Mechanism of the inhibition of cholesterol biosynthesis by 6-fluoromevalonate

Jean-François NAVE,* Hugues d'ORCHYMONT, Jean-Bernard DUCÉP, François PIRIOU and Michel J. JUNG

Department of Biochemistry, Merrell–Dow Research Institute, Strasbourg Center, 16 rue d'Ankara, 67084 Strasbourg-Cedex, France

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6-Fluoromevalonate blocks the incorporation of mevalonic acid, but not that of isopentenyl pyrophosphate, into non-saponifiable lipids in a rat liver multienzyme system. With ^3H -labelled 6-fluoromevalonate, it was found that 6-fluoromevalonate is converted to its phospho and pyrophospho derivatives in this system. The kinetics of the two kinases were studied. 6-Fluoromevalonate 5-pyrophosphate is a potent competitive inhibitor of pyrophosphomevalonate decarboxylase (K_i 37 nM). In the multienzyme assay for cholesterol biosynthesis, there is accumulation of mevalonate 5-phosphate and mevalonate 5-pyrophosphate in the presence of $5\text{ }\mu\text{M}$ -6-fluoromevalonate, and 6-fluoromevalonate 5-pyrophosphate is more effective than 6-fluoromevalonate in inhibiting cholesterol biosynthesis. We suggest therefore that 6-fluoromevalonate blocks cholesterol biosynthesis at the level of pyrophosphomevalonate decarboxylase after being pyrophosphorylated.

Hypercholesterolaemia is considered to be a major risk factor in coronary artery diseases (Kolata, 1983). This correlation, if not causal relationship, explains the constant efforts made to understand the regulation and to find inhibitors of cholesterol biosynthesis. With few exceptions (Hulcher, 1971; Shama Bhat & Ramasarma, 1979; Rowan Debold & Elwood, 1981; Miller *et al.*, 1981) the main emphasis has been put on the rate-limiting enzyme, hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34). The reports that 6-FMVA decreases the conversion of acetate and mevalonate into cholesterol in perfused rat liver (Brauser & Hermann, 1965) and also blocks the synthesis of juvenile hormone in insect larvae (Quistad *et al.*, 1981a) led us to investigate its mode of action. We report here that 6-FMVA is converted to its phospho and pyrophospho derivatives and that 6-FMVAPP is a strong competitive inhibitor of MVAPP decarboxylase.

Abbreviations used: MVA, mevalonate; MVAP, mevalonate 5-phosphate; MVAPP, mevalonate 5-pyrophosphate; 6-FMVA, 6-fluoromevalonate; 6-FMVAP, 6-fluoromevalonate 5-phosphate; 6-FMVAPP, 6-fluoromevalonate 5-pyrophosphate; IPP, isopentenyl pyrophosphate.

* To whom correspondence and reprint requests should be addressed.

Experimental Procedures

Materials

Pig and chicken livers were obtained fresh from local sources. (*R,S*)-Mevalonolactone, NADP^+ , NAD^+ , ATP, bovine serum albumin and pyruvate kinase were from Sigma; phosphoenolpyruvate was from Aldrich; glucose 6-phosphate was from Merck; NADH and lactate dehydrogenase were from Boehringer. The DEAE-Sephadex A-25 was from Pharmacia. For t.l.c., silica gel 60 F254 plates from Merck were used. R -[3- ^{14}C]Mevalonolactone and R -[2- ^{14}C]mevalonate 5-pyrophosphate were from New England Nuclear; [1- ^{14}C]isopentenyl pyrophosphate and NaB^3H_4 (9.7 Ci/mmol) were from Amersham International. Unlabelled isopentenyl pyrophosphate was synthesized by following known methods (Cornforth & Popják, 1969). All other reagents were of analytical grade.

Synthesis of 6-fluoro[5- ^3H]mevalonolactone

Fluoromethyl diallyl carbinol was synthesized as described by Quistad *et al.* (1981b). This synthesis of 6-FMVA was modified as follows.

Ozone was bubbled into a solution of 2.0 g of fluoromethyl diallyl carbinol dissolved in 80 ml of methylene dichloride and 4 ml of methanol at -78°C until persistence of the blue colour. After elimination of the excess ozone with a stream of N_2

14 ml of dimethyl sulphide was added and the mixture was stirred for 1 h at -40°C , 1 h at -20°C , and overnight at 0°C . After evaporation to dryness, the oily residue was chromatographed on silica to yield 1.4 g of a mixture showing two spots on t.l.c. and no aldehydic protons by n.m.r. A solution of 440 mg of this mixture in 10 ml of methylene dichloride was allowed to react with 41 mg of NaB^3H_4 (100 mCi) dissolved in 2.2 ml of 2-methoxyethyl ether for 2 h at $40-60^{\circ}\text{C}$. After addition of 49 mg of unlabelled NaBH_4 , the reaction mixture was stirred overnight at room temperature and quenched with 1 ml of acetic acid. Solvent evaporation and chromatography on silica gel yielded 274 mg of radiolabelled 3-fluoromethylpentane-1,3,5-triol (sp. radioactivity 10.4 mCi/mmol).

A solution of 274 mg of triol in 16 ml of acetone was treated at 0°C with 1.2 ml of Jones' reagent (2.67 M- CrO_3 in 8 M- H_2SO_4) added dropwise. After 3 h at room temperature the reaction was quenched by a few drops of propan-2-ol, diluted with 30 ml of water, concentrated under reduced pressure and the lactone was extracted with warm methylene dichloride. After drying, evaporation and silica gel chromatography, 128 mg of pure labelled 6-FMVA were obtained (sp. radioactivity 6.8 mCi/mmol). ^1H -n.m.r. (90 MHz, C^2HCl_3) δ (p.p.m.) 1.97 [3H, m, $\text{C}(\text{OH})\text{CH}_2\text{CH}_2$], 2.65 [2H, s, $\text{C}(\text{OH})\text{CH}_2\text{CO}$], 4.30 [2H, d, $\text{C}(\text{OH})\text{CH}_2\text{F}$, J 47 Hz], 4.3–4.7 (2H, m, $\text{CH}_2\text{CH}_2\text{O}$).

Unlabelled material was obtained by the method described by Quistad *et al.* (1981b).

Rat liver multienzyme system

Preparation of rat liver (Sprague-Dawley rats) 10000 g supernatant as well as cholesterol biosynthesis were conducted according to Raulston *et al.* (1980). The substrates used were either radiolabelled MVA or IPP.

For the measurement of squalene and sterol formation, the reaction mixture was saponified and extracted with light petroleum as described by Popják (1969). Total radioactivity incorporated into the nonsaponifiable extract was measured.

For the determination of water-soluble precursors of cholesterol, the incubation mixture was heated for 3 min in boiling water. After centrifugation, aliquots of the supernatant were analysed by t.l.c. with propan-1-ol/ NH_4OH /water (6:3:1, by vol.). Radioactive spots were located with a t.l.c. radioactivity scanner (Berthold model LB 2723). Spots were scraped off and transferred into counting vials for measure of radioactivity in a scintillation cocktail consisting of 6 g of 2,5-diphenyloxazole 0.2 g of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in 1 litre of toluene/methanol (8:1, v/v).

Mevalonate kinase

The enzyme was partially purified from pig liver according to Popják, (1969) up to the batch elution from calcium phosphate. The specific activity of a standard preparation varies between 0.15 and 0.25 units/mg of protein. One unit is the amount of enzyme which phosphorylates MVA at an initial rate of $1\text{ }\mu\text{mol/min}$ at 25°C under the conditions described below.

Using this procedure, 50 enzyme units (0.2 units/mg of protein) free of phosphomevalonate kinase were obtained from 125 g of liver. This preparation, stored as an $(\text{NH}_4)_2\text{SO}_4$ precipitate, was used for the kinetic experiments. In a larger scale preparation (750 g of liver), the calcium phosphate gel eluate having the highest specific activity (0.15 unit/mg of protein; 250 units) was contaminated by phosphomevalonate kinase. This large scale preparation was found suitable for biosynthesis of phosphate and pyrophosphate analogues of MVA and 6-FMVA. The spectrophotometric assay (Popják, 1969) which couples the pyruvate kinase/lactate dehydrogenase system to mevalonate kinase was used with minor modifications. All measurements were performed at 25°C with a double beam recording difference spectrophotometer. Each cuvette contained 0.4 mM-NADH, 6.5 mM-ATP, 0.85 mM-phosphoenolpyruvate, 5 mM- MgCl_2 , 10 mM-cysteine (freshly liberated from its hydrochloride with KOH), 0.1 M-Tris/HCl, pH 7.3, 12 units of lactate dehydrogenase/ml, 23 units of pyruvate kinase/ml and the enzyme in a total assay volume of 1.2 ml. The reaction was started by addition of substrate in $40\text{ }\mu\text{l}$ to the reference cell. The same volume of water was added to the assay cell. Absorbance difference was measured at 340 nm. The initial velocities were determined from the slope of the recorder tracing. The ability of the pyruvate kinase/lactate dehydrogenase system to oxidize NADH in a rapid and quantitative way following ADP addition was checked before each series of experiments.

Phosphomevalonate kinase

The pig liver enzyme was purified following the method of Bazaes *et al.* (1980) up to the DEAE-cellulose step (0.15 unit/mg of protein). This enzyme, free of pyrophosphomevalonate decarboxylase, was used for the kinetic experiments. It was stored in 10 mM-phosphate buffer, pH 7.5, containing 10 mM-2-mercaptoethanol at -20°C and kept well for many months. The enzyme was assayed at pH 7.6 using the same spectrophotometric assay as for mevalonate kinase except that ATP concentration was 3.3 mM, and the substrate was MVAP.

Pyrophosphomevalonate decarboxylase

Purification of the chicken liver enzyme was carried out according to Alvear *et al.* (1982). After the Sephadex G-200 step, the enzyme (0.11 unit/mg of protein) was kept at -20°C in 4mM-phosphate buffer, pH 7.0, containing 8mM-2-mercaptoethanol, 0.08mM-EDTA and 20% (v/v) glycerol and remained stable for at least 2 months. The radioactive assay according to Alvear *et al.* (1982) was used throughout the enzyme purification. Commercial (*R*)-[2- ^{14}C]MVAPP (55mCi/mmol) diluted with biosynthesized (*R*)-MVAPP was used as substrate. For the kinetic experiments, a spectrophotometric assay coupling pyruvate kinase and lactate dehydrogenase to MVAPP decarboxylase (Alvear *et al.*, 1982) was used. Assays were performed at 30°C and pH 7.0. One enzyme unit is defined as the amount of enzyme decarboxylating MVAPP at an initial rate of $1\mu\text{mol/min}$ at 30°C under the assay conditions.

Preparation of MVAP and MVAPP

The large scale preparation of MVA kinase was used to prepare at the same time MVAP and MVAPP. (*R,S*)-MVA (3mmol) containing 30 μCi of commercial (*R*)-[3- ^{14}C]MVA was incubated with 120units of enzyme preparation under the conditions described by Popják (1969) for 1.5h. After protein precipitation by heating at 80°C for 3min, centrifugation and extraction in 80% ethanol at pH 7.4, the reaction products were separated by ion-exchange chromatography on a DEAE-Sephadex A-25 column (60cm $\times$ 2.6cm). After washing with 1.1 litre of water the MVAP and MVAPP were eluted by a linear gradient of $(\text{NH}_4)_2\text{CO}_3$ (2 litres, 0–0.35M). MVAPP was contaminated with MVAP and needed an additional purification on a smaller column of the same resin. $(\text{NH}_4)_2\text{CO}_3$ was eliminated by lyophilization. The yields were 341mg of (*R*)-MVAP and 60mg of (*R*)-MVAPP. The compounds were homogenous

on t.l.c. (silica gel 60; propan-1-ol/ NH_4OH /water, 6:3:1 by vol.) when revealed either by a spray of reagent specific for phosphorus-containing compounds or by scanning for radioactivity.

[3- ^{14}C]MVAPP prepared as described above comigrated with a commercial sample of [2- ^{14}C]MVAPP. The two derivatives were further characterized by ^1H -n.m.r. [90MHz, $^2\text{H}_2\text{O}$, external reference sodium 3-trimethylsilyl (2,2,3,3- ^2H) propanoate]. MVAP: δ (p.p.m.) 1.3 [3H, s, C(OH)CH $_3$], 2.0 [2H, t, C(OH)CH $_2$ CH $_2$, J 6.9Hz], 2.5 [2H, s, C(OH)CH $_2$ CO $_2$ H], 4.0 (2H, td, CH $_2$ CH $_2$ OPO $_3^-$, $J_{\text{H-P}}$ 6.9Hz). MVAPP: δ (p.p.m.) 1.4 [3H, s, C(OH)CH $_3$], 2.0 (2H, t, C(OH)CH $_2$ CH $_2$, J 6.9Hz], 2.6 [2H, s, C(OH)CH $_2$ CO $_2$ H], 4.1 (2H, broad multiplet, CH $_2$ CH $_2$ OP $_2$ O $_6^{2-}$).

Miscellaneous methods

MVA and 6-FMVA were prepared as their potassium salts in solutions at pH 7.5 from the respective lactones according to Popják (1969). Stock solutions of the phosphorus derivatives of MVA and 6-FMVA were prepared in water and stored at -20°C . Their phosphorus content was determined according to Rouser *et al.* (1970). Protein determinations were made according to the method of Lowry *et al.* (1951). The solvent mixture used for separation on t.l.c. of MVA, 6-FMVA and their products of enzymic conversion was always propan-1-ol/ NH_3 /water (6:3:1, by vol.) and is referred to as the standard t.l.c. system.

Results*Localization of the site of cholesterol biosynthesis inhibition by 6-FMVA*

The 10000g supernatant of a rat liver homogenate contains all the enzymes needed to synthesize cholesterol from mevalonate (Popják *et al.*, 1978; Raulston *et al.*, 1980) or from IPP. As can be seen in Table 1, the incorporation of radioactivity

Table 1. Incorporation of [^{14}C]MVA or [^{14}C]IPP into non-saponifiable lipids by the rat liver multienzyme system (*RS*)-MVA (20nmol; 50nCi) or IPP (20nmol; 50nCi) were incubated at 37°C with 0.85ml of the 10000g supernatant from rat liver supplemented with cofactors according to Raulston *et al.* (1980) in a final assay volume of 1ml with or without 6-FMVA. Incubation was stopped with 2ml of 4M ethanolic KOH solution. After saponification for 2h at 70°C , the non-saponifiable lipids were extracted twice with 3ml of light petroleum and total radioactivity was determined in this extract.

Additions	Incorporation of [^{14}C]MVA into non-saponifiable lipids			Incorporation of [^{14}C]IPP into non-saponifiable lipids		
	Incubation time (min)	Incorporation (d.p.m.)	Inhibition (%)	Incubation time (min)	Incorporation (d.p.m.)	Inhibition (%)
None	10	18685	0	12	13060	0
	50	36805	0	40	20415	0
6-FMVA (5 μM)	10	1271	93.2	12	12840	1.7
	50	17645	52.1	40	18870	7.6

into non-saponifiable lipids is rapid during the first 10 min and is not linear with long times of incubation. Addition of $5\mu\text{M}$ -6-FMVA blocks extensively the incorporation of mevalonate into non-saponifiable lipids at the 10 min time point (93%); the effect diminishes with the incubation time (52% at 50 min). When IPP is used as precursor of sterols, 6-FMVA no longer has an effect either at short or at long time points.

In the second part of this experiment, the distribution of radioactivity among the water-soluble metabolites of MVA was determined in the absence or presence of 6-FMVA. As can be seen in Fig. 1, the elution profiles are totally different. All peaks except δ could be identified by spiking with authentic samples: α is MVAPP, β is IPP, γ is MVAP; δ could possibly be farnesyl pyrophosphate. While MVAP and MVAPP are not found in control incubations, they represent the main radioactive products in presence of 6-FMVA. On the contrary, IPP and peak δ are absent from the experiment with the inhibitor.

Formation and characterization of phosphorylated metabolites of 6-FMVA

Radiolabelled 6-FMVA was incubated with the rat liver multienzyme system under similar conditions as for mevalonate. The reaction was followed by t.l.c. Fig. 2 shows a radiochromatogram obtained after 2.5 h of incubation. Besides unchanged 6-FMVA, three major radioactive products A, B and C can be detected: they represent 3, 15 and 2.5% of total applied radioactivity respectively.

Biosynthesis of these metabolites for preparative purposes was attempted under the conditions described for MVAP and MVAPP biosynthesis above. Partially purified mevalonate kinase (60 units), which also contains phosphomevalonate kinase, were incubated with 1.5 mmol of (R,S)-6-FMVA containing 350 μCi of the ^3H -labelled compound at 30°C for 7 h. The formation of metabolites of 6-FMVA was followed by t.l.c. in the standard system. Assuming that only (R)-6-FMVA was transformed, a maximum of 65% conversion was reached after about 4 h. Separation and recovery of the individual metabolites was achieved as for the phosphorylated metabolites of MVA. Five peaks were collected, as shown in Fig. 3. Peak I (R_F 0.6) in the standard t.l.c. system corresponded to unreacted 6-FMVA. Peak II contained no fluorine (^{19}F -n.m.r.) and was discarded. Peaks III and IV needed a second DEAE-Sephadex column for complete separation. Peak V was contaminated with nucleotides and was purified by t.l.c. followed by a second DEAE-Sephadex column. Compounds IV and V were homogenous on t.l.c. either when visualized according to the method of Parker *et al.* (1978)

Fig. 1. Effect of 6-FMVA on the relative amounts of the water-soluble metabolites of MVA formed in the rat liver multienzyme system

[^{14}C]MVA (8 nmol, 0.4 μCi) was incubated for 15 min at 37°C with 0.1 ml of the 10000g supernatant from rat liver supplemented with cofactors according to Raulston *et al.* (1980) without (a) or with (b) $50\mu\text{M}$ -6-FMVA in a final volume of 0.15 ml. Incubation was stopped by immersion for 3 min in boiling water. After centrifugation to remove protein, samples were applied to silica gel plates and eluted with the standard t.l.c. system. Peaks were identified by their co-migration with authentic labelled samples. Their identity is indicated in the text. Scan (a) was recorded at a 4-fold greater attenuation than scan (b).

specific for phosphorus-containing substances or by radioscanning. They were further characterized by ^1H -n.m.r. spectroscopy (90 MHz) in $^2\text{H}_2\text{O}$ with sodium 3-trimethylsilyl (2,2,3,3- ^2H)propanoate as external reference.

Compound IV: δ (p.p.m.) 2.0 [2H, td, C(OH)(CH $_2$ F)CH $_2$ CH $_2$, J 6.6 Hz, $J_{\text{H-F}}$ 1.5 Hz], 2.6

[2H, d, C(OH)(CH₂F)CH₂CO₂H, J_{H-F} 1.4 Hz], 4.0 (2H, td, CH₂CH₂OPO₃⁻, J 6.5 Hz, J_{H-P} 6.5 Hz), 4.5 [2H, d, C(OH)CH₂F, J_{H-F} 46.8 Hz]. This spectrum corresponds unambiguously to 6-FMVAP.

Compound V: δ (p.p.m.) 2.1 [2H, dt, C(OH)(CH₂F)CH₂CH₂, J 6.2 Hz, J_{H-F} 1.0 Hz], 2.7 [2H, d, C(OH)(CH₂F)CH₂CO₂H, J_{H-F} 1.2 Hz], 4.1 (2H, broad multiplet, CH₂CH₂OP₂O₆²⁻), 4.6 [2H, d, C(OH)CH₂F, J_{H-F} 47.2 Hz]. This compound is most likely the pyrophosphate ester of 6-FMVA.

Compound III contains no fluorine; however, as it represents a major metabolite of 6-FMVA, it was attempted to characterize it further. ¹H, ¹³C, and ³¹P-n.m.r. spectroscopy and mass spectrometry suggest the following cyclic structure:

¹H-n.m.r. [90 MHz, ²H₂O, sodium 3-trimethylsilyl (2,2,3,3-²H)propanoate as external reference]: δ (p.p.m.) 2.1 (2H, m), 2.5 (2H, s), 4.0 (4H, m). ³¹P-n.m.r. (36.4 MHz, ²H₂O, H₃PO₄ as external reference): δ -59 p.p.m., pseudo-quintuplet, J 17 Hz. ¹³C-n.m.r. [22.63 MHz, ²H₂O, sodium 3-trimethylsilyl(2,2,3,3-²H)propanoate as external reference]: δ (p.p.m.) 180.0 (s, CH₂CO₂H), 73.9 [s, CH(OH)CH₂CO₂H], 72.6 [d, C(OH)CH₂OP, $J_{13C-31P}$ 4.5 Hz], 64.1 (d, CH₂CH₂OP), 46.0 (s, CH₂COOH), 43.6 [s, CH(OH)CH₂CH₂OP].

Mass spectrum: chemical ionization with NH₃ as reactant gas gave a pseudomolecular peak MH^+ at 226 and fragments at m/z 209 ($MH^+ - OH$), 182 ($MH^+ - CO_2$) and 165 ($MH^+ - OH - CO_2$).

Metabolite A of Fig. 2 was found to comigrate with 6-FMVAPP and metabolite B with 6-FMVAP. Metabolite C, however, behaves differently on t.l.c. from the cyclic phosphate ester characterized above.

Interaction of 6-FMVA and its phosphorylated derivatives with MVA kinase, MVAP kinase and MVAPP decarboxylase

The different compounds were tested as substrates or inhibitors of the partially purified enzymes. The results are summarized in Table 2; the data for the natural substrate determined under identical conditions are included for comparison.

It was confirmed that 6-FMVA and 6-FMVAP are as expected substrates of their corresponding kinases. V_{max} values are unchanged by fluorine substitution; however, affinities are reduced by a

Fig. 2. Conversion of 6-FMVA to polar metabolites by the rat liver multienzyme system

[³H]6-FMVA (4.4 μ mol, 28 μ Ci) was incubated at 37°C for 2.5 h with 0.45 ml of the 10000g supernatant from rat liver supplemented with cofactors according to Raulston *et al.* (1980) in a final volume of 0.5 ml. Samples were further treated as described in Fig. 1. 6-FMVA and its metabolites were detected by scanning the radioactivity. Quantification was achieved by scraping the silica and determining the associated radioactivity by liquid-scintillation counting. The nature of compounds A, B and C is discussed in the text.

factor of 2 for 6-FMVA and of 5 for 6-FMVAP. No substrate activity could be detected for 6-FMVAPP; however, this compound is a potent competitive inhibitor of MVAPP decarboxylase. The degree of inhibition does not change with time of incubation.

Comparison of 6-FMVA and 6-FMVAPP as inhibitors of cholesterol biosynthesis

The inhibition of cholesterol biosynthesis in the multienzyme system by 6-FMVA and 6-FMVAPP was studied as a function of time (Fig. 4). The two compounds were added at a concentration of 5 μ M. It clearly appears that 6-FMVAPP is a stronger inhibitor of cholesterol biosynthesis than 6-FMVA. The inhibition is almost total at the 10 min incubation point and seems to be relieved at longer time points. This could possibly be due to the

Fig. 3. Elution profile of $[5\text{-}^3\text{H}]6\text{-FMVA}$ metabolites obtained by using the large-scale MVA kinase preparation (see the text). The DEAE-Sephadex A-25 column ($60\text{ cm} \times 2.6\text{ cm}$) was eluted with a 2 litre linear gradient of $(\text{NH}_4)_2\text{CO}_3$ (0–0.35 M). Nucleotides were detected by measure of A_{265} (○---○). The nature of peaks I, II and III is discussed in the text.

Fig. 4. Inhibition of non-saponifiable lipid biosynthesis by 6-FMVA and 6-FMVAPP

(*RS*)- $[1\text{-}^{14}\text{C}]$ MVA (20 nmol, 50 nCi) was incubated at 37°C with 0.85 ml of the 10000g supernatant from rat liver supplemented with cofactors according to Raulston *et al.* (1980) in a final assay volume of 1 ml, without (○) or with $5\text{ }\mu\text{M}$ -6-FMVA (▲) or $5\text{ }\mu\text{M}$ -6-FMVAPP (△). Incubation was stopped by 2 ml of 4 M-ethanolic KOH solution. After saponification for 2 h at 70°C , the non-saponifiable lipids were extracted twice with 3 ml of light petroleum and total radioactivity was determined in this extract.

hydrolytic action of phosphatases on 6-FMVA PP, although this was not verified.

Discussion

In the multienzyme system of rat liver, 6-FMVA inhibits the incorporation of MVA into the non-saponifiable lipids but not that of IPP, and produces accumulation of MVAPP and to a lesser extent of MVAP. This clearly designates the decarboxylation of MVAPP as the main target for inhibition. Indeed 6-FMVA undergoes phosphorylation and pyrophosphorylation in this system or by the partially purified kinases.

In addition to the two normal phosphate ester derivatives, a cyclic ester is formed and even represents the major metabolite. The mechanism of formation of this metabolite is not explained; we were unable to demonstrate its formation from either 6-FMVAP or 6-FMVAPP under the conditions of experimentation. This cyclic compound has only a marginal effect on the kinases or the decarboxylase (results not shown). During the decarboxylation step of MVAPP, the hydroxy on C-3 is lost and is involved in ATP hydrolysis. Substitution by the electroattractive F renders the C–O

Table 2. *Kinetic parameters for substrates and inhibitors of MVA kinase, MVAP kinase and MVAPP decarboxylase* (a) The data refer to measurements at 25°C in buffer at pH 7.3 and 6.5 mM-ATP for MVA kinase and in buffer at pH 7.6 and 3.3 mM-ATP for MVAP kinase. The enzymic assays were performed as described in the Experimental procedures section. The data given for each enzyme are obtained from a single experiment. (b) The data presented were obtained at 30°C by using the spectrophotometric assay according to Alvear *et al.* (1982). The reaction mixture contained 0.1 M-Tris/HCl buffer, pH 7.0, 0.1 M-KCl, 5 mM-ATP, 5 mM-MgCl₂, 0.5 mM-phosphoenolpyruvate, 0.23 mM-NADH, 6.5 units of pyruvate kinase, 11.8 units of lactate dehydrogenase, MVAPP decarboxylase (135 µg of protein) and MVAPP at various concentrations (5–25 µM) in a final volume of 1 ml. The higher concentration of 6-FMVAPP tested was 160 nM. K_i was determined by using a plot of the slopes of Lineweaver-Burk plots at various concentrations of inhibitor versus inhibitor concentration.

Enzyme	Substrate or inhibitor (*)	K_m or K_i (*)	V_{max} (unit/mg of protein)
(a)			
MVA kinase	MVA	78 µM	0.25
	6-FMVA	152 µM	0.24
MVAP kinase	MVAP	30 µM	0.14
	6-FMVAP	158 µM	0.13
(b)			
MVAPP	MVAPP	10 µM	0.11
decarboxylase	6-FMVAPP*	37 nM*	—
	(competitive inhibition)		

bond more resistant to cleavage; therefore, 6-FMVAPP is not a substrate of MVAPP decarboxylase but a strong competitive inhibitor. The gain in affinity of almost two log units compared with MVAPP hints towards a structure of a transition state analogue. If this hypothesis can be accepted, it would mean that during the binding process of MVAPP to the decarboxylase, the C–O bond is elongated to a point where it is broken. The fluorine substitution could prevent a positive charge at C-3 from developing in the transition state; thus during binding the breaking point would not be reached. 6-FMVAPP is the most potent inhibitor of the avian liver MVAPP decarboxylase known. *p*-Coumaric and isoferulic acids, described by Shama Bhat & Ramasarma (1979), have K_i values in the millimolar range.

As stated earlier on, MVAPP decarboxylase is probably the main site of blockade of cholesterol synthesis by 6-FMVA, as seen from the accumulation of MVAPP. However MVAP is also accumulated to some extent; therefore competition of 6-FMVA with MVA for MVA kinase and of 6-FMVAP with MVAP for MVAP kinase also contributes to the delay of conversion. Such an effect has been described for 3,5-dihydroxy-3,4,4-trimethylvaleric acid, a substrate of yeast MVA and MVAP kinases (Hulcher, 1982). Introduction of the two lipophilic methyl groups on C-4 of mevalonate increases the affinity by a factor of 2 for yeast MVA kinase and by a factor of 10 for MVAP kinase; the V_{max} values have not been reported. For the mammalian enzymes, the substitution of a hydrogen atom by fluorine on C-6

does not affect V_{max} ; however, the binding constants are decreased, suggesting that the electronic factor may play a role in binding. As already mentioned, 6-FMVA inhibits juvenile hormone synthesis in insects; the 6,6-difluoromevalonate however is markedly less active. If the inhibition of juvenile hormone synthesis by 6-FMVA is also mediated through the phosphorylated metabolites, due to the effect of the fluorine atom on the binding constants to the kinases, it is likely that the difluoro analogue is poorly phosphorylated. It would be interesting to synthesize chemically the pyrophosphorylated analogue of 6,6-difluoromevalonate and to study its interaction with MVAPP decarboxylase.

Quistad *et al.* (1981a) concluded that 6-FMVA is not potent enough to be of practical utility as an insecticide. This statement is probably also valid for the control of cholesterol biosynthesis in mammals. Even though the pyrophosphorylated derivative is a powerful inhibitor of MVAPP decarboxylase, the conversion of 6-FMVA is too slow and not complete. In addition, part of the compound is diverted to the cyclic phosphate ester for which no useful activity could be found. Inhibition of MVAPP decarboxylase may however still be a valid target to control cholesterol biosynthesis. 6-FMVAPP is too prone to be inactivated by phosphatases to be seriously considered for evaluation *in vivo*. Substitution of phosphates or pyrophosphates by phosphonates or phosphonophosphates has yielded inhibitors of phosphomevalonate kinase, prenyl transferase and squalene synthetase (Corey & Volante, 1976; Parker *et al.*, 1978; Popják

et al., 1978). By analogy, analogues of that kind for 6-FMVAPP may yield potent inhibitors of MVAPP decarboxylase stable enough to be tested *in vivo*.

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