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RUBBER FORMATION BY FRESH BOTTOM FRACTION OF *HEVEA* LATEX*

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Key Word Index—*Hevea brasiliensis*; latex; *in vitro* rubber synthesis; fresh bottom fraction; freeze-dried bottom fraction; C-serum; rubber transferases; new rubber.

Abstract—The formation *de novo* of rubber was confirmed by the incorporation of ^{14}C-IDP into rubber by fresh bottom fraction (BF) and C-serum (CS) at 37 for 6 hr after pre-incubation with the substrate at 4° overnight. The incorporation of ^{14}C-IDP and rubber yield were highest in fresh BF. A control experiment with boiled BF showed practically no incorporation of ^{14}C-IDP, although it contained about 1% (w/w dried BF) of rubber corresponding to pre-existing rubber. A very small amount of rubber was formed on incubation of CS and ^{14}C-IDP. Rubber formation in fresh BF was inhibited by the addition of NaF. Freeze-dried BF formed no rubber, although it was able to form polyprenols. The radiolabelled rubber formed on incubation of fresh BF and ^{14}C-IDP showed a molecular-weight distribution (MWD) having a peak similar to the low molecular-weight fraction of the skewed unimodal distribution of the endogenous rubber. Membrane-bound particles in fresh BF are presumed to have an important role in the synthesis of new rubber molecules.

INTRODUCTION

Two mechanisms, at least, have been postulated for the conversion of isopentenyl diphosphate (IDP) to *in vitro* synthesized rubber [1]. The first is by initiation of new rubber molecules on the surface of the rubber particles and by chain extension of pre-existing rubber molecules. The second is by formation of completely new rubber particles by a process which does not involve pre-existing rubber particles [1].

By ultracentrifugation, fresh latex from *Hevea brasiliensis* can largely be separated into three fractions, i.e. rubber phase, C-serum (CS) and bottom fraction (BF). *In vitro* rubber biosynthesis has been studied by incubating whole latex [2, 3], freeze-dried latex serum (CSS) [4] or washed rubber particles (WRP) [5–7] with the expected substrate(s). The incorporation of [1-^{14}C]IDP into rubber was found to be proportional to the amount of latex added [2]. The activity in CSS was found to be highest, due to the presence of very small rubber particles [8]. WRP prepared by ultracentrifugation [5, 7] or gel-filtration [1, 9] of fresh latex or freeze-dried latex gave a higher yield of rubber and was found to be a suitable system for analysis of the enzymatic activities, both in the *H. brasiliensis* and *Parthenium argentatum* [10–14]. It has been confirmed that IDP was converted into rubber in WRP in the presence of Mg^{2+} as a cofactor, and was stimulated by the addition of allylic diphosphates. This indicates that WRP contains, at least, two enzymatic systems needed for the formation of the initiating species and chain-elongation [9, 15].

The stimulatory effect of C_5- to C_{20}-allylic diphosphates on the incorporation of ^{14}C-IDP into rubber molecules was found to decrease in the order of $C_{20} \geqq C_{15} > C_{10} > C_5$ and was almost independent of the geometric isomerism of the isoprene units for *in vitro* rubber synthesis with WRP from *Hevea* [5, 6]. These allylic diphosphates showed no significant difference in the case of *P. argentatum* [10–14]. These findings suggest that allylic diphosphates are the initiating species in rubber biosynthesis. However, this is apparently inconsistent with the structural observation showing the absence of an ω-dimethylallyl-group in *Hevea* rubber [16].

The discrepancy between the biochemical and struc-

*Part 2 in the series '*In vitro* Synthesis of *Hevea* Rubber by Bottom Fraction and C-Serum'. For part 1 see ref. [17].

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tural observation is attributable to the differences in the objectives of the two studies, i.e. the synthesis *in vitro* and the structure *in vivo*. This discrepancy may be resolved by analysis of *in vivo Hevea* rubber just after polymerization or *in vitro* rubber synthesized, as closely as possible to the *in vivo* conditions. In the previous paper, we showed that the formation of polyprenols and the activities of IDP-isomerase, as well as prenyl transferase on incubation of fresh BF or CS in the presence of ^{14}C-IDP [17]. The rubber formed on incubation of fresh BF will be used as an appropriate model to analyze the direct initiating species of rubber formation.

In the present paper, we have analyzed the yield of rubber formed on incubation of fresh BF with IDP. A proof of new rubber formation is given, based on the incorporation of ^{14}C-IDP into the new rubber. The MWDs of the radiolabelled rubber were compared to that of the endogenous rubber to elucidate the difference between *in vivo* and *in vitro* synthesized rubbers. This results proved that the new rubber formed on incubation of fresh BF with ^{14}C-IDP was synthesized by chain elongation or new initiation. Comparison of the reactivity of rubber formation in fresh BF and freeze-dried BF revealed a crucial role for a membrane-bound particle which is found only in fresh BF.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-IDP into rubber formed on incubation of fresh bottom fraction or C-serum

Table 1 shows rubber yield and incorporation of ^{14}C-IDP into rubber formed on incubation of fresh CS, BF and a mixture of both (samples A, B and C). The addition of ^{14}C-IDP into fresh BF gave the highest yield of rubber, with the highest incorporation of ^{14}C-IDP into rubber. The mixture of fresh BF and CS in the presence of ^{14}C-IDP produced about one-half of that formed on the incubation of fresh BF and ^{14}C-IDP. Fresh CS (sample A) gave a very small amount of rubber formation. It is noticeable that both the rubber yield and the incorporation of ^{14}C-IDP increased with increasing concentration of ^{14}C-IDP, for samples B and C. The rubber yields in samples B and C were higher than those expected from the amount of added ^{14}C-IDP. This indicates that the new rubber is formed from both exogenous and endogenous IDP, which pre-exist in BF and is being-formed in the course of the incubation of BF.

The incorporation of ^{14}C-IDP into the rubber formed on incubation of fresh BF with ^{14}C-IDP was 10%, at maximum. Archer and Audley [7] also reported a very small uptake rate of ^{14}C-IDP into rubber formed on incubation with WRP and CSS of *Hevea* latex, without the addition of a primer. A similar phenomenon was observed by Cornish [14] on incubation of WRP and ^{14}C-IDP. However, on addition of IDP + prenyl transferases or IDP + FDP to the incubation mixtures, the uptake rate was increased markedly.

The poor incorporation of ^{14}C-IDP into rubber may be due to the rapid dephosphorylation of ^{14}C-IDP by a phosphatase known to be present in BF [8]. It is also likely that most of the added IDP was used for the synthesis of the primers needed for the synthesis of oligo- and poly-prenols as well as rubber. This is supported by the difference in the ^{14}C-IDP incorporation ratio and rubber yield. However, the amounts of rubber formed in these experiments were extremely high. If the isoprene units were derived from the endogenous IDP, the content of IDP should be extremely high in BF, but this was not in this case. The presumed origin of the rubber formation is discussed in ref. [21].

The inhibition of the incorporation of ^{14}C-IDP in the presence of fluoride ion is shown in Table 1. The addition of fluorides resulted in a significant decrease in the incorporation of ^{14}C-IDP into rubber by all samples. The effect of the fluoride ion will be discussed elsewhere. The incorporation of ^{14}C-IDP was observed to be insignificant on incubation with boiled CS, boiled BF and boiled CS + BF mixture. The presence of about 2 and 8 mg of rubbers were observed in the boiled CS and boiled BF, respectively, and could be attributed to the endogenous rubber.

It seems reasonable to assume that rubber transferase activity is sufficient for catalyzing the chain extension on the surface of pre-existing rubber particles to form a high M_r rubber. The formation of new rubber starting from ^{14}C-FDP also seemed very likely based on the findings published in the previous paper [17].

It is necessary to analyze whether the radioactivity of the resulting rubber was derived only from rubber molecules or polyprenols formed from fresh BF, i.e. the purity of the radiolabelled rubbers. The extracted rubber and polyprenol fraction from the incubation of ^{14}C-IDP with fresh BF, together with the rubber from control experiment were analyzed, by both RP- and normal-phase TLC (data not shown). These results showed that the incorporation of ^{14}C-IDP into the new rubber was not due to the contamination of the low M_r compound, i.e. the radiolabelled rubber was sufficiently purified by reprecipitation.

Effect of concentration of ^{14}C-IDP

The relationship between the amount of added ^{14}C-IDP and incorporation as well as rubber formation after incubation of 6 hr are shown in Fig. 1. Both the incorporation of ^{14}C-IDP and the rubber yield increased as the amount of added ^{14}C-IDP increased and then levelled off about 4 nmol of ^{14}C-IDP, with a rubber yield of 120 mg. The saturation of rubber yield indicates that all of the IDP or C-5 sources were consumed after 6 hr. On the other hand, the saturation kinetics of the incorporated ^{14}C-IDP suggests that ^{14}C-IDP was not consumed for chain elongation, but predominantly used for the formation of initiating spec-

Table 1. Rubber yield and incorporation of ^{14}C-IDP into rubber formed on incubation of fresh CS or BF

Sample	Added ^{14}C-IDP (nmol)	Incorporation of ^{14}C-IDP into rubber (dps)*	Resulting rubber (mg)*
A: CS§	8.350†	35	5.4
	4.175‡	11	2.8
B: BF§	8.350	1125	144
	4.175	867	112
C: CS + BF§	8.350	750	63
	4.175	403	52
D: CS + NaF	4.175	3.3	4.5
E: BF + NaF	4.175	1.3	19.9
F: CS + BF + NaF	4.175	6.7	11.6
G: boiled CS	4.175	3.3	2
H: boiled BF	4.175	1.7	8
I: boiled (CS + BF)	4.175	3.3	5

*These values are averaged from triplicated measurements.
†16 035 dps.
‡8032 dps.
§Dried weight of 2.50 g CS, 2.50 g BF and mixture of (CS + BF) corresponds to 416, 790 and 603 mg, respectively.

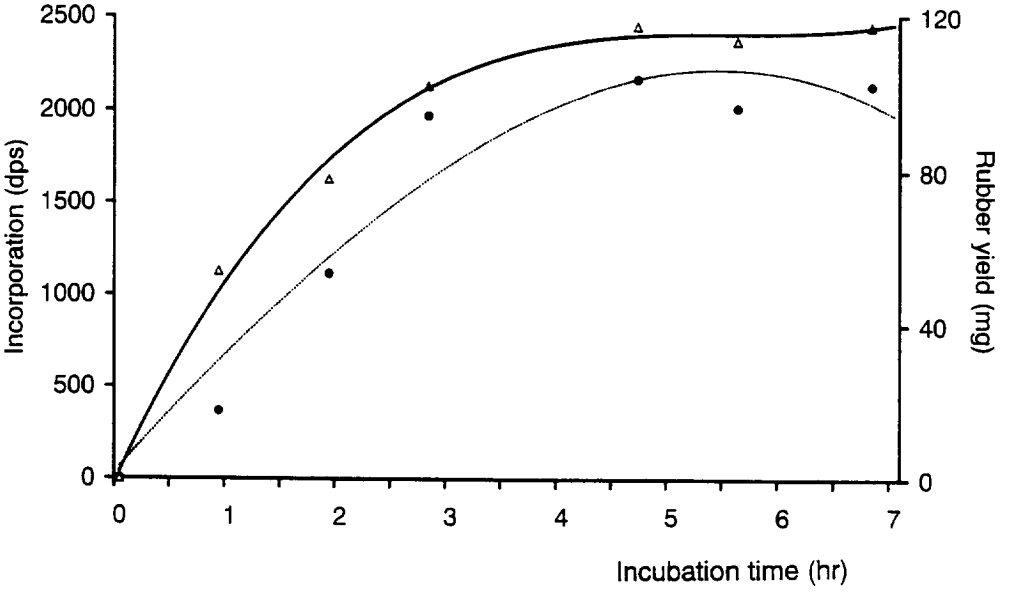

Fig. 1. The effect of the amount of added ^{14}C-IDP on incorporation (●—●) and rubber yield (△—△).

ies. In this case, the consumption of all the IDP or C-5 source in BF will result in an interruption of the usage of the initiating species in excess to form new rubber.

Rubber formation by freeze-dried BF

Table 2 shows the yield of rubber and the incorporation of ^{14}C-IDP into rubber formed on incubation of freeze-dried BF. The yield of rubber was about 1% (w/w dried BF), which was practically the same as that obtained from the control experiment. The radioactivity of these rubbers was less than 1% of that observed on incubation of ^{14}C-IDP with fresh BF (sample B in Table 1).

There was no difference in the rubber yield extracted from boiled or unboiled freeze-dried BF, after treatment with either 5 M HCl or 5 M KOH, suggesting that there are no more rubber components in BF. These results support the view that the increasing rubber yield in each incubation containing fresh BF was actually new, enzymatically synthesized rubber.

The rubbers extracted from samples J to N were analyzed by normal-phase TLC. They showed no band corresponding to radiolabelled rubber in the autoradiogram, compared with the rubber formed on

Table 2. Incorporation of ^{14}C-IDP into rubbers formed on incubation of freeze-dried BF. Incubations contain: BF suspension prepared from 100 mg freezed-dried BF (*ca* 1 mg rubber), 0.9 nmol of ^{14}C-IDP (1800 dps) and 5 μmol of primer

Sample	Incorporation of ^{14}C-IDP into rubbers (dps)	Resulting rubber (mg)
J: Primer omitted	0.5	1.1
K: +GDP	1.4	0.8
L: +FDP	0.5	1.3
M: +GGDP	0.7	1.0
N: Boiled freeze-dried BF and primer omitted	0.7	1.2

incubation of ^{14}C-IDP with fresh BF (data not shown). This confirms that only fresh BF has the enzymatic activities or cofactors needed to synthesize new rubber. Thus, it seems probable to assume the existence of a certain system such as membrane-bound particles in fresh BF, which could be destroyed in the process of freeze-drying. This system might be a vital substrate for the *in vitro* rubber synthesis. However, freeze-dried BF has the ability to synthesize the polyprenols, although the incorporation of ^{14}C-IDP was far less than that obtained on incubation of ^{14}C-IDP with fresh BF [17].

MWD of rubber formed on incubation of fresh bottom fraction with ^{14}C-IDP

Figure 2 shows the MWDs of the radiolabelled rubber formed on incubation of [1-^{14}C]IDP with fresh BF compared to that of the endogenous rubber extracted from the BF. The MWD of the endogenous rubber determined by GPC analysis was a typical skewed unimodal ranging from 7.5×10^6 to 1.0×10^4, with a weight-average M_r ($\bar{M}_w$) of 1.3×10^6 and a number-average M_r ($\bar{M}_n$) of 1.8×10^5. The MWD of the radiolabelled rubber detected by UV detector showed an apparent bimodal distribution with a $\bar{M}_w$ of 3.9×10^5 and a $\bar{M}_n$ of 5.3×10^4. On the other hand, the MWD observed by the ^{14}C-radioactive detector showed a broad unimodal distribution centered around 1.0×10^5, with a $\bar{M}_w$ of 2.7×10^5 and a $\bar{M}_n$ of 3.0×10^4. The $\bar{M}_n$ of radiolabelled rubber was about one-sixth of that obtained from the endogenous rubber. It is remarkable that the newly-formed rubber showed a MWD having a peak similar to the low M_r fraction of the bimodal MWD in ordinary rubber, which usually shows peak tops at 1.2×10^6 and 2.0×10^5. This implies that the rubber having a high M_r in the MWD of newly-formed rubber, detected by UV detector, was the endogenous rubber. Furthermore, this evidence confirms that the low M_r rubber,

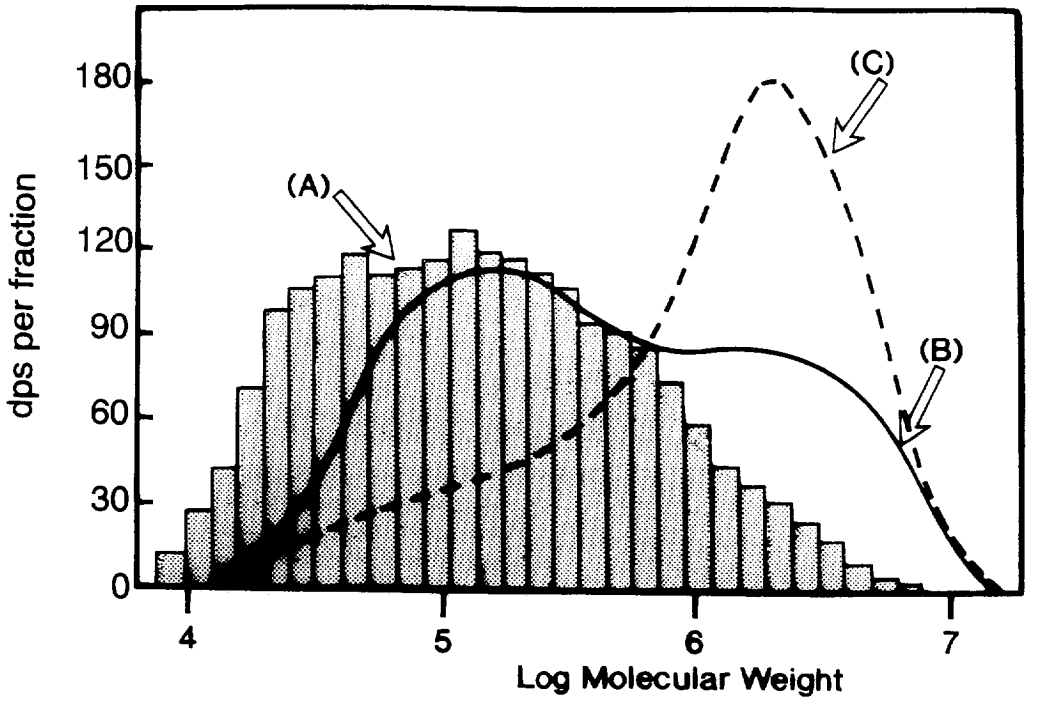

Fig. 2. MWDs of radiolabelled rubber formed on incubation of fresh BF in the presence of ^{14}C-IDP, detected by (A) ^{14}C-radioactive detector and (B) UV detector. (C) MWD of endogenous rubber existing in the boiled BF, observed by UV detector.

which increased the low M_r fraction in the MWD of this rubber, was the newly-formed rubber. Thus, the ^{14}C-IDP is not enzymatically incorporated into the high M_r rubbers, but it is preferentially incorporated into the rubbers with M_r lower than the endogenous rubber. If all the rubbers were enzymatically synthesized solely by a chain elongation mechanism, the ^{14}C-IDP would be incorporated into all of the polyisoprenes and be distributed throughout the MWD profile. The difference of MWD determined by UV- and ^{14}C-assay was also reported by Archer *et al.* [9] for the *in vitro* synthesized rubber from WRP.

The evidence mentioned above suggests that there is a clear difference in the mechanism of biosynthesis of *in vivo* and *in vitro* rubbers. Structural analysis of both rubbers will provide direct evidence on the factors controlling M_r in *Hevea* rubber and the reason why the individual molecules do not contain the ω-dimethylallyl-group found in polyprenol.

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 the other reagents were of analytical grades.

Incubation conditions. Fresh BF and CS were prepd according to the procedure in ref. [17]. The incubation mixture contained 4.175 or 8.350 nmol of [1-^{14}C]IDP (2.0 GBq mol^{-1}) and 2.50 g (wet wt) of the fresh BF, CS or a mixt. of both (1 : 1, by wt). An identical mixt. was prepd to which 200 mM NaF was added to inhibit the activity of phosphatases [18–19]. The incubation mixture was pre-incubated overnight at 4°, followed by incubation at 37° for 6 hr. The control incubations were treated in the same way except that BF, CS or a mixt. of both were boiled at 100° for 30 min prior to use. All the incubation mixtures were terminated by the addition of 3 ml EtOH, and the ethanolic soln was immediately centrifuged at 12 000 *g* for 45 min. The ppt was dissolved in hexane–toluene (1 : 1), then re-centrifuged (× 3). The supernatant was taken down in a rotary evaporator to small vol. and purified by reprecipitation from toluene soln with EtOH (× 3), followed by drying *in vacuo* and weighing. The effect of ^{14}C-IDP conc. on incorporation and rubber formation was analyzed by incubating fresh BF (2.50 g wet wt.) with 0, 0.93, 1.85, 2.78, 4.63, 5.55 and 6.46 nmol of ^{14}C IDP, under the same conditions as described above.

The incubation mixture containing freeze-dried BF contained, in a final vol of 1.0 ml, 100 μl 1 M K_2PO_4 buffer, pH 7.6, 50 μl 1 M $MgCl_2$, 50 μl 1 M 2-mercaptoethanol, 0.9 nmol [1-^{14}C]IDP (2.0 GBq mol^{-1}), 5 μmol geranyl diphosphate (*t*-GDP), farnesyl diphosphate (*t,t*-FDP) or geranylgeranyl diphosphate (*t,t,t*-GGDP), and 100 mg freeze-dried BF powder. Fresh BF and CS contained 31.6 and 16.7% (w/w) of non-volatile components, respectively. The incubation mixture was treated by the same procedures as mentioned above.

Assays of radiolabelled rubber. The radiolabelled rubber pptd with EtoH in the incubation mixture was extracted with hexane–toluene (1 : 1, × 3). The extracts were combined and concd to small vol, followed by repptn of the rubber in toluene soln with Me_2CO (× 3) and drying *in vacuo*. The purity of the radiolabelled rubbers was examined by RP and normal phase TLC. Rubber soln in toluene (1% w/v) was subjected to TLC on a precoated silica-60 TLC (Merck) developed with toluene for normal phase TLC and with a solvent system of Me_2CO–H_2O (19 : 1) for RP-TLC.

Determinations of molecular-weight distribution (MWD) of radiolabelled rubber. MWD was determined by gel-permeation chromatography, using two columns in series packed with polystyrene–divinylbenzene copolymer gels having exclusion limit of 4.0×10^7 and 4.0×10^4. Measurements were made using THF as an eluent at 35°, and monitoring by UV. The eluent from the columns was collected at 0.5 min intervals and assayed for radioactivity. The elution profile of the radiolabelled rubber was compared to the MWD profile of the endogenous rubber. Gel contents were determined in the usual way [20]. All the rubbers were found to contain less than 5% of the gel fr.

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