Pergamon

PII: S0031-9422(96)00837-0

Phytochemistry, Vol. 45, No. 2, pp. 261–267, 1997

0031-9422/97 $17.00+0.00

ISOPENTENYL DIPHOSPHATE ISOMERASE AND PRENYL TRANSFERASE ACTIVITIES IN BOTTOM FRACTION AND C-SERUM FROM *HEVEA* LATEX*

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(*Received in revised form* 11 *November* 1996)

Key Word Index—*Hevea brasiliensis*; latex; fresh bottom fraction; freeze-dried bottom fraction; C-serum; polyprenyl diphosphate; isopentenyl diphosphate isomerase; polyprenyltransferases.

Abstract—The isomerization of isopentenyl diphosphate (IDP) to dimethylallyl diphosphate (DMADP), and the formation of oligoprenyl diphosphates as well as polyprenyl diphosphates, catalyzed by IDP-isomerase and polyprenyl transferases, respectively, were observed on incubation of a mixture of ^{14}C-IDP and fresh bottom fraction (BF) (separated from centrifuged fresh *Hevea* latex) which had been preincubated at 4° overnight. The incubation of a freeze-dried BF with ^{14}C-IDP gave DMADP, farnesyl diphosphate (FDP) and polyprenyl diphosphate as predominant products, while that of fresh BF gave C_{20}- and C_{40}-diphosphates, as well as rubber as main products. FDP was not detected in the incubation of the fresh BF, suggesting that it was consumed as a primer for the formation of rubber and polyprenols. Direct evidence was obtained to show the presence of IDP-isomerase and polyprenyl transferase activities in the BF of fresh latex.

INTRODUCTION

In rubber biosynthesis, the first step has been postulated to be the isomerization of isopentenyl diphosphate (IDP) to dimethylallyl diphosphate (DMADP) catalyzed by the enzyme isopentenyl diphosphate Δ-isomerase (EC 5.3.3.2) [1]. The successive addition of IDP to DMADP has been assumed to yield rubber via C_{10}-, C_{15}-diphosphates, etc., catalyzed by enzyme(s) referred to as rubber transferase [2]. It was reported that the rubber transferase might be bound to rubber particles in *Hevea brasiliensis* [2,4–6]. A particle-bound rubber transferase has been demonstrated in rubber-producing species such as *Parthenium argentatum* [7–9] and *Ficus elastica* [10]. However, it has also been postulated that rubber biosynthesis is mediated by the association of a soluble *trans*-prenyltransferase with a small particle-bound protein called rubber elongation factor [11–14]. It was originally believed that the sequential addition of IDP to DMADP involved only *cis* additions. However, structural studies on natural *cis*-1,4-polyisoprenes revealed the presence of two to four *trans*-isoprene units at the ω-end (initiating end) of these molecules [15, 16] and more recently in the case of rubber, two [17]. Therefore, the chain elongation step in the formation of *cis*-1,4-polyisoprene has been presumed to require all-*trans* oligoprenyl diphosphate(s) as initiator molecules [3, 4].

The presence of IDP-isomerase in *Hevea* latex was reported by Bernard [1]. However, ozonolysis of the *in vitro* rubber synthesized in the presence of [4-^{14}C]IDP yielded no ^{14}C-acetone, which is expected if the dimethylallyl-group in the initiating terminal of rubber is formed from DMADP [2, 4, 18]. Nevertheless, ^{14}C-acetone was found for *in vitro* synthesized geranylgeraniol when [4-^{14}C]IDP was incubated with freeze-dried serum from latex [4]. This is good evidence that IDP-isomerase is present in the latex serum.

NMR spectroscopy provided conclusive evidence as to the initiating species of rubber formation. Two *trans*-isoprene units linked to the dimethylallyl-group, i.e. *trans-trans*-farnesyl diphosphate (FDP), is the initiating species for rubber formation in sporophores of *Lactarius* mushroom [19]. Similarly, both FDP and *trans-trans-trans*-geranylgeranyl diphosphate

*Part 1 in the series 'In vitro Synthesis of *Hevea* Rubber by Bottom Fraction and C-Serum'.

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(GGDP) were found to act as initiating species for the rubbers extracted from leaves of *Solidago altissima* and *Helianthus annuus* [20,21]. However, the dimethylallyl-group was not detected in rubber from *H. brasiliensis*, although the presence of two *trans*-isoprene units was confirmed [17,22,23]. This finding suggests that FDP is the initiating species for *in vivo* rubber formation in the case of *Hevea* natural rubber.

The initiation step of rubber biosynthesis has been extensively studied in *H. brasiliensis* and *P. argentatum* [1,24]. Archer *et al.* [24] developed a procedure for analysis of the activity of *trans*-allylic diphosphates as initiating species of rubber biosynthesis by incubation of IDP with washed rubber particles (WRP) from *Hevea* latex. The incorporation of [1-^{3}H]neryl diphosphate (NDP) and [1-^{3}H]geranyl diphosphate (GDP) into rubber molecules provided direct evidence to show that rubber formation starts from allylic diphosphates. They also demonstrated that the chain lengths of these allylic diphosphates (chain lengths C_5 to C_{20}) greatly affect the rate of rubber formation in the presence of ^{14}C-IDP, by acceleration of the uptake rate of ^{14}C-IDP into WRP by 10- to 20-fold. They assumed that this was entirely due to the formation of 90–95% new rubber molecules of which some were in the form of newly initiated molecules of *cis*-polyisoprene. Benedict *et al.* and Cornish *et al.* have studied rubber biosynthesis in a similar manner to that used to study rubber formation in *H. brasiliensis* and *P. argentatum* and obtained similar results [25–30]. It is remarkable that in these studies the allylic diphosphates have similar stimulatory effects irrespective of their geometric isomerism. These biochemical studies demonstrated the possibility of using *trans*-allylic diphosphates as initiating species. However, they afforded no direct evidence as to the true initiating molecule.

In this paper, we describe a new system in which newly formed rubber is provided by incubation of fresh BF, separated from latex instead of WRP, with IDP. The activities of IDP-isomerase and polyprenyl transferase were analyzed in fresh and freeze-dried BF as well as in C-serum (CS) from *Hevea* latex, by incubation with ^{14}C-IDP. Analysis of the resulting products confirmed the formation of DMADP and C_{10}- to C_{20}-oligoprenyl diphosphates, polyprenyl diphosphates and high-M_r rubber.

RESULTS AND DISCUSSION

IDP-isomerase activities in freeze-dried CS and BF

The isomerization of IDP to DMADP was reported to have an equilibrium constant of about nine in the direction of the allyl isomer [31]. The equilibrium concentration of DMADP would clearly militate against the formation of high M_r polyisoprenoids, because of the formation of several starter molecules. It is important to ascertain the presence of IDP-isomerase in latex, because without it, IDP can only condense with pre-existing prenyl diphosphates. IDP-isomerase activity was assayed by incubation of ^{14}C-IDP with BF, followed by radiochemical detection of any DMADP formed [1]. In addition to DMADP, radiolabelled oligo- and poly-prenols and rubber should be regarded as the compounds which are formed from DMADP as an initiating species.

The incorporation of radioactivity into C_5-, C_{10}- and C_{15}-allylic alcohols and other products by incubation of freeze-dried BF is shown in Table 1. Samples A, B and C showed similar IDP-isomerase activities independent on the addition of $MgCl_2$ or the amount of ^{14}C-IDP added. The IDP-isomerase activities observed with samples A, B and C were 10- to 15-fold higher than those of the control samples D and E.

In these experiments, the radioactivity in ^{14}C-DMADP was less than that in oligo- and poly-prenols. This is presumably due to the loss of a part of the C_5-allylic alcohol during the concentration step in the workup owing to its low boiling point. In addition, a part of DMADP will be consumed as a primer for the synthesis of oligo- and poly-prenyl DP. This implies that the amount of radioactivity in ^{14}C-DMADP should be higher than that given in Table 1. Although these counts are appreciably different in the various samples, it is enough to prove the presence of IDP-isomerase in the BF [32]. These findings show the possibility of the presence of IDP-isomerase in BF of latex.

Pre-existing polyprenols in BF and CS

The oligo- and poly-prenol compounds present in BF as free alcohols were analyzed by extraction with water-equilibrated butanol, without enzymatic dephosphorylation. Figure 1 shows the RP-TLC distribution of the free oligo- and poly-prenols. The hydrolyzed products of oligo- and poly-prenyl DP after treatment of freeze-dried BF and CS with phosphatase were extracted (lanes 2 and 3, respectively). It is clear that C_{20}-DP to high M_r polyprenols, possibly as high as C_{70}-polyprenyl-DP, were present in the freeze-dried BF, while polyprenyl DP higher than C_{50}-OH were not detected in freeze-dried CS. The compounds corresponding to C_{20}-, C_{50}- and C_{60}-OH were detected in the control experiment of untreated BF (lane 1). However, these were not found in the untreated CS. The difference between the compositions of the low M_r isoprenoid compounds before and after enzymatic dephosphorylation indicates that those pre-existing in BF and CS are not predominantly present as free alcohols.

Polyprenyl transferase activity in fresh BF and CS

Oligo- and poly-prenyl DP formed on incubation of ^{14}C-IDP with fresh CS (sample J), fresh BF (sample K) or mixtures of both (sample L) were analyzed by TLC after enzymatic dephosphorylation. The radiolabelled products formed from samples J, K and L are

Table 1. Assay of IDP-isomerase activity in freeze-dried BF

Sample	Added ^{14}C-IDP (nmole)	^{14}C-IDP incorporated into (dps)		
		C_5-OH	C_{10}- & C_{15}-	oligo- & poly-prenols
A: $MgCl_2$ omitted	0.9	3.4	3.9	177.5
B:	0.9	5.4	6.3	93.4
C:	5.2	4.9	3.8	—
D: boiled freeze-dried BF	0.9	0.3	0.5	—
E: freeze-dried BF omitted	0.9	0.5	0.4	—

Incubations A–C contained: BF suspension prepared from 100 mg freeze-dried BF, ^{14}C-IDP 0.9 nmole (1800 dps) or 5.2 nmoles (10 400 dps) as indicated, unless otherwise noted.

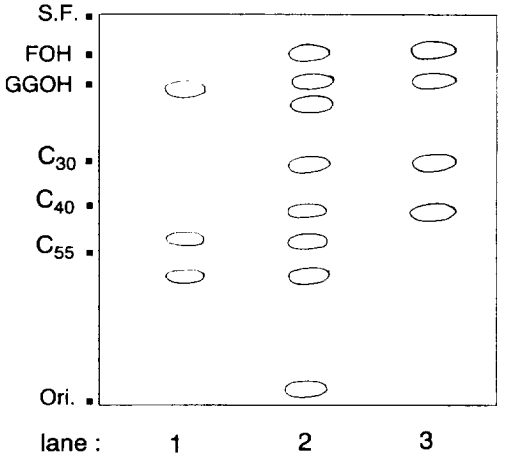

Fig. 1. RP-TLC separation of pre-existing oligo- and poly-prenols in freeze-dried BF (lanes 1 and 2: before and after enzymatic dephosphorylation, respectively) and in freeze-dried CS (lane 3: after enzymatic dephosphorylation).

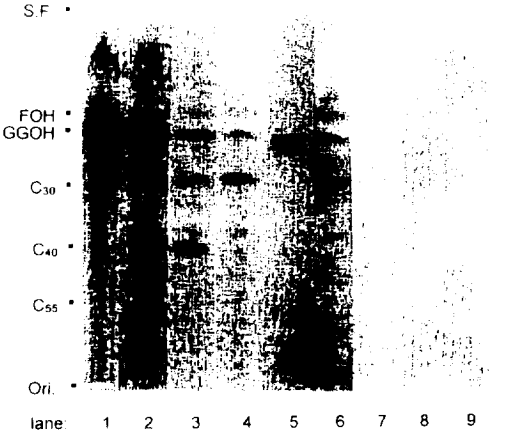

Fig. 2. Autoradiograms of the prenyl transferase products formed on incubation of substrates as indicated with 4.175 nmol [1-^{14}C]IDP. RP-TLC in Me_2CO–H_2O (19 : 1) of the products was carried out as described in the text. Lane 1—fresh CS; lane 2—fresh BF; lane 3—fresh mixture of (CS + BF); lane 4—CS + NaF; lane 5—BF + NaF; lane 6—mixture of (CS + BF) + NaF; lane 7—boiled CS; lane 8—boiled BF; lane 9—mixture of boiled (CS + BF). FOH—farnesol; GGOH—geranylgeranol; C_{45}—solanesol; S.F.,—solvent front; Ori.—origin.

shown in lanes 1, 2 and 3, respectively (Fig. 2). Farnesol (C_{15}-FOH), geranylgeranol (C_{20}-GGOH) and C_{30}- and C_{40}-polyprenols were detected in all the samples. The amount of C_{15}-OH was rather small in lanes 2 and 3, and that of C_{40}-OH was small in sample J. These findings indicate that, in addition to the IDP-isomerase, GDP-, FDP-, GGDP-, hexaprenyl-, heptaprenyl- and octaprenyl-synthetases are present in CS and BF. The formation of radiolabelled rubber was also found in the samples containing BF (samples K and L), but it was not detected in the sample containing only CS (sample J). The amount of ^{14}C-FDP detected in the incubation products of fresh BF (samples K and L) was less than in that of fresh CS (sample J). This would indicate that FDP was consumed as a primer for *in vitro* rubber synthesis, and poly-prenyl DP in fresh BF.

The inhibition effect of NaF on the conversion of IDP was analyzed. As shown in Fig. 2, samples M, N and O each gave three bands, which by increasing the sample size on TLC were identified to be C_{15}-OH, C_{20}-OH and C_{30}-OH. Control experiments with the samples boiled at 100° for 30 min before incubation, gave no detectable products (Fig. 2, lanes 7, 8 and 9).

Table 2 shows the incorporation of ^{14}C-IDP into

Table 2. Incorporation of ^{14}C-IDP into polyprenols in fresh CS and BF

Sample	Added ^{14}C-IDP (nmole)	Incorporation (dps)
J: CS	8.350	707
	4.175	337
K: BF	8.350	533
	4.175	201
L: CS + BF	8.350	2442
	4.175	723
M: CS + NaF	4.175	1.6
N: BF + NaF	4.175	4
O: CS + BF + NaF	4.175	8
P: boiled CS	4.175	0.3
Q: boiled BF	4.175	0.2
R: boiled (CS + BF)	4.175	0.3

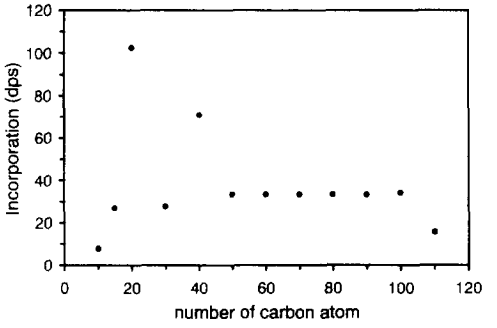

Fig. 3. Relative intensity of incorporation of ^{14}C-IDP into each fraction of the enzymatic polyprenols formed on incubation of ^{14}C-IDP with fresh BF.

polyprenyl DP in samples J–R. The highest value was observed for sample L (^{14}C-IDP+CS+BF), suggesting that prenyl transferases present in fresh CS and BF act to synthesize a large amount of polyprenyl DP. The incorporation of ^{14}C-IDP into the radiolabelled polyprenols was reduced by 50%, when the amount of ^{14}C-IDP in the incubation mixture was reduced by 50%. The addition of NaF resulted in *ca* 99% reduction of polyprenol formation, as observed in samples M, N and O. The effect of NaF will be discussed in a subsequent paper [33]. As shown in Fig. 2, the products were found to be oligoprenols, such as C_{15}-OH and C_{30}-OH, which are the predominant products resulting from IDP-isomerase, and FDP- and hexaprenyl-DP synthase activity. The activities of IDP-isomerase and prenyl transferases were lost on boiling, as indicated in the case of P, Q and R.

The relative incorporation of ^{14}C-IDP into oligo- and poly-prenols in the incubation with fresh BF (sample K) was analyzed by separation of the radiolabelled products by TLC. Nine fractions were assigned to oligo- and poly-prenols by means of authentic marker samples (Fig. 3). It is clear that the incorporation of ^{14}C-IDP was highest in the case of C_{20}-OH followed by C_{40}-OH. It is noteworthy that the amount of FDP was much smaller than that of GGDP. This may be due to the consumption of FDP for rubber biosynthesis.

Polyprenyl transferase activity in freeze-dried BF

The incorporation of ^{14}C-IDP into polyprenyl DP by freeze-dried BF in the absence or presence of *trans*-allylic primers is shown in Table 3. Sample F, which contained GDP, showed the highest conversion of ^{14}C-IDP. This indicated that the level of IDP-isomerase was not high enough to supply a sufficient amount of DMADP to saturate the FDP synthetase present in freeze-dried BF. The incorporation of ^{14}C-IDP into polyprenyl DP in sample B was similar to that in samples G and H. Thus, the addition of FDP or GGDP had no effect on the incorporation rate of ^{14}C-IDP into polyprenyl DP.

RP-TLC autoradiograms of the products from the incubation of freeze-dried BF with ^{14}C-IDP are shown

Table 3. Incorporation of ^{14}C-IDP into polyprenols formed on incubation of freeze-dried BF

Sample	Incorporated of ^{14}C-IDP into oligo- and poly-prenols (dps)
B: Starter omitted	101
F: +GDP	1211
G: +FDP	207
H: +GGDP	90
I: Boiled freeze-dried BF (control) and starter omitted	3.6

Incubations contained: BF suspension prepared from 100 mg freeze-dried BF, 0.9 nmole ^{14}C-IDP (1800 dps) and 5 μ mole starter, as indicated.

in Fig. 4 (lanes 2 and 3). In the incubation of fresh BF with IDP only (lane 1), C_{20}-OH and C_{40}-OH were predominant as mentioned above (*cf* Fig. 3). On the other hand, in freeze-dried BF (samples A and B in lanes 2 and 3), C_{15}-OH was the predominant product and C_{20}-OH was also detected in sample A. Similar distribution patterns of products were formed in the presence of GDP, FDP or GGDP (Fig. 4). A high level of C_{15}-OH was observed on addition of GDP, whereas it was low on addition of FDP or GGDP.

Another autoradiogram with a higher concentration of sample spots showed the difference of chain length of the products, i.e. the addition of GDP resulted in a high concentration of C_{15}-OH to C_{30}-OH (lane 1), and that of FDP and GGDP increased in polyprenols longer than C_{40}-OH (lanes 2 and 3) (Fig.

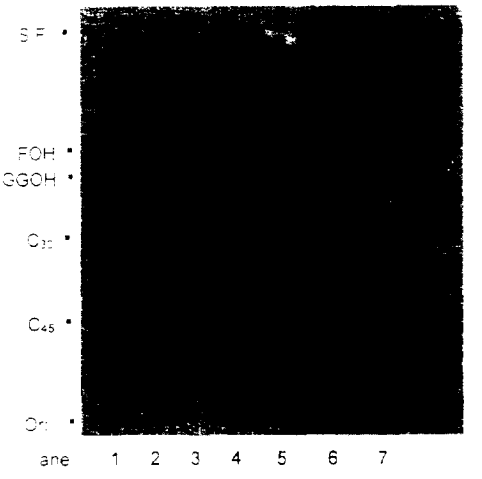

Fig. 4. Autoradiograms of the prenyl transferase products obtained from incubation with [1-^{14}C]IDP. RP-TLC with Me_2CO–H_2O (19:1) of the products was carried out as described in the text. Lane 1—fresh BF+[1-^{14}C]IDP (sample K); lane 2—freeze-dried BF+[1-^{14}C]IDP (sample A); lane 3—freeze-dried BF+[1-^{14}C]IDP (sample B); lane 4—freeze-dried BF+[1-^{14}C]IDP+GDP (sample F); lane 5—freeze-dried BF+[1-^{14}C]IDP+FDP (sample G); lane 6—freeze-dried BF+[1-^{14}C]IDP+GGDP (sample H); lane 7—freeze-dried BF alone.

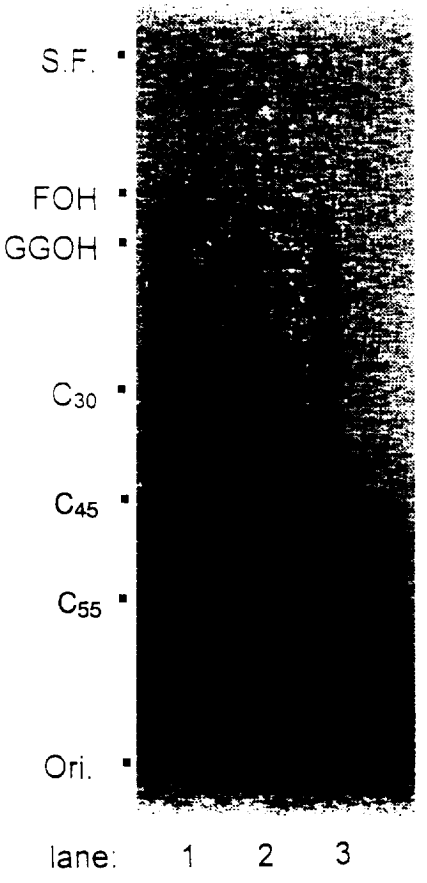

Fig. 5. Autoradiograms of the prenyl transferase products formed on incubation of the substrates indicated, in the present of 0.9 nmol [1-^{14}C]IDP with allylic primers. RP-TLC of the products was carried out with Me_2CO–H_2O (19 : 1), as described in the text. Lane 1—freeze-dried BF + GDP (sample F); lane 2—freeze-dried BF + FDP (sample G); lane 3—freeze-dried BF + GGDP (sample H).

5). This implies that the FDP synthetase activity was very high in the presence of GDP. On the other hand, octaprenyl synthetase and long-chain prenyl transferases were very active in the presence of FDP or GGDP.

The difference of the distribution of polyprenyl DP between freeze-dried and fresh BF strongly suggests that FDP formed on incubation of fresh BF was utilized as a primer for the *in vitro* biosynthesis of rubber and polyprenyl DP in the presence of membrane-bound particles which exist only in fresh BF, but not in freeze-dried BF.

EXPERIMENTAL

Chemicals. [1-^{14}C]IDP (2.0 GBq mol^{-1}) was purchased from Amersham. Unlabelled IDP, *t*-GDP, *t,t*-FDP and *t,t,t*-GGDP were purchased from Sigma Chemical Company. All other reagents were of analytical grades.

Preparation and incubation of bottom fraction and C-serum of H. brasiliensis. Fresh *Hevea brasiliensis* latex was collected in ice-chilled flasks by tapping. Latex exuded in the first 10 min was discarded to minimize contaminants. Several mature trees (RRIM 600), tapped on alternate days, were used throughout the experiments. The trees were grown at the Prince of Songkla University's experiment station, Thailand. The latex was filtered through a muslin cloth to remove some coaggulants and then immediately centrifuged at 49 000 *g* for 45 min at 4°. The rubber in the upper phase was separated from the middle clear CS and the bottom yellowish fraction (BF). Both CS and BF were centrifuged twice to separate them completely from each other.

A part of the resulting fresh BF was immediately lyophilized to yield a white-creamy powder. These frs were stored at −20° until use. Incubations were carried out using fresh or freeze-dried CS, BF and a mixture of CS and BF, immediately after prepn.

Unless stated otherwise, the incubation mixt. contained 4.175 or 8.350 nmol [1-^{14}C]IDP (2.0 GBq mol^{-1}) and 2.50 g (wet wt) of fresh CS, fresh BF or a mixture of both in a ratio of 1 : 1, by wt. Another mixt. was prepd similarly and 100 μl 200 mM NaF was added to inhibit the activity of phosphatase [34]. The mixtures were pre-incubated overnight at 4°, followed by incubation at 37° for 6 hr. The control incubations contained CS or BF preheated at 100° for 30 min. The reaction was terminated by the addition of 3 ml EtOH and the ethanolic soln was immediately centrifuged at 13 000 *g* for 30 min. The polyprenyl diphosphates and rubber fraction were extracted (× 3) with hexane–toluene (1 : 1), followed by drying up *in vacuo*. The rubber fr. in toluene soln was purified by reprecipitation (× 5) with Me_2CO. The polyprenyl diphosphates remaining in the Me_2CO fr. was collected and dried up.

Freeze-dried BF was incubated in a similar way as described above. The mix. contained 100 μl 1 M K_2PO_4 buffer soln, pH 7.6, 50 μl 1 M $MgCl_2$, 50 μl 1 M 2-mercaptoethanol, 0.9 or 5.2 nmol of [1-^{14}C]IDP (2.0 GBq mol^{-1}), 100 μl 5 mM *trans*-allylic diphosphate (*t*-GDP, *t,t*-FDP or *t,t,t*-GGDP), and 100 mg freeze-dried BF powder, in a final vol. of 1.0 ml. The mix. was treated immediately after prepn in a similar way to that above.

Assay of ^{14}C-*labelled polyprenols.* The polyprenyl diphosphates remaining in the Me_2CO supernatant, see above, were extracted with 1-BuOH equilibrated with H_2O, followed by washing the extracts with the lower phase (× 3). The radiolabelled polyprenyl diphosphates were hydrolyzed to the corresponding alcohols with 22 units of potato acid phosphatase in 60% MeOH and 0.1% Triton X-100, pH 4.7, with acetate buffer. These mixs were incubated at 37° overnight. The resulting polyprenols were extracted (× 3) with pentane, followed by washing (× 3) with H_2O. Under these conditions, potato acid phosphatase hydrolyzes polyprenyl diphosphates of at least C_{55} [35,36]. The composition of the hydrolysates extracted from freeze-dried BF was analyzed by RP-LKC-18

(Whatman) TLC developed Me_2CO–H_2O (19 : 1). The radiolabelled oligo- and poly-prenols were identified by comparison with commercial authentic standards and visualized with I_2 vapour.

The polyprenyl transferases were assayed by measurement the incorporation of ^{14}C-IDP into the polyprenols. The radiolabelled polyprenols were dissolved in pentane and assayed for radioactivity in an LS-5000TD Beckman liquid scintillation spectrometer. The hydrolysates were analyzed by RP-LKC-18 (Whatman) TLC developed with Me_2CO–H_2O (19 : 1). The positions of the commercial authentic standards were visualized with I_2 vapour. The distribution of radioactivity was detected by autoradiography with a Fuji BAS 2000 bioimage analyzer.

The radiolabelled polyprenols formed on incubation of fresh BF and ^{14}C-IDP were fractionated into 9 frs by recovery from a RP-TLC plate. These samples were then assayed to determine the relative intensity of radioactivity.

Isolation of pre-existing polyprenols in BF and CS. The unlabelled oligo- and poly-prenols pre-existing in the freeze-dried BF and CS were isolated by extraction with 1-BuOH and then treatment the products in the same procedure as described above for the radiolabelled polyprenyl diphosphates.

Assay of IDP-isomerase. The assay of IDP-isomerase was carried out using freeze-dried BF mixtures prepared in the same method as mentioned above. The enzymatic reaction was terminated by chilling the reaction mixture in an ice bath. To remove all the long-chain polyprenyl diphosphates, the mixture was washed with Et_2O until the ^{15}C-counts residing in this washing became less than 10 dps. At this stage, only IDP, DMADP, GDP and FDP remain in the aq. phase. The mixture was acidified with 2 ml 2 M HCl, followed by incubation at 37° for 15 min (IDP is not hydrolyzed by this procedure) to give a mixture of the corresponding primary and tertiary alcohols. These compounds were extracted with Et_2O ($\times 3$), then washed by an aq saturated NaCl ($\times 3$). The Et_2O sol was evap at 10° in a centrifugation evaporator. The C_5-allylic alcohol was separated from the C_{10}- and C_{15}-allylic alcohols by extraction with H_2O–hexane (1 : 1). The former was solubilized in the aq. phase, and the others were solubilized in the hexane layer. The resulting radiolabelled alcohols were assayed for radioactivity in an LS-5000TD Beckman liquid scintillation spectrometer.

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