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Biosynthesis of *trans,trans,trans*-Geranylgeranyl Diphosphate by the Cytosolic Fraction from Rat Tissues

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The cytosolic fractions from rat liver, brain, kidney, spleen and testis demonstrate the capacity to synthesize two products from [^{3}H]isopentenyl diphosphate, i.e., farnesyl diphosphate and geranylgeranyl diphosphate. The highest rate of geranylgeranyl diphosphate synthesis was found in brain, testis and spleen, accounting for up to 30% of the total incorporation of radioactivity under optimal conditions. In all tissues examined the geranylgeranyl diphosphate formed was identified as the *trans,trans,trans*-isomer. The ratio of geranylgeranyl diphosphate to farnesyl diphosphate produced was specific for the tissue investigated and could be altered by the addition of divalent cations. The results in this study demonstrate the presence of a specific *trans,trans,trans*-geranylgeranyl diphosphate synthetase showing high affinity for farnesyl diphosphate.

The synthesis of farnesyl diphosphate (FPP) from IPP involves two enzymes, i.e., IPP isomerase [E. C. 5.3.3.2] and FPP synthetase [E.C. 2.5.1.1]. FPP synthetase plays a central role in the mevalonate pathway, since it is the last enzyme in the common, initial portion of this biosynthetic sequence. This enzyme catalyzes sequential additions of IPP units to DMAPP and/or *t*-GPP to produce *t,t*-FPP (1). FPP serves as the common substrate for the first committed enzymes in the biosynthesis of cholesterol, dolichol and the side-chain of ubiquinone (2-4). FPP synthetase has been purified from a number of sources, both prokaryotic and eukaryotic, and extensively studied (1,5). Recently, the gene for this protein has been identified and cloned from yeast, rat and human (6-8).

Abbreviations:
IPP; isopentenyl diphosphate, DMAPP; dimethylallyl diphosphate, GPP; geranyl diphosphate, FPP; farnesyl diphosphate, GGPP; geranylgeranyl diphosphate, *t*; trans-, *c*; cis-, TLC; thin-layer chromatography, HPLC; high performance liquid chromatography

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Not only is FPP converted to cholesterol, dolichol and ubiquinone, but this compound is also involved in protein modification. The attachment of polyisoprenoid moieties to proteins is well established (9-14) and protein-farnesyl transferases have been observed in the cytosolic fractions of several tissues (15-17). A protein-farnesyl transferase from rat brain cytosol has recently been purified and characterized (18). Cytosolic protein-geranylgeranyl transferases have also been described (19,20).

In contrast to the situation in plants, yeast and in several prokaryotes, the biosynthesis of GGPP has been investigated to only a limited extent in mammalian systems. The biosynthesis of *t,t,c*-GGPP has been described in rat liver microsomes as an intermediate step in dolichol biosynthesis (21). Furthermore, it has been reported that the cytosolic fraction from pig liver can synthesize *t,t,t*-GGPP from FPP and IPP (22), although the enzyme activity was low and incubation times of up to 12 hours were necessary in order to detect any product. It has also been shown that the FPP synthetase purified from liver has the capacity to condensate an additional molecule of IPP with FPP, although at a low rate (1). Recently, it has been reported that rat brain cytosol has the capacity to convert [^{3}H]mevalonic acid into a geranylgeranyl moiety bound to an acceptor peptide, indicating the presence of a *t,t,t*-GGPP synthetase, as well as of a protein-geranylgeranyl transferase, in this fraction (23).

In the present study we have analyzed the synthesis of *t,t*-FPP and *t,t,t*-GGPP in the cytosolic fractions from different rat tissues.

Materials and Methods

Chemicals- [^{3}H]IPP and the unlabeled phosphorylated isoprenoids were prepared as described earlier (24). The polyisoprenoid alcohols used as standards were kindly provided by Dr T. Takegawa, Kururay Co., Okayama, Japan. All other chemicals used were of reagent grade.

Subfractionation of tissues- Non-starved male Sprague-Dawley rats (150-180 g) were used. Tissue homogenates (20% w/v) were prepared in 0.25 M sucrose containing 2.5 mM immidazole-Cl, pH 6.5, and 0.1 mM dithiothreitol, using a Turrax blender. The homogenates were centrifuged for 10 minutes at 350 *g* in order to remove unbroken cells. The resulting supernatant was centrifuged at 105 000 *g* for 60 minutes. The middle layer of this supernatant was recentrifuged in the same manner and the resultant particulate-free cytosol was used as enzyme source.

Incubations- Prenyltransferase activities were measured in 300 µl incubation mixtures containing 25 mM immidazole-Cl, pH 6.5, 1 mM $MgCl_2$, 10 mM KF, 0.1 mM dithiothreitol and 26 µM [^{3}H]IPP (1.8 x 10^5 dpm/nmol). When indicated, the reaction mixtures were supplemented with 50 µM *t*-GPP. The reactions were started by the addition of 5-400 µg freshly prepared cytosolic protein and were allowed to continue for 15 minutes at 37°C. The reaction was stopped by the addition of 2.0 ml *n*-butanol saturated with water and the lipids extracted by extensive vortexing. After the addition of 1.0 ml 2 M KCl, the samples were centrifuged in order to separate the organic and aqueous phases. The organic phases were removed and the aqueous phases reextracted in the same manner. The pooled organic phases were dried under N_2, the residues redissolved in 200 µl β-octyl-*n*-glycopyranoside (5 % w/v) and the radioactivity determined by scintillation counting. For further analyses, the reaction products were enzymatically dephosphorylated by the method of Wong and Lennarz (25).

HPLC analyses-The dephosphorylated products were analyzed by reversed phase HPLC using a C-18 column (Hewlet Packard Hypersil ODS 3μm). A linear gradient from 70 % (v/v) to 90 % methanol in water was run for 20 minutes at a flow rate of 1.5 ml/min. The absorption of the eluate was monitored at 210 nm and radioactivity was detected using a radioactivity flow detector (Radiomatic Instruments, Tampa, Fl.).

TLC analyses- The labeled reaction products were also analyzed using silica plates (Merck). The phosphorylated derivatives were developed using diisobutylketone:acetic acid:water (8:5:1, solvent system A), while the dephosphorylated products were developed using benzene:ethyl acetate (4:1, solvent system B) as the mobile phase. For autoradiography, the plates were sprayed with En^{3}Hance spray (NEN Research Products, Boston) and exposed to x-Omat AR Film (Eastman, Kodak, Rochester) at -80°C for approx. 48 hours.

Protein was determined using the Lowry procedure (26).

Results

Prenyltransferase activities- The incorporation of [^{3}H]IPP into butanol-extractable products by cytosolic fractions from various rat tissues was investigated (Table 1). The activities in the various tissues differed substantially. Using [^{3}H]IPP, in the absence of any allylic substrate, the highest incorporation was observed with liver cytosol, followed by testis, brain, spleen and kidney, in that order. The specific activity in liver was as much as 12-fold higher than that of kidney. The total incorporation with all cytosolic fractions was stimulated more than 5-fold by the addition of *t*-GPP to the reaction mixture.

Product identification-The butanol extracts were evaporated and analyzed by silica TLC using solvent system A (Figure 1). The radioactivity comigrated with short-chain isoprenoid diphosphate standards (R_f=0.12). No radioactivity was associated with isoprenoid monophosphates or free alcohols. In order to further characterize the nature of the reaction products, these were dephosphorylated enzymatically and analyzed using a reversed-phase HPLC system equipped with a radioactivity flow detector. In this system two individual

Table 1. *Prenyltransferase activities in the cytosolic fractions from various rat tissues in the absence and presence of t-GPP*

Tissue	Total incorporation* with [^{3}H]IPP	Total incorporation* with [^{3}H]IPP+ *t*-GPP
Liver	2500	17600
Brain	361	2570
Kidney	206	990
Spleen	288	1350
Testis	760	3660

The total incorporation of [^{3}H]IPP into butanol-extractable products by the different cytosolic fractions in the absence and presence of *t*-GPP was analyzed .
*dpm/μg protein/15 min.

Fig. 1. *TLC analysis of the reaction products formed from [^{3}H]IPP by cytosolic fractions.* The cytosolic fraction isolated from rat brain was incubated with [^{3}H]IPP and the reaction products extracted into *n*-butanol and analyzed on silica TLC in solvent system A, as described under Materials and Methods. The plate was placed onto x-ray film and exposed for 48 hours at -80°C. The arrows indicate the migration of **PP**: farnesyl diphosphate, **P**: farnesyl monophosphate and **OH**: farnesol standards.

products could be separated (Figure 2). The first peak (Rt=7.3 min) coeluted with farnesol, while the second peak (Rt=15.3 min) coeluted with geranylgeraniol, added as internal standards. These two products were produced by all cytosolic fractions investigated.

The dephosphorylated products were also analyzed by silica TLC using solvent system B. The resulting autoradiogram revealed two individual products (Figure 3). The major product (R_f=0.47) was identified as *t,t*-farnesol, and the minor product (R_f=0.56) comigrated with *t,t,t*-geranylgeraniol. No radioactivity was associated with *t,t,c*-geranylgeraniol.

Product distribution- The biosynthesis of *t,t*-FPP and *t,t,t*-GGPP from [^{3}H]IPP by the different cytosolic fractions was investigated (Table 2). The highest rate of synthesis of *t,t*-FPP was observed with the cytosolic fractions prepared from liver and testis followed by brain, spleen and kidney.

The highest incorporation into *t,t,t*-GGPP was observed with the cytosolic fractions prepared from testis, brain and spleen. In these tissues the incorporation into *t,t,t*-GGPP corresponded to 12, 24 and 28%, respectively, of the total products. In kidney the rate of *t,t*-FPP synthesis was low, although as much as 22% of the radioactivity was associated with *t,t,t*-GGPP. The rate of *t,t,t*-GGPP synthesis by the liver cytosolic fraction was similar, but somewhat lower than in brain, spleen and testis. Since the synthesis of FPP was much higher in liver than in the other tissues, the radioactivity associated with *t,t,t*-GGPP was only 2% of the total.

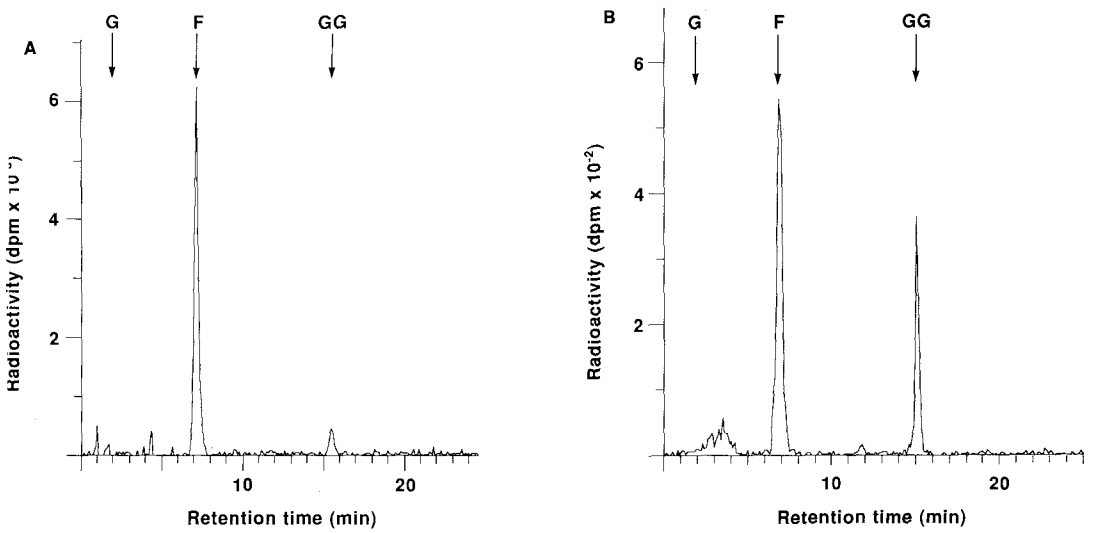

Fig. 2. *Separation of the reaction products formed from [^{3}H]IPP by cytosolic fractions using HPLC.*
Cytosolic fractions from **(A)** liver and**(B)** brain were incubated with [^{3}H]IPP and the reaction products were extracted, dephosphorylated and analyzed using a reversed phase HPLC system equipped with a radioactivity flow detector.The arrows indicate the elution of **G**: geraniol, **F**: farnesol and **GG**: geranylgeraniol standards.

When *t*-GPP was included in the reaction medium, the overall synthesis of *t,t,t*-GGPP by the different cytosolic fractions was unaffected, although the synthesis of FPP was increased 5- to 9-fold (not shown).

Effects of divalent cations- In order to optimize the synthesis of *t,t,t*-GGPP, the brain cytosolic fraction was incubated with [^{3}H]IPP in the presence of different divalent cations (Table 3). As expected, the total incorporation was low in the absence of any divalent cations. However, under these conditions over 50 % of the total incorporation was associated with *t,t,t*-GGPP. With Mn^{2+} at a concentration of 0.1 mM, the total incorporation was stimulated 4-fold, even though incorporation into *t,t,t*-GGPP was unchanged. In the presence of 1.0 mM Mg^{2+} the total activity was stimulated 9-fold, while the incorporation into *t,t,t*-GGPP was only stimulated 3-fold. Higher concentrations of Mg^{2+} did not increase the incorporation of radioactivity. The addition of Zn^{2+} decreased the total incorporation, although more than 70% of the products was identified as *t,t,t*-GGPP under these conditions. Interestingly, when low concentrations of Mn^{2+} were present in the reaction medium together with 1.0 mM Mg^{2+}, the total biosynthetic activity was significantly suppressed.

Discussion

The cytosolic fractions from all tissues investigated had the capacity to synthesize both *t,t*-FPP and *t,t,t*-GGPP. The highest rate of synthesis of FPP was observed with the

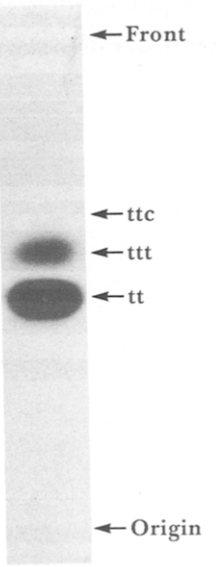

Fig. 3. *Determination of the trans/cis configuration of the short-chain isoprenoid reaction products.*
The cytosolic fraction from rat brain was incubated with [^{3}H]IPP and the reaction products were extracted, dephosphorylated and analyzed on silica TLC using solvent system B and the radioactivity was subsequently detected by autoradiography as described under Materials and Methods. The arrows indicate the migration of **tt**: *trans,trans*-farnesol, **ttt**: *trans,trans,trans*-geranylgeraniol and **ttc**: *trans,trans,cis*-geranylgeraniol standards.

cytosolic fraction from liver. This clearly reflects the high rates of cholesterol, ubiquinone and dolichol synthesis in this tissue. However, the highest rate of *t,t,t*-GGPP synthesis was associated with the cytosolic fractions from the brain, spleen and testis. The high rate of *t,t,t*-GGPP synthesis in these latter tissues might reflect a high demand for protein-prenylation. In fact, several investigations have shown that covalent attachment of a geranylgeranyl moiety

Table 2. *t,t-FPP and t,t,t-GGPP biosynthesis in the cytosolic fractions from different rat tissues*

Tissue	Product formation*		
	t,t-FPP	*t,t,t*-GGPP	% GGPP
Liver	2450	53.5	2.1
Brain	276	86.3	24.1
Kidney	159	46.4	22.2
Spleen	207	80.9	27.8
Testis	673	89.0	11.7

Cytosols isolated from different rat tissues were incubated with [^{3}H]IPP and the reaction products were extracted, dephosphorylated and identified using an HPLC system equipped with a radioactivity flow detector, as described under Materials and Methods.
*dpm/μg protein/15 min.

Table 3. *The effect of divalent cations on t,t-FPP and t,t,t-GGPP synthesis by the rat brain cytosolic fraction*

Ion added[a] Concentration (mM)	Product formation[b] *t,t*-FPP	*t,t,t*-GGPP
None	17	25
Mn^{2+}		
0.1	127	28
1.0	63	23
Zn^{2+}		
0.1	0.6	8.4
0.3	3.6	12
1.0	0.9	12
Mg^{2+}		
0.1	48	18
0.3	179	49
1.0	279	87
3.0	255	67
1.0 mM Mg^{2+}		
and 0.1 mM Mn^{2+}	32	36
and 0.1 mM Zn^{2+}	106	45

The cytosolic fraction isolated from brain was incubated with [^{3}H]IPP in the presence of various divalent cations and the reaction products were extracted, dephosphorylated and identified using an HPLC system equipped with a radioactivity flow detector, as described under Materials and Methods.

[a] as the chloride salt.

[b] dpm/μg protein/15 min.

to proteins is a very active process in the brain (20,23,27). Furthermore, when the prenylcystein compositions of mouse tissues were investigated, brain tissue was found to contain approximately 4 times more prenylcystein than liver and the dominant protein-bound isoprenoid was identified as *t,t,t*-geranylgeraniol (28).

The rate of hepatic biosynthesis of *t,t,t*-GGPP was low compared to that of FPP synthesis. One explanation for this finding could be that *t,t,t*-GGPP synthetase is saturated with FPP even at low concentrations. Another possibility is the presence of a larger endogenous pool of FPP in this cytosolic fraction. In this case newly synthesized [^{3}H]FPP would be diluted to a larger extent than in the other tissues, giving rise to a lower apparent specific radioactivity. In order to resolve this question in the future, the synthesis of *t,t,t*-GGPP must be investigated using an excess of FPP as substrate.

In testis, kidney, brain and spleen 11, 22, 24 and 28 %, respectively, of the total incorporation of radioactivity from [^{3}H]IPP was recovered as *t,t,t*-GGPP, which demonstrates the presence of a specialized *t,t,t*-GGPP synthetase in the cytosolic fractions of these tissues. Previously, it was found that purified FPP synthetase from pig liver catalyzes the additional condensation of FPP with IPP at a low rate (1). Therefore, it is unlikely that the synthesis of *t,t,t*-GGPP in these tissues is explained by this enzyme activity. With respect to liver, however, we cannot rule out the possibility that *t,t,t*-GGPP synthesis is catalyzed to some extent by FPP synthetase.

It is noteworthy that when *t*-GPP was included in the reaction mixture, giving rise to a more than 5-fold increase in the rate of FPP synthesis, the rate of *t,t,t*-GGPP synthesis was unchanged for all organs. Moreover, when the incorporation of IPP was low, i.e., in the absence of divalent cations or in the presence of both Mg^{2+} and Mn^{2+}, the ratio of *t,t,t*-GGPP to FPP produced was shifted in favor of *t,t,t*-GGPP synthesis. These observations also imply that *t,t,t*-GGPP synthetase has a high affinity for FPP and is saturated even under conditions which result in a low rate of FPP synthesis.

In the present investigation we have thus demonstrated that *t,t,t*-GGPP can be formed by all the cytosolic fractions investigated and that this compound is a major product in the cytosolic portion of the mevalonate pathway of rat brain, spleen, kidney and testis.

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