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Rate of Acetate and Mevalonate Incorporation by Extracts of *Pinus pinaster* Seedlings *

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E. GARCIA-PEREGRIN and F. MAYOR. *Rate of Acetate and Mevalonate Incorporation by Extracts of* Pinus pinaster *Seedlings.* R. esp. Fisiol., 27, 15-22, 1971.

The conditions for obtaining cell-free extracts of pine seedlings able to acetate and mevalonate incorporation have been investigated. The whole sequence of reactions which relate acetate to «active isoprene» in pine seems to be identical to the pathway in animal cells and microorganisms.

Acetate is incorporated by the enzymes present in the extract and is transformed into MVA derivatives. Incorporation only takes place when CoA-SH and glutathion are added. In the absence of CoA-SH the reaction rate is very slow and its intensity is greatly reduced when NADPH is not supplied. The addition of malonate has no effect on the reaction.

Incorporation of mevalonate by cell-free extracts of *P. pinaster* seedlings has been also demonstrated. The addition of fluoride to the reaction mixture as inhibitor of the phosphatases prevents its action on P-MVA and PP-MVA and allows a greater accumulation of intermediates and a better visualization of the reactions of the metabolic system.

Using mevalonate-1-^{14}C and 2-^{14}C, the rate of its incorporation has been studied. The reaction is extremely rapid. Highest levels of P-MVA and PP-MVA are found after 15-30 minutes. As incubation time increases (0-10 hours) the production of further metabolites (geranyl-PP or neryl-PP?) also increases.

The biosynthesis of «active isoprene», precursor of all the polyprene compounds, starts at the acetyl-CoA level, which reacts with acetoacetyl-CoA to form hydroxymethylglutaril-CoA (HMG-CoA). HMG-CoA is then reduced to mevalonic acid (MVA) (2, 3, 6), NADPH being required for the reduction, as well as -SH group protectors such as glutathion or cysteine.

Carbon dioxide, acetate and mevalonate incorporation in β-carotene of *Phycomyces* has been studied by BRAITHWAITE and GOODWIN (1) showing that the incorporation of acetate decreases in the presence of mevalonate and leucine. The rate of MVA incorporation is some fifteen fold greater than that of acetate.

GOODWIN (5) has investigated the incorporation of carbon dioxide-^{14}C, acetate-2-^{14}C and mevalonate-2-^{14}C by etiolated

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maize seedlings. The same author (7, 8) has followed the incorporation of carbon dioxide and MVA in the phytol side chain of clorophyll and of MVA in squalene and the major phytosterols of maize and pea leaves (4).

In *Pinus*, VALENZUELA *et al.* (13) have studied the phosphorylated intermediates of the biosynthesis of terpenes in *P. radiata*, as well as the incorporation of ^{14}C-CO_2 in α- and β-pinene and limonene (14). SANDERMAN (11) has studied the incorporation of labelled precursors in the gum of *P. jeffrey*, and that of MVA-2-^{14}C in *P. nigra* (9) and *P. pinea* (10), while STANLEY did so in *P. attenuata* (12). WIECKOWSKI and GOODWIN (15) have investigated the incorporation of mevalonate-2-^{14}C in β-carotene and in the phytol chain of chlorophyll using extracts of cotyledons from 4 species of germinating pine seeds: *P. silvestris*, *P. contorta*, *P. radiata* and *P. jeffrey*.

In this paper the results found in the incorporation of acetate-^{14}C and mevalonate-^{14}C by cell-free extracts from *Pinus pinaster* seedlings, as well as the corresponding rates, are presented.

Materials and Methods

P. pinaster seedlings 15-25 days old have been used grown from seeds stratified at 5° C for 6 weeks.

A Nuclear-Chicago Actigraph III system, model 1002, has been used for radioactivity measurement of the products separated by chromatography on Whatman paper n.° 1. A detector, model 470, a sample changer, model M-5, and a recording scale, model 8168, all from Nuclear-Chicago, have been used for the measurement of radioactivity in solid samples. A «Hanovia-Chromatolite» 260 mμ monochromatic lamp has been used for the observation of chromatograms under UV.

The products for the preparation of extracts and for chromatography were supplied by BDH and Riedel. MVA (1 and 2-^{14}C) was supplied by «The Radiochemical Centre» of Amersham (England) and sodium acetate-1-^{14}C by the «Junta de Energía Nuclear», Madrid. Coenzymes and effectors tested were from the firms Sigma, Nutritional Biochemicals and Boehringer.

Extracts were obtained by grinding the seedlings in an ice-cold mortar, with tris-ClH 0.5 M buffer, pH 7.9, so that the final plant/buffer ratio is 1/1. The extract is filtered through a cloth and centrifuged at 2000×*g* for 5 minutes at 4° C.

Enzymatic reactions have been carried out incubating the extracts at 37° C for different periods of time, with the addition of coenzymes and cofactors as detailed in each case. Reactions were stopped by heating at 90° C for 2 minutes. The precipitate was centrifuged off at 2000×*g* for 5 minutes. The metabolites formed were investigated in the supernatant by means of paper chromatography, using the solvents shown in Table I.

Table I. *Chromatographic solvents used in the identification of derivatives of acetate and mevalonate.*

Solvent n.°	Components	Proportion (V/V)	Time (hrs)
1	n-butanol: formic acid: water	77:10:13	12
2	t-butanol: formic acid: water	20: 5: 8	10
3	t-amylic alcohol: acetic acid: water	4: 1: 2	8
4	isobutyric acid: ammonia: water	22: 1:10	10
5	n-propanol: ammonia: water	6: 3: 1	10

Results

a) *Incorporation of acetate-1-^{14}C.* — The incorporation of acetate by extracts of *P. pinaster* seedlings, as well as the influence of the different cofactors, was in-

Table II. *Incorporation of acetate-1-^{14}C by extracts of P. pinaster seedlings with or without some cofactors of the pathway.*
Activities in c.p.m. of the metabolites formed from acetate. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 300; Spd. = 30 (*).

	Solv.	Complete incubation	INCUBATION WITHOUT			
			CoA-SH	NADPH	G-SH	Malonic acid
P-MVA	1	270	45	90	60	165
	4	270	45	65	40	210
PP-MVA	4	110	—	60	—	120
Ip-PP	1	75	35	45	—	45
	4	50	—	—	—	50
	5	180	—	65	40	150
Dal-PP	5	120	—	60	—	80
Unidentified compounds	1	75	—	45	—	50
	1	60	—	30	—	—
	4	60	—	45	—	60

(*) Abbreviations: P-MVA = phosphomevalonic acid; PP-MVA = pyrophosphomevalonic acid; Ip-PP = Isopentenylpyrophosphate; Dal-PP = dimethylallylpyrophosphate; Solv. = Chromatographic solvent; Col. = width of collimator in mm; TC. = time constant; Sc. = range of the scale in c.p.m. Spd. = scan. speed in cm/h.

vestigated in the following reaction system: ATP (8×10^{-3} M); $MnCl_2$ (4×10^{-3} M); $MgCl_2$ (4×10^{-3} M) and acetate-1-^{14}C (16.6 μC/ml; 5×10^{-5} M). The cofactors tested were: CoA-SH (10^{-4} M), NADPH (2×10^{-3} M), glutathion (10^{-3} M) and malonic acid (5×10^{-4} M). Incubation time was 30 minutes to 3 hours.

The results found when measuring the radiochromatograms are given in Table II, in which it can be seen that acetate is incorporated by the enzyme systems present in the extract, being transformed into MVA derivatives. These reactions only occur when CoA-SH and glutathion are added (Fig. 1). In the absence of CoA-SH, incorporation is practically nil and decreases very much when NADPH is omitted. The reaction does not take place at all in the absence of glutathion. At the concentration tested, malonic acid does not appear to have any effect on the reaction.

In order to check the release of labelled CO_2 from the C-1 of the acetate, a piece in the form of an inverted U with a bulb at one end was adapted to the tube in which the complete reaction system was placed. 1 ml of a solution of NaOH 20 % was placed in the side arm to collect the released CO_2 as carbonate. The carbonate formed is precipitated with $BaCl_2$ producing $Ba^{14}CO_3$, with which a pan is prepared for the measurement of radioactivity (Table III). The release of $^{14}CO_2$ shows the incorporation of acetate and its transformation into isopentenylpyrophosphate (Ip-PP) by decarboxylation of the PP-MVA previously formed.

Table III. *Activity of $Ba^{14}CO_3$ formed from $^{14}CO_2$ released in the production of Ip-PP from acetate-1-^{14}C.*

Weight of $Ba^{14}CO_3$ measured (g)	C.p.m.	C.p.m. corrected by	
		background	self-absorption
4	186	165	785

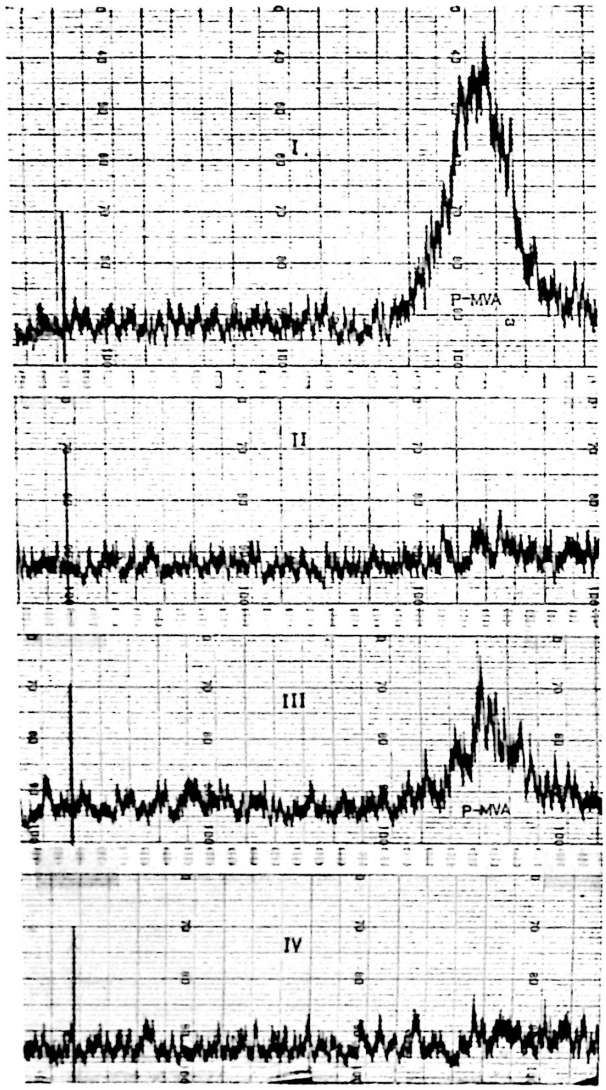

Fig. 1. *Incorporation of acetate-1-^{14}C by extracts of* P. pinaster *seedlings.*
I. Incubation with CoA-SH, NADPH and glutathion. II. Incubation without CoA-SH. III. Incubation without NADPH. IV. Incubation without glutathion. Chromatography run in solvent n.° 2, t-butanol: formic acid: water (20:5:8).

The results obtained incubating the complete system for 1, 3 and 7 hours are shown in Table IV.

b) *Incorporation of mevalonate.* The incorporation of labelled MVA by extracts of *P. pinaster* seedlings has been investigated in reactions carried out with ATP (8×10^{-3} M); $MnCl_2$ (4×10^{-3} M); $MgCl_2$

Table IV. *Rate of acetate incorporation by extracts of* P. pinaster *seedlings.*
Activities in c.p.m. of the metabolites formed from acetate-1-^{14}C. Conditions of measurements: Col. = 12; TC. = 10; Sc. = 300; Spd. = 30.

	Solv.	INCUBATION TIME (hrs.)			
		0	1	3	7
P-MVA	2	Tra.*	70	85	110
	4	Tra.	90	110	170
PP-MVA	3	Tra.	60	60	140
Ip-PP	5	—	90	105	130

* Abbreviations: Tra. = traces.

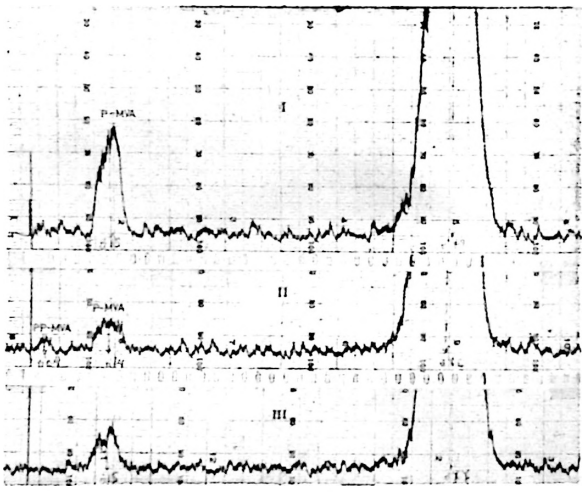

Fig. 2. *Rate of MVA-1-^{14}C incorporation to P-MVA by extracts of* P. pinaster *seedlings.* Incubation times: I, 1 hr; II, 3 hrs; III, 7 hrs. Chromatography run in solvent n.° 1, n-butanol: formic acid: water (77:10:13).

(4×10^{-3} M) and MVA-2-^{14}C (10 μC/ml; 7×10^{-5} M). In order to check the influence of the F^- ion, NaF (10^{-2} M) was added in some experiments with or without glutathion (2×10^{-3} M) as activator and iodoacetamide (10^{-3} M) as inhibitor. Incubation time was usually of 3 hours. The results obtained are given in Table V, which shows the effect of NaF on the MVA derivatives either through a direct action or increasing the activating effect of glutathion of decreasing the weak inhibit-

Table V. *Incorporation of mevalonate by extracts of* P. pinaster *seedlings.* Activities in c.p.m. of the metabolites formed from MVA-2-^{14}C. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30.

	Solv.	EFFECTORS (*)						
		—	F^-	Iod.	G-SH	F^- + Iod.	F^- + G-SH	Iod. + G-SH
P-MVA	5	70	75	60	125	60	150	115
PP-MVA	1	250	250	200	500	225	500	500
	5	—	Tra.	—	50	—	60	—
Ip-PP	5	60	70	50	110	70	160	160
Dal-PP	5	70	70	60	110	65	190	190

(*) Abbreviations: Iod. = Iodoacetamide (10^{-3} M); G-SH = Glutathion (2×10^{-3} M); Tra. = traces.

ing action of iodoacetamide at the concentration tested.

The incorporation of mevalonate after incubation for 1, 3 and 7 hours is shown in Table VI. The experiments were performed as follows: MVA-1-^{14}C (6.5 μC/ml; 1.3×10^{-4} M); ATP (8×10^{-3} M); $MnCl_2$ (4×10^{-3} M) $MgCl_2$ (4×10^{-3} M); glutathion (2×10^{-3} M) and NaF (10^{-2} M). The formation of P-MVA reaches its maximum level after 1 hour, decreasing afterwards as incubation time increases (Fig. 2), probably due to transformation into other P-MVA derivatives not measurable in the radiochromatograms as they lack ^{14}C in their molecule due to decarboxylation of the labelled carbon.

Table VI. *Rate of mevalonate incorporation by extracts of* P. pinaster *seedlings.* Activities in c.p.m. of the P-MVA formed from MVA-1-^{14}C. Conditions of measurements: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30.

Solv.	Incubation time (hrs)						
	0	1		3		7	
		G-SH	F^- + G-SH	G-SH	F^- + G-SH	G-SH	F^- + G-SH
1	—	170	180	70	60	75	100
2	—	75	85	40	80	40	50
4	—	110	125	65	90	65	80
5	—	150	170	70	100	80	100

In order to follow more precisely the transformations which occur through the incubation time, parallel experiments were carried out with MVA-1-^{14}C (6.5 μC/ml;

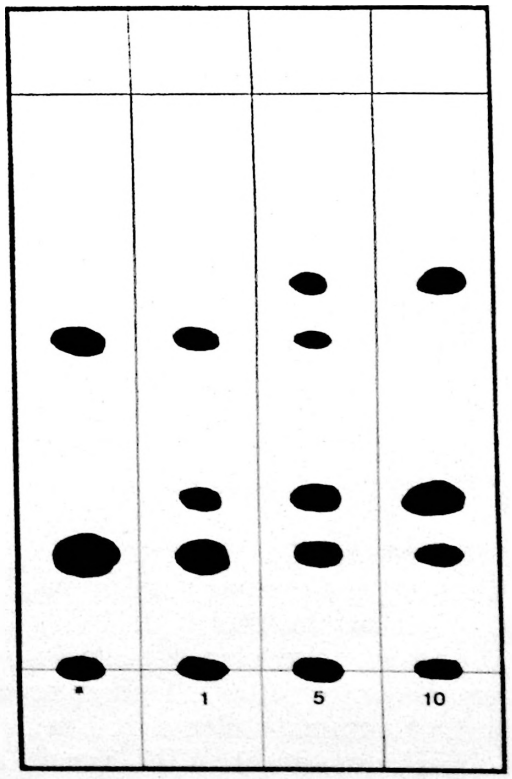

Fig. 3. *Influence of incubation time (in hours) on ATP and its derivatives.* Observation at 260 mμ. Chromatograms run in solvent n.° 5, n-propanol: ammonia: water (6:3:1).

Table VII. *Rate of mevalonate incorporation by extracts of* P. pinaster *seedlings.* Activities in c.p.m. of the metabolites formed from MVA-2-^{14}C. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 1.000; Spd. = 30.

	Solv.	INCUBATION TIME (hrs.)						
		0	1/4	1/2	1	3	5	10
P-MVA	1	40	480	1,000	700	500	400	400
	3	—	700	960	820	600	550	200
	5	—	200	150	90	80	—	—
PP-MVA	1	—	80	80	—	—	—	—
	3	—	120	100	90	—	—	—
Ip-PP	5	—	50	100	80	—	—	—
	5	—	450	850	700	500	350	270
Unidentified	3	—	80	120	200	370	400	430
	4	—	Tra.	Tra.	80	125	170	220
	5	—	—	150	180	250	350	350

Table VIII. *Rate of mevalonate incorporation by extracts of* P. pinaster *seddlings.* Activities in c.p.m. of the metabolites formed from MVA-1-^{14}C. Conditions of measurements: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30.

	Solv.	INCUBATION TIME (hrs.)				
		0	1/4	1/2	1	3
P-MVA	1	40	100	110	Tra.*	Tra.
	2	—	150	125	50	—
	3	50	120	100	Tra.	—
	4	50	120	150	50	—
	5	Tra.	120	140	60	Tra.
PP-MVA	1	—	40	50	Tra.	—
	3	—	60	75	50	—
	4	—	50	75	—	—

* Abbreviations: Tra. = traces.

1.3×10^{-4} M) and MVA-2-^{14}C (10 μC/ml; 7×10^{-5} M) for periods varying between 0 and 10 hours (Tables VII, VIII). The changes which take place during incubation can be also visualized on observing the chromatograms under a 260 mμ UV lamp. The amount of ATP clearly decreases as its derivatives increase (Fig. 3). The results obtained in the reaction stopped by heating at 0 time indicate that the incorporation of MVA is extremely rapid as the brief seconds that elapse before reaching 90° C are sufficient for this incorporation to take place in some extent. The highest levels of P-MVA and PP-MVA are found after 15-30 minutes, other peaks appearing in the radiochromatogram corresponding to further metabolism of P-MVA such as Ip-PP (Figs. 4 and 5) and others non identified compounds, which amount increases as incubation time increases.

Discussion

The incorporation of acetate to the precursors and intermediates of the biosyn-

Fig. 4. *Rate of MVA-2-^{14}C incorporation by extracts of* P. pinaster *seedlings.* Chromatography run in solvent n.° 3, t-amylic alcohol: acetic acid: water (4:1:2). Incubation times: A: I, 15 mins; II, 30 mins; III, 60 mins; B: I, 3 hrs; II, 5 hrs; III, 10 hors.

Fig. 5. *Rate of MVA-2-^{14}C incorporation by extracts of* P. pinaster *seedlings.* Chromatography in solvent n.° 5, n-propanol: ammonia: water (6:3:1). Incubation times: A: I, 15 mins; II, 30 mins; III, 60 mins; B: I, 3 hrs; II, 5 hrs; III, 10 hrs.

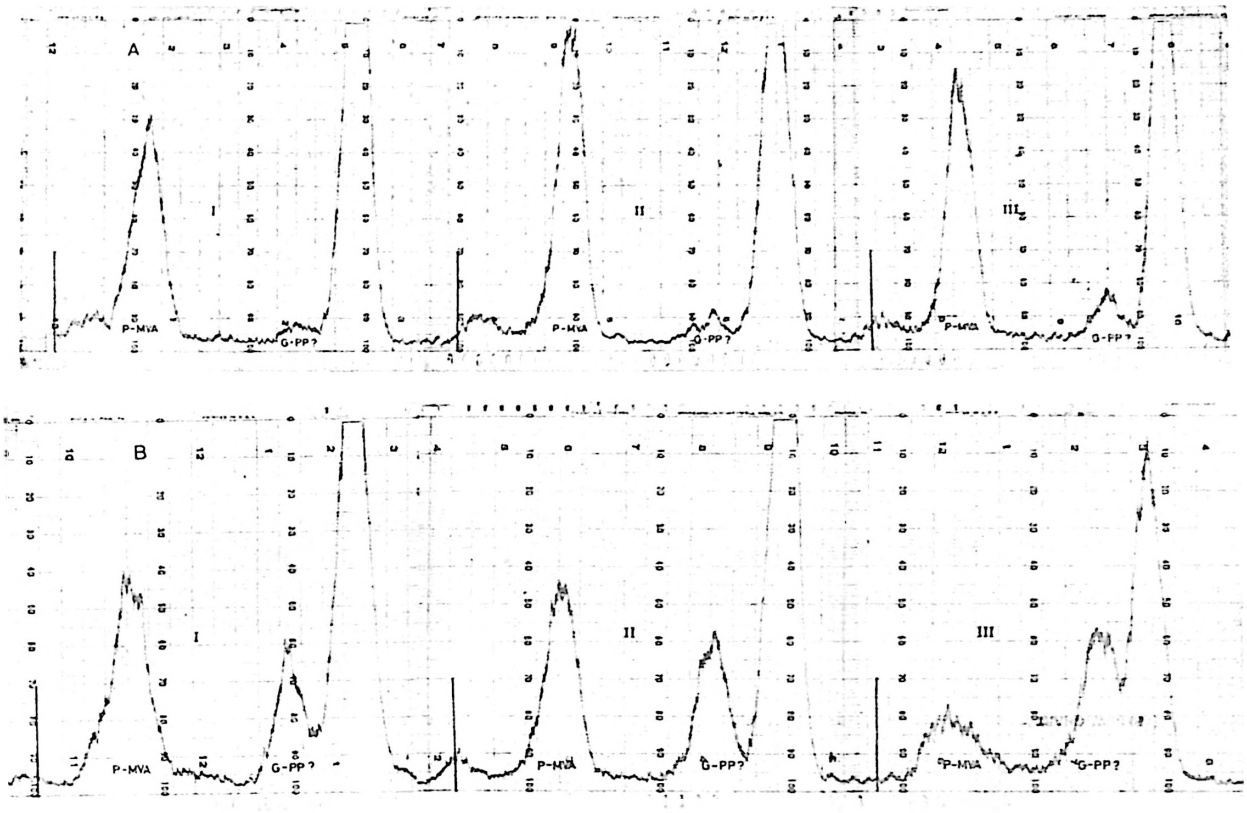

Fig. 4, *A* and *B*.

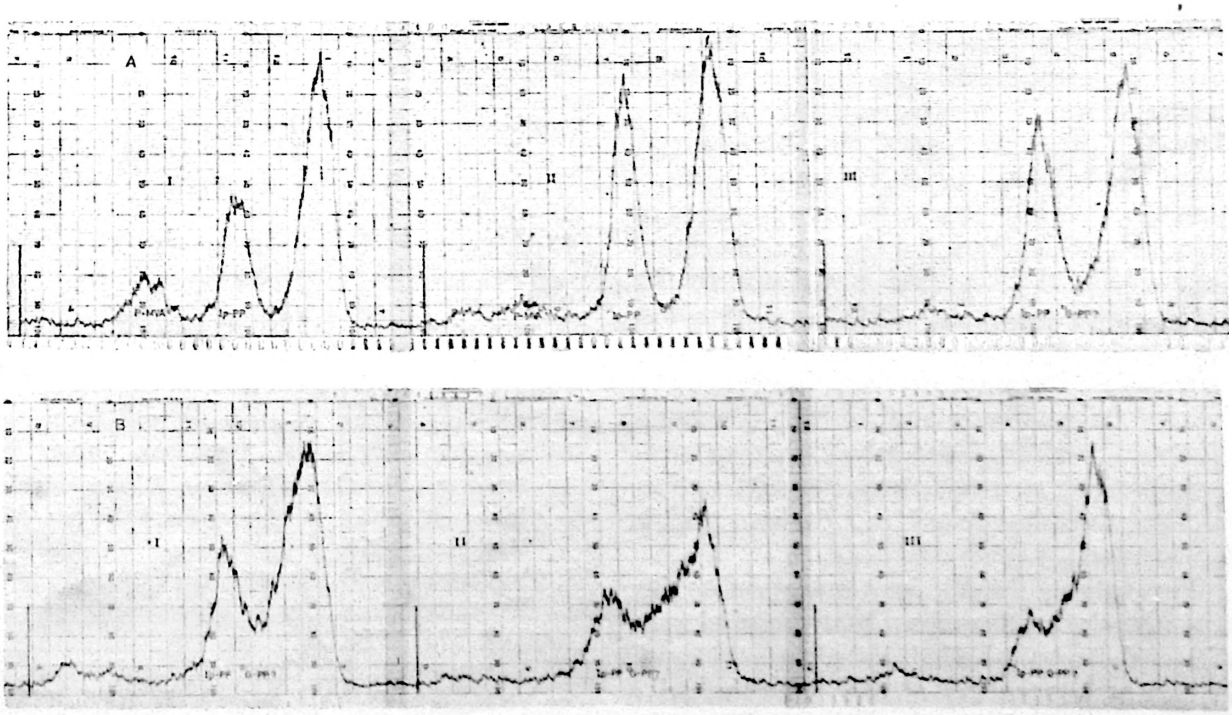

Fig. 5, *A* and *B*.

thesis of terpenes is reportted for the first time in cell-free extracts of pine seedlings. Most authors who have previously worked in plants on this subject have used carbon dioxide or mevalonate as substrates. Fixation of optimum conditions for obtaining cell-free extracts from pine seedlings made possible to test the influence of effectors on this pathway in seedlings. The whole sequence of reactions which relate acetate to «active isoprene» in pine seems to be identical to the pathway in animal cells and microorganisms. As expected, acetate incorporation into intermediates increases as the time of incubation (1-7 hours) is longer.

The decarboxylation of PP-MVA has been shown by collecting, in the form of $Ba\text{-}^{14}CO_3$, the $^{14}CO_2$ released. Incorporation of acetate only takes place when CoA-SH and glutathion are present. In the absence of CoA-SH the reaction is very slight and its intensity is also greatly reduced when NADPH is not added. On the other hand, the addition of malonate does not appear to have any effect on the reaction.

The results obtained in cell-free extracts of *P. pinaster* seedlings when mevalonate was used are in good agreement with those found in other species of pine by STANLEY (12), VALENZUELA *et al.* (13) and SANDERMAN (9). A wider study on requirements of effectors which could influence the reactions from MVA would contribute to the better understanding of this pathway in plants. Among the substances tested, the effect of F^- ion is particularly interesting to follow the sequence and identify intermediates, as inhibits the phosphatases present in the extracts, preventing its action on the phosphorylated metabolites which are formed.

The rate of MVA incorporation to its phospho-derivatives has been carried out with substrate labelled in carbons 1 and 2. The incorporation of MVA by the cell-free extracts of *P. pinaster* seedlings is extremely rapid. The highest levels of P-MVA and PP-MVA are reached after 15-30 minutes. In experiments carried out with MVA-1-^{14}C, labelled Ip-PP production is not detected, while in those in which MVA-2-^{14}C is used the labelled carbon remains in the Ip-PP molecule. A radioactive spot of high Rf which does not correspond to metabolites identified as P-MVA, PP-MVA, Ip-PP or dimethylallylpyrophosphate (Dal-PP) is found in these chromatograms. The size of the spot increases according to the incubation time. The results suggest that it corresponds to a further metabolite of terpene biosynthesis, before cyclation, possibly geranyl-PP or a similar compound (neryl-PP?).

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