

Microbial Degradation of Natural Rubber Vulcanizates

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An actinomycete, *Nocardia* sp. strain 835A, grows well on unvulcanized natural rubber and synthetic isoprene rubber, but not on other types of synthetic rubber. Not only unvulcanized but also various kinds of vulcanized natural rubber products were more or less utilized by the organism as the sole source of carbon and energy. The thin film from a latex glove was rapidly degraded, and the weight loss reached 75% after a 2-week cultivation period. Oligomers with molecular weights from 10^4 to 10^3 were accumulated during microbial growth on the latex glove. The partially purified oligomers were examined by infrared and ^1H nuclear magnetic resonance and ^{13}C nuclear magnetic resonance spectroscopy, and the spectra were those expected of *cis*-1,4-polyisoprene with the structure, $\text{OHC}-\text{CH}_2-[-\text{CH}_2-\text{C}(\text{CH}_3)=\text{CH}-\text{CH}_2-]_n-\text{CH}_2-\text{C}(=\text{O})-\text{CH}_3$, with average values of n of about 114 and 19 for the two oligomers.

Although many reports have been submitted on the microbial breakdown of natural rubber, few reports are available on the mechanisms involved (10). One reason may be that it is quite resistant in comparison with other natural polymers, and the actions of microorganisms are very slow under normal ecological conditions.

Microbial degradation of unvulcanized natural rubber hydrocarbon was confirmed by Spence and Van Niel (15). They showed that microbial degradation caused a loss in the weight of the rubber hydrocarbon and a decline in the relative viscosity of the polymer solution. The molecular weight reduction of natural rubber was also estimated by gel permeation chromatography (1).

The changes in many physical properties, including the decrease of tensile strength, and the change of network chain density or infrared spectrum caused by the microbial deterioration of vulcanized natural rubber have been reported (3,19). However, it is not certain whether the rubber is directly attacked by microorganisms without undergoing some degree of oxidation and whether it is utilized as the carbon source.

We have isolated a *Nocardia* sp. capable of utilizing natural rubber vulcanizates as a sole source of carbon and report the spectral evidence for the production of isoprene oligomers which were accumulated during the microbial degradation of natural rubber.

MATERIALS AND METHODS

Unvulcanized rubber. Various kinds of commercial rubber were used without purification. They were natural rubber (Malaysian pale crepe; RSS no. 1); synthetic isoprene rubber A (*cis*-polyisoprene, 97 to 98% of the *cis*-1,4 structure; Aldrich Chemical Co., Inc.); synthetic isoprene rubber B (Maxprene IR-900, 92 to 93% of the *cis*-1,4 structure; Seitetsu Chemical Industries Co., Ltd.); chloroprene rubber (10% *cis*, 85% *trans*; Aldrich); butadiene rubber (BR-01, 98% *cis*; JSR); styrene butadiene rubber (SBR, 23.5% styrene; Aldrich).

Natural rubber products. The compositions of many vulcanized rubber products commercially available were examined by infrared spectroscopy of their pyrolysis products, and those made of natural rubber were selected and used for the study: latex gloves (SD gloves no. G-262; Sanko Chemical Industries Co., Ltd.); rubber bands (O-band no. 16;

Kyowa Co. Ltd.); rubber stopper (MS-101 no. 8; Kyowa); rubber tubing A (light brown; Kyowa); latex tubing TL-005; rubber tubing B (black, Crown Spray; Komine Rubber Manufacturing Co. Ltd.); bicycle tire tread A (26 by 1 3/8 in; IRC); bicycle tire tread B (TK K6332, 26 by 1 3/8, TK K6332; Maruishi).

Microorganisms and culture. Many microorganisms capable of utilizing natural rubber were isolated from soils and deteriorated rubber. Of these, strain 835A, which was the strongest decomposer of solid rubber, was used throughout this study. The strain was a slightly acid-fast actinomycete with moderate aerial hyphae. The fragmentation of its substrate mycelium occurred after cultivation for 2 or 3 days. The strain had *meso*-diaminopimelic acid in the cell, but no spore formation was observed. From these characteristics, it was considered as belonging to the genus *Nocardia* (6). The taxonomical results were very similar to those of *Proactinomyces ruber* reported by Nette et al. (12). A detailed description of the organism will be presented elsewhere. The strain was preserved on an agar medium containing glucose, malt extract, and yeast extract.

Methods of the degradation tests. To examine the microbial degradation of various kinds of rubber, growth experiments in which various types of rubber were used as the sole carbon substrate for the organism were carried out. Each type of rubber other than the glove rubber was cut into strips with a diameter of about 0.5 mm and a length of 120 mm, and one of the strips (60 to 70 mg) was added to 100 ml of mineral salt medium. The composition of the mineral salt medium was reported previously (17). Latex glove rubber (about 0.1 mm thick) was extracted by acetone and CHCl_3 before use and cut into filmlike strips with a width of 2 mm and a length of 120 mm. About 200 mg of the film was added to 200 ml of the mineral salt medium. After the flasks containing the mixture of rubber and medium were autoclaved, the organism was injected into them. The flasks were aerated by stirring with a magnetic stirrer at 600 rpm and 30°C for 2 to 8 weeks. After the incubation period, the medium was filtered through gauze, and the mixture of rubber and cells was collected and weighed after being dried in vacuo. The mixture was then extracted by boiling in 1 N NaOH for 5 min. The total protein in the filtered medium and the alkaline extract was then determined by the method of Lowry et al. as modified by Herbert et al. (8) and used to express cell growth.

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TABLE 1. Microbial degradation of various kinds of unvulcanized rubber and vulcanized natural rubber products^a

Type of rubber	% rubber weight loss ^b	Cell growth (protein %) ^c
Unvulcanized rubber		
Natural rubber	100	27
Isoprene rubber A	100	26
Isoprene rubber B	50	17
Butadiene rubber	1	1
Chloroprene rubber	4	3
SBR	4	2
Vulcanized natural rubber		
Rubber band	88	26
Tubing A	56	15
Tubing B	95	18
Stopper	30	10
Tire tread A	17	5
Tire tread B	7	3

^a The incubation period was 8 weeks, and all data are averages of duplicate experiments.

^b Weight loss is expressed as $100 \times (\text{initial weight of rubber added} - \text{final weight of rubber recovered})/\text{initial weight of rubber}$.

^c Growth is expressed as $100 \times \text{total protein weight}/\text{initial weight of rubber}$.

Determination of the degree of cross-linking in vulcanizate.

The thin film from the latex glove was preserved in benzene for 2 days at 30°C. The volume fraction (V_r) of the rubber network in the swollen gel was estimated from the change of swollen film area, assuming that the change in thickness was proportional to the change in length. The cross-link density ($N_c = \rho/2M_c$), or network-chain-average molecular weight (M_c), is calculated from V_r by the modified Flory-Rehner equation, $-\ln(1 - V_r) + V_r + \mu V_r^2 = \rho V_s(V_r - V_r/2)/M_c$, where μ is the interaction parameter, V_s is the molar volume of the solvent, and ρ is the vulcanizate density (5).

Isolation of the degradation products. The mixture of rubber film and organisms was extracted with CHCl_3 by using a Knoepfler apparatus before extraction with NaOH . The CHCl_3 extracts were subjected to gel permeation chromatography (GPC). The conditions for GPC were reported previously (17). Standard polyisoprenes (Polymer Laboratories Ltd.), each of which has a very narrow molecular weight distribution, were used for the calibration of GPC. The extracts were then subjected to chromatography on layers of Kieselgel 60 F₂₅₄ (thickness, 2 mm; Merck & Co.) with benzene-acetone (20:1, vol/vol) as the developing solvent. In this system, two distinct spots of oligomers, fraction A (R_f , 0.97) and fraction B (R_f , 0.41), could be obtained and were located on the chromatogram with uv light. The fractions were further purified by another preparative thin-layer chromatography with hexane-acetone (20:1, vol/vol) as the developing solvent. The positions of fraction A (R_f , 0.31) and fraction B (R_f , 0.08) were indicated by spraying with a Schiff reagent. The purple color given with the reagent was evidence that the oligomers possess aldehyde groups.

Spectral analysis. The infrared spectra were recorded with a JASCO A-302 infrared spectrometer. Nuclear magnetic resonance (NMR) spectra in CDCl_3 were recorded with a JEOL JNM-GX 270 FT-NMR spectrometer, with trimethylsilane as the internal standard. ¹H-NMR spectra were obtained at 270 MHz, and ¹³C-NMR spectra were obtained at 68 MHz. Neryl acetone (*cis*-6,10-dimethylundeca-5,9-diene-2-one; Fluka AG) and dolichol (from porcine liver; Sigma Chemical Co.) were used as the standard for the peak assignment.

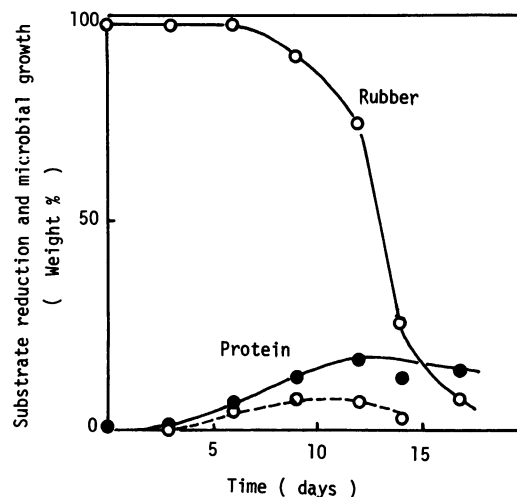


FIG. 1. Time course of the degradation of latex glove by strain 835A. Symbols: ○—○, rubber film recovered ($100 \times \text{weight of recovered rubber film}/\text{initial weight}$); ●—●, cell growth ($100 \times \text{total protein weight}/\text{initial weight of rubber film added}$); ○—○, CHCl_3 -soluble fraction ($100 \times \text{weight of } \text{CHCl}_3 \text{ extract}/\text{initial weight of rubber}$).

RESULTS

Microbial degradation of various kinds of unvulcanized rubber. Strain 835A grew well on natural rubber (pale crepe) and synthetic isoprene rubber. The weight loss of natural rubber and synthetic isoprene rubber A (97 to 98% *cis*) were about 100% after an 8-week cultivation period, whereas that of isoprene rubber B (92 to 93% *cis*) was about 50% (Table 1). Other types of synthetic rubber examined were hardly attacked by the strain. These results were consistent with the deterioration of various kinds of rubber vulcanizates reported by Leeftang (11).

Degradation of natural rubber vulcanizates. Not only unvulcanized but also various kinds of vulcanized natural rubber products were utilized as the sole carbon source by the strain. It grew well on the soft-type products, such as rubber bands and tubing, and the weight loss of these

TABLE 2. Analytical data of microbial degradation of latex gloves^a

Sample	Incubation period (days)	% Rubber film recovered ^b	% Loss by alkali treatment ^c	% CHCl_3 soluble ^d	Rubber volume fraction (V_r)
Uninoculated control	0	97	0.2	0.6	0.18
Uninoculated control	14	97	0	1.3	0.18
Inoculated with strain 835A	12	74	49	7.0	ND ^e
Inoculated with strain 835A	14	25	2.5	2.9	ND

^a All data are averages of duplicate experiments.

^b Expressed as $100 \times \text{final weight of rubber recovered}/\text{initial weight of rubber added}$.

^c Expressed as $100 \times (\text{rubber weight before extraction by } 1 \text{ N NaOH} - \text{rubber weight after the extraction})/\text{initial weight of rubber added}$.

^d Expressed as $100 \times \text{the weight of } \text{CHCl}_3 \text{ extract}/\text{initial weight of rubber}$.

^e ND, Not determined.

products was 50 to 95% after an 8-week cultivation period (Table 1). Growth on the hard-type products was poor, and the weight loss of these products was only 7 to 30%. These values were not corrected for biomass present, and therefore represent the minimal decomposition values.

Time course of the degradation of latex gloves. Among the various kinds of natural rubber vulcanizates examined, the glove made of natural rubber latex was degraded most rapidly by the strain, probably because the film was very thin (about 0.1 mm). The typical time course is shown in Fig. 1, and the analytical results are listed in Table 2. The cell growth started after a lag time of 3 to 6 days and attained its maximum on day 14. The weight loss was about 75% for the 14-day period and over 90% for the 17-day period. On the contrary, the weight loss of the uninoculated control after the 14-day incubation period was only 3%, which was the same as at time zero. The treatment of the rubber film by boiling 1 N NaOH caused a substantial weight loss of degraded rubber film. The weight loss of the uninoculated control caused by the alkali treatment, however, was negligible (Table 2). The results showed that the colonization of strain 835A occurred at the surface of the rubber film and that the mycelium of the organism attached tightly to the film in the early stage of growth.

Isolation of the degradation products from the latex glove.

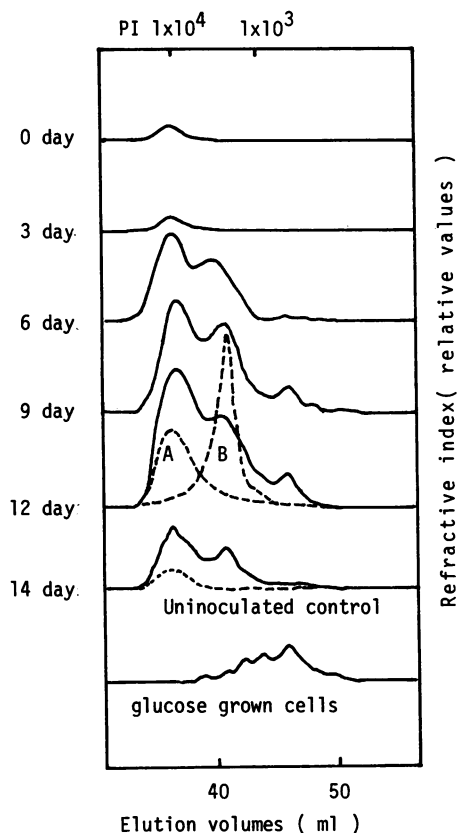


FIG. 2. GPC of the CHCl_3 extracts of the latex glove. The lines at the top of the figure indicate the elution volumes of standard polyisoprenes, with molecular weights of 1.0×10^4 and 0.9×10^3 . Dotted lines at 12 days show the purified fraction A and B. "Glucose-grown cells" indicates the CHCl_3 extract of the cells of strain 835A grown on a glucose medium.

TABLE 3. $^1\text{H-NMR}$ data for the purified oligomers

Positions of bands (ppm)	Assignment ^b	Relative areas for:			
		Fraction A		Fraction B	
		Found	Expected ($n = 114$)	Found	Expected ($n = 19$)
9.77 (t) ^a	CHO	1.0	1	1.0	1
5.13 (br)	H_B	114	114	19.3	19
2.49, 2.44 (t-d), (t)	$\text{H}_\alpha, \text{H}_\alpha'$	5.1	4	4.8	4
2.35, 2.24 (t), (q)	$\text{H}_\beta, \text{H}_\beta'$	8.2	4	5.2	4
2.13 (s)	H_α''	2.4	3	3.6	3
2.04 (br)	H_C and H_D	473	452	71.0	72
1.68 (br)	H_E	359	332	62.0	57
1.26 (br)		38		2.0	
0.88 (br)		16		0.6	

^a Symbols: s, singlet; d, doublet; t, triplet; q, quartet; br, broad band.

^b Assignment according to the formula in Fig. 4.

With the progress of degradation, the amount of CHCl_3 -soluble fraction, which may contain the cellular lipids and low-molecular-weight fragments of rubber, increased (Fig. 1). Molecular weight distributions of the CHCl_3 extracts of the latex glove are shown in Fig. 2. The oligomers with molecular weights from 10^4 to 10^3 appeared after a 6-day cultivation period. The amount of the oligomers reached the maximum during days 9 through 12 and then gradually decreased. In the extract of glucose-grown cells, the main peak was present at a molecular weight of about 500, and the content of the higher-molecular-weight fraction was small. Although the oligomers have a molecular weight of about 10^4 also appeared in the extract of the uninoculated control, the amount was very small even after a 14-day incubation period. The oligomers with molecular weights from 10^4 to 10^3 were then considered the degradation products of the latex glove by the organism.

Two fractions (A and B) of the partially purified oligomers were obtained by the two-step preparative thin-layer chromatography. Each of the fractions had a very narrow molecular weight distribution, and no indication of a low-molecular-weight component like cellular lipids was observed by GPC (Fig. 2). The average molecular weights of fractions A and B were 1.0×10^4 and 1.6×10^3 , respectively, by GPC.

Infrared spectra of the purified oligomers. The infrared spectrum of fraction B was almost identical to that of the aldehyde derivative of dolichol (2) and had values of ν_{max} at 1,720, 1,445, 1,375, 1,090 and 835 cm^{-1} . Although the spectrum of fraction A resembled that of B as a whole, the intensity of the carbonyl absorption band relative to the rest of the spectrum suggests that fraction A has a much larger average molecular weight than fraction B. The minor bands of fraction A at 1,740 and $1,260 \text{ cm}^{-1}$, which were not present in the spectrum of fraction B, might be due to the lipid impurities.

$^1\text{H-NMR}$ spectra of the purified oligomers. The positions of the bands and their relative areas of $^1\text{H-NMR}$ spectra are listed in Table 3. The strong bands at 5.13 ppm ($=\text{CH}-$), 2.04 ppm ($-\text{CH}_2-\text{C}=\text{C}-$, plus $-\text{CH}_2-\text{CH}=\text{C}-$), and 1.68 ppm ($\text{H}_3\text{C}-\text{C}=\text{CH}-$) fit the *cis*-1,4-polyisoprene structure. The small band at 9.77 ppm (triplet) is clearly due to the aldehyde proton attached to a methylene group, and

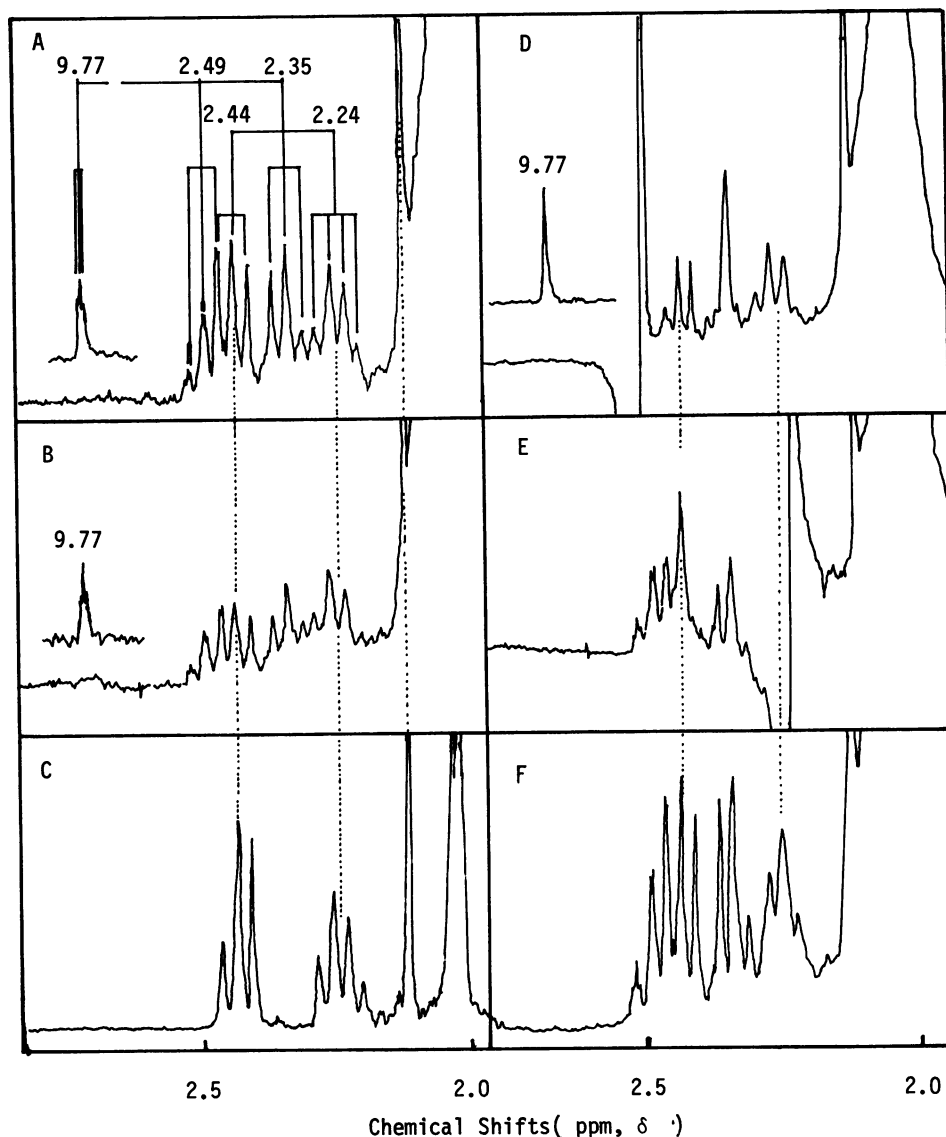


FIG. 3. $^1\text{H-NMR}$ spectra and spin decoupling experiments. A, fraction B under nondecoupling conditions; B, fraction A, nondecoupling; C, neryl acetone, nondecoupling; D, fraction B irradiated at 2.49 ppm (δ); E, fraction B irradiated at 2.24 ppm; F, fraction B irradiated at 5.13 ppm.

the small singlet at 2.13 ppm fits the protons of the methyl ketone. In both fractions, the area under the methyl ketone proton band was nearly three times that under the aldehyde proton band, indicating that the content of the aldehyde group $-\text{CHO}$ was almost equal to that of the methyl ketone group $\text{CH}_3-\text{C}(=\text{O})-$.

There were about 12 small peaks between 2.24 and 2.49 ppm in the spectra of the two fractions. Of these, the triplet at 2.44 ppm and the quartet at 2.24 ppm (and singlet at 2.13 ppm) were almost identical with those of the neryl acetone (Fig. 3A to C). Confirmation of these agreements was obtained from the spin decoupling experiments, which showed that the quartet at 2.24 ppm was coupled to the triplet at 2.44 ppm and the broad band at 5.13 ppm (Fig. 3E and F). The results showed that the oligomers and neryl acetone have the same structure, namely, $\text{CH}_3-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$.

Irradiation at the triplet of doublet at 2.49 ppm causes the triplets at 9.77 and 2.35 ppm to collapse to singlets, indicat-

ing the mutual coupling of these bands (Fig. 3D). The

structure $\text{OHC}-\text{CH}_2-\text{CH}_2-\text{C}=\text{}$ fits the results. In conclusion, we propose the formula $\text{OHC}-\text{CH}_2-[-\text{CH}_2-\text{C}(\text{CH}_3)=\text{CH}-\text{CH}_2-]_n-\text{CH}_2-\text{C}(=\text{O})-\text{CH}_3$, as a whole. From the relative areas given in Table 3, we can estimate the average values of n to be 114 for fraction A and about 19 for fraction B. The number-average molecular weights calculated from these values were 7,852 and 1,392, respectively. The absorption in the region from 0.88 to 1.26 ppm fit the protons of the saturated alkyl group and may be due to the lipid impurities.

$^{13}\text{C-NMR}$ spectroscopy. The spectra and their tentative peak assignments are shown in Fig. 4. The strong bands at 135 ppm ($-\text{C}=\text{}$), 125 ppm ($=\text{CH}-$), 32 ppm ($-\text{CH}_2-\text{C}=\text{}$), 26 ppm ($=\text{CH}-\text{CH}_2-$), and 23 ppm ($\text{CH}_3-\text{C}=\text{}$) are consistent with that of *cis*-1, 4-polyisoprene. Although only one

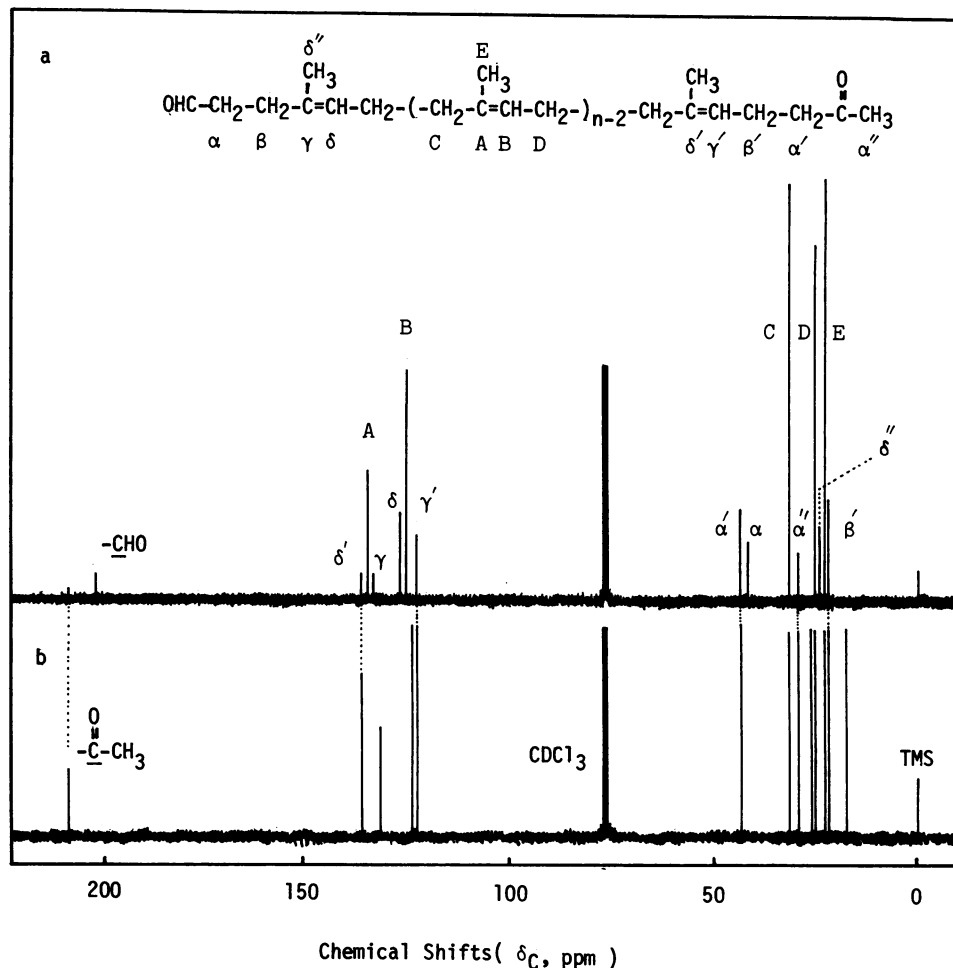


FIG. 4. ¹³C-NMR spectra of the fraction B. a, fraction B under the ¹H complete decoupling conditions; b, neryl acetone under the same conditions.

small band at 29.5 ppm can be seen in the spectrum of fraction A, 11 small bands were observed in the ¹³C-NMR spectrum of fraction B. The bands at 208.7 and 202.2 ppm are consistent with the carbonyl carbon of the methyl ketone and aldehyde, respectively. The positions of the six small bands at 208.7, 136, 122.5, 43.5, 29.5, and 22 ppm are exactly identical with those of neryl acetone, and these bands assigned to the terminal six carbons

$\text{CH}_3-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-\text{CH}=\text{C}-$. The other five small bands may then be attributed to another terminal group, $\text{OHC}-\text{CH}_2-\text{CH}_2-\text{C}(\text{CH}_3)=\text{CH}-$, where the chemical shifts of the β -carbon attached to the aldehyde may be about 29.5 ppm, equal to that of the methyl carbon attached to carbonyl.

DISCUSSION

Natural rubber consists of hydrocarbon polymers with molecular weights of 10⁵ to 10⁶, and the vulcanizate has a network structure insoluble in any organic solvent. Besides, natural rubber is a highly unsaturated polymer, and by itself is quite susceptible to oxidative degradation. Therefore, in the microbial deterioration of rubber vulcanizates, low-molecular-weight fragments produced by a nonbiological degradation were thought to be metabolized by the organism (4). As a result of the latex glove experiment shown in Table

2, however, the content of the CHCl₃-soluble fraction of the uninoculated control was only 1.3% and more than 95% of rubber film remained as an insoluble gel after a 14-day incubation period. Furthermore, the V_r of the rubber film was constant during the incubation period, indicating that the degree of cross-linking was the same. The role of the physicochemical reaction was considered to be not so important in the degradation of the latex glove by *Nocardia* sp. strain 835A. These results suggest that the network of the vulcanizate was directly attacked by the biological action.

During the assimilation of the latex glove rubber, CHCl₃-soluble isoprene oligomers with molecular weights between 10⁴ and 10³ were accumulated. These oligomers were probably produced by the enzymatic cleavage of polymeric chains in the network structure. The largest molecular weight of the extractable oligomers corresponds well to the average-chain-segment molecular weight (M_c of about 10⁴) between adjacent cross-links estimated from the V_r of the glove. The fragments with molecular weight larger than M_c must have cross-links, and their extraction from the network may be prevented by the chain entanglements. On the contrary, we have previously reported that the oligomers of isoprene or butadiene with a molecular weight up to about 1,000 can be rapidly utilized by microorganisms, but the oligomers with a molecular weight higher than about 2,000 were attacked very slowly (16, 17). Short oligomers with

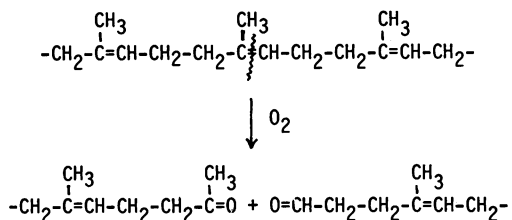


FIG. 5. Schematic diagram of natural rubber degradation by strain 835A.

molecular weights less than 1,000 may be produced by the scission of longer polymeric chains of natural rubber, but are rapidly consumed by the organism.

^1H -NMR spectra of the partially purified oligomers were those expected of the *cis*-1,4-polyisoprene mixtures with the structure $\text{OHC-CH}_2\text{-[CH}_2\text{-C(-CH}_3\text{)=CH-CH}_2\text{-]}_n\text{-CH}_2\text{-C(=O)-CH}_3$, and the infrared and ^{13}C -NMR spectra confirmed this structure. Furthermore, the molecular weights derived from GPC and ^1H -NMR were in sufficiently close accord. The proposed chemical structure of oligomers suggests that *Nocardia* sp. strain 835A cleaved natural rubber at the double bond shown by a wavy line in the formula in Fig. 5.

The attack of natural rubber by ozone at the double bond to form levulinolaldehyde was reported by Harries (7). The attack of the isoprenoid compound squalene at the double bond by *Arthrobacter* sp. to give geranyl acetone has also been reported (18). Besides, various kinds of methyl ketones, aldehydes, and the corresponding alcohols are widely distributed in plants, cyanobacteria, and fungi. One group of them, norcarotenoids, are the flavoring isoprenoids, which may have derived from carotenes by oxidative cleavage of double bond. An oxygenase in *Microcystis* spp., which cleaves β -carotene to form β -cyclocitral and crocetindial, was described quite recently (9).

A second group of volatile compounds contains C_6 and C_9 aldehydes in fruits and vegetables. These aldehydes are biosynthesized by enzymatic splitting of the double bond of C_{18} polyunsaturated fatty acids. Two enzymes, lipooxygenase and hydroperoxide lysase, are involved (14). The conversion of β -carotene into retinal in rat liver was reported as a dioxygenase reaction (13).

Natural rubber may also be degraded by an oxygenase reaction of *Nocardia* sp., but the mechanism of chain scissions and the properties of enzymes involved require further study.

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