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STRUCTURE OF *IN VITRO* SYNTHESIZED RUBBER FROM FRESH BOTTOM FRACTION OF *HEVEA* LATEX*

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Key Word Index—*Hevea brasiliensis*; latex; fresh bottom fraction; new rubber; M_r distribution; *trans*-isoprene unit; dimethylallyl-group; ^{13}C NMR.

Abstract—Bottom fraction (BF) separated from fresh *Hevea brasiliensis* latex by ultracentrifugation, after pre-incubation at 4° overnight, produced new rubber on incubation at 37° for 6 hr. The rubber yield was increased about 3.2- and 4.7-fold by the addition of isopentenyl diphosphate (IDP) and farnesyl diphosphate (FDP), respectively. The rubber formed on incubation in the presence of FDP showed a clearly bimodal molecular-weight distribution (MWD) with $\bar{M}_n$ of 9.6×10^4 and $\bar{M}_w$ of 1.0×10^6; which were about 1/4 of that of the endogenous rubber. This indicates that the formation of new rubber in BF uses FDP as a direct initiator. The presence of the dimethylallyl-group of the initiating species was observed in the new rubber formed on incubation of fresh BF with FDP, although it was not detected in *in vivo* rubber. The new rubber formed on incubation contained no long-chain fatty acid groups esterified to the rubber molecules, as is the case in *in vivo Hevea* rubber. These facts show the differences in the mechanism of initiation and termination between the *in vitro* and *in vivo* rubber formation reactions.

INTRODUCTION

Conclusive evidence that allylic diphosphates act as initiating species for rubber formation was reported by Archer and Audley [1, 2]. They showed the incorporation of [1-^{3}H]neryl diphosphate (NDP) and also [1-^{3}H]geranyl diphosphate (GDP) into rubber in the presence of unlabelled isopentenyl diphosphate (IDP) by washed rubber particles (WRP) from *Hevea brasiliensis*. It was also confirmed that the addition of allylic diphosphates showed a stimulatory effect on rubber formation in a system containing IDP and WRP from *H. brasiliensis* [1, 2], *Parthenium argentatum* [3, 4] and *Ficus elastica* [5]. The efficiency of allylic diphosphates was found to increase with increasing chain length, i.e. $C_{20} \geqq C_{15} > C_{10} > C_5$, and that the geometric isomerism of the isoprene units had little effect [1, 2]. These findings suggest that the direct initiator will be FDP and/or GGDP for *H. brasiliensis*. Similarly, the addition of C_5- to C_{20}-diphosphates, including *cis*-neryl diphosphate (NDP) and *trans*-geranyl diphosphate (GDP), gave almost the same stimulatory effects in the case of *P. argentatum* [6]. These findings imply that the rubber transferase can use any short-chain allylic diphosphates as an initiator, independent of chain length or geometric isomerism.

The initiating species expected from biochemical studies would result in the formation of a characteristic ω-dimethylallyl-group in rubber. This group was clearly detected in the ^1H and ^{13}C NMR spectra for wild rubbers from leaves of *Solidago altissima* [7], *Helianthus annuus* [8] and from sporophores of *Lactarius volemus* [9]. It has also been confirmed that two or three *trans*-isoprene units are linked to the ω-dimethylallyl-group. This structural evidence strongly supports the idea that FDP or GGDP acts as the initiating species in rubber formation [7, 8]. However, the characteristic ^{13}C NMR signals of the expected dimethylallyl-group were not detected in rubber from *H. brasiliensis*, although signals corresponding to small amounts of *trans*-isoprene units in the *trans-trans*- or dimethylallyl-*trans*-sequence were observed [7, 8, 10–15]. This suggests that allylic diphosphates are not direct initiators in *Hevea* rubber or the occurrence of some modification of the ω-dimethylallyl-group after polymerization [16]. This point is resolvable by analysis of newly synthesized rubber rather

*Part 3 in the series '*In Vitro* Synthesis of *Hevea* Rubber by Bottom Fraction and C-Serum'. For part 2 see ref. [27].

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than those obtained from latex vessels and which have been stored for a long time after synthesis.

Archer and Audley [17, 18] reported that the ^{14}C-labelled rubber synthesized by incubation of WRP with ^{14}C-IDP had a lower M_r ($\bar{M}_n$: 5.6×10^4 and $\bar{M}_w$: 1.3×10^5) than the endogenous rubber ($\bar{M}_n$: 2.6×10^5 and $\bar{M}_w$: 6.2×10^5) [2, 17, 18]. Likewise, Benedict *et al.* [6, 19], showed that the ^{14}C-IDP incorporated into rubber by WRP of *P. argentatum*, had a lower M_r than the endogenous rubber. It is well-known that rubber from fresh *Hevea* latex shows a bimodal MWD [20]. Interestingly, the radiolabelled rubbers formed in both cases showed a MWD having a peak similar to the low M_r fraction of the bimodal MWD of *in vivo Hevea* rubber. These findings may be simply due to the condition employed for *in vitro* polymerization. However, if it is a fundamental difference between *in vivo* and *in vitro* rubbers, structural analysis of both rubbers will provide direct evidence of the factors controlling M_r in *Hevea* rubber, as well as the reason why newly synthesized rubber has terminal groups different from those wild rubbers.

In the previous paper [21], we reported on the polyprenols and rubbers formed on incubation of fresh BF with ^{14}C-IDP. The BF system was found to contain the substrates and enzymes, including IDP-isomerase, needed to form new rubber as well as polyprenol. The incorporation of ^{14}C-IDP into rubber was found to proceed effectively. ^{13}C NMR analysis indicated that the newly-formed rubber was distinct from *in vivo* rubber, by the absence of fatty acid ester groups. The new rubber formed on incubation of fresh BF with ^{14}C-IDP, on the whole, was similar to the rubber obtained from *Hevea* seedling [21].

In the present paper, we show rubber formation on incubation of fresh BF with IDP in the presence of FDP, and analyze the stimulatory effect of FDP on rubber yield. The MWD of the new rubber formed in these incubations was shown to be different from the rubber formed by chain-extension of pre-existing rubber molecules. A structural analysis was carried out to clarify the characteristics of the newly-formed rubber, in connection with the mechanism controlling the molecular weight of rubber.

RESULTS AND DISCUSSION

Effect of temperature and storage time for cold stimulation

Rubber biosynthesis in *P. argentatum* was reported to be increased after exposing the plants to low temperatures of 5–7° [22]. A similar phenomenon was also observed for the *Hevea* tree; a peak of natural rubber production is always obtained during the cold-month period (January in Thailand). Our experiments showed that rubber biosynthesis by fresh BF after pre-incubation at 4° for 12 hr, gave a rubber yield higher than that of the non-pretreated control (Fig. 1). In addition, rubber formation was not observed on pre-cooling fresh BF at −20°. Inactivation of the enzymatic activity was also observed in lyophilized BF or freeze-dried BF [23]. This indicates that some necessary requirements for rubber biosynthesis, such as membrane-bound particles, were destroyed by freezing.

Formation of rubber in fresh bottom fraction and C-serum

Yields of rubbers formed on incubation of fresh BF and CS are given in Table 1. BF gave the highest rubber yield, whereas the mixture of (BF + CS) gave about 50% less, suggesting that rubber formation in CS is insignificant. The actual new rubber yield was estimated from the difference between the resulting yield of each experiment with the control one, i.e. 35, 17 and 3 mg for BF alone, BF + CS mixture and CS alone, respectively.

The BF consists mostly of the lutoid particles of Homans and Van Gils [24], but also includes varying amounts of lipid-containing Frey-Wyssling particles [25], mitochondria and other particulate components of normal plant cells having a density greater than that of the serum, e.g. ribosomes [26]. The results mentioned above clearly show that BF contains all of the enzymes, carbon sources and co-factors necessary to synthesize rubber molecules. The new rubber formed on incubation of fresh BF was confirmed by radiochemical studies reported in a previous paper [27].

It is to be expected that CS would contain IDP, precursors of IDP, water-soluble enzymes and small rubber particles. The oligo- and poly-prenyl DP could be formed on incubation of CS and ^{14}C-IDP [23, 27]. However, CS gave a small amount of rubber and the mixture of BF and CS yielded about one-half of the rubber of that obtained from BF alone. This may be due to the preferential synthesis of oligo- and polyprenols in CS, and these compounds might then have an inhibitory effect on the conversion of IDP into rubber. A similar phenomenon was observed on incubation of whole latex and IDP [28].

Formation of rubber in bottom fraction following the addition of IDP or FDP

The rubber yields obtained on incubation of fresh BF with IDP or FDP and that of boiled BF are shown in Fig. 2. The rubber yield of unboiled BF increased markedly with incubation time following the addition of IDP or FDP. This indicates the presence of all the necessary enzymes required to produce new rubber in fresh BF. The rubber yield increased approximately 3.2-fold following the addition of IDP into the fresh BF. The addition of FDP would be expected to accelerate new rubber formation, if FDP is a direct initiating species as in the case of rubber from *Lactarius*

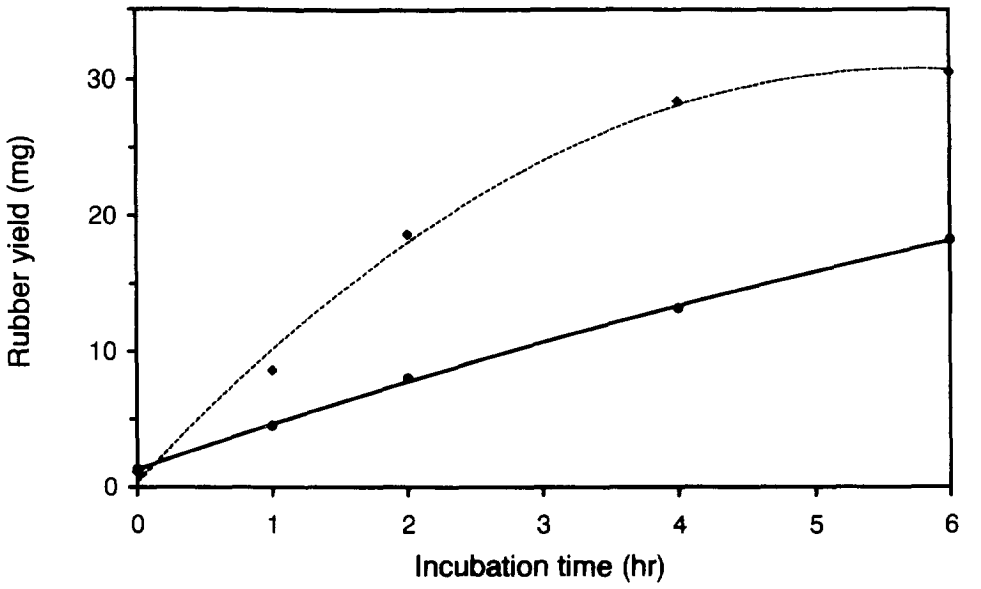

Fig. 1. The yield of rubber formed (A) after pre-incubation of fresh BF at 4 overnight before incubation at 37 for various incubation times (—◆—), and (B) without pre-incubation (—●—).

Table 1. Yield of rubber formed on incubation of fresh BF or CS (no starter added)

Sample	Dried weight of sample (mg)	Resulting rubber*(mg)
CS	417	5
BF	790	45
Mixture of CS + BF	603	28
Boiled CS	417	2
Boiled BF	790	10
Boiled mixture of CS + BF	603	11

*Data averaged from triplicated experiments.

volemus [9, 14]. The highest rubber yield was obtained on addition of FDP; about 4.7-fold of that formed on incubation of fresh BF alone. This also agrees with our observation that FDP in fresh BF was presumed to completely utilized as an initiator in rubber biosynthesis [23, 27].

The amount of rubber formed by the addition of FDP was as high as 160 mg from 790 mg dry weight of BF. This indicates that the endogenous IDP, synthesized in the course of the incubation, as well as pre-existing IDP, is consumed to form new rubber. The formation of IDP in BF is necessary to explain the rubber yield in this case, as well as that of rubber formation in BF with ^{14}C-IDP, if IDP is the sole origin of all the isoprene units. However, it is not easy to assume the presence of effective carbon sources and energy sources to form IDP in BF, at present. The possibility that a C-5 carbon source, other than IDP, could account for the high rubber yield in BF has to be considered, although there is no direct evidence to support this idea.

The yield of new rubber increased rapidly at the initial stage, but it appeared to level off around 6 hr with BF alone. This may be due to the limitation of the endogenous IDP or initiating species present in fresh BF. However, such a saturation phenomenon was not observed in the cases of incubation of fresh BF with FDP or IDP.

The addition of NaF to the incubation containing fresh BF and FDP decreased appreciably the rate of rubber formation, and the final rubber yield was lower than even that obtained on incubation of fresh BF alone. A possible explanation for the inhibition is that essential Mg^{2+} ion is removed by complexation as magnesium fluorophosphate, as happens in the inhibition of the glycolytic enzyme, enolase, by fluoride ion [29]. It has been reported that inorganic phosphate is present in substantial quantities in BF.

Molecular weight of rubber formed in bottom fraction

The rubbers extracted from BF alone, boiled BF and cream phase showed a typical MWD with main- and shoulder-peaks corresponding to high and low M_r rubbers (Fig. 3). It is clear that the rubber formed on incubation of BF (sample D) shows a significant increase in low M_r fraction and the number-average M_r (M_n), determined by GPC was about one-half of that in boiled BF (sample E). It is also remarkable that the polydispersity ($\bar{M}_w/\bar{M}_n$) of the rubber formed on incubation of fresh BF alone was 1.8-fold, higher than in the control sample. The MWD of rubbers formed on incubation of fresh BF + IDP (sample A) and fresh BF + FDP (sample B) showed a typical bimodal distribution, while those of fresh BF + FDP + NaF (sample C), fresh BF alone (sample D) and boiled BF (sample E) showed a skewed unimodal distribution rich in the high M_r fraction. This implies that the M_r of the new rubber formed by the

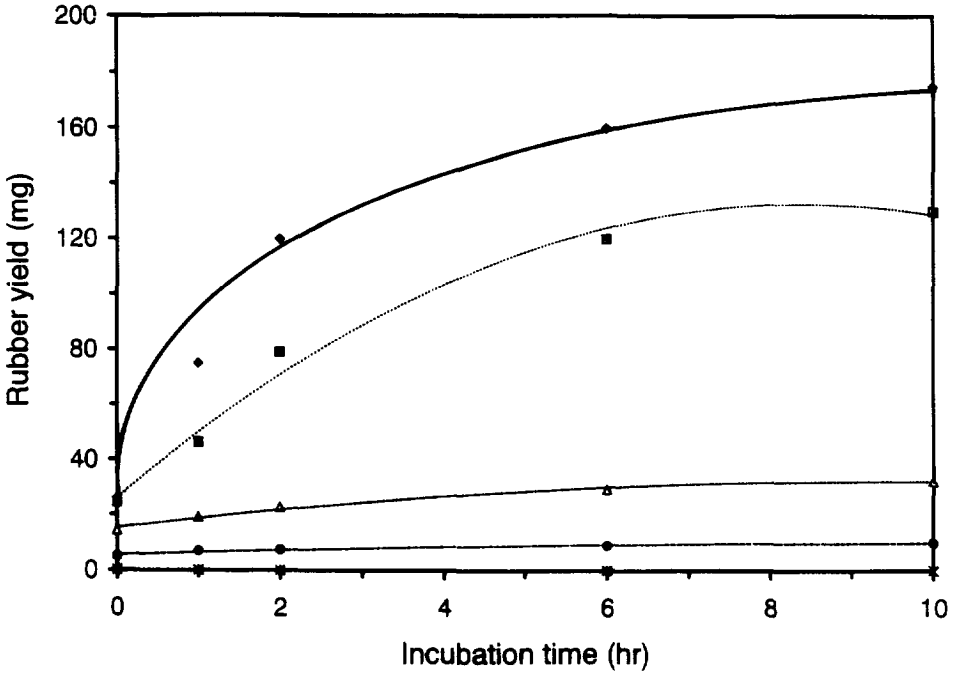

Fig. 2. Yield of rubber formed on incubation of (A) BF + 4.2 nmol IDP (■—■), (B) BF + 3.5 μ mol FDP (◆—◆), (C) BF alone (△—△), (D) sample B + 0.5 mmol NaF (●—●) and (E) boiled BF (*—*).

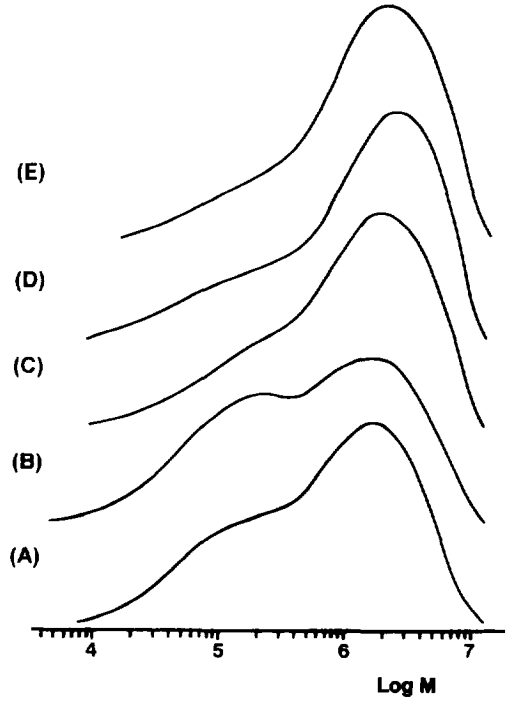

Fig. 3. Molecular-weight distribution of rubber formed on incubation of (A) BF + 4.2 nmol IDP, (B) BF + 3.5 μ mol FDP, (C) BF alone, (D) sample B + 0.5 mmol NaF and (E) boiled BF.

addition of FDP is lower than that of the endogenous rubber, and on the addition of IDP.

Table 2 shows the $\bar{M}_w$ and $\bar{M}_n$ of the rubbers formed on incubation of fresh BF with/without IDP or FDP, together with the relative intensity of the low and high M_r peaks in MWD. It is clear that the addition of FDP decreased $\bar{M}_n$ about 30% and $\bar{M}_w/\bar{M}_n$ increased 2-fold, compared with the control experiment (sample E). The addition of IDP to fresh BF showed a similar effect on $\bar{M}_n$ and $\bar{M}_w/\bar{M}_n$, but to a lesser degree than observed for the addition of FDP. However, this effect was not seen when NaF was added to the incubation mixture.

These findings indicate that new rubber formed on incubation of fresh BF contains low M_r rubber. The addition of FDP is expected to initiate directly the formation of new rubber more effectively than the formation of the initiating species via isomerization of IDP to DMADP. If the new rubber was formed predominantly by chain extension, the resulting rubber should have a M_r higher than the control one. This would provide confirmatory evidence that the rubber formed on incubation of fresh BF consists of new rubber and pre-existing rubber obtained by chain-extension by the condensation of IDP.

Archer and Audley [18] reported that the new rubber formed on incubation of washed rubber particles with ^{14}C-IDP and neryl diphosphate (NDP) had a low M_r. This could be attributed to a large amount of initiating species for rubber biosynthesis, so that the low M_r rubbers were mainly formed.

Structure of rubber formed on incubation of fresh bottom fraction

It was reported that *Hevea* rubber from fresh latex and commercial solid rubber contains about 5 mmol kg^{-1} rubber of long-chain fatty acids esterified to rubber molecules, even after extensive deproteinization [13]. Figure 4 A and B show the aliphatic signals in

Table 2. Molecular weight and relative ratios of high and low M_r fraction of rubbers formed from fresh BF in the presence of IDP or FDP (Fig. 3)

Sample	$\bar{M}_{w,RI} \times 10^{-6}$	$\bar{M}_{n,RI} \times 10^{-5}$	$\bar{M}_w/\bar{M}_n$	Ratio of high to low M_r fraction
A: BF+IDP	1.1	1.2	8.6	69:31
B: BF+FDP	1.0	0.9	10.2	60:40
C: BF+FDP+NaF	1.6	2.5	6.5	82:18
D: BF alone	1.6	1.8	8.8	77:23
E: Boiled BF	1.8	3.6	5.2	85:15

*Values estimated from relative intensity of high and low M_r peaks.

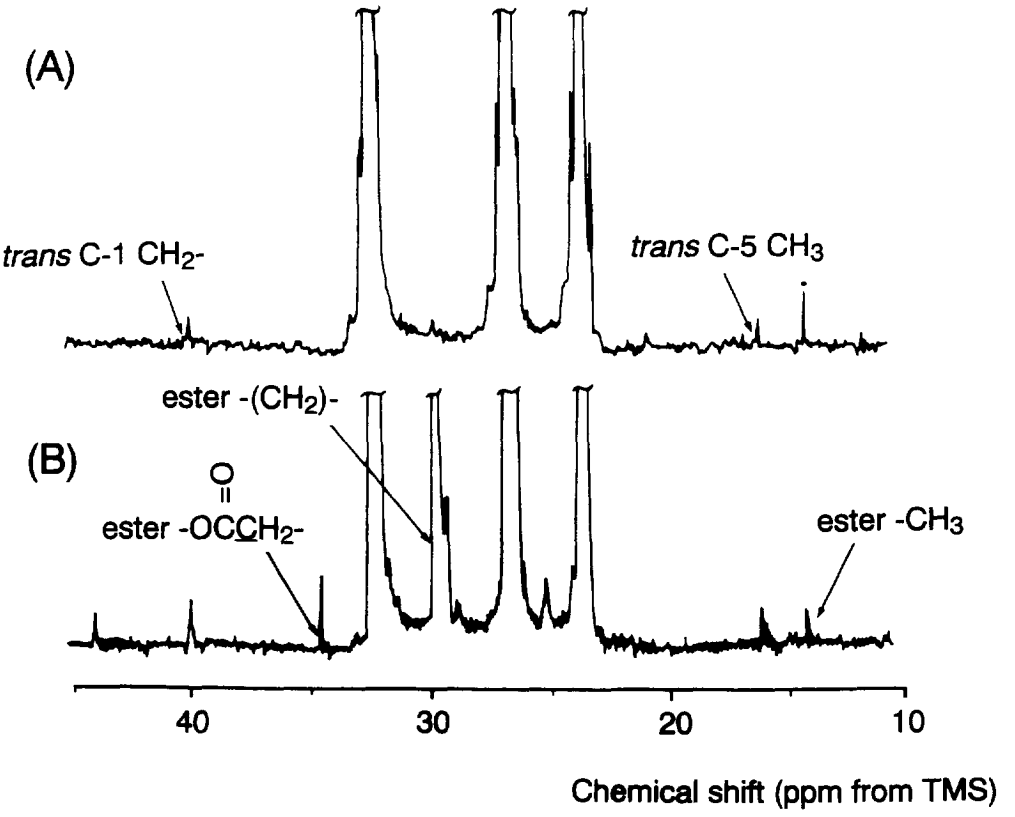

Fig. 4. ^{13}C NMR spectra of rubber from (A) incubation of fresh BF alone, and (B) cream phase.

^{13}C NMR spectra of the rubber formed on incubation of fresh BF alone and cream rubber. It is noteworthy that the newly formed rubber shows no signals at δ 29.7 and 14.0, which are due to the methylene carbon atoms (-($\underline{C}H_2$)$_n$-) and methyl carbon atoms ($\underline{C}H_3$-) in the long-chain fatty acid groups, respectively [14], showing that this rubber contains no long-chain fatty acids. This indicates that the presence of the appropriate enzymes needed to esterify long-chain fatty acids to rubber molecules in latex, in the termination process of rubber biosynthesis. It has been estimated that about 1–2 long-chain fatty acid groups are linked with rubber molecules, presumably at the α-terminal end, independent of the M_r of rubber [30]. It is well-known that the naturally occurring polyprenols are esterified with the long-chain fatty acids at the α-terminal end [31], whereas this is not the case in *Hevea* rubber [16]. The terminal -CH_2-O carbon atom esterified to a long-chain fatty acid was not detected in the ^{13}C NMR spectrum of *Hevea* rubber [16]. The detailed structure of the α-terminal end will be reported in a subsequent paper.

The low M_r rubber (1.0×10^4) from the rubber formed on incubation of fresh BF and FDP, was fractionated by GPC. A characteristic signal of the methyl carbon atom in the dimethylallyl-group was clearly

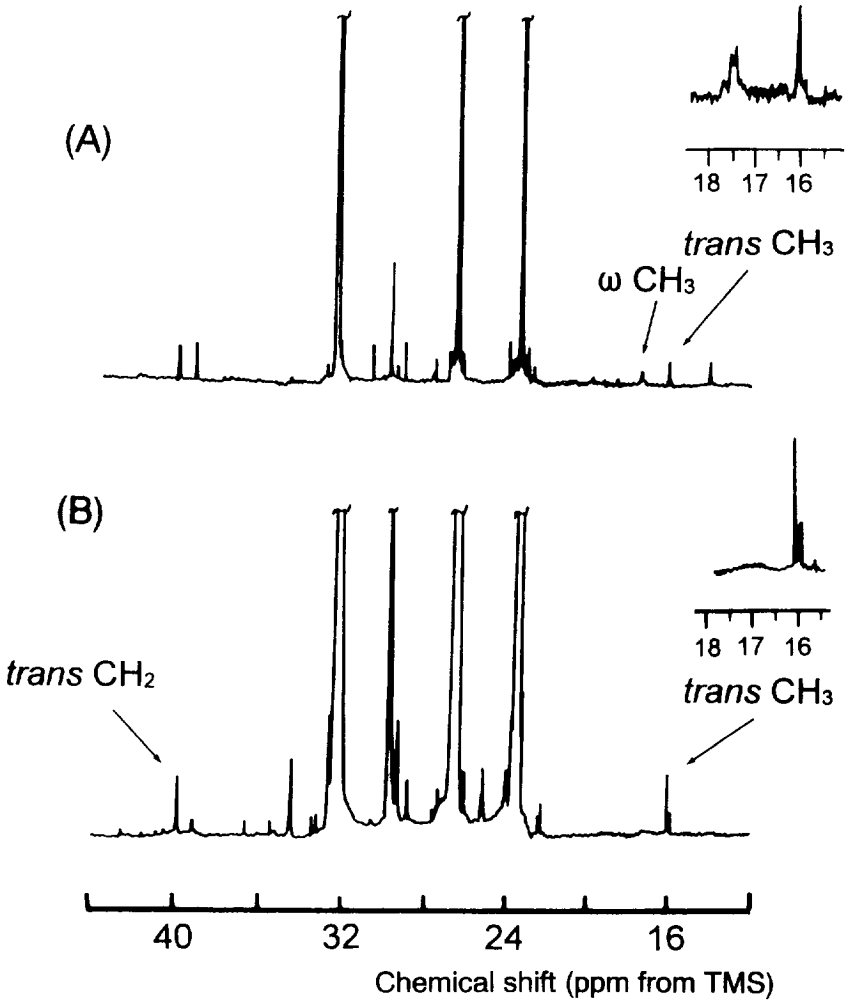

Fig. 5. ^{13}C NMR spectra of the lowest M_r rubber from (A) rubber formed on incubation of fresh BF and FDP and (B) cream phase.

detected at δ 17.59 in the ^{13}C NMR spectrum (Fig. 5A). This signal was not detected in the lowest M_r rubber obtained from the cream phase (Fig. 5B), and the lowest M_r rubber formed on incubation of fresh BF and IDP. The signals due to the C-5 methyl and C-1 methylene carbon atoms in *trans*-isoprene units were observed at δ 16.04 and 39.81, respectively, with a relative intensity about 2-fold higher than the signal at δ 17.59. This implies a difference in the initiating species in the rubbers formed *in vitro* and *in vivo*, i.e. *in vitro* rubbers is synthesized directly using FDP as a primer whereas *in vivo* rubber is not synthesized directly from FDP, but an unidentified initiating species, having two *trans*-isoprene units.

EXPERIMENTAL

Chemicals. IDP and *t,t*-FDP were purchased from Sigma Chemical Company. All other reagents were of analytical grades.

Incubation conditions. The BF and CS from fresh *Hevea* latex were prepd according to the procedure in ref. [23]. Firstly, the incubation was carried out for 2.50 g (wet wt) of CS or BF alone and a mixt. of CS and BF in a ratio of 1:1 (w/w). Each sample was pre-incubated overnight at 4 , followed by incubation at 37 for 6 hr. The control experiments were prepd in a similar procedure, except for boiling each sample at 100 for 30 min before cold stimulation.

Secondly, the incubation was carried out by addition of unlabelled IDP or *t,t*-FDP in fresh BF. The incubations A and B contained 4.2 nmole IDP and 3.5 μmol FDP in 2.50 g (wet wt, *ca* 790 mg dried wt) fresh BF, respectively. The incubation C was prepd in the same way as B, except the addition 0.5 mmol NaF. The incubation D contained 2.50 g of fresh BF alone. All these incubation mixts were pre-incubated overnight at 4 , followed by incubation at 37 for 0, 1, 2, 6 and 10 hr. The incubation E was a control experiment prepd by boiling 2.50 g of fresh BF at 100 for 30 min before cold stimulation. All the incubations were terminated by the addition of EtOH and the ethanolic soln was immediately centrifuged at 12 000 *g* for 45 min. The ppt was extracted by hexane–toluene (1:1, 2 ml × 3). The extracts were concd by a rotary evaporator to a small vol. and purified by

repptn from toluene solution with EtOH (3×), followed by drying up *in vacuo* and weighing.

GPC, NMR measurements. MWD was determined by GPC using two columns in series, packed with polystyrene–divinylbenzene copolymer gels having exclusion limit of 2.0×10^7 and 5.0×10^4. Measurements were made by using THF as an eluent at 35 , monitoring with a TOSOH LS-8000 with refractive index and low-angle-laser light-scattering detectors. Commercially obtained standard polystyrenes were used for calibration. Samples were prepared in concentration of 0.01–0.02% g dl^{-1} and were filtered through a Millipore LS prefilter and an 0.2 μm membrane filter. Gel content was determined in the usual way [21] and it was estimated less than 5% in all the samples.

The fractionation of rubber was carried out by GPC, using two columns in series having exclusion limit of 5×10^4 and 3×10^3. The resulting rubber fractions were saponified with 15% KOH in hexane–2-propanol–H_2O (7:2:1) by refluxing at 70 for 1 hr under N_2 atmosphere in the dark.

The ^{13}C-NMR spectra were obtained using a JEOL ALPHA-500 NMR spectrometer at 125 MHz in $CDCl_3$ solution at 50 , with a pulse repetition time of 7 sec. The chemical shifts were measured by taking the central signal in $CDCl_3$ as a reference and converted to TMS scale.

REFERENCES

1. Archer, B. L. and Audley, B. G., *Botanical Journal of the Linnean Society*, 1987, **94**, 181.
2. Audley, B. G., Archer, B. L., in *Natural Rubber Science and Technology*, ed. A. D. Roberts. Oxford University Press, London, 1988, p. 25.
3. Madhavan, S., Greenblatt, G. A., Foster, M. A. and Benedict, C. R., *Plant Physiology*, 1989, **89**, 506.
4. Cornish, K. and Backhaus, R. A., *Phytochemistry*, 1990, **29**, 3809.
5. Siler, D. J. and Cornish, K., *Phytochemistry*, 1993, **32**, 1097.
6. Venkatachalam, K. V., Greenblatt, G. A. and Benedict, C. R., in *Secondary-Metabolite Biosynthesis and Metabolism*, ed. R. J. Petroski and S. P. McCormick Plenum Press, NY, 1992, p. 351.
7. Tanaka, Y., Sato, H. and Kageyu, A., *Rubber Chemistry and Technology*, 1983, **56**, 299.
8. Tanaka, Y., in *Proceedings of the International Rubber Conference*, Kyoto, Japan, 1985.
9. Tanaka, Y., Mori, M. and Takei, A., *Journal of Applied Polymer Science, Applied Polymer Symposium*, 1992, **50**, 43.
10. Tanaka, Y., in *Proceedings of the International Rubber Conference*, Kuala Lumpur, 1987, Vol. 2, p. 73.
11. Tanaka, Y., in *NMR and Macromolecules*, ed. J. C. Randall. ACS Symposium Ser. 247, American Chemical Society, Washington DC, 1984, p. 233.
12. Tanaka, Y., Mori, M., Ute, K. and Hatada, K., *Rubber Chemistry and Technology*, 1990, **63**, 1.
13. Tanaka, Y., Kawahara, S., Eng, A. H., Shiba, K. and Ohya, N., *Phytochemistry*, 1995, **39**, 779.
14. Eng, A. H., Kawahara, S. and Tanaka, Y., *Rubber Chemistry and Technology*, 1995, **67**, 159.
15. Tanaka, Y., in *Methods in Plant Biochemistry, Vol. 7 Terpenoids*, ed. P. M. Dey and J. B. Harborne. Academic Press, London, 1991, p. 519.
16. Tanaka, Y., Eng, A. H., Ohya, N., Nishiyama, N., Tangpakdee, J., Kawahara, S. and Witsuwannakul, R., *Phytochemistry*, 1996, **41**, 1501.
17. Archer, B. L. and Audley, B. G., in *Advances in Enzymology*, Vol. 29, ed. F. F. Nord. NY, 1967, p. 221.
18. Archer, B. L. and Audley, B. G., in *Biochemistry and Regulation of* Cis-*Polyisoprene in Plants*, ed. C. R. Benedict. Proceedings of a workshop held at Texas A&M University, 1986, p. 35.
19. Benedict, C. R., Rosenfield, C. L., Gale, M. A. and Foster, M. A. in *Biochemistry and Regulation of* Cis-*Polyisoprene in Plants*, ed. C. R. Benedict. Proceedings of a workshop held at Texas A&M University, 1986, p. 85.
20. Subramaniam, A., *Rubber Chemistry and Technology*, 1972, **45**, 346.
21. Tangpakdee, J., Tanaka, Y., Witsuwannakul, R. and Chareonthiphakorn, N., *Phytochemistry*, 1996, **42**, 353.
22. Ji, W., Benedict, R. and Foster, M. A., *Plant Physiology*, 1993, **103**, 535.
23. Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Witsuwannakul, R. and Witsuwannakul, D., *Phytochemistry*, 1997, **45**, 261.
24. Homans, T. L. S. and Van Gils, G. E., *Proceedings of the 2nd Rubber Technology Conference*, 1948, London, p. 292.
25. Jacob, J-L., *Proceedings of the International Rubber Conference*, 1975, Kuala Lumpur p. 470.
26. Archer, B. L., Audley, B. G., McSweeney, G. P. and Tan, C. H., *Journal of the Rubber Research Institute, Malaya*, 1969, **21**, 560.
27. Tangpakdee, J., Tanaka, Y., Ogura, K., Koyama, T., Witsuwannakul, R., Witsuwannakul, D. and Asawatreratanakul, K., *Phytochemistry*, 1997, **45**, 269.
28. Bernard, D., *Proceedings of the Natural Rubber Production Association Jubilee Conference*, Cambridge, 1964, p. 89.
29. d'Auzac, J. and Jacob, J-L., in *Physiology of Rubber Tree Latex*, Chap. 2.1, ed. J. d'Auzac, J-L. Jacob and H. Chrestin. CRC Press, FL, 1989.
30. Eng, A. H., Ejiri, S., Kawahara, S. and Tanaka, Y., *Journal of Applied Polymer Science, Applied Polymer Symposium*, 1994, **53**, 5.
31. Hemming, F. W., in *Lipids and Lipid Polymers in Higher Plants*, Chap. 10., ed. M. Tevini and H. K. Lichtenthaler Springer, Berlin, 1977.