Inhibition of the Biosynthesis of Sterols by Phenyl and Phenolic Compounds in Rat Liver

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The presence of mitochondria increased the incorporation of [2-¹⁴C]mevalonate into sterols in a cell-free system from rat liver. Various phenyl and phenolic compounds inhibited the incorporation of mevalonate when added *in vitro*. *p*-Hydroxycinnamate, a metabolite of tyrosine, was the most powerful inhibitor among the compounds tested. Catechol, resorcinol and quinol were inhibitory at high concentrations. Organic acids lacking an aromatic ring were not inhibitory. Two hypocholesterolaemic drugs, Clofibrate (α -*p*-chlorophenoxyisobutyrate) and Clofenapate [α ,4-(*p*-chlorophenyl)phenoxyisobutyrate], which are known to affect some step before the formation of mevalonate in the biosynthesis of cholesterol *in vivo*, showed inhibition at a step beyond the formation of mevalonate *in vitro*. The presence of the aromatic ring and the carboxyl group in a molecule appears to be necessary for the inhibition.

It has been shown that phenylalanine and its deaminated metabolites inhibited the biosynthesis of sterols from [2-¹⁴C]mevalonate by homogenates of rat liver and brain when added *in vitro* (Shah *et al.*, 1968, 1969). The phenyl acids were more effective and phenylpyruvate was the most powerful inhibitor among them. The site of action was identified as the step of decarboxylation of mevalonate 5-pyrophosphate, yielding isopentenyl pyrophosphate.

In experimental phenylalaninaemia the concentration of phenylalanine was found to be increased in the brain and the liver (Shah *et al.*, 1968). Since it is known that the metabolism of tyrosine is also impaired under these conditions it was decided to study whether the phenolic acids derived from tyrosine show similar inhibition. Therefore a study of the specificity of the inhibition of incorporation of [2-¹⁴C]mevalonate into sterols by a cell-free system from rat liver was undertaken. The results showed that a number of phenolic acids were inhibitory whereas phenols and aliphatic acids were not. The presence of an aromatic ring and a carboxyl group in the molecule appears to be the structural requirement for the inhibition. Decarboxylation of mevalonate 5-pyrophosphate or one of the earlier kinase reactions appears to be inhibited by these compounds.

Experimental

Materials

DL-[2-¹⁴C]Mevalonic acid lactone (5.8 mCi/mmol) and DL-[1-¹⁴C]mevalonic acid lactone (6 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Phenylalanine, tyrosine, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-

lactic acid, *p*-hydroxyphenylacetic acid, *p*-hydroxybenzoic acid and ATP were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. *p*-Hydroxycinnamic acid was obtained from Koch-Light Laboratories, Colnbrook, Bucks., U.K. The samples of Clofibrate and Clofenapate were gifts from Dr. J. M. Thorp, Imperial Chemical Industries, Macclesfield, Cheshire, U.K. All other chemicals used were of AnalaR grade, obtained from BDH Ltd.

Methods

Preparation of homogenates. Young male albino rats weighing about 40–50 g were used in all the experiments. The rats were killed by cervical dislocation and the livers were quickly removed and chilled in ice. The livers were homogenized in a Potter-Elvehjem glass homogenizer with a Teflon pestle in 9 vol. of freshly prepared 0.25 M-sucrose solution. Post-nuclear supernatant was obtained by centrifuging the homogenate at 800g for 10 min. Post-mitochondrial supernatant was obtained by centrifuging the homogenate or the post-nuclear supernatant at 8500g for 10 min. The sediment obtained after centrifuging the 800g supernatant at 8500g for 10 min was suspended in 0.25 M-sucrose (2 ml/g of liver) and used as the mitochondrial fraction. All centrifugations were carried out in a Sorvall RC-2B refrigerated centrifuge and the operations were carried out at 0–5°C.

Incubation procedures. (a) Biosynthesis of sterols from [2-¹⁴C]mevalonate. The incubation mixture consisted of post-nuclear (unless otherwise stated) supernatant (1 ml), potassium phosphate buffer, pH 7.4 (250 μ mol), ATP (5 μ mol), MgCl₂ (20 μ mol), nicotinamide (60 μ mol) and DL-[2-¹⁴C]mevalonate

(15 nmol) (2×10^5 c.p.m.) in a total volume of 2.5 ml. The incubation was carried out in 50 ml Erlenmeyer flasks in a metabolic shaker at 37°C for 1 h. The reaction was stopped by adding 0.5 ml of 10M-NaOH and 20 ml of ethanol. The contents were then saponified under reflux for 20 min on a boiling-water bath. The non-saponifiable lipids were extracted with two 15 ml portions of light petroleum (b.p. $40-60^\circ\text{C}$), the combined extracts were washed free of alkali with water, followed by aq. 75% (v/v) ethanol, and the extract was evaporated to dryness.

(b) Release of $^{14}\text{CO}_2$ from $[1-^{14}\text{C}]$ mevalonate. The reaction mixture was essentially the same as that used to study the biosynthesis of sterols. Nicotinamide was omitted and $[1-^{14}\text{C}]$ mevalonate was used instead of $[2-^{14}\text{C}]$ mevalonate. The total volume was made up to 2 ml. The incubation was carried out in closed Warburg flasks at 37°C without shaking. The central well contained 0.2 ml of 1M-KOH and the side arm 0.5 ml of 1M- H_2SO_4 . At the end of the incubation for 30 min the contents of the side arm were tipped into the reaction mixture and the flasks were left at room temperature for 30 min before samples were taken for measurement of radioactivity.

Measurement of radioactivity. The radioactivity in the samples was measured in a Beckman model LS-100 liquid-scintillation spectrometer in 5 ml of a scintillation fluid consisting of 0.5% 2,5-diphenyloxazole in toluene. The extracts were transferred quantitatively by using 2 ml of light petroleum. To count radioactivity in $^{14}\text{CO}_2$, the contents of the central well of the Warburg flasks were quantitatively transferred to 5 ml of scintillation liquid, consisting of 0.2% 2,5-diphenyloxazole and 0.002% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene-methanol (1:1, v/v) (Yardley, 1964). The counting efficiency, which was about 95%, was not altered by the presence

of light petroleum or KOH in the respective scintillation fluids.

The changes in the radioactivity in the non-saponifiable lipids are considered equivalent to that of sterols, especially in view of the site of inhibition in the present system being before the formation of the lipid intermediates and also because more than 85% of the radioactivity in this fraction was found to be associated with sterols.

The results are represented as c.p.m. of radioactivity incorporated under the experimental conditions or as percentage change compared with the control.

Results

Cell-free system for the incorporation of $[2-^{14}\text{C}]$ mevalonate into sterols

Unsupplemented rat liver homogenate gave very poor incorporation of $[2-^{14}\text{C}]$ mevalonate into sterols. The incorporation was increased by the addition of ATP and Mg^{2+} , with which a maximum was reached at 2 mM and 8 mM concentrations respectively. The requirement for ATP could be replaced by succinate and ADP together; the incorporation was more than that obtained with ATP. As a routine ATP was used as the energy source in the test system.

With the above test system, consistent time-dependent and enzyme concentration-dependent incorporations were obtained with whole homogenate, post-nuclear supernatant and post-mitochondrial supernatant. However, of the three, post-mitochondrial supernatant showed relatively low activity, which could be stimulated to nearly the value obtained with the homogenate by addition of isolated mitochondria. The results also showed that the stimulation increased with the amount of added mitochondria.

Table 1. *Effect of mitochondria on the incorporation of $[2-^{14}\text{C}]$ mevalonate into non-saponifiable lipids*

Where mentioned 1 ml each of homogenate, post-nuclear supernatant or post-mitochondrial supernatant was used. Addition of 0.2 ml of mitochondrial suspension corresponds to the equivalent amount present in 1 ml of the homogenate. The reaction mixture is as given in the Experimental section. The reaction was carried out for 1 h at 37°C with shaking.

Cell fraction	Mitochondria (ml)	Incorporation (c.p.m.)
Homogenate	—	21 000
Post-nuclear supernatant	—	15 900
Post-mitochondrial supernatant	—	9 000
Post-mitochondrial supernatant	0.2	18 000
Post-nuclear supernatant	—	19 200
Post-mitochondrial supernatant	—	10 200
Post-mitochondrial supernatant	0.1	14 500
Post-mitochondrial supernatant	0.2	18 300
Post-mitochondrial supernatant	0.4	20 800

dria and was nearly maximal when the amount of mitochondria added was equal to that normally present in the equivalent volume of post-mitochondrial supernatant used (Table 1). Addition of twice the amount of mitochondria did not further stimulate the incorporation significantly. Experiments with [1-¹⁴C]mevalonate showed that the addition of mitochondria stimulated the release of ¹⁴CO₂, suggesting that the stimulatory effect of mitochondria on the biosynthesis of sterols is at the level of decarboxylation of mevalonate 5-pyrophosphate or earlier (Table 2). The effect of mitochondria was observed even when the concentration of ATP in the system was saturating (Fig. 1), indicating that mitochondria do not stimu-

late the reaction by providing more ATP to the system. However, mitochondria damaged by freezing and thawing were unable to show the effect.

Effect of phenolic and phenyl acids

The effect of addition of some phenolic acids on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids was tested and compared with that of phenyl acids (Table 3). Under these conditions tyrosine also inhibited and the inhibition was even higher than that obtained with phenylalanine. This apparent contradiction with the results of Shah *et al.* (1969) may be explained if some keto acid acceptors were present in the liver extracts used, which may have resulted in the formation of some of the phenolic acids by way of the transamination reaction. The results in Table 3 show that the deaminated products of phenylalanine and tyrosine are more effective inhibitors than the amino acids themselves.

Comparison of the inhibitory action of the two deaminated products, phenylpyruvate and *p*-hydroxyphenylpyruvate, is shown in Fig. 2. At 1.6mM concentration, *p*-hydroxyphenylpyruvate was not inhibitory, whereas phenylpyruvate inhibited 75%.

Table 2. Effect of mitochondria on the release of ¹⁴CO₂ from [1-¹⁴C]mevalonate

Addition of 0.2ml of mitochondrial suspension corresponds to the equivalent amount of mitochondria present in 1ml of the homogenate. Where mentioned, 1ml of post-nuclear supernatant or post-mitochondrial supernatant was used. Other details of the experiments are as given in the Experimental section of the text.

Cell fraction	¹⁴ CO ₂ released (c.p.m.)
Post-nuclear supernatant	11 200
Post-mitochondrial supernatant	7 600
Post-mitochondrial supernatant +0.2ml of mitochondria	11 800
Post-mitochondrial supernatant +0.4ml of mitochondria	12 000

Table 3. Effect of various phenyl and phenolic acids on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids

Post-nuclear supernatant (1 ml) was used in the assay system. The compounds were added as sodium salts. The details of the experiments are as given in the Experimental section. The results are represented as percentage change compared with control. —, Not tested.

Additions	Concn. (mm)	Inhibition (%)		
		2	4	8
Phenylalanine	—	Nil	Nil	3
Phenylacetate	50	68	93	93
Tyrosine	9	48	84	84
<i>p</i> -Hydroxyphenylacetate	—	—	—	85
<i>p</i> -Hydroxyphenyl-lactate	—	—	—	94
<i>p</i> -Hydroxycinnamate	93	97	98	98
<i>p</i> -Hydroxybenzoate	77	97	97	97

Fig. 1. Effect of mitochondria on the release of ¹⁴CO₂ from [1-¹⁴C]mevalonate, with increasing concentrations of ATP

For the systems with mitochondria (●) and without mitochondria (○) 1 ml of post-nuclear supernatant or 1 ml of post-mitochondrial supernatant was used respectively. Other details of the experiments are given in the Experimental section.

Of the compounds tested, *p*-hydroxycinnamate was the most powerful inhibitor (Fig. 3). At a concentration as low as 0.2 mM, this compound inhibited the activity by nearly 50%. The other metabolites,

Fig. 2. Effect of phenylpyruvate and *p*-hydroxyphenylpyruvate on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids *in vitro*

Post-nuclear supernatant (1 ml) was used for incubation. ●, Phenylpyruvate; ○, *p*-hydroxyphenylpyruvate. The experimental details are given in the text.

Fig. 3. Inhibition of incorporation of [2-¹⁴C]mevalonate into nonsaponifiable lipids by phenyl and phenolic acids *in vitro*

Post-nuclear supernatant (1 ml) was used. The experimental details are given in the text. ●, *p*-Hydroxycinnamate; ○, cinnamate; ■, *p*-hydroxybenzoate; □, benzoate.

which all gave inhibition to different extents, are implicated in the degradative pathway of the two aromatic amino acids. The inhibition observed, particularly with *p*-hydroxybenzoate, has significance in biosynthetic regulation. It is noteworthy that phenolic acids derived from tyrosine are the possible intermediates in the formation of *p*-hydroxybenzoate, the precursors of the aromatic ring of ubiquinone, a molecule whose isoprene-side-chain formation depends on the very mevalonate pathway that is inhibited by the phenolic acids including *p*-hydroxybenzoate. This offers a peculiar model wherein an intermediate in one branch inhibited the other branch in a convergent pathway leading to the synthesis of the end product.

Effect of phenols

In view of the inhibition obtained by phenolic and phenyl acids, it was decided to investigate whether the inhibition was due to simple phenols or to organic acids. Of the phenols tested, some showed inhibition only at very high concentration relative to phenolic acids (Table 4). The results indicate that a phenolic group itself is probably not responsible for the inhibition. Catechol at low concentrations gave significant stimulation (Table 4) in both the presence

Table 4. Effect of phenols on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids

Post-nuclear supernatant (1 ml) was used for the assay. The experimental details are as given in the text.

Additions	Concn. (mM)	Incorporation (c.p.m.)
None		8200
Phenol	4.0	6000
2,4-Dinitrophenol	0.2	3700
	0.4	4200
	0.8	3000
	2.0	1900
2-Nitrophenol	1.0	3800
	2.0	1900
None		9800
Catechol	0.8	13600
	2.0	22400
	4.0	7500
	4.0	3700
Resorcinol	1.4	11000
Quinol	0.8	7300
	2.0	7500
	4.0	3700
	4.0	17600
None		17600
Catechol	2.0	26300
<i>p</i> -Hydroxycinnamate	0.4	7800
Catechol + <i>p</i> -hydroxycinnamate	2.0	5400
	0.4	

Table 5. *Effect of various compounds on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids, in the presence and absence of mitochondria*

The compounds were added as sodium salts. Post-nuclear supernatant represents the system with mitochondria, and post-mitochondrial supernatant the system without mitochondria. Where mentioned, 1 ml of post-nuclear supernatant or post-mitochondrial supernatant was used. Other details of the experiments are as given in the Experimental section. +, Stimulation; –, inhibition.

Additions	Concn. (mm)	Change in activity (%)	
		Post-nuclear supernatant	Post-mitochondrial supernatant
Benzoate	4	–67	–60
<i>p</i> -Hydroxybenzoate	2	–84	–85
<i>p</i> -Hydroxycinnamate	2	–92	–91
2,4-Dinitrophenol	0.4	–76	–50
Citrate	4	+29	+2
Malate	4	+36	+14
Catechol	1.4	+130	+74

Table 6. *Effect of organic acids lacking the aromatic ring on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids*

Post-nuclear supernatant (1 ml) was used for incubation. Sodium salts of the acids were used. The experimental details are as given in the text.

Addition	Concn. (mm)	Incorporation (c.p.m.)
Nil		16000
Succinate	6	19500
Nil		31900
Citrate	4	41000
Malate	4	43300
Oxaloacetate	4	43800
Glycine	4	30100
Nil		20800
2-Oxoglutarate	6	20600

and the absence of mitochondria (Table 5). Addition of *p*-hydroxycinnamate inhibited catechol-stimulated activity also (Table 4).

2,4-Dinitrophenol was inhibitory, but the inhibition was not concentration-dependent (Table 4). The inhibitory effect was more pronounced when mitochondria were present, presumably because of the stimulation of mitochondrial adenosine triphosphatase and a consequent rapid depletion of ATP.

Effect of aliphatic acids

On testing the effect of some aliphatic acids it became apparent that the inhibition by phenyl acids could not be accounted for by the mere presence of carboxylic groups. Intermediates of the tricarboxylic

Table 7. *Effect of Clofibrate and Clofenapate on the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids*

Experimental details are given in the text. —, Not tested.

Concn. (mm)	Inhibition (%)	
	Clofibrate	Clofenapate
0.2	—	31
0.6	22	93
1.2	41	97
3.0	62	100
6.0	94	—

acid cycle, such as citrate, malate and oxaloacetate, stimulated the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids. Succinate showed only slight stimulation whereas 2-oxoglutarate and glycine were without effect (Table 6). The stimulatory effect of citrate and malate was obtained only when mitochondria were present in the incubation system, indicating that the stimulation was mediated through an effect on the mitochondria (Table 5).

Effect of chlorophenoxy compounds

Two of the hypocholesterolaemic drugs, Clofibrate (Thorp & Waring, 1962) and Clofenapate (Thorp, 1970), when fed to rats are known to inhibit the synthesis of cholesterol in the liver from acetate but not from mevalonate *in vivo* (Krishnaiah & Ramasarma, 1970). However, on addition *in vitro*, in the incubation system they gave considerable inhibition of incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids (Table 7). Clofenapate appeared to be only

Table 8. Effect of increasing concentrations of ATP on the inhibition of incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids by Clofibrate and *p*-hydroxycinnamate

Post-nuclear supernatant (1 ml) was used for incubation. Other experimental details are as given in the Experimental section. —, Not tested.

Concn. of ATP (mM)	Incorporation (c.p.m.)		
	Without inhibitor	With 0.2mM- <i>p</i> -hydroxy- cinnamate	With 1.2mM-Clofibrate
Nil	2100	330	350
0.8	10700	6300	5100
2.0	11800	—	5900
4.0	11700	6700	6600
8.0	8400	3000	4300

Table 9. Effect of phenolic acids on the release of ¹⁴CO₂ from [1-¹⁴C]mevalonate

Details of the experiments are given in the Experimental section.

Additions	Concn. (mM)	Inhibition (%)
Tyrosine	4	Nil
<i>p</i> -Hydroxyphenylpyruvate	2	51
	4	65
<i>p</i> -Hydroxyphenyl-lactate	2	33
	4	65
<i>p</i> -Hydroxycinnamate	0.2	72
	0.4	92
<i>p</i> -Hydroxybenzoate	1	64
	2	88

about fourfold more effective than Clofibrate in the present experiments *in vitro*, whereas in the feeding experiments *in vivo* it was found to be active at one-hundredth the concentration of Clofibrate. These observations imply that the mechanism of action of the drugs *in vivo* is complex and most probably does not involve any direct participation of these drugs beyond the formation of mevalonate in the biosynthesis of sterols.

Competition with ATP

It is possible that the observed inhibition *in vitro*, in some cases anomalous from the known responses *in vivo*, may be due to simple competition with ATP, which is an absolute requirement in the system *in vitro*. Such a possibility was indicated by the work of Walsh *et al.* (1969), who found that Clofibrate inhibited mevalonate kinase in bovine aorta by competing with ATP and that addition of excess of ATP could counteract this inhibition. The present work with the liver system showed that excess of ATP was ineffective

in reversing the inhibition obtained with both Clofibrate and *p*-hydroxycinnamate. Although 0.8mM-ATP caused a considerable drop in the inhibition when compared with the system without ATP, addition of larger amounts of ATP did not reverse the inhibition completely (Table 8). Therefore the inhibition would become effective provided that sufficient concentrations of these compounds reach the liver, and the absence of the effect may be taken as an indirect evidence that the necessary amounts may not have reached the liver or been formed *in vivo*.

Site of inhibition

To find the site of action of these inhibitors, the effect of these compounds on the liberation of ¹⁴CO₂ from [1-¹⁴C]mevalonate was studied. The results given in Table 9 show that these phenolic compounds inhibit the release of ¹⁴CO₂ from [1-¹⁴C]mevalonate, suggesting that the inhibition is at the level of mevalonate 5-pyrophosphate decarboxylase or earlier. Tyrosine, however, did not affect the liberation of ¹⁴CO₂; its effect on the biosynthesis of sterols appears to be at a step beyond the decarboxylation of mevalonate 5-pyrophosphate.

Discussion

All the steps in the biosynthesis of cholesterol from mevalonate are localized in the soluble and microsomal fractions. None of the enzymes is contributed by mitochondria. In the light of this it is interesting to know how the significant stimulation is obtained by adding mitochondria. Since sufficient ATP was added in the incubation mixtures the function of the mitochondria appears to be other than to supply ATP. The lack of effect with damaged mitochondria implicates some energy-dependent reaction. Nevertheless this observation has opened an interesting control aspect of cholesterol biosynthesis by mitochondria.

Of the many types of compounds tested only those with the aromatic ring and the carboxyl group were inhibitory to the incorporation of [2-¹⁴C]mevalonate into non-saponifiable lipids *in vitro*. Phenols or organic acids lacking an aromatic group were not inhibitory. The presence of a hydroxyl group in the ring gave variable effects. Thus phenylpyruvate was more powerful as an inhibitor than *p*-hydroxyphenylpyruvate, whereas the opposite was true of cinnamate and *p*-hydroxycinnamate.

It is known that in phenylketonuria the metabolism of tyrosine is also affected in addition to that of phenylalanine. For example, it was found that phenylketonuric subjects excreted large quantities of *p*-hydroxyphenyl-lactate and *p*-hydroxyphenylacetate (Boscott & Bickel, 1954), and this diversion in the metabolic pathway may be due to a possible block by excess of phenylalanine in the formation of acetoacetate from tyrosine (Bickis *et al.*, 1957). Under these conditions *p*-hydroxycinnamate may also be derived from *p*-hydroxyphenyl-lactate, which on administration to the rat was found to give rise to the former compound (Booth *et al.*, 1960). The present study brings out the importance of the metabolites of

tyrosine as potential regulating agents in the synthesis of cholesterol.

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