identified as products of the enzyme reaction (Table 2). The phosphates of MVA could be accounted for by the action of kinase enzymes of orange previously reported.[10] Only the decarboxylation of MVAPP could produce IPP in the reaction mixture.

Isomerization of IPP to DMAPP

The presence of IPP isomerase (isopentenyl pyrophosphate-Δ^3,Δ^2-isomerase, E.C. 5.3.3.2) in orange was demonstrated by using IPP-1-^{14}C as the substrate. DMAPP, the product of the reaction, is readily dephosphorylated in acid to DMA, which can be removed from the reaction mixture by ether extraction. The orange enzyme preparation utilized IPP-1-^{14}C as shown in Fig. 2, from the disappearance of label from the reaction mixture. Paper chromatographic analyses of the reaction mixture in *n*-propanol–NH_3–water system[12] confirmed that DMAPP was formed from IPP. About 40 per cent of the IPP-1-^{14}C was isomerized in 30 min before the reaction slowed noticeably.

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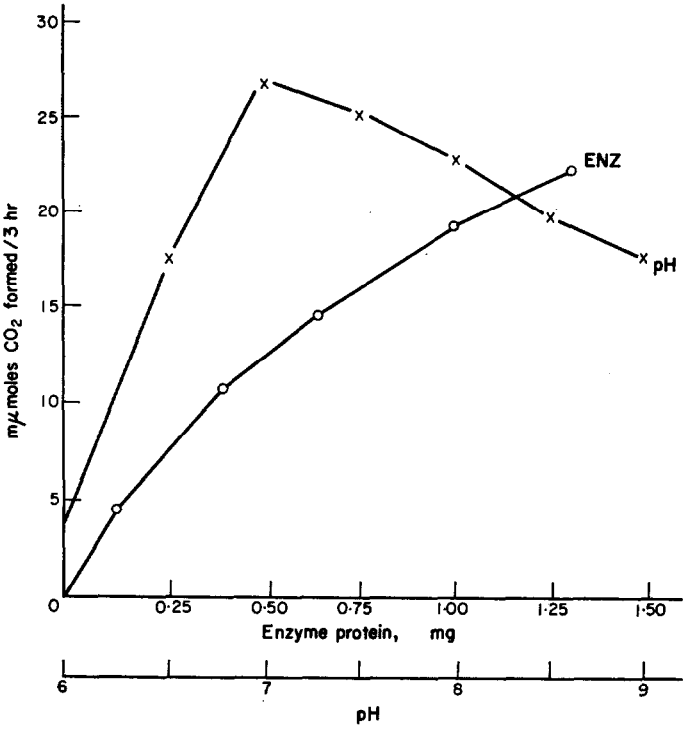

FIG. 1. EFFECTS OF pH AND ENZYME CONCENTRATION ON DECARBOXYLATION OF MVA-1-^{14}C

TABLE 2. CHROMATOGRAPHY OF MVA METABOLITES ON DOWEX-1 (FORMATE)

Stepwise elution with	No. of radioactive peaks	*Ratio of Ve of radioactive peak / Ve of reference peak: AMP	ADP	ATP	Peak identity†
250 ml 4 M HCO_2H	2	0·67	0·20	—	MVA Lactone
		6·00	1·20	—	MVAP
250 ml 0·4 M $(NH_4)_2CO_3$ in 4 M HCO_2H	1	9·50	1·90	0·9	IP
500 ml 0·8 M $(NH_4)_2CO_3$ in 4 M HCO_2H	2	13·5	2·7	1·35	MVAPP
		21·5	4·30	2·15	IPP

* Ve refers to elution volume. The references used were AMP, ADP and ATP whose elution volumes were determined by absorption measurements at 254 nm.

† Peaks were identified by comparing elution volume ratios for reaction mixture to those for the pure compounds. Peak identity was confirmed by paper chromatographic analyses.

Formation of 10-*Carbon Alcohols*

The products of the action of the orange enzyme preparation and ATP on MVA-2-^{14}C were separated on DEAE-cellulose columns and identified by paper chromatography. Figure 3 shows the elution pattern of the products of the reaction, MVAP, MVAPP, IP, IPP

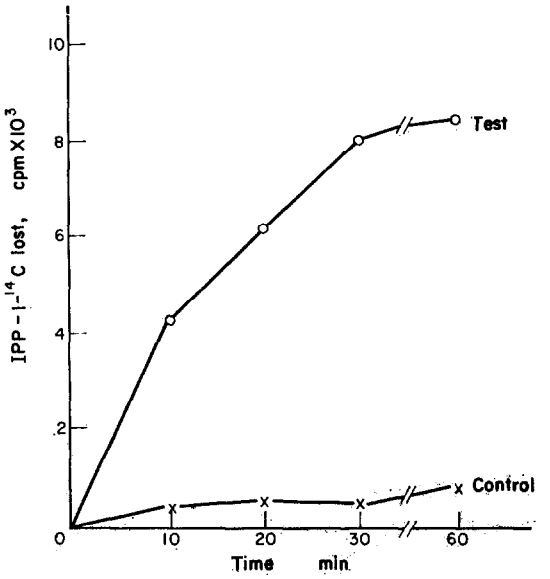

FIG. 2. UTILIZATION OF IPP-1-^{14}C BY ORANGE ENZYME SYSTEM
Heat inactivated enzyme was used in control experiments.

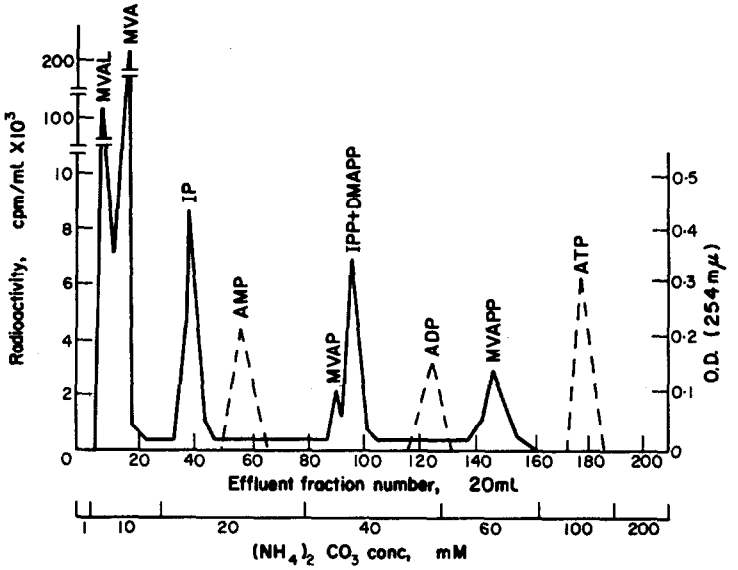

FIG. 3. DEAE-CELLULOSE CHROMATOGRAPHY OF MVA METABOLITES
—— radioactivity; - - - O.D. (254 mμ).

and DMAPP and the adenosine phosphates, ATP, ADP and AMP. Neither GPP nor farnesyl pyrophosphate (FPP) was detected in the effluents. IPP and DMAPP were not resolved by this method. However, they were separated by paper chromatography in *n*-propanol–NH_3–water.[12]

To ascertain whether IPP and DMAPP react to form a 10-carbon product, MVA-2-^{14}C was incubated with ATP and the enzyme preparation for 3 hr at 37°. After enzymatic hydrolysis of the pyrophosphates and extraction into ether, the products were separated by gas chromatography. Figure 4 shows the excellent separation of linalool, geraniol and nerol

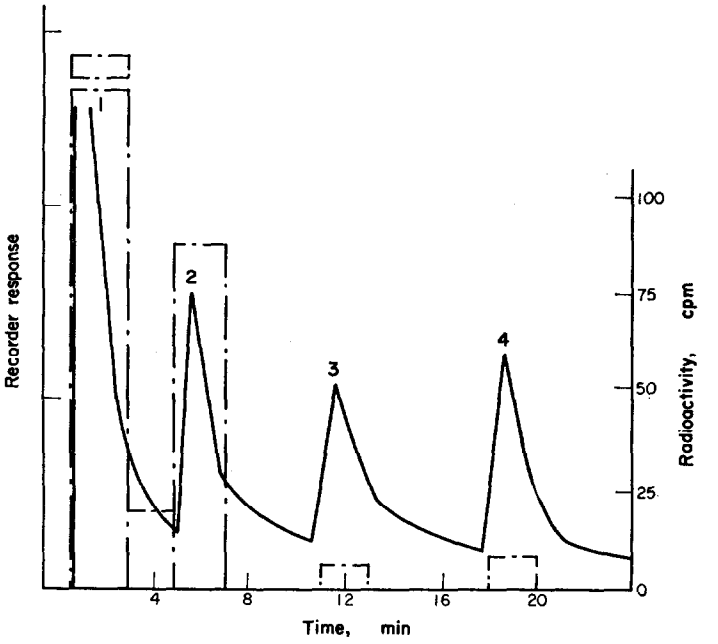

FIG. 4. GAS CHROMATOGRAPHIC SEPARATION OF 10-CARBON ALCOHOLS FROM MVA.
Peak 1 contained the solvent and 5-carbon alcohols. Peaks 2, 3 and 4 represent linalool, geraniol and nerol. Bars of dotted lines indicate radioactivity in fractions collected in the corresponding time.

achieved with GLC. Five carbon alcohols, eluting out first, contained sizeable levels of radioactivity. Among 10-carbon alcohols, only the linalool fraction had significant radioactivity. The nerol and geraniol fractions showed insignificant activity.

Phosphorylation of Linalool by ATP

Citrus fruit contain relatively high concentrations of linalool compared to other C_{10} terpene alcohols. The fruit probably activates these alcohols by phosphorylating them to their pyrophosphates. Therefore the enzyme preparation was tested for this activity using ATP and linalool-3-^{14}C. Table 3 shows that the unreacted linalool was eluted with 0·001 M $(NH_4)_2CO_3$. A significant amount of radioactivity was eluted with 0·06 M $(NH_4)_2CO_3$ which elutes pyrophosphates.[13] GLC analysis of the ether extract of this fraction after alkaline phosphatase treatment recovered linalool-3-^{14}C.

DISCUSSION

Orange fruit vesicles contain a complement of enzymes that convert MVA to a number of isoprenoid derivatives which are intermediates in the metabolic pathway from MVA to linalool. MVA is activated to MVAP and MVAP to MVAPP by their respective kinases

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TABLE 3. DEAE-CELLULOSE CHROMATOGRAPHY OF LINALOOL-3-^{14}C AND ATP REACTION PRODUCTS

$(NH_4)_2CO_3$ used for elution (M)	ml Effluents collected	* Radioactivity in effluent (cpm/ml)	
		Control	Test
0·001	10	7709	7434
0·001	25	73	64
0·001	25	29	33
0·01	25	23	18
0·06	5	18	169

* Radioactivity readings were corrected for quenching. Heat-inactivated enzyme was used in the control reaction mixture.

which were shown by Potty and Bruemmer[10] to be present in orange fruit vesicles. MVAPP decarboxylase is responsible for the formation of IPP with concommitant release of CO_2. Liberation of $^{14}CO_2$ from MVA-1-^{14}C and identification of IPP as a product from MVA activation by the vesicle preparation is evidence for the presence of this enzyme in orange juice vesicles. The presence of IP among the products of MVA metabolism by orange preparations was due to enzymatic dephosphorylation of IPP by the phosphatase present in these preparations. Similar observations were also made by Chesterton and Kekwick[14] in *Hevea brasiliensis* latex. Studies on MVAPP decarboxylase from plant materials have been confined to identification of IPP as a product formed in incubation mixtures capable of synthesizing terpenyl pyrophosphates[15,16] or rubber.[17] Also liberation of CO_2 from MVA-1-^{14}C has been used as evidence for the presence of this enzyme in microorganisms.[11]

IPP isomerase, which catalyzes the isomerization of IPP to DMAPP, was also demonstrated in the orange. This enzyme has been isolated from yeast, but not from higher plants. Indirect evidence has pointed to its presence in *Rhodospirillum rubrum*,[18] *Mycoplasma*[11] and also in tomato fruits.[19] IPP isomerase has been highly purified from pig liver by Shah *et al.*[12] who found the reaction readily reversible, the equilibrium ratio of DMAPP to IPP being 87:13. The crude preparations used in our studies, however, used only 40% of IPP-1-^{14}C supplied to the system.

The biosynthetic intermediates and the enzymes involved in the step-wise transformation of MVA to DMAPP in the orange appear to be identical to those established in plant and animal systems. However, the formation of linalool from MVA is unique to citrus. In an animal system GPP was identified as the condensation product of IPP and DMAPP.[20] In some plant systems, too, GPP was implicated as the 10-carbon intermediate from MVA.[14,21,22] However, recently Beytia *et al.*[8] found NPP as the major C_{10} acyclic intermediate obtained from MVA by the action of enzyme extracts from pine seedlings. In the orange, neither geraniol nor nerol was formed from MVA; only linalool was detected among enzymatically hydrolyzed products of MVA metabolism. This finding does not rule out the probability

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that GPP and NPP are also formed in citrus fruits but rather suggests that LPP is a natural intermediate in terpene synthesis in the orange. The reported conversion of linalool-3-^{14}C into α-terpineol *in vivo* by citrus fruits[23] also supports this supposition. A possible mechanism for the formation of LPP from IPP and DMAPP through the mediation of an hypothetical enzyme LPP synthetase was proposed by Attaway *et al.*[9] This involves addition of DMAPP across the double bond in IPP followed by elimination of pyrophosphate anion from IPP.

The relative abundance of linalool in citrus[24–26] also indicates that linalool might have an important role in the metabolism of terpenes. A direct correlation was found between linalool and limonene levels in some citrus cultivars. Thus, in tangerine, the increase in concentration of (+)-limonene during maturation was accompanied by a proportional decrease in linalool levels.[9]

For linalool to be useful in terpene synthesis, it must be transformed to its pyrophosphate which requires mediation of a kinase enzyme. Such an enzyme system was demonstrated in the orange preparations which phosphorylated linalool-3-^{14}C to LPP in presence of ATP. However, it is not clear whether this enzyme system is the same as that involved in the activation of mevalonate. There are reports indicating a specific kinase for geraniol in germinating peas[27] and in peppermint leaves.[28] The presence of a specific kinase for linalool may provide a mechanism for the control and regulation of terpene synthesis through LPP in the fruit. In this scheme, LPP would be controlled by competing activities of the kinase and the phosphatase.

The above studies clearly establish the capability of orange fruit vesicles to transform MVA to LPP. The role of LPP in terpenoid metabolism in the fruit is not known, but it could be as the precursor for cyclization. We plan to investigate this possibility with labeled LPP.

EXPERIMENTAL

Materials

Valencia oranges with Brix/acid ratio 6:1 and above were obtained through the generosity of Dr. W. Grierson from the Florida Citrus Experimental Station groves.

Dowex-1 (Cl^-) of 100–200 mesh, was converted to its formate form by successive washings with 1 N alkali and 1 N formic acid. DL-MVA-1-^{14}C was provided by Nuclear Chicago, Des Plaines, Illinois, as a benzene solution. After evaporating the benzene in a stream of N_2, the residue was taken up in a known volume of water. DL-MVA-2-^{14}C was obtained as its N,N′-dibenzylethylene diamine salt, from New England Nuclear Corporation, Boston, Massachusetts. An aqueous solution of the salt was treated with an excess $NaHCO_3$ and the free DBED was removed by ether extraction. IPP-1-^{14}C was prepared enzymatically in this laboratory using MVA-2-^{14}C as substrate with a 35–55% $(NH_4)_2SO_4$ fraction from pig-liver acetone-powder (Sigma Co.) according to the method of Dugan *et al.*[13] Linalool-3-^{14}C was a generous gift from Dr. J. A. Attaway from the Florida Citrus Commission.

Enzyme Preparations

Juice vesicles from orange fruit were separated from other tissues as described previously[29] and stored at −96°.

Insoluble polyvinyl pyrrolidone, sodium ascorbate and neutralizing amounts of tris buffer were added to the frozen vesicles to prepare a 10,000 *g* supernatant as described elsewhere.[10] This supernatant was treated

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with $(NH_4)_2SO_4$ to 90% saturation, centrifuged and the residue suspended in 0·01 M tris buffer, pH 7·0. After dialysis against distilled water for 2 hr, the clear supernatant was treated with pre-swelled Dowex-1 (Cl^-) resin (50%, w/v) and filtered. The filtrate, containing approximately 2 mg protein, as estimated by modified Lowry's procedure,[30] served as enzyme source.

Enzyme Assays

MVAPP decarboxylase was assayed using MVA-1-^{14}C as substrate and monitoring the formation of $^{14}CO_2$. Enzymatically liberated $^{14}CO_2$ was trapped in hyamine base for counting according to a modified procedure of Albers and Brady.[31] The reaction mixtures containing 200 μmoles tris buffer, pH 7·0, 1·3 μmoles MVA-1-^{14}C ($\sim 2\,\mu C$), 20 μmoles $MgCl_2$, 20 μ moles KF, 10 μmoles mercaptoethanol and about 2 mg enzyme protein in a final volume of 2 ml were incubated in Warburg flasks at 30° for 3 hr. The center well of the flasks contained 0·4 ml 1 M hyamine hydroxide (in methanol) while the side-arm carried 0·5 ml 5 N H_2SO_4. At the end of the incubation period, the acid was tipped into the flask. After 30 min the liberated $^{14}CO_2$, trapped in hyamine, was counted by quantitatively transferring the center-well contents to glass vials containing 20 ml of the toluene-based PPO-Dimethyl POPOP scintillation cocktails. Heat inactivated enzyme was used in control experiments.

IPP isomerase activity was assayed by measuring the conversions of labeled IPP to DMAPP according to a modification of the method described by Agranoff *et al.*[6] The reaction mixture containing 200 μmoles tris buffer, pH 7·0, IPP-1-^{14}C (20,000 cpm), 20 μmoles $MgCl_2$, 20 μmoles KF, 10 μmoles mercaptoethanol and about 2 mg enzyme protein in a final volume of 2 ml was incubated at 30°. Aliquots were withdrawn at 10-min intervals into centrifuge tubes containing 0·5 ml 10% trichloracetic acid. After removing the precipitates, the supernatants were extracted with ether (10 ml × 3). The aqueous phase was then counted by liquid scintillation in Dioxane-cellosolve system.[32] The amount of IPP converted to DMAPP was computed from the difference in readings between control and test systems. For identification of the product of IPP isomerization, the reaction mixture was concentrated by lyophilization and analyzed by paper chromatography in *n*-propanol–NH_3–water.[12]

Ion-exchange Chromatography of MVA Metabolites

When MVA-2-^{14}C was used as substrate, the reaction products were analyzed by ion-exchange chromatography using Dowex-1 (formate) and DEAE-cellulose.

Dowex-1 (formate) column. The de-proteinized reaction mixture was co-chromatographed with 2 μmoles each of AMP, ADP and ATP on a Dowex-1 (formate) column (1·5 × 12 cm) as described by Dugan *et al.*[13] Eluants were collected in 20-ml fractions and aliquots of each fraction counted by liquid scintillation using the Dioxane-cellosolve system. Peak fractions were lyophilized and components of the fractions were identified by paper chromatographic analyses in multiple solvent systems as well as by their elution pattern with reference to that of AMP, ADP and ATP.

DEAE-cellulose column. The reaction mixture, after diluting to an ion concentration below 0·005 M, was loaded on a DEAE-cellulose column (3 × 20 cm) with 2 μmoles each of AMP, ADP and ATP and the column developed by stepwise elution with ammonium carbonate solution of increasing molarity (0·01 to 0·2 M and pH 8·3). Column effluents were collected in 20-ml fractions and radioactive peaks were located and identified as described with Dowex chromatography.

Paper chromatography. Beckman No. 1 filter paper strips (20 × 3 cm) were developed in *n*-BuOH–HCO_2H–H_2O, *t*-BuOH–HCO_2H–H_2O and *n*-ProH–NH_3–H_2O as described earlier.[10] Radioactive spots were located on the strips by cutting them into 0·5 cm wide pieces and counting by liquid scintillation.

Gas chromatography. A 3 hr reaction mixture from MVA-2-^{14}C, after heat inactivation of the enzymes, was treated with 1 mg alkaline phosphatase (calf intestine) at 30°. After 24 hr the mixture was extracted with 25 ml ether and the ether washed with 2 vol. dilute alkali and then with 2 vol. water. After drying, the ether was evaporated and the residue taken up in 0·05 ml dimethyl pentane. One aliquot was counted by liquid scintillation. Another aliquot was co-chromatographed (Perkin–Elmer Vapor Fractometer, Model 154) with 1 μl each of linalool, geraniol and nerol on a 10 ft × ¼ in. aluminum column packed with diethylene glycol succinate coated (6%, w/w), acid-washed Chromosorb W. The helium flow rate was 60 ml/min and the column temperature was 150°. The alcohols were collected as they emerged from the column by bubbling the effluents through 20 ml of cold, toluene-based scintillation fluid and were assayed for radioactivity.

Phosphorylation of linalool. The phosphorylation of linalool by the enzyme preparation was tested in a 1·2 ml reaction mixture made up of 50 μmoles tris buffer, pH 7·0, 1 μmole linalool-3-^{14}C (36 μc/mM), 10 μmoles ATP, 10 μmoles $MgCl_2$, 10 μmoles KF, 10 μmoles mercaptoethanol and about 1 mg enzyme protein (90% $(NH_4)_2SO_4$ fraction). After incubation for 1 hr at 30°, the reaction mixture was diluted to 12 ml with 0·001 M $(NH_4)_2CO_3$ and loaded on an 8 × 1·5 cm DEAE-cellulose column. The column was developed with 0·001 M, 0·01 M and 0·06 M $(NH_4)_2CO_3$. The effluents were assayed for radioactivity by liquid scintillation.

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The fraction eluted with 0·06 M $(NH_4)_2CO_3$ was concentrated by lyophilization and then treated with alkaline phosphatase before being extracted with ether. The ether extract was analyzed by gas chromatography as described in the previous section.

Radioassay. Radioactivities in all samples were assayed by liquid scintillation in a Packard Tricarb Scintillation Spectrometer, Model 4322. For non-aqueous samples, counting fluid contained 5 g PPO and 0·1 g dimethyl POPOP per liter of toluene. Aqueous samples were counted in scintillation cocktail containing 1% PPO, 0·05% POPOP and 5% naphthalene in a mixture of 5 parts dioxane and 1 part ethyl collosolve.[32] All readings were corrected for quenching by automatic external standardization technique.