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# INITIATION OF RUBBER BIOSYNTHESIS IN *HEVEA BRASILIENSIS*: CHARACTERIZATION OF INITIATING SPECIES BY STRUCTURAL ANALYSIS*

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**Key Word Index**—*Hevea brasiliensis*; seedlings; natural rubber; biosynthesis; initiation; $^{13}C$ NMR; $^{1}H$ NMR; farnesyl diphosphate; *trans*-1,4; *cis*-1,4.

**Abstract**—The initiating molecular species for rubber formation in *Hevea brasiliensis* has been investigated by structural characterization of the terminal group in the rubber by means of $^{13}C$ NMR and $^{1}H$ NMR spectroscopy. The presence of *trans*-isoprene units in *trans–trans* linkage was confirmed by the characteristic C-1-methylene-carbon signal of the *trans*-isoprene unit. The number of *trans*-isoprene units was estimated to be about two from the relative intensity of this signal, and the degree of polymerization of various fractions from transesterified deproteinized natural rubber. Two methyl-proton signals in the *trans–trans* sequence were detected by $^{1}H$ NMR spectroscopy. This spectral evidence shows that the *trans*-isoprene units are in the *trans–trans–cis* sequence at the initiating terminal. However, the expected methyl- and olefinic-carbon signals and methyl-proton signal of the dimethylallyl group were not detected in natural rubber from regularly tapped trees and from a *Hevea* seedling. Thus, the direct initiating species of rubber formation is either a $C_{15}$ allylic diphosphate consisting of two *trans*-isoprene unites as in the case of farnesyl diphosphate (FDP), but which is modified at the dimethylallyl group, or FDP, the dimethylallyl group of which is modified after polymerization.

## INTRODUCTION

Earlier work on the biosynthesis of *Hevea* rubber was carried out using mainly radiotracer techniques. The incorporation of [1-$^{14}C$]isopentenyl diphosphate (IDP) into rubber *in vitro* showed that this was the molecule used for polymerization [1,2]. Biosynthesis was initially thought to start from dimethylallyl diphosphate (DMADP), which is an isomerization product of IDP, by analogy with the biosynthesis of terpenes. Subsequent chain-elongation was postulated to proceed via successive '*cis*' addition of IDP to DMADP [1,3]. The rubber molecule was thus expected to be composed of a dimethylallyl group and a long sequence of *cis*-isoprene units. However, no direct evidence was available at that time to support the hypothesis of this initiation step. On the contrary, ozonolysis of the rubber yielded no [$^{14}C$] acetone, a product derived from the dimethylallyl group, which it was predicted would be formed by isomerization of [$^{14}C$] IDP [1,3,4].

The discovery of *trans*-isoprene units in dimethylallyl-*trans* and *trans–trans* arrangements in naturally occurring *cis*-polyisoprenes [5–7], by means of $^{13}C$ NMR studies, provided new information on the initiating species of rubber formation. The detailed work on the initiating species of *in vitro* formation of rubber clearly demonstrated the incorporation of [$^{3}H$]neryl diphosphate (NDP) on incubation of this substrate and IDP with washed rubber particles [8,9]. However, it was also observed that (*trans*)-[$^{3}H$]geranyl diphosphate (GDP) as well as its *cis* isomer (NDP) initiated rubber formation [8,9]. The stimulatory effect of allylic diphosphates in the *cis*- and *trans*-configuration on rubber formation was studied by incubation of IDP with washed rubber particles from *H. brasiliensis* [8–10], *Parthenium argentatum* [10,11] and *Ficus elastica* [12]. The efficiency of the stimulatory effect increased slightly with the chain length of the allylic diphosphate in the order $C_5 < C_{10} < C_{15} < C_{20}$, rather than in relation to the geometric isomerism of the isoprene units [8,9]; on the other hand, the $C_5$- to $C_{20}$-compounds were found to be used equally well as initiators [13]. It is known that a water-soluble enzyme which catalyses the *trans*-addition of IDP to

*Part 1 in the Series 'Initiation of Rubber Biosynthesis in *Hevea brasiliensis*.

DMADP to form $C_{15}$- *trans–trans*-FDP is present in *Hevea* latex [8, 9, 14, 15]. Despite this clear evidence on the utilization of allylic diphosphates, it is difficult to identify the actual initiator involved in rubber biosynthesis *in vitro*.

Early $^1$H NMR structural studies on natural rubber indicated that *cis*-1,4-isoprene is the only repeating unit of *Hevea* and Guayule ( *P. argentatum*) rubbers [17, 18]. Later, on the basis of signal assignments of polyprenols, $^{13}$C NMR studies on rubbers from Goldenrod (*Solidago altissima*) and Sunflower (*Helianthus annuus*) revealed that two to three *trans*-isoprene units linked to a terminal dimethylallyl group are present [5–7]. In the case of *Hevea* rubber, the characteristic $^{13}$C NMR signals of the expected terminal dimethylallyl and hydroxyl groups were not detected, although the signals corresponding to small amounts of *trans*-isoprene units in the *trans–trans* or dimethylallyl-*trans* sequence were observed [7].

Since the number of *trans*-isoprene units per linear rubber chain indicates the type of initiator in rubber biosynthesis, it is necessary to determine this number in the study of the initiating mechanism of rubber formation. The relative intensity of the $^{13}$C NMR signals between the *trans*-isoprene units and the dimethylallyl group provides direct information on the number of *trans*-isoprene units. However, this method is not sufficient to distinguish clearly between the two-*trans* and three-*trans* types, because of the low accuracy of the $^{13}$C NMR analysis on the very small amounts of terminal groups in the high-$M_r$ polymer. These problems have now been solved by the development of more detailed assignments of signal splittings in the $^{13}$C NMR and $^1$H NMR spectra, which reflect the triad sequences of *trans*- and *cis*-isoprene units, measured at 100–125 and 400–500 MHz, respectively [18, 19]. In the case of *cis*-polyisoprene from *Lactarius* mushrooms, we have observed the $^{13}$C NMR signals corresponding to the dimethylallyl-*trans–trans* sequence and demonstrated that the initiating species is *trans–trans*-FDP [20]. Our recent studies on the *trans*-isoprene units in *cis*-polyisoprene from leaves of Goldenrod and Sunflower revealed that *trans–trans*-FDP and *trans–trans–trans*-GGDP are the initiators in the biosynthesis of both of these rubbers [19]. In this paper, we have tried to characterize the initiating species of *cis*-polyisoprene biosynthesis in *H. brasiliensis* by the use of both $^{13}$C NMR and $^1$H NMR techniques.

## RESULTS AND DISCUSSION

### *trans-Isoprene units in natural rubber*

The presence of *trans*-isoprene units in natural rubber has been inferred from the $^{13}$C NMR signals of low-$M_r$ rubber obtained by fractionation [7, 21]. The present work has further demonstrated this for various fractions from rubber from field latex, which have been purified by deproteinization (DPNR) followed by transesterification. These fractions showed $^{13}$C NMR signals characteristic of C-1 methylene and C-5 methyl carbon atoms in *trans*-isoprene units at $\delta$ 39.8 and 16.0, respectively (Fig. 1). Here, the carbon atoms of the isoprene units are designated as follows:

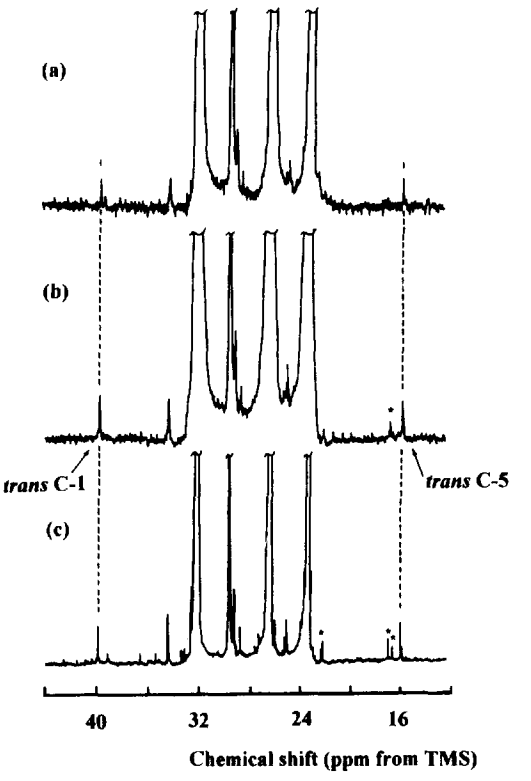
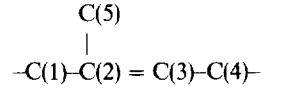



Fig. 1. $^{13}$C NMR spectra of fractionated TE-DPNR from field latex with $\bar{M}_n$ of (a) $2.7 \times 10^5$, (b) $0.93 \times 10^5$, and (c) $0.30 \times 10^5$. The asterisked signals are due to impurities.




The chemical shift of the C-1 methylene-carbon signal of *trans*-isoprene units indicates that this carbon is linked to the dimethylallyl terminal unit and/or another *trans*-isoprene unit. The validity of this structural inference has been extensively demonstrated by $^{13}$C NMR studies of acyclic terpenes and polyprenols of known sequential structure [7, 22, 23]. It has been confirmed that these *trans*-isoprene units are not derived from isomerization of *cis*-isoprene units [24]. This indicates that the *trans*-isoprene units are a true constituent of the rubber chain and are derived from a *trans*-allylic diphosphate initiator.

The degree of polymerization can be determined by the intensity ratio between the *cis* and *trans* signals in the $^{13}$C NMR spectrum, and the number of *trans*-isoprene units per molecule in the case of a linear polymer chain. In other words, the number of *trans*-isoprene units per rubber molecule is determinable by the measurement of the number-average $M_r$ by osmometry and the intensity ratio between *cis* and *trans* signals in the $^{13}$C NMR spectrum. It has been found that linear rubber molecules

Table 1. Number of *trans*-isoprene units in various fractions from transesterified deproteinized natural rubber (TE-DPNR) isolated from field latex

| Sample | *Cis/trans* ($^{13}$C NMR) | $\bar{M}_n$ (osmometry) | *Trans*-units per molecule* |
|---|---|---|---|
| 1 | 613 | $0.91 \times 10^5$ | 2.2 |
| 2 | 838 | $1.23 \times 10^5$ | 2.2 |
| 3 | 1030 | $1.40 \times 10^5$ | 2.0 |
| 4 | 1180 | $1.50 \times 10^5$ | 1.9 |
| 5 | 2500 | $3.50 \times 10^5$ | 2.1 |

* Calculated from $\bar{M}_n$ and *cis/trans* ratio.

can be obtained by transesterification of DPNR [25]. Consequently, transesterified DPNR (TE-DPNR) is an appropriate material to analyse the number of *trans*-isoprene units per linear rubber molecule. The results obtained for the low-$M_r$ fractions of TE-DPNR suggest the presence of about two *trans*-isoprene units per rubber molecule (Table 1).

*Dimethylallyl group in rubber from fresh field latex and a* Hevea *seedling*

It is remarkable that the $^{13}$C NMR signals due to the dimethylallyl group have not been detected even in low-$M_r$ fractions of rubber from transesterified DPNR from fresh field latex (Fig. 1). The absence of the dimethylallyl group in rubber from several clones was confirmed to be independent of the method of coagulation of the latex, i.e. coagulation by formic acid, calcium chloride, or centrifugation followed by ethanol. Detailed analysis of rubber samples prepared under different conditions will be presented in a subsequent paper. If the *trans*-isoprene units were derived from the usual *trans*-allylic diphosphate(s), it would be reasonable to expect the dimethylallyl group of the initiating molecule(s) to be present in the rubber. In the case of rubber from *L. volemus*, a decrease in the number of dimethylallyl groups per molecule was observed during the ageing of the sporophores of the fungus [20]. If a similar disappearance of dimethylallyl groups proceeds during storage of the rubber in the laticifers of *Hevea* between tappings, then it is obviously necessary to analyse freshly synthesized rubber.

We have analysed the rubber extracted from one-month- to three-year-old seedlings of *H. brasiliensis*. The $M_r$ of the rubber increased with increasing the age of the seedlings. The gel content of these rubbers was *ca* 1%. This indicates that the $M_r$ and structure are representative of the rubber from seedlings. The rubber from the one-month-old seedling was of low-$M_r$ with $\bar{M}_n = 6.5 \times 10^4$ and $\bar{M}_w = 2.6 \times 10^5$, by GPC-LALLS measurement using standard polystyrenes. The rubber showed $^{13}$C NMR signals due to the C-5-methyl and C-1-methylene carbon atoms in *trans*-isoprene units, at $\delta$ 16.0 and 39.8, respectively, but the C-5-methyl carbon signal in the dimethylallyl group, which is expected to resonate at $\delta$ 17.6, was not detected (Fig. 2). Detailed analysis of rubber from the seedlings will be presented in a subsequent paper.

*Arrangement of the trans-isoprene units in natural rubber*

Polyprenols containing two *trans*-isoprene units were distinguished from those containing three *trans*-isoprene units by characteristic methyl-proton signals observed at 500 MHz [26]. The $^1$H NMR method will give definite evidence of the number and arrangement of *trans*-isoprene units in the rubber molecule.

The $^1$H NMR spectrum of the low-$M_r$ fraction obtained from transesterified DPNR was compared with those of polyprenol-16 and ficaprenol-11 (Fig. 3). Here, the signal due to the methyl-proton in *cis–cis–cis* arrangement was taken as the reference to be $\delta$ 1.750, in order to eliminate the effect of concentration on the chemical shift of the methyl-proton signals of interest. Apart from this major signal, six other methyl-proton signals of a similar intensity were found in the $^1$H NMR spectrum of polyprenol-16. Ficaprenol-11 showed an additional methyl proton signal due to the *trans*-isoprene unit in a *trans–trans–trans* arrangement. This signal is close to, but distinguishable from, that in a *trans–trans–cis* arrangement. Detailed assignments of these signals have been reported previously, and therefore are used here without further discussion [19, 26]. The low-$M_r$ fraction from transesterified DPNR showed two signals at $\delta$ 1.639 and 1.654, corresponding to the methyl-protons of the *trans*-isoprene unit in dimethylallyl-*trans–trans* and *trans–trans–cis* arrangements in the spectrum of polyprenol-16. It has been confirmed that the *trans*-isoprene unit in the dimethylallyl-*trans–trans* and *trans–trans–trans* arrangements shows the same methyl-proton signal at $\delta$ 1.640 [24].

In the $^1$H NMR spectrum of the rubber from the one-month-old seedling, the methyl proton in the *trans*-isoprene units showed very small signals at $\delta$ 1.639 and 1.654, although that due to the dimethylallyl group was not detected. This may be partly a result of overlapping of a $^{13}$C-satellite signal of the *cis–cis–cis* sequence when measurement was made at 400 MHz.

*Initiating species of rubber formation*

The results thus obtained from $^{13}$C and $^1$H NMR studies demonstrate that the initiating species of rubber formation in *Hevea* is a derivative of *trans–trans*-FDP, presumably modified at the methyl-carbon of the dimethylallyl group, which is abbreviated here as $\omega'$. This assumption is consistent with the finding that the methyl-proton of the *trans*-isoprene unit in the $\omega'$-*trans–trans* group showed a similar chemical shift as that of the dimethylallyl-*trans–trans* arrangement. This suggests that the $\omega'$ group has a structure and magnetic shielding effect on the subsequent *trans*-isoprene unit similar to those of the dimethylallyl group.

Although the dimethylallyl group was not detected, even in rubber isolated from a very young seedling, it is still not possible to deduce if a molecule derived from

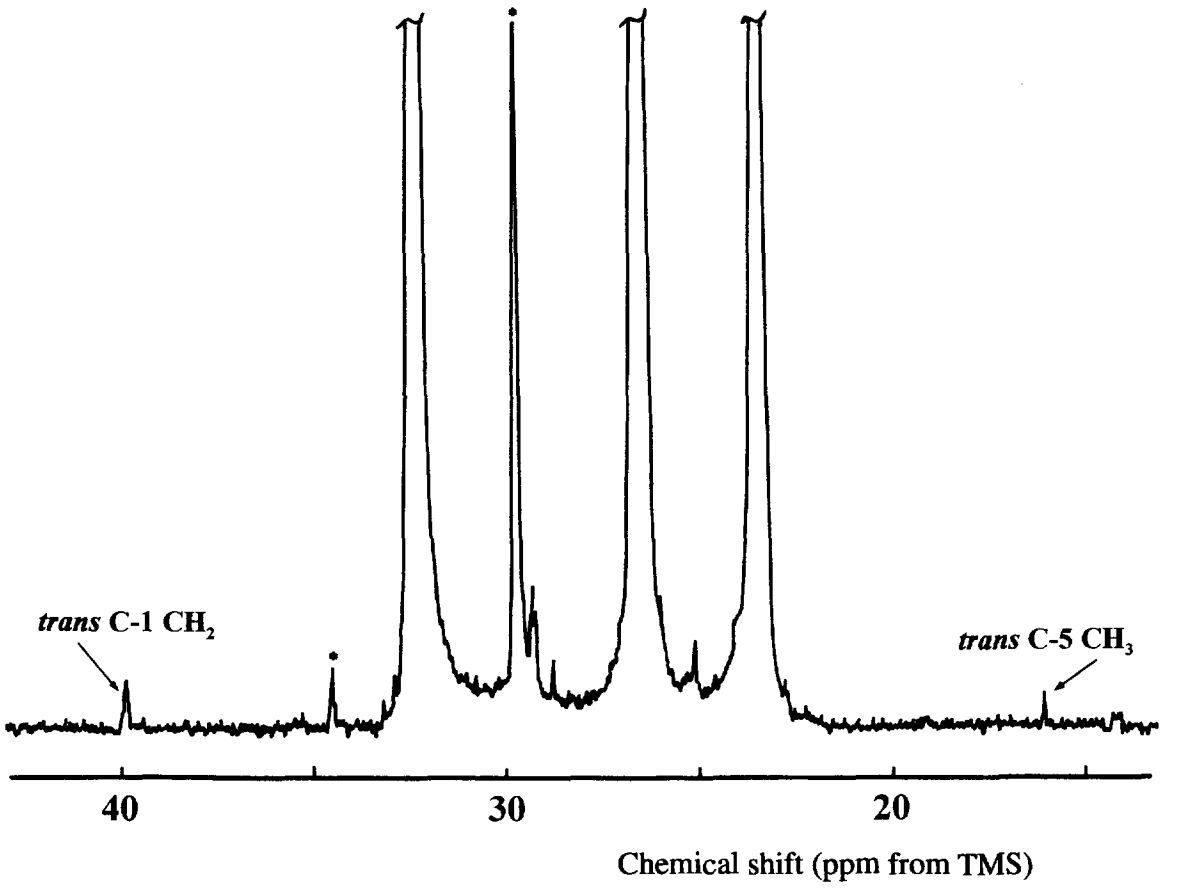



Fig. 2. $^{13}C$ NMR spectrum of rubber from one-month-old *Hevea* seedling.

FDP initiates polymerization, or alternatively, that FDP itself is the initiator, and subsequently the resulting polyisoprene undergoes complete selective modification of the dimethylallyl group. The occurrence of such a modification reaction has already been mentioned in the case of rubber from *L. volemus* [20]. Modification post-polymerization is supported by the finding that FDP stimulates *in vitro* rubber formation on incubation with IDP and washed rubber particles [8–11], and that under similar conditions the incorporation of [$^3$H] NDP is observed [8, 9]. The *trans*-isoprene units, as well as the dimethylallyl group, were not observed in wild rubbers occurring as latex [25], nor in rubbers from *Lactarius* [20]. This further implies that the occurrence of modification at the dimethylallyl group may be accompanied by a decrease of *trans*-isoprene units as well. However, the NMR analysis shows this is unlikely in the case of rubber from *H. brasiliensis*, because the dimethylallyl group was apparently absent even in the low-$M_r$ fractions of rubber from field latex and the seedling, despite the fact that just two *trans*-isoprene units were clearly detected.

This apparent conflict between biochemical information and structural evidence might result from the difference in conditions between *in vitro* and *in vivo* polymerization. It is noteworthy that the $M_r$ of the *in vitro* rubber obtained by incubation of washed rubber particles and IDP was considerably lower than that of the pre-existing rubber molecules, i.e. $\bar{M}_n = 5.6 \times 10^4$ and $\bar{M}_w = 1.3 \times 10^5$ [8, 9]. A similar tendency was reported in the case of Guayule rubber [10]. This may simply reflect the ratio of the concentration of the initiator to that of IDP, *in vitro* versus *in vivo*. It is also conceivable that the rubber transferase(s) operating *in vivo* is different from that on the washed rubber particles used in the *in vitro* work.

## EXPERIMENTAL

*Materials.* DPNR was prepd by treating 1% (w/v) Na dodecyl sulphate-stabilized field latex with 0.04% (w/v) Alcalase 2.0T (Novo Nordisk Bioindustry), at pH 9.5 and 37° for 24 hr, followed by centrifugation ( × 2) at 16000 *g* for 30 min. The N content of this sample, as analysed by the Kjeldahl method, was 0.009% (w/w) [27].

DPNR was further purified by transesterification with MeONa to remove the bonded fatty acids. Freshly prepd 1.0 M MeONa in MeOH was added dropwise to a gently stirred toluene soln of DPNR (1% w/v) in the ratio of 1:15 (v/v). The mixt. was stirred for 3 hr at room temp. and neutralized with methanolic HCl; the soln was then centrifuged at 16000 *g* for 30 min and the rubber pptd with MeOH. The transesterified rubber (TE-DPNR) was purified by re-pptn from toluene with MeOH, and dried under red. pres. to constant wt. Fractionation of

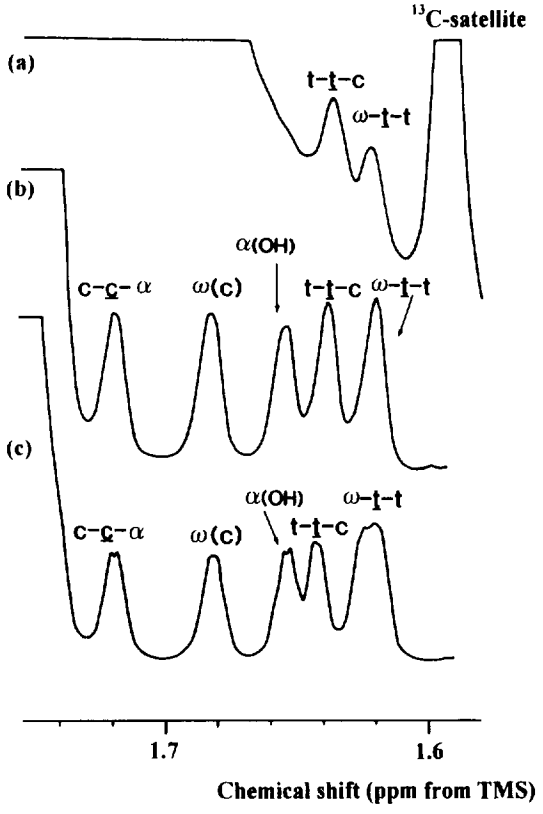



Fig. 3. Methyl-proton NMR spectra of (a) a low-$M_r$ fraction of TE-DPNR from field latex, (b) polyprenol-16, and (c) ficaprenol-12.

TE-DPNR was performed by adding MeOH to a toluene soln of the rubber at 25° in the usual way.

The rubber from the 1-month-old seedling (grown at Hat-Yai, Thailand) was obtained by dripping latex from the cut end of the stem into hexane. The hexane soln was washed with $H_2O$ followed by centrifugation (× 3) at 16000 *g* for 30 min. This rubber was purified by re-pptn from toluene soln with MeOH and was subjected to analysis without deproteinization or transesterification.

*NMR and* $M_r$ *measurements.* $^{13}$C NMR: 100 MHz, $CDCl_3$, TMS as int. standard, 50°, 10000 MHz sweep width, 32000 data points, 10 sec pulse interval, 20000 scans; $^1$H NMR: 400 MHz, $^2H_6$-benzene, TMS as int. standard, 50°, 3000 MHz sweep width, 32000 data points, 4 sec pulse interval and 1000 scans.

Measurements of the number-average $M_r$ were made using a Wescan 231 Membrane Osmometer. Triplicate analyses was carried out on each rubber soln and the average taken. The accuracy of the instrument was tested by the use of 3 commercial standard polystyrene samples, and the results indicated that the osmometer performed within a deviation of 4–6% in $\bar{M}_n$ measurement.

## REFERENCES


1. Lynen, F. and Henning, U. (1960) *Angew. Chem.* **72**, 820.
2. Archer, B. L., Ayrey, G., Cockbain, E. G. and McSweeney, G. P. (1961) *Nature* **189**, 663.
3. Lynen, F. (1969) *J. Rubb. Res. Int. Malaya* **21**, 389.
4. Henning, U., Moslein, E. M., Arreguin, B. and Lynen, F. (1961) *Biochem. Z.* **333**, 534.
5. Tanaka, Y., Sato, H. and Kageyu, A. (1983) *Rubb. Chem. Technol.* **56**, 299.
6. Tanaka, Y. (1985) *Proc. Int. Rubb. Conf., Kuala Lumpur* **2**, 73.
7. Tanaka, Y. (1984) in *NMR and Macromolecules* (Randall, J. C., ed.), ACS Symposium Series 247, p. 233. American Chemical Society, Washington, DC.
8. Archer, B. L. and Audley, B. G. (1987) *Bot. J. Linnean Soc.* **94**, 181.
9. Audley, B. G. and Archer, B. L. (1988) in *Natural Rubber Science and Technology* (Roberts, A. D., ed.), p. 35. Oxford University Press, U.K.
10. Madheaven, S., Greenblatt, G. A., Foster, M. A. and Benedict, C. R. (1989) *Plant Physiol.* **89**, 506.
11. Cornish, K. and Backhaus, R. A. (1990) *Phytochemistry* **29**, 3809.
12. Siler, D. J. and Cornish, K. (1993) *Phytochemistry* **32**, 1097.
13. Venkatachalam, K. V., Greenblatt, G. A. and Benedict, C. R. (1992) in *Secondary-Metabolite Biosynthesis and Metabolism*, (Pentroski, R. J. and McCormick, S. P., eds), p. 351. Plenum Press, New York.
14. Light, D. R. and Dennis, M. S. (1989) *J. Biol. Chem.* **264**, 18589.
15. Cornish, K. (1993) *Eur. J. Biochem.* **218**, 267.
16. Chen, H. Y. (1966) *J. Polym. Sci.* **B4**, 891.
17. Campos-Lopez, E. and Paracios, J. (1976) *J. Polym. Sci., Polym. Letters Ed.* **14**, 1561.
18. Tanaka, Y., Mori, M., Ute, K. and Hatada, K. (1990) *Rubb. Chem. Technol.* **63**, 1.
19. Tanaka, Y., Kawahara, S., Eng, A. H., Shiba, K. and Ohya, N. (1995) *Phytochemistry* **39**, 779.
20. Tanaka, Y., Mori, M. and Takei, A. (1992) *J. Appl. Polym. Sci., Appl. Polym. Symp.* **50**, 43.
21. Tanaka, Y. (1989) *J. Appl. Polym. Sci., Appl. Polym. Symp.* **44**, 1.
22. Tanaka, Y., Sato, H. and Kageyu, A. (1982) *Polymer* **23**, 1087.
23. Ibata, K., Mizuno, M., Takigawa, T. and Tanaka, Y. (1983) *Biochem. J.* **213**, 305.
24. Eng, A. H., Kawahara, S. and Tanaka, Y. (1994) *Rubb. Chem. Technol.* **67**, 159.
25. Eng, A. H., Ejiri, S., Kawahara, S. and Tanaka, Y. (1994) *J. Appl. Polym. Sci., Appl. Polym. Symp.* **53**, 5.
26. Tanaka, Y. and Hirasawa, H. (1989) *Chem. Phys. Lipids* **51**, 163.
27. Eng, A. H., Tanaka, Y. and Gan, S. N. (1992) *J. Nat. Rubb. Res.* **7**, 152.