Inhibition of Rat Liver Mevalonate Pyrophosphate Decarboxylase and Mevalonate Phosphate Kinase by Phenyl and Phenolic Compounds

By Charavu SHAMA BHAT and T. RAMASARMA

Department of Biochemistry, Indian Institute of Science, Bangalore-560 012, India

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1. Mevalonate pyrophosphate decarboxylase of rat liver is inhibited by various phenyl and phenolic acids. 2. Some of the phenyl and phenolic acids also inhibited mevalonate phosphate kinase. 3. Compounds with the phenyl-vinyl structure were more effective. 4. Kinetic studies showed that some of the phenolic acids compete with the substrates, mevalonate 5-phosphate and mevalonate 5-pyrophosphate, whereas others inhibit uncompetitively. 5. Dihydroxyphenyl and trihydroxyphenyl compounds and *p*-chlorophenoxyisobutyrate, a hypocholesterolaemic drug, had no effect on these enzymes. 6. Of the three mevalonate-metabolizing enzymes, mevalonate pyrophosphate decarboxylase has the lowest specific activity and is probably the rate-determining step in this part of the pathway.

Incorporation of mevalonate into cholesterol by homogenates of rat liver and of rat brain is inhibited by phenyl and phenolic acids (Shah *et al.*, 1968, 1969; Ranganathan & Ramasarma, 1973, 1974). The site of inhibition was found to be between mevalonate and isopentenyl pyrophosphate. In experimental phenylalaninaemia, the concentration of phenylalanine increases in the brain and in the liver, and the incorporation of [2-¹⁴C]mevalonate into non-saponi-

fiable lipids and into sterols by homogenates of the brain is decreased (Shah *et al.*, 1968). These experiments were done with the whole enzyme system shown in Scheme 1 and not with the individual enzymes. A study was therefore undertaken on the specificity and mechanism of the inhibition by phenyl and phenolic compounds of the three cytosolic mevalonate-metabolizing enzymes that convert mevalonate into isopentenyl pyrophosphate. The results showed that a variety of phenyl and phenolic acids inhibit mevalonate pyrophosphate decarboxylase [ATP-5-diphosphomevalonate carboxylase (dehydrating); EC 4.1.1.33] and under specified conditions, mevalonate phosphate kinase (ATP-5-phosphomevalonate phosphotransferase; EC 2.7.4.2), but not mevalonate kinase (ATP-mevalonate 5-phosphotransferase; EC 2.7.1.36).

Materials and Methods

Materials

(*RS*)-[1-¹⁴C]Mevalonic acid lactone (6 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Phenyl-lactic acid, phenylpyruvic acid, L-phenylalanine, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl-lactic acid, *p*-hydroxybenzaldehyde, *p*-hydroxyphenylpropionic acid, resorcinol (1,3-dihydroxybenzene), catechol (1,2-dihydroxybenzene), protocatechuic acid (3,4-dihydroxybenzoic acid), L-dopa (3,4-dihydroxyphenylalanine), *p*-chlorophenylalanine, DL-3,4-dihydroxyphenyl glycol, and ATP were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Cinnamic acid (3-phenyl-2-propenoic acid), *p*-coumaric acid (4-hydroxycinnamic

Scheme 1. Metabolism of mevalonate

acid), *m*-coumaric acid (3-hydroxycinnamic acid), ferulic acid (4-hydroxy-3-methoxycinnamic acid), isoferulic acid (3-hydroxy-4-methoxycinnamic acid), anisic acid (4-methoxybenzoic acid), phloroglucinol (1,3,5-trihydroxybenzene) and syringic acid (4-hydroxy-3,5-dimethoxybenzoic acid) were obtained from Koch–Light Laboratories, Colnbrook, Bucks., U.K. *p*-Chlorophenoxyisobutyrate (clofibrate) was a gift from Dr. J. M. Thorp, ICI, Macclesfield, Cheshire, U.K. All other chemicals used were of analytical reagent grade.

Animals

Male albino rats, weighing 80–100g, obtained from the Institute colony were used.

Preparation of the 38000g supernatant

The rats were killed by cervical dislocation between 09:00 and 11:00h. The livers were quickly removed, chilled and homogenized (20%, w/v, homogenates) in 0.25M-sucrose. The homogenate was centrifuged at 38000g for 40min and the resultant supernatant was used as the source of enzymes.

Preparation of (R)-[1-¹⁴C]mevalonate 5-phosphate and (R)-[1-¹⁴C]mevalonate 5-pyrophosphate

[1-¹⁴C]Mevalonate 5-phosphate and [1-¹⁴C]mevalonate 5-pyrophosphate were synthesized by using the 30–60%-satd.-(NH₄)₂SO₄ fraction of rat liver supernatant. The assay mixture consisted of 50mM-Tris/HCl buffer, pH 7.4, 10mM-ATP, 12.5mM-MgCl₂, 12.5mM-2-mercaptoethanol, 2mM-*p*-coumaric acid and 2.5mM-(*RS*)-[1-¹⁴C]mevalonate (specific radioactivity 0.2μCi/μmol). *p*-Coumaric acid inhibits mevalonate pyrophosphate decarboxylase and the rationale in using the inhibitor is discussed below. Mevalonic acid lactone was delactonized by incubating the mevalonic acid lactone in the presence of 0.05M-KOH for 30min at room temperature and neutralized with 1M-HCl. The potassium salt of mevalonate was used for the synthesis of the phosphate derivatives. The incubation was carried out for 45min at 37°C and the protein was precipitated by heating at 100°C for 2min. The precipitate was removed by centrifugation at 800g for 5min. Mg²⁺ was removed from the incubation mixture by precipitation as NH₃-Mg²⁺-phosphate. The supernatant was adjusted to pH 6 with 1M-HCl. Ethanol was added to a concentration of 75% (v/v), and the mixture was cooled to -20°C and kept overnight. The precipitate, which contained ATP and ADP, was sedimented, washed twice with small volumes of ice-cold 75% (v/v) ethanol and the supernatants were pooled.

The supernatants and the washings were concentrated on a rotary flash evaporator at 45°C and the concentrated solution was applied to Whatman no. 1 filter paper. The system was developed by ascending

paper chromatography (25cm) in the solvent system butan-1-ol/formic acid/water (77:10:13, by vol.) for about 11h. The spots were identified by cutting the paper into 1cm pieces with a reference spot and counting the radioactivity. The *R_F* values for mevalonate 5-pyrophosphate, mevalonate 5-phosphate, mevalonate and *p*-coumaric acid are 0.06, 0.24, 0.72 and 0.92 respectively. The paper strips corresponding to mevalonate 5-phosphate and mevalonate 5-pyrophosphate were eluted with water. Since the inhibitor *p*-coumaric acid was used, the formation of isopentenyl pyrophosphate was only 5% of the mevalonate 5-phosphate formed, as measured by the liberated ¹⁴CO₂. Even this trace amount of isopentenyl pyrophosphate formed will not be radioactive, since the labelled carbon of [1-¹⁴C]mevalonate is lost as ¹⁴CO₂. The minute amount of isopentenyl pyrophosphate will not affect the mevalonate phosphate kinase assay.

Mevalonate 5-phosphate and mevalonate 5-pyrophosphate were identified by other chromatographic systems (Bloch *et al.*, 1959; Porter & Anderson, 1961; Shah *et al.*, 1965). Other criteria for the identification were the substrate-product relationship, i.e. the effect of time on the formation of the products mevalonate 5-phosphate and mevalonate 5-pyrophosphate. It is also identified by hydrolysis of mevalonate 5-phosphate and mevalonate 5-pyrophosphate by alkaline phosphatase (Bloch *et al.*, 1959). The conversion in this system was 35–40% of the mevalonate used, which is equivalent to 70–80%, considering that only the (*R*)-isomer of the mixture of (*RS*)-forms was used by the mevalonate kinase. The *R_F* value for *p*-coumaric acid is 0.92, and the remaining mevalonate can be recovered free from *p*-coumaric acid.

Release of ¹⁴CO₂ from [1-¹⁴C]mevalonate in vitro

This was measured by the method of Shama Bhat & Ramasarma (1977), except that the enzyme used was that in the 38000g supernatant of liver homogenate instead of that in the postnuclear supernatant fraction. The specific activity is expressed as nmol of ¹⁴CO₂ liberated/h per mg of protein.

Assay of mevalonate kinase

The assay system contained 50mM-Tris/HCl, pH 7.4, 10mM-ATP, 12.5mM-MgCl₂, 12.5mM-2-mercaptoethanol and 0.6mg of 38000g supernatant protein. Preincubation was for 5min and the reaction was started by adding 0.5mM-[1-¹⁴C]mevalonate (0.2μCi/μmol). Mevalonate was delactonized before use. The total volume of the incubation mixture was 200μl. The reaction rate was linear up to 5min and 0.6mg of enzyme protein, although there will be a certain amount of formation of mevalonate 5-phosphate, which is the substrate for mevalonate phosphate kinase. Incubation was at 37°C for 5min

and the reaction was stopped by heating at 100°C for 2 min. The precipitated protein was removed by centrifugation at 800g for 5 min, and 100 μ l of the sample was applied to Whatman no. 1 paper. N.evalonate and mevalonate 5-phosphate were separated by ascending chromatography as described above. The area corresponding to mevalonate 5-phosphate (R_F 0.24) was removed and the radioactivity measured. The specific activity is expressed as nmol of mevalonate 5-phosphate formed/h per mg of protein.

Assay of mevalonate phosphate kinase

The assay system is essentially the same as that described for mevalonate kinase. Preincubation was done for 5 min (except where specified otherwise) and the incubation time was 10 min. The reaction was started by adding 150 μ M-[1- 14 C]mevalonate 5-phosphate (0.2 μ Ci/ μ mol). The mevalonate 5-pyrophosphate formed from mevalonate 5-phosphate was separated, and the area corresponding to mevalonate 5-pyrophosphate (R_F 0.06) was removed and the radioactivity was measured as described above. The time and enzyme concentration were chosen such that they were within the range of linearity. Since rate of mevalonate pyrophosphate decarboxylase is only half of that of kinase, it is unlikely to effect the rate of mevalonate phosphate kinase significantly, although some decarboxylation occurs. The specific activity is expressed as nmol of mevalonate 5-pyrophosphate formed/h per mg of protein.

Assay of mevalonate pyrophosphate decarboxylase

The assay mixture was as described above for mevalonate kinase. The total volume of the incubation mixture was 1.0 ml. The incubation mixture was placed in the outer well of a Warburg flask. The central well contained 0.2 ml of 1 M-KOH and the side arm contained 0.5 ml of 1 M-H₂SO₄. After preincubation for 5 min the reaction was started by adding [1- 14 C]mevalonate 5-pyrophosphate (0.05 mm, 0.2 μ Ci/ μ mol) and the flasks were stoppered. At the end of 20 min at 37°C the reaction was stopped by tipping the contents of the side arm into the flasks,

which were kept at room temperature (26°C) for 1 h without opening the stoppers. Portions from the central well were taken and the radioactivity was measured. The specific activity is expressed as nmol of CO₂ liberated/h per mg of protein.

Measurement of radioactivity

Radioactivity was measured in a Beckman model LS-100 liquid-scintillation spectrometer. For Whatman strips that contained mevalonate 5-phosphate or mevalonate 5-pyrophosphate, 5 ml of scintillation fluid consisting of 0.5% (w/v) PPO (2,5-diphenyl-oxazole) in toluene was used. To measure the radioactivity in 14 CO₂, 5 ml of scintillation fluid consisting of 0.2% PPO and 0.002% POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] in toluene/methanol (1:1, v/v) was used (Yardley, 1964).

Protein was determined by the method of Gornall *et al.* (1949), with bovine serum albumin as the standard.

Results

Effects of *p*-coumaric acid on mevalonate-metabolizing enzymes

Ranganathan & Ramasarma (1973) have shown that *p*-coumaric acid and cinnamic acid were potent inhibitors of the incorporation of mevalonate into non-saponifiable lipids with post-nuclear supernatant fraction of rat liver as the enzyme system. The site of inhibition was also found to be between mevalonate and isopentenyl pyrophosphate. We further screened the three mevalonate-metabolizing enzymes separately for the inhibitory activity of *p*-coumaric acid (Table 1). By using 38000g supernatant of rat liver as the source of the enzymes, it was found that liberation of 14 CO₂ from [1- 14 C]mevalonate was inhibited by 70% at a concentration of 1.25 mm. Mevalonate kinase was not affected. Mevalonate pyrophosphate decarboxylase was inhibited by up to 71%, which is comparable with the effect on the overall reaction from mevalonate to isopentenyl pyrophosphate. Mevalonate phosphate kinase was inhibited significantly only after preincubation with the inhibitor. The effect of time of preincubation

Table 1. Effect of *p*-coumaric acid on mevalonate-metabolizing enzymes of liver

The 38000g supernatant was used as enzyme source. The concentration of *p*-coumaric acid used was 1.25 mm, and preincubated for 5 min before the addition of the substrate. The details of the experiments are given in the text.

Test system	Enzyme activity (nmol/h per mg of protein)		Percentage inhibition
	No inhibitor	+ <i>p</i> -Coumaric acid	
[1- 14 C]Mevalonate $\rightarrow$ 14 CO ₂	26.2	7.9	70
Mevalonate kinase	112.0	114.2	Nil
Mevalonate phosphate kinase	66.5	36.2	46
Mevalonate pyrophosphate decarboxylase	28.3	8.2	71

with 2mM-*p*-coumaric acid on the inhibition of mevalonate phosphate kinase is shown in Fig. 1. At 5 min, 50% inhibition had occurred, and on increasing the preincubation time up to 15 min a maximum inhibition of only 54% was obtained. Hence the rest of the experiments were done after

Fig. 1. Effect of time of preincubation with *p*-coumaric acid on inhibition of mevalonate phosphate kinase

The assay system consisted of Tris/HCl (pH 7.4, 50mM), ATP (10mM), MgCl₂ (12.5mM), 2-mercaptoethanol (12.5mM), protein (0.6mg) and mevalonate 5-phosphate (0.15mM) in a total volume of 200μl. The incubation time was 10 min. *p*-Coumaric acid (2.0mM) was incubated with the enzyme protein for 0–15 min at 37°C before the addition of mevalonate 5-phosphate. No inhibitor, the enzyme activity in the absence of inhibitor.

Table 2. Effect of phenyl and phenolic acids on mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase
The assay system was as described in Figs. 1 and 2. Compounds were added at a concentration of 2.5mM and preincubated for 5 min; +, activation; –, inhibition.

Additions (2.5 mM)	Mevalonate pyrophosphate decarboxylase		Mevalonate phosphate kinase	
	Activity (nmol/h per mg of protein)	Percentage inhibition	Activity (nmol/h per mg of protein)	Percentage change
None	28.3	—	66.5	—
Isoferulic acid	3.8	87	24.0	–64
Ferulic acid	7.4	74	—	—
<i>m</i> -Coumaric acid	5.4	84	16.0	–70
<i>p</i> -Coumaric acid	4.8	81	27.5	–59
<i>p</i> -Hydroxybenzoic acid	15.4	46	48.2	–28
<i>p</i> -Hydroxybenzaldehyde	14.0	51	59.1	–11
<i>p</i> -Hydroxyphenylpropionic acid	14.1	50	36.0	–46
None	28.3	—	56.2	—
<i>p</i> -Hydroxyphenylacetic acid	19.6	31	58.5	+3
<i>p</i> -Hydroxyphenyl-lactic acid	22.0	23	54.0	–4
L-Tyrosine	17.1	40	—	—
L-Tryptophan	15.1	47	—	—
Anisic acid	18.4	36	41.0	–27
Cinnamic acid	10.0	65	40.5	–28
Phenylpyruvic acid	20.5	28	—	—
Phenyl-lactic acid	19.4	32	—	—
<i>p</i> -Chlorophenoxyisobutyrate	27.4	3	59.1	+5
Phloroglucinolcarboxylic acid	27.0	4	—	—
Syringic acid	25.0	11	—	—
None	28.3	—	56.2	—
Resorcinol	26.3	6	—	—
Phloroglucinol	22.5	20	—	—
Catechol	24.7	12	62.2	+11
Protocatechuic acid	22.1	21	63.0	+12
None	26.0	2	80.4	—
L-Dopa	25.1	5	76.4	–5
<i>p</i> -Chlorophenylalanine	24.2	7	81.0	+1
DL-3,4-Dihydroxyphenyl glycol	25.8	1	77.1	–4

Fig. 2. Double-reciprocal plot for inhibition by phenyl and phenolic acids of mevalonate pyrophosphate decarboxylase

The assay system consisted of Tris/HCl (pH 7.4, 50mM), ATP (10mM), MgCl₂ (12.5mM), 2-mercaptoethanol (12.5mM), protein (0.8mg) and mevalonate 5-pyrophosphate (0.05mM). The incubation period was 20min. The compounds were added as neutral solutions and preincubated for 5min at 37°C before the substrate was added. The concentration of the inhibitors used was 2.5mM. (a) ●, No inhibitor; ○, *p*-hydroxyphenyl-lactic acid; △, *p*-hydroxybenzoic acid; ▲, *m*-coumaric acid; ■, ferulic acid. (b) ●, No inhibitor; △, *p*-hydroxyphenylpropionic acid; ○, isoferulic acid; ○, *p*-coumaric acid; ▽, cinnamic acid. (c) △, *p*-Hydroxyphenylacetic acid and L-tryptophan;

preincubation with the inhibitors for 5 min. There was no significant difference in the inhibition of mevalonate pyrophosphate decarboxylase with or without preincubation. Since mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase are the two enzymes affected, we studied these two enzymes in detail.

Effects of phenyl and phenolic compounds on mevalonate pyrophosphate decarboxylase

The effects of addition of some phenolic compounds on liver mevalonate pyrophosphate decarboxylase were tested and compared with those of phenyl acids. The effects of various phenyl and phenolic compounds at a concentration of 2.5mM are given in Table 2. The inhibition was progressive with increasing concentrations (1.25–5.0mM) of phenyl and phenolic acids. Of the phenolic compounds tested *p*-coumaric acid, *m*-coumaric acid, isoferulic acid, ferulic acid, tyrosine, *p*-hydroxyphenyl-lactic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid, anisic acid, *p*-hydroxybenzaldehyde and *p*-hydroxyphenylpyruvic acid were all inhibitory. Of these, *m*-coumaric acid, isoferulic acid, ferulic acid and *p*-coumaric acid showed higher degrees of inhibition. The phenyl acids, cinnamic acid, phenylalanine, phenyl-lactic acid and phenylpyruvic acid, were also inhibitory.

A kinetic study of inhibition of the phenyl and phenolic compounds is shown in Fig. 2. Ferulic acid, isoferulic acid, *m*-coumaric acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, *p*-hydroxyphenyl-lactic acid, cinnamic acid and *p*-hydroxyphenylpropionic acid compete with the substrate mevalonate 5-pyrophosphate. Uncompetitive inhibition was observed with *p*-hydroxyphenylacetic acid, *p*-hydroxybenzaldehyde, anisic acid, L-phenylalanine, tryptophan and *p*-hydroxyphenylpyruvic acid. All these compounds showed no competition with MgATP (results not shown). V_{max} , apparent K_m and K_i values are given in Table 3.

Effects of phenyl and phenolic acids on mevalonate phosphate kinase

Mevalonate phosphate kinase was also inhibited by phenyl and phenolic acids after preincubation (Table 2). The degree of inhibition was less compared with that of decarboxylase. There was no significant inhibition by *p*-hydroxyphenyl-lactic acid, *p*-hydroxybenzaldehyde and *p*-hydroxyphenylacetic acid. This is in contrast with the result for decarboxylase, which is inhibited by all of these compounds. The

▲, *p*-hydroxybenzaldehyde; ■, anisic acid; ○, *p*-hydroxyphenylpyruvic acid; ▽, L-phenylalanine; ●, no inhibitor.

Table 3. Kinetic constants for the inhibition of phenyl and phenolic compounds on mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase

$V_{\max.}$, apparent K_m and K_i are calculated from Figs. 2 and 3.

	Kinetic constants		
	$V_{\max.}$ (nmol/h per mg of protein)	$10^{-7} \times$ Apparent K_m (M)	$10^{-5} \times K_i$ (M)
Mevalonate pyrophosphate decarboxylase			
None	50	125	—
Ferulic acid	50	400	110
<i>m</i> -Coumaric acid	50	333	157
<i>p</i> -Coumaric acid	50	333	149
Isoferulic acid	50	312	165
<i>p</i> -Hydroxybenzoic acid	50	208	373
<i>p</i> -Hydroxyphenyl-lactic acid	50	217	337
<i>p</i> -Hydroxyphenylpropionic acid	50	208	374
<i>p</i> -Hydroxyphenylacetic acid	17	42	374
<i>p</i> -Hydroxybenzaldehyde	17	42	374
Cinnamic acid	50	400	114
L-Tryptophan	17	42	374
Anisic acid	13	39	360
<i>p</i> -Hydroxyphenylpyruvic acid	14	40	365
L-Phenylalanine	17	43	378
Mevalonate phosphate kinase			
None	71	357	—
Isoferulic acid	71	588	385
Cinnamic acid	71	769	248
<i>p</i> -Coumaric acid	71	794	239

Fig. 3. Double-reciprocal plot for inhibition by phenyl and phenolic acids of mevalonate phosphate kinase

The assay system was as described in Fig. 1. The concentration of the inhibitor used was 2.5 mM. ●, No inhibitor; ○, cinnamic acid; ◐, isoferulic acid; △, *p*-coumaric acid.

inhibition was progressive with increasing concentration (1.25–7.5 mM) of the inhibitor. The kinetics of inhibition by isoferulic acid, *p*-coumaric acid and cinnamic acid are shown in Fig. 3. All of these compounds compete with mevalonate 5-phosphate. The apparent K_m and K_i values are shown in Table 3.

Effects of *p*-chlorophenoxyisobutyrate on mevalonate kinase, mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase

p-Chlorophenoxyisobutyrate, a hypocholesterolemic drug, is known to inhibit the incorporation of mevalonate into cholesterol when added *in vitro* (Ranganathan & Ramasarma, 1973), although it had no effect *in vivo* (Krishnaiah & Ramasarma, 1970). Walsh *et al.* (1969) had found that mevalonate kinase from bovine aorta was inhibited by this drug. However, when tested *in vitro*, it had no effect on mevalonate kinase (results not shown), mevalonate phosphate kinase (Table 2) or mevalonate pyrophosphate decarboxylase (Table 2) up to a concentration of 7.5 mM. The observed inhibition by *p*-chlorophenoxyisobutyrate on mevalonate incorporation into cholesterol may therefore be at a later step, beyond isopentenyl pyrophosphate.

Effects of dihydroxy, trihydroxy and other substituted phenyl compounds on mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase

It was shown in this laboratory that phenol, 2,4-dinitrophenol, 2-nitrophenol and quinol all gave slight inhibition, whereas catechol and resorcinol at low concentrations stimulated the incorporation of

Cinnamic acid

Syringic acid

p-Coumaric acid

Resorcinol

m-Coumaric acid

Catechol

Ferulic acid

Phloroglucinol

Isoferulic acid

Protocatechuic acid

Anisic acid

L-Dopa

p-Chlorophenoxyisobutyrate (clofibrate)

p-Chlorophenylalanine

Phloroglucinolcarboxylic acid

DL-3,4-Dihydroxyphenyl glycol

mevalonate into non-saponifiable lipids (Ranganathan & Ramasarma, 1973). It was also shown that aliphatic acids, such as citrate, malate and oxaloacetate, stimulated the incorporation of mevalonate into non-saponifiable lipids. We tested the effect of a variety of phenolic compounds on mevalonate phosphate kinase and decarboxylase (Table 2). Except for phloroglucinol, which caused 20% inhibition of the decarboxylase at 2.5mM, the other compounds tested, phloroglucinolcarboxylic acid, syringic acid, resorcinol, catechol, protocatechuic acid, L-dopa, *p*-chlorophenylalanine and DL-3,4-dihydroxyphenyl glycol, caused no significant change in either of the enzyme activities.

Discussion

Cinnamic acid, ferulic acid, isoferulic acid, *p*-coumaric acid and *m*-coumaric acid all showed a high degree of inhibition. All these compounds have a benzene ring and a side chain, $-\text{CH}=\text{CH}-\text{CO}_2\text{H}$, in common. Therefore it suggests that, for effective inhibition, a phenyl-vinyl structure is essential. All these compete with the substrates, mevalonate 5-phosphate and mevalonate 5-pyrophosphate. Dihydroxy- and trihydroxy-phenyl compounds have no effect. Side chains with pyruvate, lactate, propionate or acetate groups are all inhibitory, but the degree of inhibition is less than those with $-\text{CH}=\text{CH}-\text{CO}_2\text{H}$. Two types of inhibition were seen, competitive and uncompetitive. The compounds that showed competitive inhibition probably have structural similarities to the mevalonate phosphates in solution. Those compounds showing uncompetitive inhibition may affect the enzyme activity by another mechanism.

In phenylketonuria the metabolism of tyrosine is affected in addition to that of phenylalanine (Bickis *et al.*, 1957). It was found that phenylketonuric subjects excreted large quantities of *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid (Boscott & Bickel, 1954). Phenylalanine and phenylpyruvate at a concentration of 2mM were shown to inhibit the formation of acetoacetate from tyrosine (Bickis *et al.*, 1957). The concentration of phenylalanine in the plasma of phenylketonuric patients varies

from 1 to 4mM. From this it is evident that, in addition to the abnormal metabolism of brain lipids, the phenyl and phenolic acids might also affect liver cholesterol biogenesis by affecting mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase.

The specific activity of mevalonate pyrophosphate decarboxylase was the lowest of the three mevalonate-metabolizing enzymes (Table 1) and may act as the rate-determining enzyme in this part of the pathway. This study also provides us with information on the additional regulatory mechanism. It is well known that the major regulatory enzyme in the biogenesis of cholesterol is 3-hydroxy-3-methylglutaryl-CoA reductase. However, other regulatory sites are also known. Long-term feeding of cholesterol has been shown to inhibit the incorporation of mevalonate, in addition to acetate, into cholesterol (Bucher *et al.*, 1959; Gould & Swyryd, 1966; Krishnaiah *et al.*, 1967). Incorporation of mevalonate into non-saponifiable lipids by the liver system was low in suckling rats compared with that in weaned rats (Shah, 1973), and the decarboxylation of mevalonate was decreased by 50–80% in rats weaned on to cholesterol-supplemented diets (Rarnachandran & Shah, 1976). It has also been shown that mevalonate decarboxylation (Shama Bhat & Ramasarma, 1977) and mevalonate pyrophosphate decarboxylase exhibit circadian variation. The relative specific activities of 3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate kinase, mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase at noon and midnight are given in Table 4. When expressed per g of the liver tissue, the specific activity of 3-hydroxy-3-methylglutaryl-CoA reductase is about 20 times lower than that of decarboxylase. Therefore it is unlikely that the mevalonate-metabolizing enzymes operate at maximum rate, since the substrate is expected to be limiting. In view of the relative excess of decarboxylase it can have a controlling effect on the overall metabolism only if about 70–80% inhibition of its activity is obtained. It may be noted that, at a concentration of 2mM, some of the phenyl or phenolic compounds can produce such an inhibition.

Table 4. Comparison of the activities of 3-hydroxy-3-methylglutaryl-CoA reductase, mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase of rat liver
The data for hydroxymethylglutaryl-CoA reductase are from Rajan (1977).

Enzymes	Enzyme activity (nmol/h per g of liver)	
	12:00h	24:00h
Hydroxymethylglutaryl-CoA reductase	150	525
Mevalonate kinase	11800	11900
Mevalonate phosphate kinase	7100	7350
Mevalonate pyrophosphate decarboxylase	2970	5900

The phenolic acids derived from tyrosine are the possible intermediates in the formation of *p*-hydroxybenzoic acid, the precursor of the aromatic ring of ubiquinone. The isoprene side chain of the ubiquinone molecule is derived from mevalonate. It is noteworthy that the mevalonate pathway is inhibited by *p*-hydroxybenzoic acid and other phenolic compounds. This offers a model wherein the intermediates of one branch inhibit the other branch in a convergent pathway leading to the synthesis of the end product.

In the present experiments a 4-fold higher concentration of the inhibitor than in previous experiments (Ranganathan & Ramasarma, 1973) was needed to obtain equivalent inhibition. This is due to the fact that the substrate used in the present experiments is at saturation concentration, unlike the trace amounts used earlier.

This is the first report on the inhibition of mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase by phenyl and phenolic compounds. This study has provided two classes of inhibitors, competitive and uncompetitive, for mevalonate phosphate kinase and mevalonate pyrophosphate decarboxylase, and offers potential candidates for the development of drugs affecting cholesterol biogenesis after the mevalonate part of the pathway.

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