

Enhancement of Terpenoid Biosynthesis from Mevalonate in a Fraction of the Latex from *Euphorbia lathyris*

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ABSTRACT

A latex pellet fraction from *Euphorbia lathyris* incorporates mevalonate into triterpenols and their fatty acid esters. Conditions for improved incorporation were determined. CaCl_2 or CaCl_2 plus MnCl_2 stimulated biosynthesis, and the metal ion chelator, ethylenediaminetetraacetic acid (EGTA) enhanced stimulation. Ethylenediaminetetraacetic acid was almost as effective as EGTA, but phthalic acid and citric acid were relatively poor stimulators. The concentration of the Ca^{2+} -EGTA complex was directly measured, and the incorporation data are best fitted by a curve that shows that the receptor for the complex is saturable. In the presence of the metal-chelate complex, the addition of fructose, 1,6-bisphosphate plus aldolase (triose-P) or malate provided additional stimulation. Incorporation was maximum at 40 micromolar R-mevalonate, and inhibition occurred at higher concentrations. The apparent K_m for R-mevalonate was 15 micromolar. Under improved reaction conditions, the rate of triterpenoid biosynthesis from mevalonate is 25 times faster than was previously observed (GJ Piazza, EJ Saggese, KM Spletzer [1987] Plant Physiol 83: 177-180).

The tapped latex of *Euphorbia lathyris* can convert acetate to several structurally different tetracyclic triterpenes (cycloartenol, 24-methylene cycloartenol, euphol, lanosterol, and an isomer of lanosterol) (5). In prior studies, two major classes of triterpenes were found to be synthesized *in vitro*: triterpenols (TOH¹) and their fatty acid esters (TE) (5). Biosynthetic activity was optimum in diluted latex with 0.4 M sorbitol as a component of the diluting buffer, indicating that an osmotically sensitive organelle is involved in biosynthesis. Ponsinet and Ourisson (8) have shown that simple low speed centrifugation of the latex from several *Euphorbia* species disrupts biosynthesis when acetate is the precursor, presumably by separating portions of the biosynthetic pathway. The pellet from such a simple centrifugation will incorporate mevalonate into triterpenes.

The latex pellet affords a convenient system for the assessment of inhibitors or stimulators of plant triterpenoid biosynthesis (7). However, a systematic investigation of the influence of reaction conditions on reaction rate has never been conducted. It is reported here that significant enhancement of mevalonate utilization can be achieved by using appropriate

¹ Abbreviations: TOH, triterpenols; TE, fatty acid esters of triterpenols; FBP, D-fructose, 1,6-bisphosphate.

conditions. At an early stage in this investigation, the use of isopentenyl-PP as a precursor was ruled out because the latex pellet contains phosphatase activity, and the two most commonly used phosphatase inhibitors, sodium fluoride and sodium molybdate, were found to be inhibitory toward the biosynthesis of triterpenoids from mevalonate.

MATERIALS AND METHODS

Materials

R-[5-³H]mevalonic acid, triethyl ammonium salt (27.6 Ci/mmol) was from Dupont NEN Research Products.² DL-Mevalonic acid, dibenzylethylenediamine salt was from Calbiochem. EGTA and KH-phthalate were from Kodak. EDTA (gold label) was from Aldrich. Isocitric acid (trisodium salt) was from ICN Pharmaceuticals. All other biochemical reagents were from Sigma. Metabolites were purchased as their sodium salts, except α -glycerol-P was the di(monocyclohexylammonium) salt.

Triterpene Biosynthesis Assay

Latex was collected from *Euphorbia lathyris*, and the latex pellet was prepared as previously described (7). Briefly, freshly tapped latex (100 μL per assay) was diluted threefold with ice-cold buffer containing 10 mM NaOH-KHphthalate (pH 5.5), 0.4 M sorbitol, 30 mM CaCl_2 , 10 mM MgCl_2 , and 10 mM KCl. The diluted latex was centrifuged at 8800g for 5 min (including starting and stopping time) in an Eppendorf model 5413 desk top centrifuge. The supernatant was removed and extracted with diethyl ether to give carrier triterpenes. The pellet was resuspended in reaction buffer containing 10 mM Mes-NaOH (pH 6.5) and 0.4 M sorbitol. Each assay contained 10 μCi R-[5-³H]mevalonic acid, and if necessary unlabeled DL-mevalonic acid was added to raise the concentration as indicated in the text, assuming that only one isomer was utilized. The pH of all chelator solutions was adjusted to 6.5 with NaOH. All reactions were conducted in disposable borosilicate glass culture tubes (12 \times 75 mm). The reaction volume was 0.25 mL, and the reaction time was 3 h. Data were analyzed as before and compared to a control set at 100%. All errors are reported as standard errors except as indicated. One-half of selected samples of triterpenols were subjected to

² Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

HPLC analysis as described (5). The radioactivity recovered by collecting the peaks corresponding to the endogenous triterpenols was 80 to 110% of the injected radioactivity. The pattern of labeling was very insensitive to reaction conditions; under very high rate conditions the radiolabel was somewhat more evenly distributed between the individual triterpenols.

Calcium Ion Measurements

Free Ca^{2+} concentration was measured directly in latex pellet fractions using a Radiometer calcium ion selectrode (F 2112 Ca) with a SCE type calomel electrode (K 4040) as reference. Calibration curves were insensitive to the presence of Mg^{2+} , but were shifted in the presence of free Mn^{2+} . The calcium-chelator concentration is equal to the decrease in free Ca^{2+} upon the addition of chelator.

RESULTS

Influence of EGTA

In prior work, the latex pellet isolation buffer and the biosynthesis buffer consisted of phthalate-NaOH containing sorbitol, KCl, MgCl_2 , and CaCl_2 (7). The phthalate buffer was chosen because its pK_a was in the range of interest and because it did not interfere with analysis of tricarboxylic acids. Since a study of the influence of ions upon biosynthesis was one of the goals of this project, attempts were made to isolate the latex pellet in Mes-sorbitol with no added ions; ion salts were added later in the biosynthesis reaction. Observed biosynthesis was very poor, and ion effects were not reproducible between latex batches. Presently there is no marker for the site of triterpene biosynthesis, except biosynthesis *per se*. Therefore it was not possible to know whether the poor results were caused by a failure in the isolation procedure or by poor conditions for biosynthesis. Subsequently, the latex pellet was isolated in phthalate-sorbitol with ions, as before; after resuspending the latex pellet in Mes-sorbitol, ion salts were again tested. Biosynthesis was generally poor under most tested conditions. (The possibility that Mes was inhibitory was eliminated by adding it to the phthalate biosynthesis buffer.) In examining the results it was noted that when EGTA was added to the Mes-sorbitol reaction buffer, biosynthesis was generally higher. Due to this observation, experiments were conducted over a range of EGTA concentrations; no ion salts were present, except for those carried over from the isolation buffer. As shown in Figure 1 (circles) the addition of EGTA remarkably stimulated biosynthesis.

Ion Salts

It was not known whether EGTA was functioning to stimulate biosynthesis by chelating trace amounts of highly inhibitory ions or working by another mechanism. It was reasoned that if EGTA were acting only in the former way, then the addition of EGTA-binding salts would cause inhibition because these salts would compete with inhibitory ions for binding to EGTA. As shown in Table I, most tested ions either inhibited or did not stimulate, but both CaCl_2 and MnCl_2 actually enhanced biosynthesis over that given by EGTA alone. Therefore, the enhancement of biosynthesis by

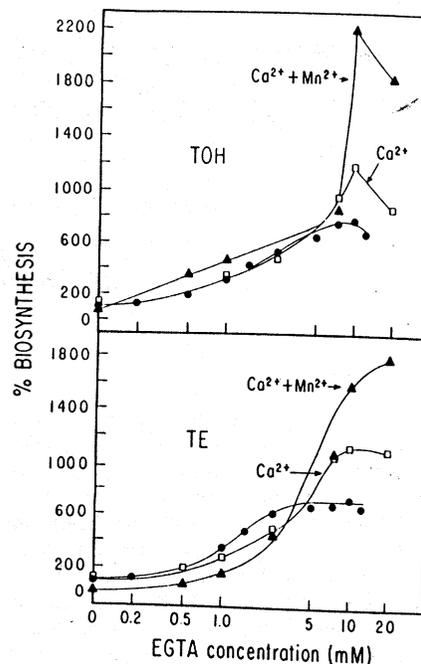


Figure 1. Influence of EGTA on TOH and TE biosynthesis. (●) no additions, (□) 10 mM CaCl_2 , (▲) 10 mM CaCl_2 + 10 mM MnCl_2 . All assays contained 10 mM Mes-NaOH (pH 6.5), 0.4 M sorbitol, 3 mM DTT, 100 μM 100 μM S-adenosylmethionine, and 1.6 μM mevalonate. Points are the average of duplicate determinations. Without the addition of EGTA or salts the incorporation rates were TOH, 0.028 to 0.57 and TE, 0.10 to 0.27 $\text{pmol h}^{-1} \text{mg}^{-1} \text{protein}$.

EGTA is not due merely to the removal of inhibitory ions, some component of the reaction mixture must also be acting to alter the rate of biosynthesis. It should be noted that in this experiment, control levels of TE predominated over TOH by 4.3 ± 0.4 (SE), $N = 15$). Although the salts FeCl_3 and $\text{Na}_2\text{B}_4\text{O}_7$ caused TOH levels to be higher than the control, the decrease in TE more than compensated for this; overall biosynthesis was reduced to 90% (FeCl_3) and 56% ($\text{Na}_2\text{B}_4\text{O}_7$) of the control level. Clearly these salts can influence the partitioning of carbon between the two major classes of triterpenes in latex, but they are not stimulators of biosynthesis.

Figures 2 and 3 report complete concentration curves of CaCl_2 and MnCl_2 with EGTA fixed at a concentration of 10 mM. While CaCl_2 stimulated both TE and TOH biosynthesis equally, MnCl_2 enhanced TOH biosynthesis more than TE biosynthesis. When no ions are added, the absolute rate of TE synthesis predominates by a factor of 4 to 10 over TOH biosynthesis. Thus when the concentration of MnCl_2 is greater than 10 mM, the absolute rate of TOH biosynthesis becomes approximately equal or greater than TE biosynthesis.

Metal Ion-Chelator Complex

The influence of changing EGTA concentrations in the presence of fixed levels of CaCl_2 or the combination of CaCl_2 and MnCl_2 is shown in Fig. 1. Compared with the curve of EGTA alone, the added ion salts stimulated only at relatively high levels of EGTA. However, since the carryover ion salt concentration from the isolation medium was not known, it

Table I. Influence of Salts on Triterpene Biosynthesis

Salt ^a	Triterpene	Triterpene Biosynthesis ^b
		%
NaCl	TOH	106 ± 4 (6) ^c
	TE	95 ± 18
KCl	TOH	100 ± 2 (5)
	TE	95 ± 16
ZnCl ₂	TOH	109 ± 21 (5)
	TE	108 ± 16
MgCl ₂	TOH	99 ± 6 (5)
	TE	114 ± 16
CuCl ₂	TOH	32 ± 16 (5)
	TE	38 ± 13
FeCl ₂	TOH	39 ± 6 (3)
	TE	16 ± 9
CoCl ₂	TOH	47 ± 17 (5)
	TE	12 ± 5
CaCl ₂	TOH	397 ± 75 (8)
	TE	343 ± 62
MnCl ₂	TOH	899 ± 98 (8)
	TE	377 ± 60
FeCl ₃	TOH	206 ± 73 (7)
	TE	67 ± 28
NaB ₄ O ₇	TOH	212 ± 74 (9)
	TE	48 ± 13

^a All assays contained 10 mM EGTA, 10 mM salt, and other additions as in Figure 1. ^b Mean control incorporation rates: TOH, 0.51; TE 2.2 pmol h⁻¹ mg⁻¹ protein. ^c Number in parentheses is the number of experimental repetitions.

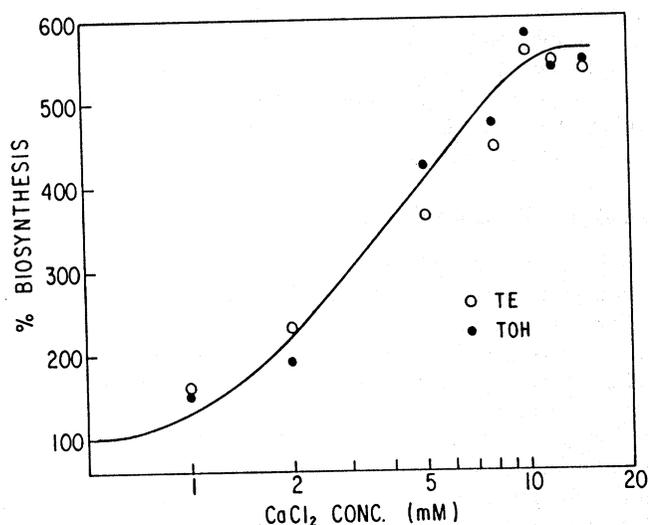


Figure 2. Influence of CaCl₂ on TOH and TE biosynthesis. All assays contained 10 mM EGTA and other additions as in Figure 1. Points are the average of duplicate determinations. With no added CaCl₂ the average incorporation rates were TOH, 0.32 and TE, 1.76 pmol h⁻¹ mg⁻¹ protein.

was difficult to interpret the data. Consequently, the levels of free Ca²⁺ and Ca²⁺-EGTA complex were determined in equivalent latex samples using a Ca²⁺ selective electrode as described in "Materials and Methods." As expected, the concentration of free Ca²⁺ decreased when EGTA increased, the

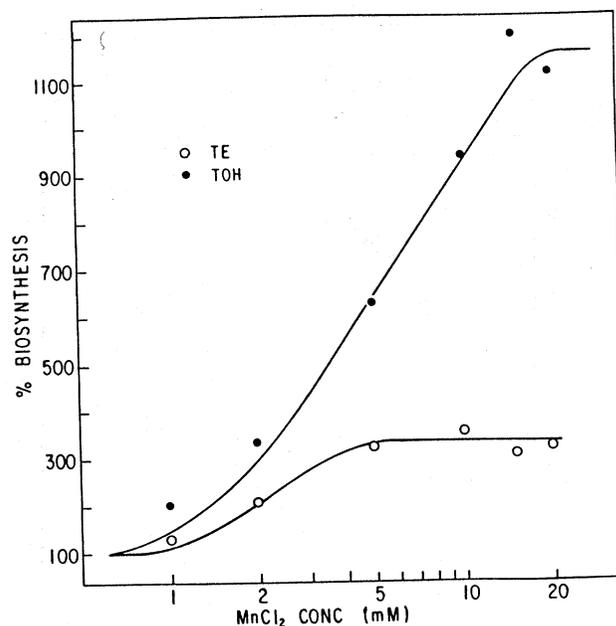


Figure 3. Influence of MnCl₂ on TOH and TE biosynthesis. All assays contained 10 mM EGTA and other additions as in Figure 1. Points are the average of duplicate determinations. With no added MnCl₂ the average incorporation rates were TOH, 0.18 and TE, 0.76 pmol h⁻¹ mg⁻¹ protein.

result of Ca²⁺-EGTA complex formation. Figure 4 shows that a replot of the rates of TE and TOH biosynthesis *versus* the concentration of Ca²⁺-EGTA is well fitted by a computer-generated binding curve that assumes that only one type of binding site is present, and that the binding sites are noninteractive. The apparent dissociation constant of Ca²⁺-EGTA from the site of either TOH or TE biosynthesis is about 11 mM.

EGTA has a good affinity for Mn²⁺, and it is likely that MnCl₂ is also acting through its complex with EGTA. As shown in Figure 1, the effectiveness of MnCl₂ as a stimulator increases as EGTA is increased. However free Mn²⁺ inhibits biosynthesis when no EGTA is added (Fig. 1), and inhibition by free Mn²⁺ (above 1 mM concentration) was observed when Ca²⁺-EGTA was kept fixed at 5 mM (data not shown). Since inhibition by free Mn²⁺ complicates data analysis, further experimentation was not performed.

A comparison of four chelators is shown in Table II. Using the Ca²⁺ selective electrode, the free Ca²⁺ concentration of the latex pellet fraction was brought to 11 mM, and then chelator was added until the concentration of the calcium-chelator complex was 5 mM. Both EGTA and EDTA were much superior to either phthalate or citrate in stimulating biosynthesis. Apparent equilibrium binding constants at pH 6.5 for Ca²⁺ show no correlation with the amount of stimulation. It is probable that the structure of the complex and/or its net charge are important in determining the degree of interaction with the receptor. The data in Table II were obtained under conditions where Ca²⁺ is in excess of EGTA. Thus the uncomplexed EGTA concentration is negligible, and these data again demonstrate the importance of the Ca²⁺-EGTA complex.

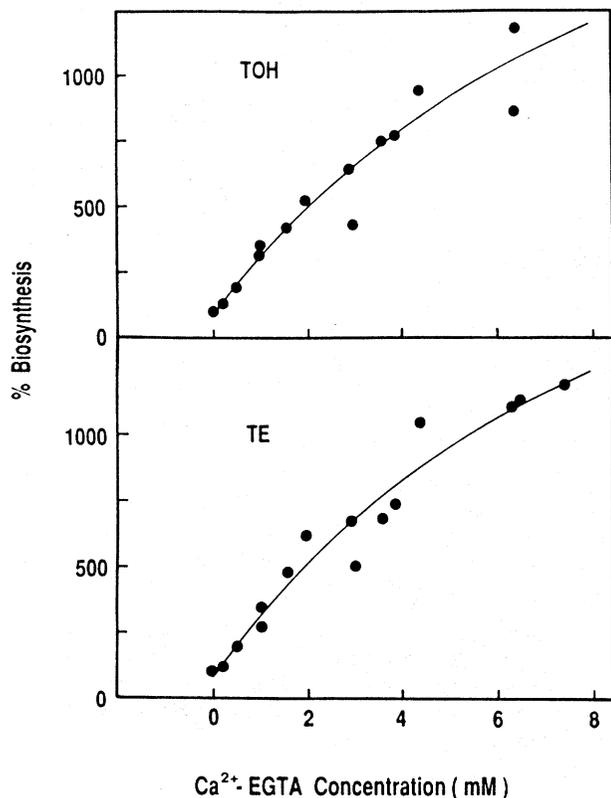


Figure 4. Influence of Ca^{2+} -EGTA on TE and TOH biosynthesis. The incorporation data were from no additions and 10 mM CaCl_2 , Figure 1. The curves were computer-generated for binding to noninteractive sites.

Table II. Effect of Calcium Chelator Structure on Biosynthesis

Chelator	Biosynthesis ^a		Log Apparent Binding Constant (Ca^{2+} , pH 6.5) ^b
	TOH	TE	
	<i>pmol h⁻¹ mg⁻¹ protein</i>		
Control	1.9 ± 0.1	10.4 ± 0.1	
Phthalate	2.5 ± 0.8	10.9 ± 1.6	2.40
Citrate	2.5 ± 0.6	13.4 ± 1.1	4.76
EGTA	12.8 ± 2.1	87.7 ± 5.8	5.70
EDTA	8.7 ± 3.1	58.9 ± 5.8	6.74

^a All assays contained 1 mM malate and 10 μM mevalonate and other additions as given in Figure 1; the control contained 11 mM free Ca^{2+} . The other assays contained 6 mM free Ca^{2+} and 5 mM Ca^{2+} -chelator. Data are the mean of three repetitions. ^b Apparent calcium binding equilibrium constants were calculated according to (6) using data from (10).

Phosphorylated Compounds and Organic Acids

Malate and fructose 1,6-bisphosphate (FBP) plus aldolase stimulated biosynthesis (Table III). Aldolase alone was very slightly inhibitory. Presumably, NAD(P)H is generated at the site of synthesis via malate dehydrogenase or malic enzyme and by triose-P dehydrogenase. The action of malic enzyme in the production of pyruvate and NADPH has been proposed as the source of reducing power for lipid biosynthesis in developing soybean seeds (1). Sometimes it was observed that

Table III. Influence of Phosphorylated Compounds and Organic Acids on Triterpene Biosynthesis

Compound ^a	Triterpene	Triterpene Bio-synthesis ^b
		%
FBP	TOH	109 ± 12 (3) ^c
	TE	108 ± 8
FBP + aldolase	TOH	130 ± 15 (5)
	TE	305 ± 47
α -Glycerol-P	TOH	111 ± 14 (3)
	TE	154 ± 16
ATP	TOH	113 ± 17 (3)
	TE	88 ± 8
NADP	TOH	94 ± 5 (3)
	TE	91 ± 11
NADPH	TOH	57 ± 4 (3)
	TE	52 ± 12
L-Malate	TOH	170 ± 29 (4)
	TE	381 ± 49
Pyruvate	TOH	44 ± 6 (3)
	TE	35 ± 6
DL-Isocitrate	TOH	97 ± 15 (3)
	TE	78 ± 11

^a All compounds tested at a concentration of 1 mM. All assays contained 10 mM EGTA, 25 mM MnCl_2 , 5 mM CaCl_2 , and other additions as given in Figure 1. Aldolase (4 units) and FBP were incubated in 1 mM HEPES (pH 7.4) for 10 min and then added to the reaction. ^b Mean control incorporation rates: TOH, 2.6; TE 2.7 $\text{pmol h}^{-1} \text{mg}^{-1} \text{protein}$. ^c Number in parentheses is the number of experimental repetitions.

the addition of FBP plus aldolase on top of malate gave no more stimulation than malate alone. Since triose-P can yield ATP through the concerted action of dehydrogenase and phosphoglycerate kinase, but malate can only produce NAD(P)H, it is concluded that endogenous ATP levels can be sufficient in some latex samples.

Mevalonate Concentration

Figure 5 shows the effect of increasing mevalonate concentration on biosynthesis. Maximum biosynthesis was achieved at 30 to 40 μM mevalonate. Maximum incorporation into TE plus TOH is about 75 $\text{pmol hr}^{-1} \cdot \text{mg}^{-1} \text{protein}$ when malate and triose-P are added. Without these additions the maximum rate of biosynthesis is reduced by a factor of about 2. When the mevalonate concentration is higher than 40 μM , the rate of biosynthesis decreases, probably as a result of enzyme inhibition by excessively high intermediate levels. An estimate of K_m can be obtained by replotting the data in inverse form. The mean K_m given by all four curves is $15 \pm 4 \mu\text{M}$ (SD). This value compares favorably with the lower estimates for the K_m of isolated mevalonate kinase, the first enzyme of the biosynthetic pathway: rat ovary, 1.8 μM ; pig liver, 9.3 μM ; *Euglena gracilis*, 30 μM ; *Hevea brasiliensis*, 75 μM ; *Sarcophaga bullata*, 310 μM ; rabbit liver, 2600 μM (9).

DISCUSSION

Under optimal conditions total triterpenoid biosynthesis lies in the range of 75 to 100 $\text{pmol hr}^{-1} \text{mg}^{-1} \text{protein}$ (Fig. 5

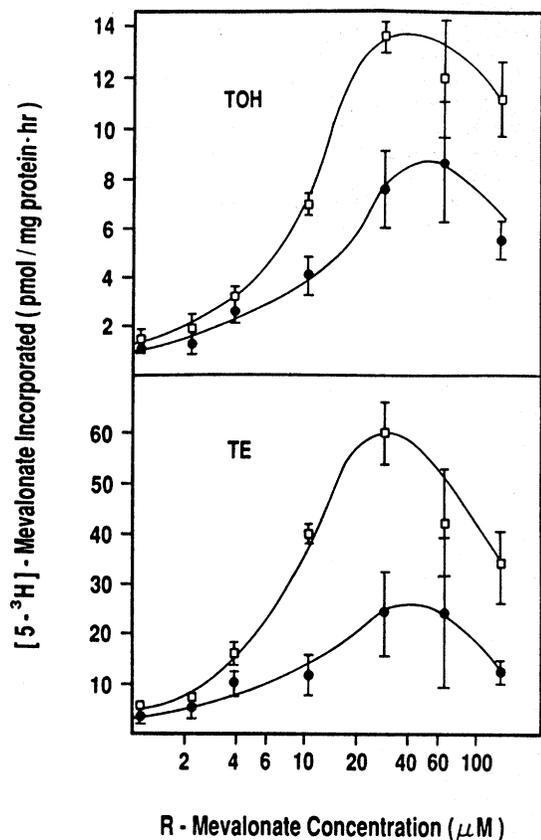


Figure 5. Influence of R-mevalonate concentration on the rate of TOH and TE biosynthesis. (●) four units aldolase, (□) 4 units aldolase + 1 mM FBP + 1 mM malate. All assays contained 11 mM EGTA, 10 mM CaCl_2 , 2 mM MnCl_2 , and other additions as in Figure 1. Values are the mean \pm SE of three determinations.

and Table II). In each assay the latex pellet fraction was derived from 100 μL of whole latex and contains approximately 0.1 mg protein. Thus, the rate of biosynthesis can also be expressed as 75 to 100 $\text{pmol hr}^{-1} \text{mL}^{-1}$ latex. From Figure 2 of Nemethy *et al.* (5), the rate of biosynthesis in whole latex with 0.3 mM acetate is 165 $\text{pmol hr}^{-1} \text{mL}^{-1}$ latex (0–3 h). However, another worker has reported rates of biosynthesis from acetate approximately 7-fold higher (12) (Table II). Therefore, the incorporation capacity of the isolated latex pellet is 10 to 60% of whole latex. The best incorporation rates for the latex pellet reported here are about 25 times faster than those previously reported by this laboratory.

There is a large body of data indicating that changes in calcium concentration can influence cellular metabolism (11). It is possible that the rate of latex triterpenoid biosynthesis is controlled by calcium *in vivo* in conjunction with a putative "natural" chelator. The advantage of having the complex as the active species, rather than free Ca^{2+} , is that the cytoplasmic concentration of free Ca^{2+} can be kept very low. The role that calcium might play in biosynthesis is unknown. However, those enzymes directly involved in the isoprenoid pathway that have been studied *in vitro* do not require Ca^{2+} . Therefore it is tentatively concluded that calcium interacts with biosynthesis indirectly. Possible sites of action could include mem-

brane ion pumps or enzymes involved with the generation of reducing power.

We have suggested that triterpene biosynthesis in the latex pellet fraction occurs in an organelle (7). The evidence for this is as follows: (a) good biosynthesis requires the presence of 0.4 M sorbitol, presumably to regulate osmotic pressure; (b) the site of synthesis can be pelleted by low speed centrifugation; and (c) when centrifugation is conducted at forces above 20,000g, biosynthesis is lost, presumably because the site of synthesis is damaged. The data presented here are consistent with the notion that the site of biosynthesis is a vesiculated nonporous membrane structure. Particularly striking is the observation that exogenously added ATP, Mg^{2+} -ATP (data not shown), or NADPH do not stimulate biosynthesis, although they are directly utilized by the isoprenoid pathway. Thus it appears that the membrane of the putative latex organelle selectively admits external metabolites. The membrane of the chloroplast is also known to be selective and contains transporters that are specific for certain types of substances, *e.g.* phosphate or phosphorylated metabolites, dicarboxylic acids, and adenosine phosphates (4).

Although the chelator-ion complex may be transported, it is important to remember that the data indicate only that the Ca^{2+} -chelator receptor is saturable (Fig. 4). Several years ago it was discovered that EDTA strongly stimulated photosynthesis in isolated chloroplasts from sunflower and wheat (3). The idea that EDTA was passing through the membrane was not considered. It was suggested that EDTA was complexing cations on the chloroplast envelope because the addition of divalent cations along with EDTA results in inhibition. Unfortunately, the divalent ions were added in large excess over EDTA yielding a high concentration of free cations. Since the free cations are by themselves inhibitory, it is not possible to tell if the ion-chelator complex would provide stimulation. Thus, it remains unclear whether there is any similarity in the mechanism of chelator action with regard to chloroplasts metabolism and triterpenoid biosynthesis.

In conclusion, good triterpenoid biosynthesis can be achieved in the latex pellet fraction when appropriate ingredients are added to the reaction mixture. Key ingredients are CaCl_2 and MnCl_2 together with a carboxylate chelator. Malate or malate plus triose-P are also beneficial. The optimal R-mevalonate concentration is about 40 μM .

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