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Influence of Some -SH Group Effectors on the Incorporation of Mevalonic Acid by *Pinus pinaster* Seedlings *

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F. GARCIA-PEREGRIN and F. MAYOR. *Influence of Some -SH Group Effectors on the Incorporation of Mevalonic Acid by* Pinus pinaster *Seedlings.* R. esp. Fisiol., 26, 209-216, 1970.

A study has been made of the action of some -SH group effectors on the incorporation of mevalonic acid (MVA) by extracts of *Pinus pinaster* seedlings. Among the protecting substances of thiol groups tested, glutathion has a markedly favourable effect on the phosphorylation of MVA, weak at a concentration of 10^{-3} M, but especially strong at 10^{-2} M. A similar effect, although less marked, is shown by cysteine. Both are sufficiently strong to be able to be followed observing the decrease of ATP at 260 mμ, and the simultaneous appearance of their derivatives.

In the study of some inhibiting substances of -SH groups, the addition of iodoacetamide shows that it not only inhibits the production of dimethylalylpyrophosphate by the blocking of the isopentenylpyrophosphate-isomerase activity, but that it acts in the same way on the kinases which catalyse the phosphorylation of MVA, the production of P-MVA and PP-MVA decreasing at the same time. This inhibition is clearly found at concentrations of 5×10^{-3} M and very strong at 10^{-2} M. Its effect has been studied with and without glutathion. This compound, at concentrations of 1.66×10^{-3} M, counteracts the inhibiting effect of iodoacetamide at similar concentrations.

p-Hydroxymercuribenzoate completely inhibits the enzyme activity at concentrations of 10^{-3} M. Its effects is weak, but perceptible at 10^{-5} M. The presence of glutathion 1.66×10^{-3} M does not counteract this action when the concentration of the inhibitor is 10^{-3} M, but does so at a concentration of 10^{-5} M.

The effect of ethylmaleimide is somewhat less than that of p-hydroxymercuribenzoate at 10^{-5} M, and it must be pointed out that the inhibition found at 10^{-3} M is completely counteracted by the addition of glutathion at the same concentration.

Most of the studies on the metabolism of mevalonic acid (MVA) have been directed towards the reactions of biosynthesis of the derivatives of cyclopentanoperhydrophenantrene in animals. Little research has been carried out on the process in plants, and especially on the biosynthesis of terpenes (12, 15, 17). The requirements of effectors which control the first steps of the pathway in plants have not been established.

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POPJAK *et al.* (10, 11) studied the coenzyme requirements of the synthesis of squalene in rat liver, showing that, in anaerobic conditions, the addition of a reducing agent was not necessary. WILLIAMSON and KEKWICK (19, 20) found that mevalonate kynase of the latex of *Hevea brasilliensis* does not need cysteine or glutathion (G-SH) for its activation in aerobic conditions, while the inhibitors of thiol group which were tested showed a strong inhibition of enzymatic activity, with the exception of iodoacetamide. HENNING (6) mentioned the inhibition of isopentenil pyrophosphate-isomerase of yeasts and *Mycobacterium* by iodoacetamide. The same enzyme of pig was studied by SHAH (13), as well as the effect of iodoacetamide and of p-hydroxymercuribenzoate at different concentrations.

The present investigation was undertaken to examine the influence of the different —SH group effectors on the basic biosynthetic process, in pine seedlings.

Materials and Methods

Pinus pinaster seedlings have been used grown from seeds previously stratified at 5°C for 6 weeks, and grown in suitable conditions of light and temperature for 25 days.

The coenzymes were supplied by Sigma and the other products of the enzymatic reactions were from BDH and Riedel. Most of the effectors tested were purchased from Sigma and Boehringer. The mevalonic acid-2-^{14}C (MVA-2-^{14}C) was supplied in the form of lactone by «The Radiochemical Centre», with a specific activity of 4.82 mC/m M. The potassium salt was prepared by treating the lactone at 36°C for 30 minutes with a KOH solution containing an excess of potassium hydroxide. The pH of the solution was approximately 10.

The extracts are obtained by grinding the seedlings in a mortar with a tris-ClH buffer 0.5M, pH 7.9, placed in an ice-bath (the temperature must be kept between 0-5°C). The final optimum proportion of plant/buffer is 1/1. The extract obtained in this way is passed through a cloth filter and the filtrate centrifuged at 2.000 × *g* for 5 minutes at 4°C.

The reactions have been carried out by incubating the extracts at 37°C with ATP (8×10^{-3}M), $MgCl_2$ (4×10^{-3}M), $MnCl_2$ (4×10^{-3}M), FNa (10^{-2}M) and MVA-2-^{14}C (8×10^{-5}M), plus the effectors being tested. Effects are clearly shown after 30 minutes incubation. However, the normal time used was 3 hours for a better resolution.

The reactions are stopped inactivating the enzymes by heating at 90°C for 3-4 minutes. The precipitate is separated by centrifugation at room temperature at 2.000 × *g* for 10 minutes. Chromatographic analysis of the supernatant was made using Whatman n.° 1 paper in the following solvents: n.° 1, n-butanol:formic acid:water (77:10:13) (16, 18); n.° 2, t-butanol:formic acid:water (20:5:8) (3); n.° 3, t-amylic alcohol:acetic acid:water (4:1:2) (6); n.° 4, isobutyric acid:ammonium:water (22:1:10) (18) and n.° 5, n-propanol:ammonium:water (6:3:1) (14, 2).

An Actigraph III system of Nuclear-Chicago has been used for the detection and measurement of the radioactivity in the chromatograms.

Results

ACTIVATORS. Having shown (4) the activating effect of NaF on the enzymatic systems which catalyse the reactions of incorporation of mevalonic acid by extracts of *Pinus pinaster* seedlings, tests were made with some possible effectors of —SH groups, such as glutathion, cysteine and ascorbic acid, as well as co-factors of the NADH and NADPH type.

The results obtained (mean values) are shown in Table I, in which the activity is

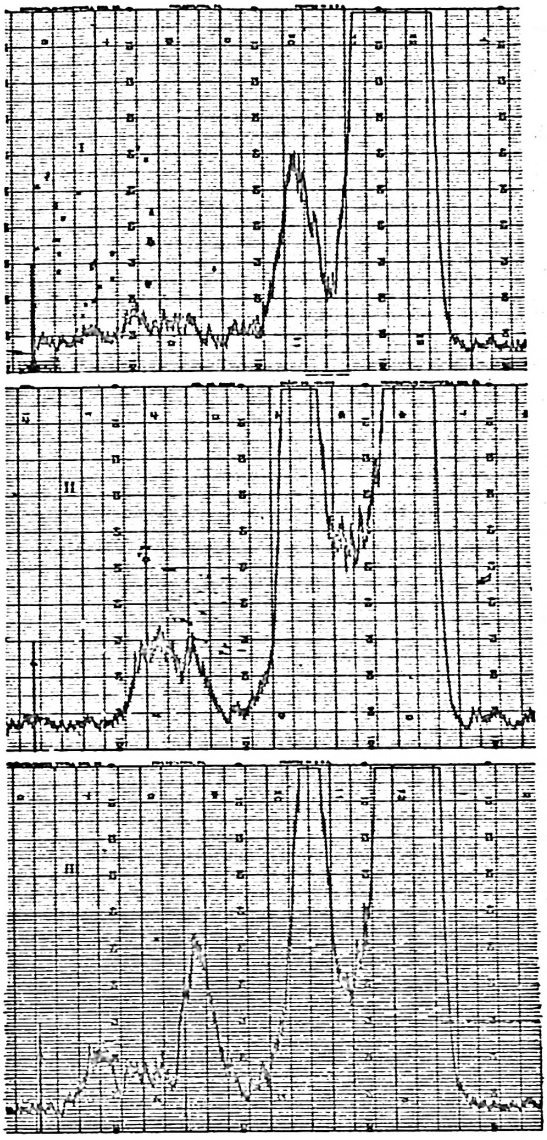

Fig. 1. *Effect of glutathion and cysteine on the incorporation of MVA-2-^{14}C by extracts of seedlings of* P. pinaster.
I. Incubation without glutathion neither cysteine. II. Incubation with glutation 10^{-3} M. III. Incubation with cysteine 10^{-3} M. Chromatograms run with solvent n.° 5, n-propanol-ammonia-water (6:3:1).

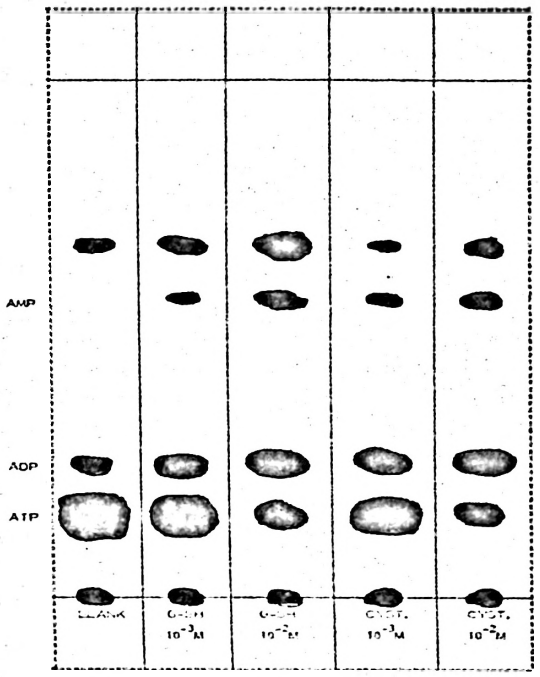

Fig. 2. *Influence of the concentration of glutathion and cysteine on the adenine nucleotides present at the end of the reaction.*
Observation at 260 mμ. Chromatograms run with solvent n.° 5, n-propanol-ammonia-water (6:3:1).

given in c.p.m. of each of the derivatives of MVA-2-^{14}C previously separated by chromatography using aliquots of 25 μl of the total of 3 ml of the reaction tubes. Glutathion clearly increases the phosphorylation of MVA at a concentration of 10^{-3}M, the effect being so strong at a concentration of 10^{-2}M that the response goes over the limit of the scale employed (in these cases the results are expressed approximately in mm. of the width of the peak in the limit zone) (Fig. 1). Cysteine produces a similar effect. This effect is so clear that the decrease of ATP and the increase of the phosphorilated derivatives can be visualized by observing the chromatograms at 260 mμ (Fig. 2).

INHIBITORS. The effect has been studied which different inhibitors of —SH groups, such as iodoacetamide, p-hydro-

xymercuribenzoate (P.H.M.B.) and ethylmaleimide have on the incorporation of MVA. The intensity of this inhibition with an activator such as glutathion at a concentration of 1.66×10^{-3}M has also been investigated.

The results, summarized in Table II, show that iodoacetamide at concentrations of 10^{-2}M produces almost complete inhibition, while at 10^{-3}M inhibition is much less. Glutathion at 1.66×10^{-3}M counteracts the inhibition found in the presence of 10^{-3}M iodoacetamide, and reduces the effect of a 10^{-2}M inhibitor concentration.

P.H.M.B. has a weak effect at a con-

Table I. *Effect of some -SH group inhibitors on the incorporation of mevalonic acid (MVA) by pine seedlings.*
Activity in c.p.m. of the derivatives of MVA-2-^{14}C. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30.*

	Ld.	Blank	G-SH 10^{-3}M	G-SH 10^{-2}M	Cysteine 10^{-3}M	Cysteine 10^{-2}M	Ascorbic acid 10^{-3}M	Ascorbic acid 10^{-2}M	NADH 10^{-3}M	NADH 10^{-2}M	NADPH 10^{-3}M	NADPH 10^{-2}M
P-MVA	1	380	350	14 mm	280	13 mm	240	180	170	140	175	200
	2	150	200	10 mm	170	500	125	125	75	150	120	120
	3	140	125	360	125	325	140	90	80	80	120	120
PP-MVA	3	150	150	400	110	340	150	100	100	100	120	120
	5	80	65	150	65	110	60	—	—	—	—	—
Ip-PP	2	110	80	200	100	280	75	60	70	70	75	100
	3	250	150	300	150	260	200	100	160	75	125	125
	5	290	240	15 mm	220	8 mm	200	140	100	90	75	160

* Abbreviations: P-MVA = phosphomevalonic acid; PP-MVA = pyrophosphomevalonic acid; Ip-PP = isopentenylpyrophosphate; Ld. = chromatography solvent solution; Col. = width of collimator in mm; TC. = time constant; Sc. = extent of the scale in c.p.m.; Spd. = speed of registering chart.

Table II. *Effect of some -SH group inhibitors on the incorporation of mevalonic acid (MVA) by pine seedlings.*
Activity in c.p.m. of the derivatives of MVA-2-^{14}C. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30 *.

	Ld.	Blank	G-SH **	Iodoacetamide 10^{-3}M	Iodoacetamide 10^{-2}M	Iodoac. + G-SH ** 10^{-3}M	Iodoac. + G-SH ** 10^{-2}M	P.H.M.B. 10^{-3}M	P.H.M.B. 10^{-2}M	P.H.M.B. + G-SH 10^{-3}M	P.H.M.B. + G-SH 10^{-2}M	Ethylmal. 10^{-3}M	Ethylmal. 10^{-2}M	Ethylmal. + G-SH ** 10^{-3}M	Ethylmal. + G-SH ** 10^{-2}M
P-MVA	2	90	60	50	40	190	50	75	—	200	—	120	—	130	90
	4	80	60	—	—	90	—	90	—	90	—	90	—	100	—
	5	90	50	—	—	95	—	65	—	110	—	100	—	110	—
PP-MVA	1	180	125	125	50	225	100	140	—	500	—	180	—	250	175
	2	60	—	—	—	60	40	50	—	90	—	60	—	65	45
	5	90	70	50	40	85	50	50	—	125	—	70	—	80	60
Ip-PP	5	125	130	—	—	100	55	—	—	180	—	150	—	170	90
Dal-PP*	5	100	110	75	50	175	70	80	—	200	—	120	—	150	120

* Abbreviations: Dal-PP = dimethylalypyrophosphate. ** Molarity of glutation = 1.66×10^{-3}M.

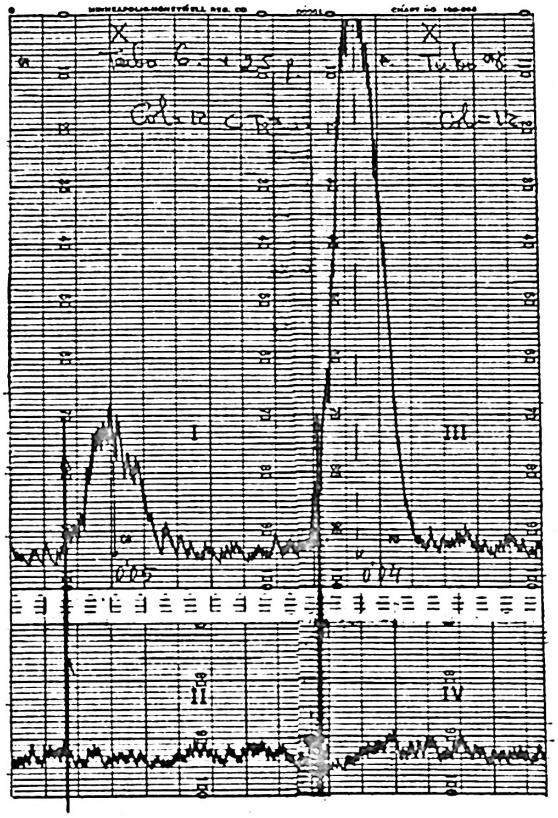

Fig. 3. *Effect of P.H.M.B. on the incorporation of MVA-2-^{14}C to PP-MVA by extracts of seedlings of* P. pinaster.
I. Incubation with P.H.M.B. 10^{-5} M. II. Incubation with P.H.M.B. 10^{-3} M. III. Incubation with P.H.M.B. 10^{-5} M and glutathion 1.66×10^{-3} M. IV. Incubation with P.H.M.B. 10^{-3} M and glutation 1.66×10^{-3} M. Chromatograms run with solvent n.° 1, n-butanol-formic acid-water (77:10:13).

centration of 10^{-5}M, inhibition being complete at 10^{-3}M. The presence of glutathion 1.66×10^{-3}M does not prevent this inhibiting action, but does so at 10^{-5}M P.H.M.B. (Fig. 3).

Ethylmaleimide has somewhat less effect than P.H.M.B. when added at a final concentration of 10^{-5}M, although in this case the inhibition at 10^{-3}M is counteracted by glutathion at the concentration mentioned above (Fig. 4).

With the aim of obtaining a better knowledge of the inhibitor concentration needed to influence the reaction, the effect of a wide-range of iodoacetamide concentration has also been tested. The results shown in Table III corroborate that inhibition is clearly found at concentrations of 5×10^{-3}M and is very strong

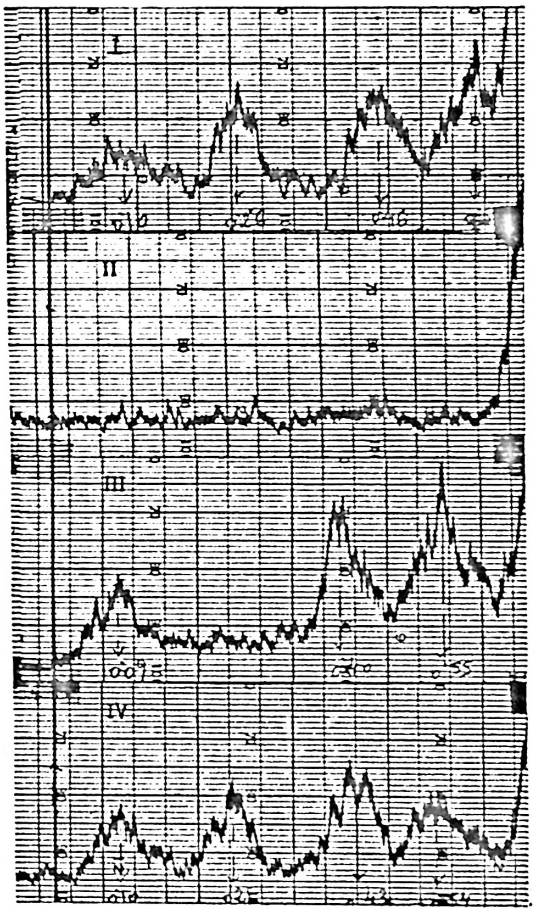

Fig. 4. *Effect of ethylmaleimide (E.M.) on the incorporation of MVA-2-^{14}C by extracts of seedlings of* P. pinaster.
I. Incubation with E.M. 10^{-5} M. II. Incubation with E.M. 10^{-3} M. III. Incubation with E.M. 10^{-5} M and glutathion 1.66×10^{-3} M. IV. Incubation with E.M. 10^{-3} M and glutathion 1.66×10^{-3} M. Chromatograms run with solvent n.° 5, n-propanol-ammonia-water (6:3:1).

Table III. *Effect of the concentration of iodoacetamide on the incorporation of mevalonic acid (MVA) by pine seedlings.*
Activity in c.p.m. of the derivatives of MVA-2-^{14}C. Conditions of measurement: Col. = 12; TC. = 10; Sc. = 500; Spd. = 30.

	Ld.	Iodoacetamide × 10^{-3}M					
		—	0.5	1	2	5	10
P-MVA	4	60	50	45	35	Ind.*	—
	5	70	50	45	40	Ind.	—
PP-MVA	1	250	200	200	185	150	100
	5	45	35	30	Ind.	Ind.	—
Ip-PP	5	70	70	60	45	40	Ind.

* Abbreviations: Ind. = Traces.

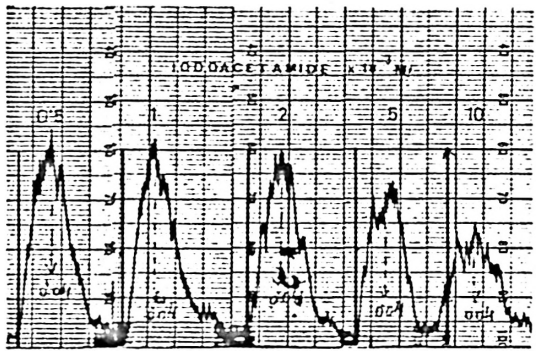

Fig. 5. *Effect of the concentration of iodoacetamide on the formation of PP-MVA from MVA by extracts of seedlings of* P. pinaster. Chromatograms run with solvent n.° 1, n-butanol-formic acid-water (77:10:13).

at 1×10^{-2}M. At lesser concentrations the effect of iodoacetamide is weak (Fig. 5).

Discussion

The localization of the products of the reactions of biogenesis present in the incubation mixture, in accordance with the conventional Rf values, is extremely difficult because the original compounds can undergo changes according to the solvents used. This has made it necessary to employ several systems to confirm the probable situation of some derivatives (5).

The results obtained with —SH group effectors, using cell-free extracts of pine seedlings are generally in good agreement with those found in animals and microbes on the same biosynthetic pathway.

Among the protecting compounds of thiol groups tested, glutathion and cysteine show a clear activating effect of the phosphorilation of MVA. As far as we know, only yeast extracts (1) seems not to require the addition of any reducing agent for the synthesis of squalene from mevalonate, in both aerobic and anaerobic conditions. Works made using very different enzyme sources agree on the favourable effect of reducing agents and, in most cases, show that their presence is imperative. Thus, the cell-free and dialysed preparations of rat liver need the addition of ATP and of a reducing agent (cysteine, glutathion of ascorbic acid) for the synthesis of squalene and steroids from mevalonate. The action which we have observed in pine extracts suggests that ascorbic acid has a certain activating effect on mevalonate-kinase although it inhibits the enzymatic systems which act later on. On the other hand NADH and NADPH appear to have a slight inhibiting effect on the first steps of the reaction at the concentrations tested.

The partial protecting action of glutathion (not of ascorbic acid) on the inhibiting effect of mercuric chloride and of p-chloromercuribenzoate on the synthesis of squalene in rat liver (10, 11) agrees with the results found by us in the preparations of *Pinus pinaster*. The protecting action of the compounds which counteracts the oxydation of the apoenzyme thiol groups confirms the presence of different —SH enzymes in the metabolic pathway. Experiments carried out with isolated enzymes corroborates these conclusions obtained in the over all process, p.e. liver mevalonate-kinase is a —SH enzyme requiring cysteine for its activation in aerobic conditions (8, 9).

Also the results obtained in pine seedlings extracts with some inhibitors of —SH groups are in complete agreement with the previous data. However, the action of iodoacetamide shows that not only is the production of dimethylalylpyrophospate inhibited by the blocking of the isopentenylpyrophosphate-isomerase activity (6, 13), but that it acts in the same way on the kinase which catalyses the phosphorylation of MVA, the production of P-MVA and PP-MVA decreasing simultaneously. The inhibiting action of iodoacetamide is counteracted by the addition of glutathion at similar concentrations. The strong inhibition produced by p-hydroxymercuribenzoate confirms the results previously found in liver by SHAH *et al.* (13). These authors obtained an inhibition of 94% with p-chloromercuribenzoate at the concentration of 10^{-4}M. It is interesting to point out that at lower concentrations inhibition decreased sharply. Therefore, the functionning of the pathway in *Pinus pinaster* is essentially very similar, as regards the inhibiting action on —SH groups, to the whole system or isolated enzymes obtained from mammalian tissue extracts (7).

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