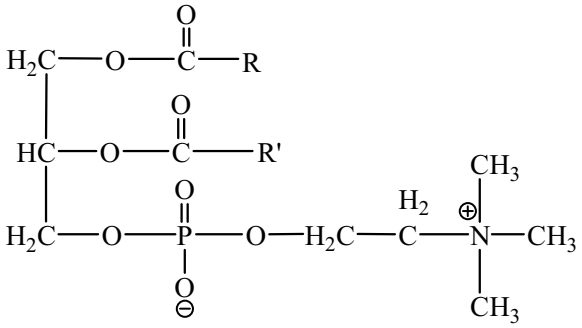
3.2 ^{1}H-, ^{13}C- and ^{31}P-NMR Analysis of Phospholipids as a Model for Structural Characterization of Natural Rubber

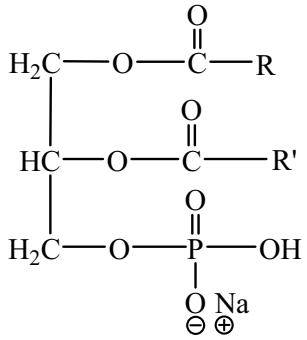
3.2.1 ^{1}H-NMR spectroscopy of phospholipids

The chemical structures of L-α-phosphatidylcholine (PC) and L-α-phosphatidic acid sodium salt (PA) are shown in **Figure 3.9a** and **3.9b**, respectively.

(a)



(b)



R, R' = Alkyl groups (C_xH_y)

Figure 3.9 Chemical structure of (a) L-α-phosphatidylcholine and (b) L-α-phosphatidic acid sodium salt.

The 750 MHz ^{1}H-NMR spectra of PC and PA are given in **Figure 3.10**. Both phospholipids are assumed to form inverse micelle in C_6D_6. Assignments of signals were made by referring to the studies by Machaelis and Schlieper [97] and Sander [98],

which gave information on the chemical shift range, although the ^{1}H-NMR spectra were not measured in C_6D_6. The assignments for both compounds are shown in **Table 3.2**. All the signals are aligned in a similar order with shift by 0.1-0.6 ppm compared with those in the previous reports [99, 100]. Almost all the PA signals showed down field shift compared to those of PC. The methyl and long chain methylene protons of the fatty acid ester groups were clearly seen with the chemical shift of 0.94 and 1.37-1.90 ppm, respectively, in both PC and PA. The resonances at 2.16 and 2.93 ppm were assigned to the methylene protons next to the unsaturated and allylic bonds, respectively. The vinyl protons were observed at 5.53 ppm for PC and 5.55 ppm for PA.

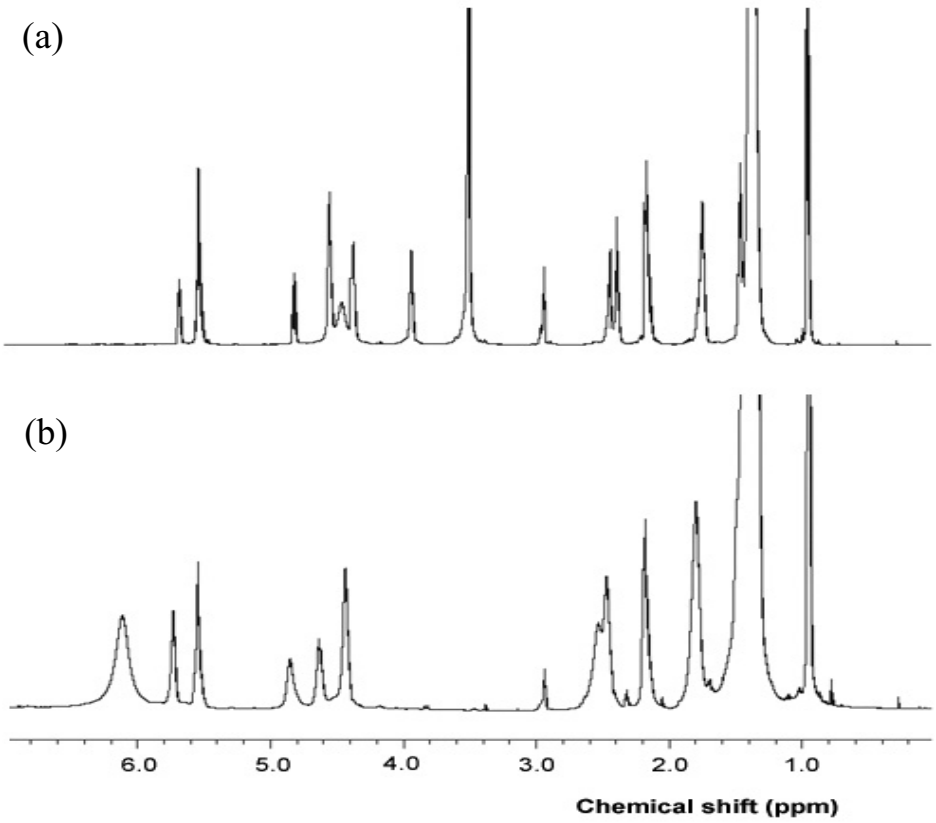


Figure 3.10 ^{1}H-NMR spectra at 750 MHz of L-α-phosphatidylcholine (a), and L-α-phosphatidic acid sodium salt (b) in C_6D_6 at 50°C.

The two methylene protons in C$\underline{H_2}$OC=O group of glyceride backbone of PC are magnetically nonequivalent, which resonated at 4.53 and 4.80 ppm. These results are in accordance with study by Neumann *et al.* [100] for sonicated lecithin, showing

two different resonances at 4.18 and 4.38 ppm. The methylene protons of $C\underline{H}_2OC=O$ in PA resonated at 4.63 and 4.85 ppm. The signal of the $C\underline{H}$-OC=O proton in glyceride backbone of PC and PA appeared at 5.67 and 5.73 ppm, respectively. The resonance due to the $C\underline{H}_2OP$ protons of glyceride backbone gave rise to a unique resonance appeared at 4.36 ppm for PC and 4.44 ppm for PA.

The polar head group showed a unique resonance characteristic of each phospholipid. The resonance at 5.23 ppm of PC was assigned to the $C\underline{H}_2$-OP of choline group overlapping with $C\underline{H}_2OC=O$ protons of glyceride backbone. The resonance of $N(C\underline{H}_3)_3$ protons was clearly observed at 3.50 ppm. These proton resonances were not observed for PA due to the lack of choline head group. All the assignments were confirmed by two-dimensional ^1H-^1H COSY and ^1H-^{13}C HMQC measurements as described subsequently.

Table 3.2 ^1H-NMR assignments of L-α-phosphatidylcholine and L-α-phosphatidic acid sodium salt measured in C_6D_6 at 50°C

Assignments	Chemical shift (ppm)	
	L-α-phosphatidylcholine	**L-α-phosphatidic acid**
$C\underline{H}_3$	0.937	0.944
$-C\underline{H}_2-$	1.385	1.374
$COCH_2C\underline{H}_2$	1.736	1.797
$C\underline{H}_2CH=CH$	2.165	2.166
$CH_2OCOC\underline{H}_2CH_2$	2.390	2.470
$CHOCOC\underline{H}_2CH_2$	2.427	2.543
$C\underline{H}_2-(C=C)_2$	2.933	2.940
$N(C\underline{H}_3)_3$	3.498	-
$NC\underline{H}_2$	3.926	-
$C\underline{H}_2$-OP (glyceride)	4.359	4.436
$C\underline{H}_2$-OC=O and $C\underline{H}_2OP$ (choline)	4.528	4.630
$C\underline{H}_2$-OC=O	4.804	4.852
$C\underline{H}$=CH	5.527	5.545
$C\underline{H}$-OC=O	5.669	5.730

3.2.2 ^{13}C-NMR spectroscopy of phospholipids

The ^{13}C-NMR spectra of PC and PA in C_6D_6 are shown in **Figure 3.11**. The detailed assignments are listed in **Table 3.3**.

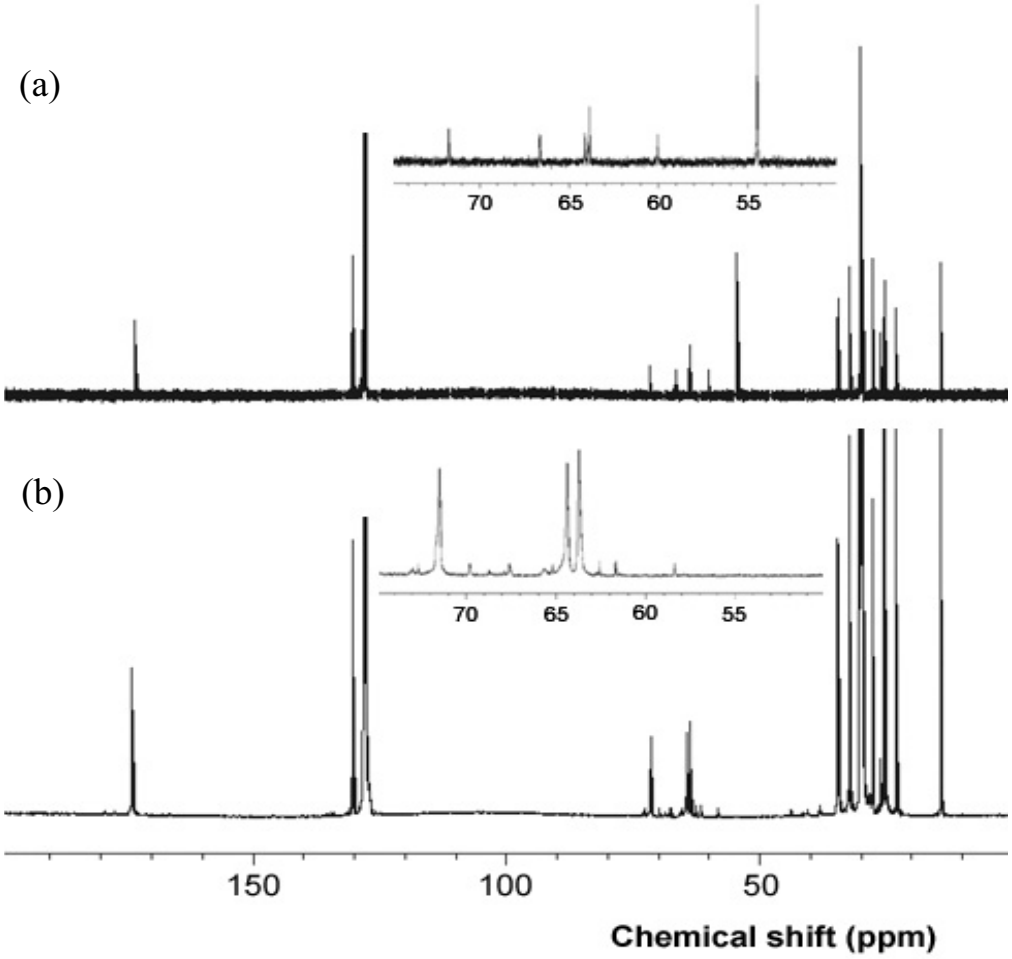


Figure 3.11 ^{13}C-NMR spectra at 188 MHz of L-α-phosphatidylcholine (a), and L-α-phosphatidic acid sodium salt (b) in C_6D_6 measured at 50°C.

The assignments of the aliphatic carbons of the fatty acid ester group are consistent with those in the previous work [101], although the solvent system and phospholipid were different. The signals due to methyl and long chain methylene carbons of fatty acid ester group were clearly observed at 14.22-14.38 ppm and 29.85-30.14 ppm, respectively. The signals around 130.3-130.5, 27.75 and 26.19 ppm were assigned to the C=C, CH_2C=C and $CH_2(C=C)_2$ carbons of fatty acid ester group, respectively. The carboxyl carbon, OC=O, of PC gave two signals at 173.2 and 173.5

ppm, while that of PA showed a singlet signal at 174.0 ppm. The head group of PC resonating at 60.04, 66.62 and 54.78 ppm are assigned to the carbon atoms in $\underline{C}H_2OP$, $\underline{C}H_2N$ and $N(\underline{C}H_3)_3$, respectively. These signals were not found in PA. The glyceride backbone carbons consistently resonated at 63.85-63.86 ppm, 71.64-71.70 ppm and 64.12-64.50 ppm, which are assigned to $\underline{C}H_2OC{=}O$, $\underline{C}HOC{=}O$ and $\underline{C}H_2OP$ carbons, respectively, for both PC and PA. It is clear that there is no significant change in the chemical shift of carbon glyceride backbone, although the polar head group was different. This is in contrast to the proton chemical shifts of glyceride backbone.

Table 3.3 ^{13}C-NMR assignment of L-α-phosphatidylcholine and L-α-phosphatidic acid sodium salt measured in C_6D_6 at 50°C

Assignments	Chemical shift (ppm)	
	L-α-phosphatidylcholine	**L-α-phosphatidic acid**
$\underline{C}H_3$	14.22	14.38
$-\underline{C}H_2-CH_3$	22.94-23.05	23.18
$COCH_2\underline{C}H_2$	25.45	25.37
$\underline{C}H_2(C{=}C)_2$	26.19	26.33
$\underline{C}H_2C{=}C$	27.75	27.77
$-(\underline{C}H_2)_n-$	29.85	30.14
$CO\underline{C}H_2CH_2$	34.54-34.73	34.45-34.94
$N(\underline{C}H_3)_3$	54.78	-
$\underline{C}H_2$-OP (choline)	60.04	-
$\underline{C}H$-OC=O	63.86	63.85
$\underline{C}H_2$-OC=O	64.12	64.50
$N\underline{C}H_2$	66.62	-
$\underline{C}H_2$-OP (glyceride)	71.70	71.64
$\underline{C}{=}C$	130.4-130.5	130.5-130.6
$O\underline{C}{=}O$	173.2-173.5	174.0

3.2.3 ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC spectroscopy of L-α-phosphatidylcholine

The ^{1}H- and ^{13}C-NMR assignments of glyceride and choline head group in PC were confirmed by two-dimensional ^{1}H-^{1}H COSY and ^{1}H-^{13}C HMQC measurements shown in **Figures 3.12** and **3.13**, respectively.

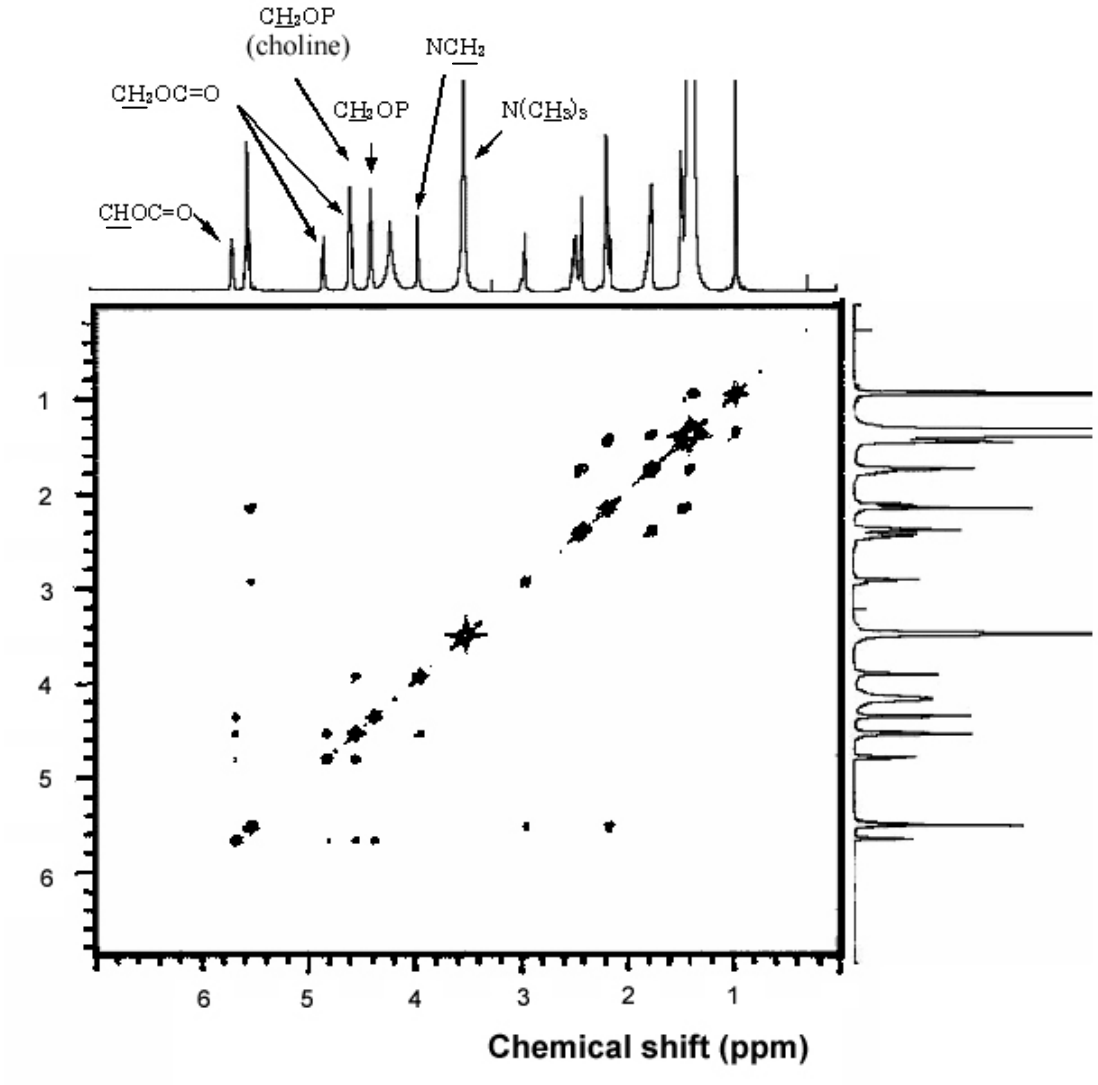


Figure 3.12 ^{1}H-^{1}H COSY spectrum at 750 MHz of L-α-phosphatidylcholine in C_6D_6 measured at 50°C.

In the ^{1}H-^{1}H correlation spectrum, the singlet proton signal at 3.50 ppm was assigned to $N(C\underline{H}_3)_3$ in choline head group due to the absence of correlation with any

other signals. The signal at 4.53 ppm corresponding to C<u>H</u>$_2$OP proton of choline head group correlated with the proton signal at 3.93 ppm from NC<u>H</u>$_2$. In addition, it was related with proton signal of C<u>H</u>OC=O of glyceride backbone at 5.67 ppm. This correlation clearly indicates the overlapping between C<u>H</u>$_2$OC=O of glyceride backbone and C<u>H</u>$_2$OP of choline head group. The proton signal of C<u>H</u>OC=O glyceride backbone at 5.67 ppm showed the correlation with C<u>H</u>$_2$OC=O and C<u>H</u>$_2$-OP at 4.80, 4.53 and 4.36 ppm, respectively.

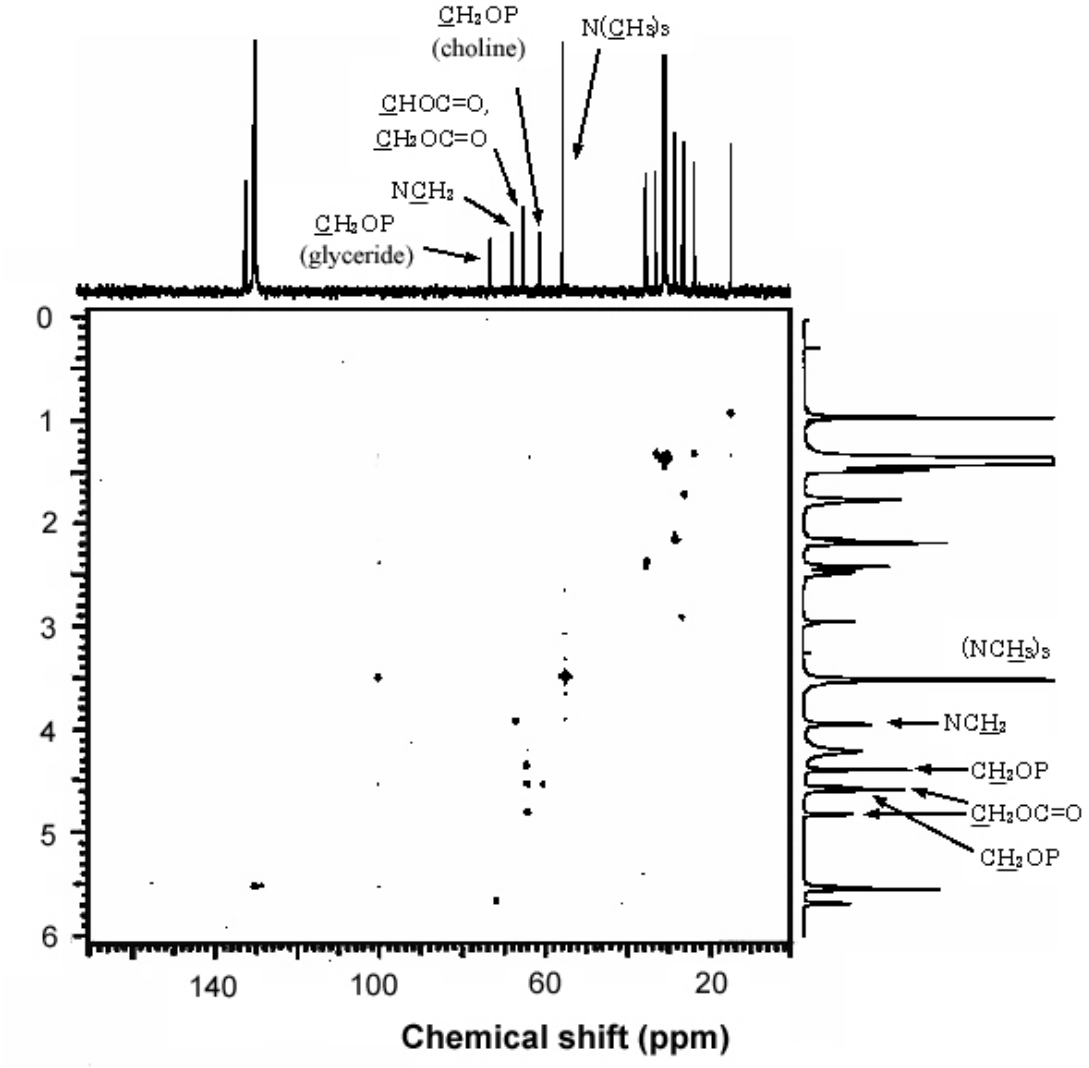


Figure 3.13 ^{1}H-^{13}C heteronuclear correlation spectrum (HMQC) at 750 MHz of L-α-phosphatidylcholine in C_6D_6 measured at 50°C.

In the ^{1}H-^{13}C HMQC spectrum shown in **Figure 3.13**, the proton signal due to $N(C\underline{H}_3)_3$ correlated with the carbon signal at 54.78 ppm of $N(\underline{C}H_3)_3$. Similarly, the proton signal at 3.93 ppm from $NC\underline{H}_2$ had the unique correlation with carbon the signal of $N\underline{C}H_2$ at 66.62 ppm. The proton signal at 4.53 ppm correlated with the carbon signals of $\underline{C}H_2OC{=}O$ at 63.86 ppm and $\underline{C}H_2OP$ of choline head group at 60.04 ppm. It is noteworthy that the carbon signal of $\underline{C}H_2OC{=}O$ of glyceride backbone had the strong correlation with two proton signals at 4.53 and 4.80 ppm due to magnetically nonequivalent $C\underline{H}_2OC{=}O$ protons. The proton signal at 4.36 ppm due to $C\underline{H}_2OP$ of glyceride backbone correlated to the carbon signal at 64.12 ppm, similar to the proton signal of $C\underline{H}OC{=}O$ at 5.67 ppm that correlated with the carbon signal at 71.70 ppm.

3.2.4 ^{31}P-NMR spectroscopy of phospholipids

It is known that the line-width of ^{31}P-NMR signal is very sensitive to the composition of the solvent system [102]. This indicates that the stability of phospholipid micelle is modulated by solvent. It is noteworthy that the micelle is expected to form in organic solvents. In this case, the chemical shift of phospholipid may vary significantly compared to vesicle (bilayer) structure. In addition, the composition of fatty acid ester group can also affect the interactions among the phosphate group and chemical shift. The previous study [102] showed that the $CDCl_3$: CD_3OD: D_2O solvent system gave higher dispersion and better reproducibility of the chemical shifts for ^{31}P-NMR measurement of crude lipid extracts. However, phospholipid dissolved in this solvent system is not useful as a model compound for the analysis of NR, because NR is insoluble in this solvent system. Thus, C_6D_6 was used instead.

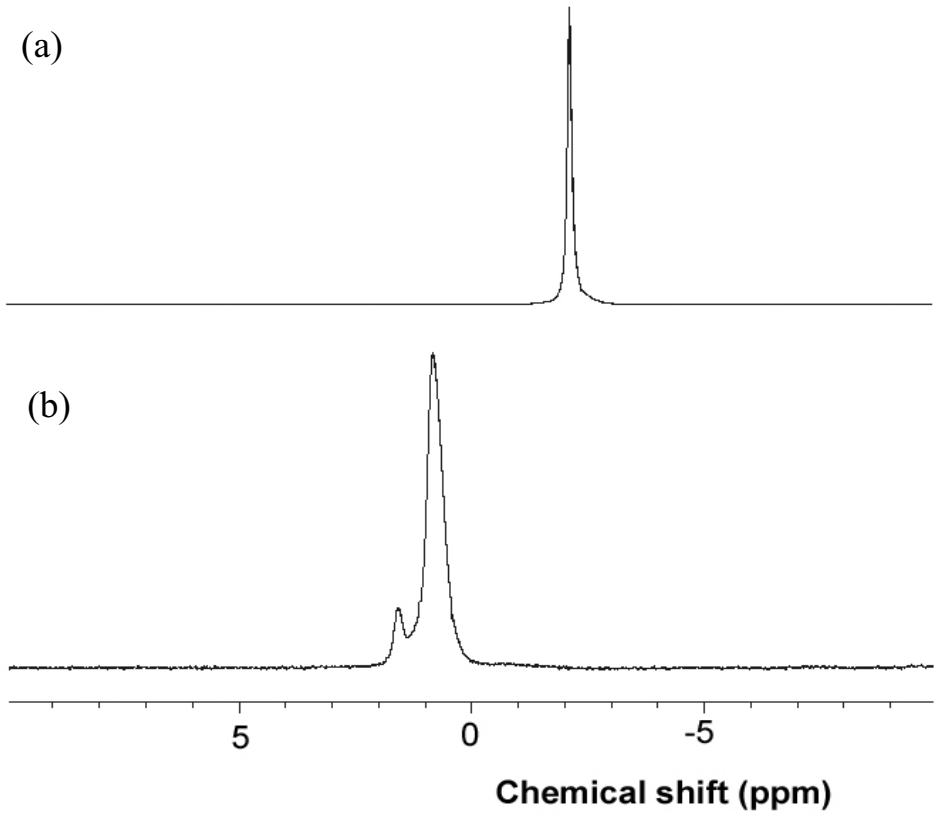


Figure 3.14 ^{31}P-NMR spectra at 303 MHz of L-α-phosphatidylcholine (a), and L-α-phosphatidic acid sodium salt (b) in C_6D_6 measured at 50°C.

Figure 3.14 shows the ^{31}P-NMR spectra of PC and PA dissolved in C_6D_6. The phosphate group showed a singlet signal in PC, while it showed a signal split into two peaks in PA. The ^{31}P nucleus of PA resonated at 0.83 ppm down field shift from H_3PO_4, while that of PC showed upfield shift by 2.13 ppm. It was reported that the ^{31}P-NMR spectrum of a mixture of sonicated dipalmitoyl lecithin and detergents in D_2O showed two resonance signals, separated by 0.15 ppm at 50°C [98]. This was explained to be arisen from the phosphate group in phospholipid molecules on the inside and outside layers of the spherical bilayer vesicles. However, the origin of the difference in chemical shift between inside and outside layers is not clear. In aqueous solution, this is expected to be arisen from the difference in bulk susceptibility between the aqueous and lipid phases. It can produce a slightly different magnetic field between the inner and external aqueous phases [103]. A more feasible explanation would be the alternation of electrostatic interactions, which will thus

affect the chemical shift [104]. This explanation would be consistent with the effect of high salt concentration on the phosphorus chemical shift. The splitting of ^{31}P-NMR signal is supposed to be due to the Na^+ counter ions present in PA.

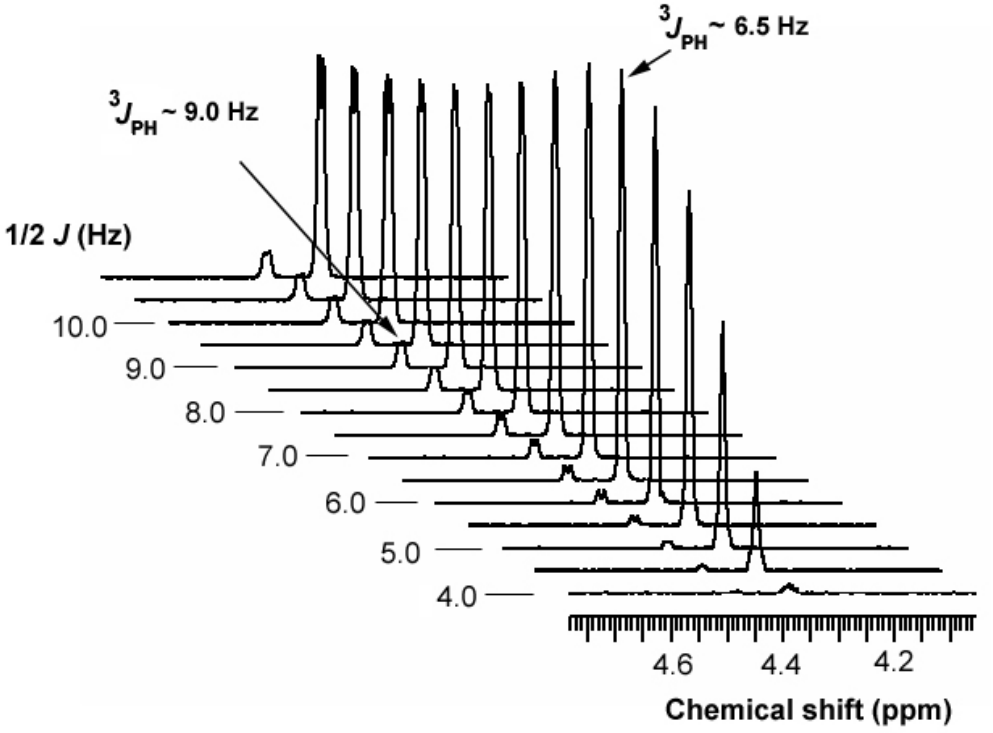


Figure 3.15 One-dimensional $^1H\{^{31}P\}$ HMBC spectrum of L-α-phosphatidylcholine as a function of the mixing time (τ) in the pulse sequence.

Three-bond scalar coupling constant ($^3J_{PH}$) between the phosphorous nucleus and C$\underline{H_2}$OP protons was determined by one-dimensional $^1H\{^{31}P\}$ HMBC experiment using the array of delay parameter $\tau = 1/2J_{PH}$. The highest intensity of singlet signal is the $^3J_{PH}$ constant as illustrated in **Figure 3.15**. The signal at 4.36 ppm was assigned to the correlation signal due to C$\underline{H_2}$OP of glyceride backbone. The $^3J_{PH}$ for the C$\underline{H_2}$OP linkage was estimated to be 6.5 Hz, because the intensity of the correlation signal reached the maximum at $1/2J_{PH}$ = 0.077 s. In a similar way, the $^3J_{PH}$ for the C$\underline{H_2}$OP linkage in choline group present at 4.53 ppm was determined to be about 9.0 Hz.

3.3 Structural Characterization of α-Terminal Group of Natural Rubber obtained by GPC Fractionation

3.3.1 GPC fractionation of acetone-extracted deproteinized natural rubber

Hydroxyl and fatty acid ester groups are common terminal groups in polyprenol derived by biochemical hydrolysis of monophosphate or diphosphate terminal groups. However, it is remarkable that the α-terminal group of NR is neither hydroxyl group nor its simple derivatives. The ^{13}C-NMR spectrum of DPNR shows no signal due to C-4 $\underline{C}H_2OH$ group, corresponding to the terminal isoprene unit, and its fatty acid ester [65]. It is noteworthy that DPNR was found to contain about 1 to 2 phosphorus atoms per rubber chain, while it disappeared after transesterification and saponification [61]. In addition, the content of ester groups linked to rubber molecules was found to be 2 moles of fatty acid ester residues per chain for fractionated DPNR from FL-latex [62]. These findings support the idea that the purified NR contains a phospholipid at the α-terminal end, although there is no direct evidence at present to indicate the linkage between rubber chain and phospholipid molecule.

In order to characterize the functional end group of DPNR, the free fatty acid ester and phospholipid molecules must be removed completely. However, most of phospholipids resist solubilization in polar as well as non-polar solvents by the formation of micelles or inverse micelle, respectively, due to their amphiphilic nature. This problem leads to the difficulty to purify DPNR from non-linked or free phospholipids. Based on the fact that the molecular weight of DPNR is much higher than that of phospholipids, thus non-linked phospholipids can be eluted completely from rubber molecules by the use of preparative GPC fractionation with a low-exclusion limited column using chloroform as eluent by considering the fact that non-linked phospholipids dissolved in chloroform due to the formation of inverse micelle structure.

Figure 3.16 shows the GPC curve of PC, acetone-extracted DPNR (AE-DPNR) and AE-DPNR mixed with PC as a model. It was observed clearly that PC was eluted at the retention volume of about 95 ml. The AE-DPNR mixed with PC

sample showed a similar elution behavior as AE-DPNR. The mixture of AE-DPNR and PC was separated completely after GPC fractionation. The former was eluted at the retention volume starting from 45 to 70 ml with the peak top value of 55 ml, while the latter was eluted after the retention volume of 70 ml. As described above, free phospholipids are expected to form micelle or aggregate structure in non-polar solvent. These free phospholipids may also associate with linked phospholipid in rubber molecule, which cannot be removed from rubber fraction by acetone extraction.

In **Figure 3.16** it is clear that free phospholipids are completely removed from the rubber fraction by GPC fractionation. In other words, the rubber fraction is free from mixed phospholipids after GPC separation. The AE-DPNR was then fractionated into two fractions, the first is the high molecular-weight DPNR fraction eluting at the retention volume from 45 to 70 ml having the $\overline{M}_n$ greater than 2.3×10^5. The second fraction is low molecular-weight DPNR that may contain both linked and free phospholipid compounds excluding at the retention volume from 70 to 100 ml having the $\overline{M}_n$ less than 2.3×10^5.

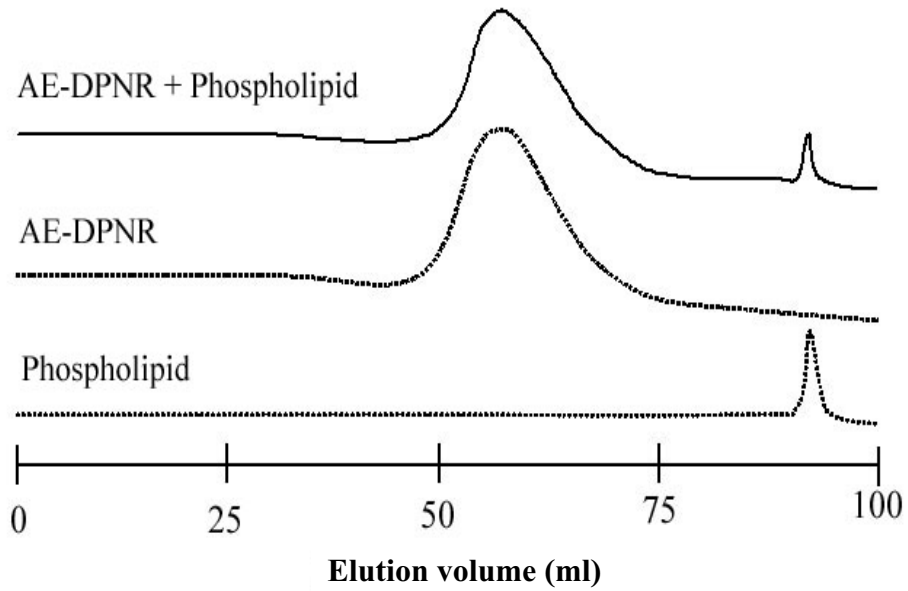


Figure 3.16 GPC of L-α-phosphatidylcholine (PC), AE-DPNR and AE-DPNR mixed with PC in chloroform at room temperature (ca ~ 25°C).

3.3.2 ^{13}C- and ^{1}H-NMR spectroscopy of high molecular-weight fraction in acetone-extracted DPNR

Figure 3.17 shows the ^{13}C-NMR spectrum of high molecular-weight AE-DPNR fraction. The methyl and methylene carbon signals of fatty acid ester were clearly observed at 14.3 and 30.3 ppm. As usual, DPNR shows the two small signals at 54.8 and 64.3 ppm, which were clearly observed for low molecular-weight fraction of DPNR fractionated by molecular weight using toluene/methanol. The signal appeared at 54.8 ppm is assignable to the methyl carbon next to nitrogenous compound, $N(\underline{C}H_3)_3$, while the signal observed at 64.3 ppm corresponded to $\underline{C}H_2OC{=}O$ of glyceride structure based on the assignment of phospholipid or it might be the methylene carbon next to mono- or diphosphate group, $\underline{C}H_2OP$, of polyprenyl phosphate compound. These findings indicate that rubber molecule contains a kind of phospholipid containing nitrogen atom as a component and also mono- or diphosphate group, expecting at the α-terminal. It is interesting that these two small signals were also observed in this high molecular-weight fraction of GPC fractionated DPNR, although the signal-to-noise ratio (S/N ratio) of spectrum was not high due to the high molecular-weight of sample. In addition, a signal observed at 173.0 ppm is assignable to the carboxyl carbons of acylglycerol compounds, $O\underline{C}{=}O$. It has been confirmed that NR contains about two *trans*-isoprene units per rubber single chain independent of molecular weight [25]. The C-1 methylene carbons from the *trans*-isoprene unit in ω–***trans*** and *trans*–***trans*** linkages resonated at 40.4 ppm, the relative intensity of which was about one-half of the signal due to the $O\underline{C}{=}O$ carbon. This indicates the presence of 4 carboxyl carbons in rubber chain. The presence of 4 carboxyl carbons corresponded to two phospholipid molecules associating with a single rubber chain. These findings are strong supporting evidence to confirm the presence of about two phospholipid molecules at the α-terminal end as reported previously [61, 62].

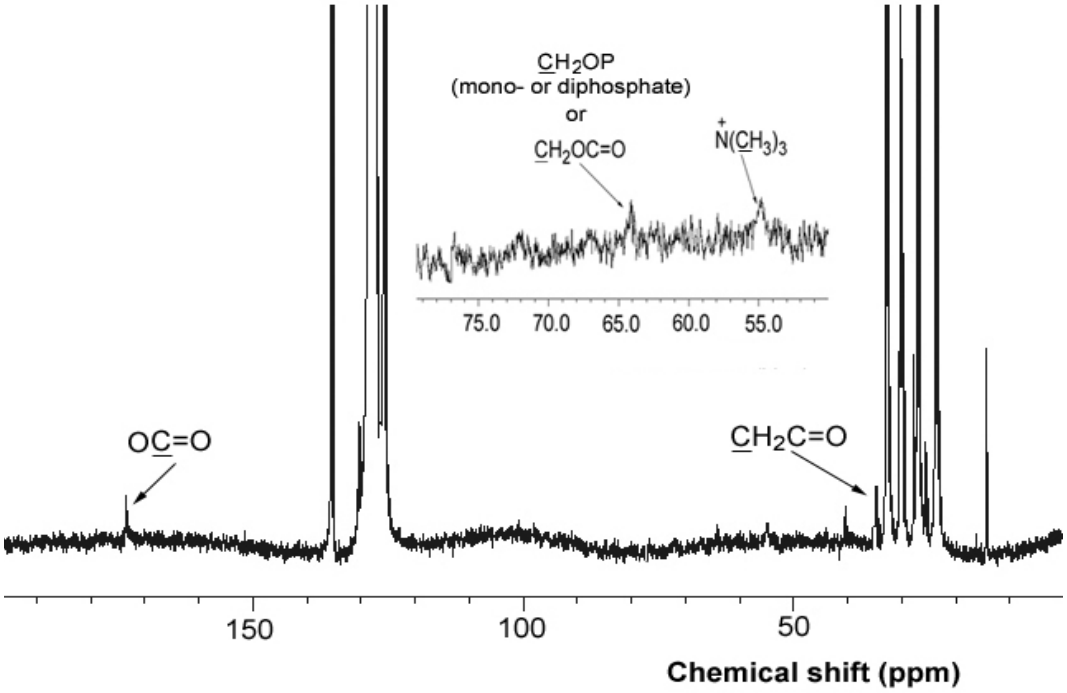


Figure 3.17 ^{13}C-NMR spectrum of high molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 188 MHz.

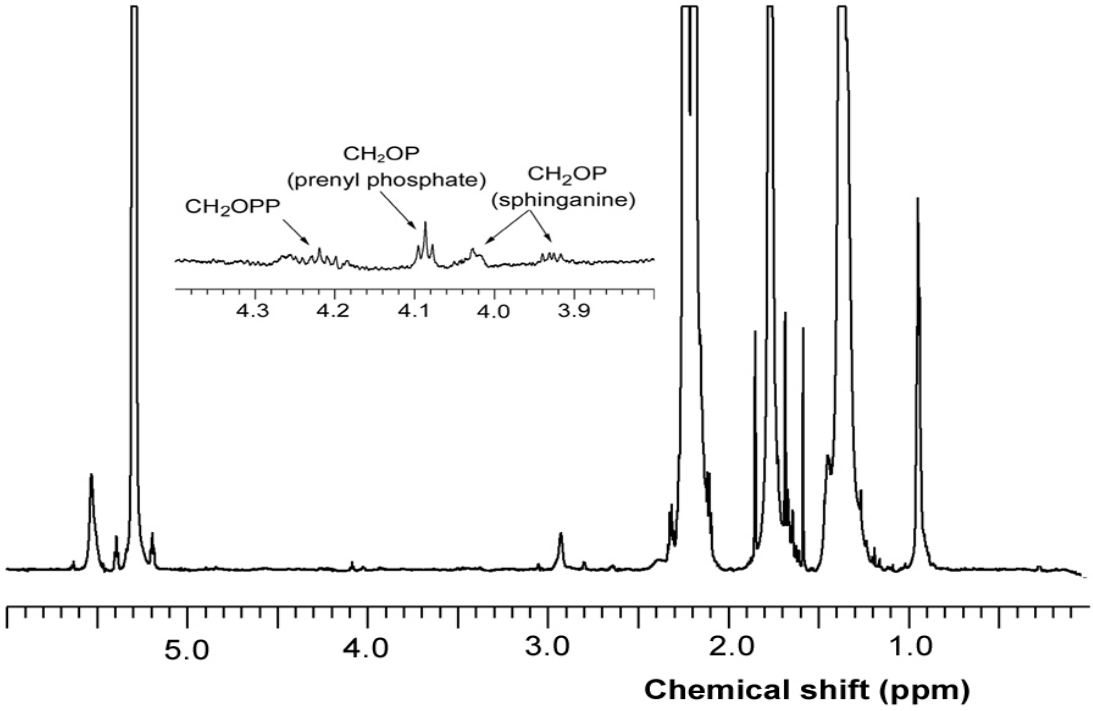


Figure 3.18 ^{1}H-NMR spectrum of high molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 750 MHz.

The presence of phospholipid at the α-terminal group of high molecular-weight rubber fraction was further confirmed by ^{1}H-NMR spectrum as shown in **Figure 3.18**. The methyl protons, C<u>H</u>$_3$, and long chain methylene protons, (C<u>H</u>$_2$)$_n$, of fatty acid ester groups were resonated at 0.95 and 1.37 ppm, respectively. The signal resonating at 2.93 ppm was assignable to the methylene proton next to double bond, C<u>H</u>$_2$-(C=C)$_2$, of fatty acid ester groups. The presence of ^{1}H-NMR signals of fatty acid ester in the high molecular-weight fraction of GPC fractionated AE-DPNR is supporting evidence to confirm that some fatty acid ester groups are linked or associated to rubber molecule at the α-terminal group. It is remarkable that small signals appearing in the region between 3.50 and 5.00 ppm corresponded to the α-terminal group of polyisoprene including rubber molecule [105]. Two multiplet signals resonating at 3.92 and 4.04 ppm were expected to be due to the non-equivalent methylene protons linked to phosphate group, C<u>H</u>$_2$OP, of phospholipid containing amide and hydroxyl groups such as phosphosphingolipid [106]. A triplet proton signal of methylene protons linked to phosphate group, C<u>H</u>$_2$OP, of polyprenyl phosphate was resonated at 4.09 ppm and indicated that monophosphate group might be linked to rubber molecule, expecting at the rubber chain-end based on the previous study [105]. It is noticeable that a small triplet signal was also observed at 4.20 ppm, which can be derived from methylene protons linked to diphosphate group, C<u>H</u>$_2$OPP. In addition, the singlet signal resonating at 5.53 ppm was assumed to be corresponded to C<u>H</u>OC=O of the glyceride backbone of phospholipid.

The information from ^{13}C- and ^{1}H-NMR spectra indicates the presence of three functional groups at α-terminal group of high molecular-weight rubber molecule, which are monophosphate, diphosphate groups and phospholipids containing nitrogenous as well as hydroxyl group. About two moles of phospholipid containing nitrogenous compound were present in the high molecular-weight rubber fraction based on the relative intensity of the signal due to O<u>C</u>=O.

3.3.3 ^{13}C- and ^{1}H-NMR spectroscopy of low molecular-weight fraction in acetone-extracted DPNR

The presence of free and linked phospholipids in NR was further confirmed by the analysis of low molecular-weight DPNR fractionated by GPC. In this rubber fraction, both low molecular-weight rubber molecules and free phospholipids are expected to be mixed together. The ^{13}C-NMR spectrum of low molecular-weight DPNR fraction is shown in **Figure 3.19**. The carbon signals due to methyl, long-chain methylene carbons and methylene carbon next to carboxyl group of fatty acid ester were clearly observed at 14.3, 30.3 and 34.6 ppm, respectively. The carboxyl carbon, O<u>C</u>=O, of fatty acid ester appeared at 173.3 ppm. A small signal at 179.0 ppm was expected to be the carbonyl carbon next to nitrogen of phospholipid containing amide functional group such as phosphosphingolipid [107]. The presence of a nitrogenous group in the rubber molecule was confirmed by the presence of a small signal resonating at 54.6 ppm, which was assigned to the methyl carbon linked to nitrogen atom, N(<u>C</u>H$_3$)$_3$, based on the assignment of phospholipid model compounds. However, carbon signal due to N<u>C</u>H$_2$ group was not detected in the low molecular-weight rubber fraction. The methyl and methylene carbons of *trans*-isoprene unit in *trans*-***trans***-*cis* alignment, which are expected to resonate at around 17 and 40 ppm, were also not observed. This may be due to the small amount of fractionated sample obtained by GPC compared to that of high molecular-weight rubber fraction.

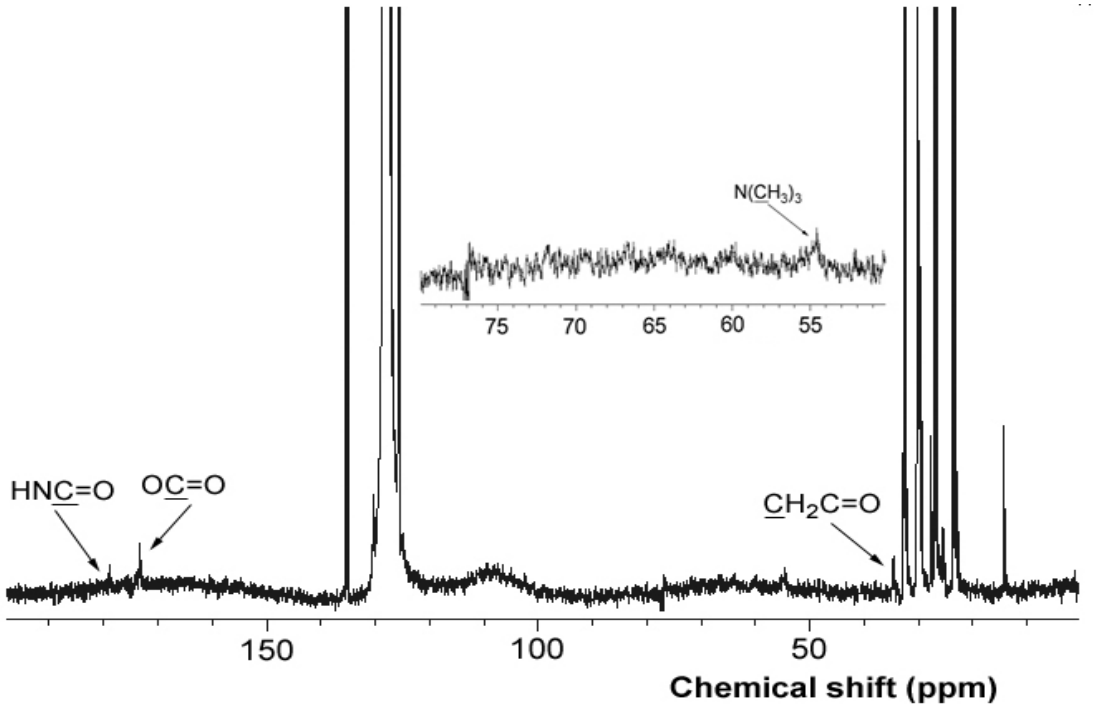


Figure 3.19 ^{13}C-NMR spectrum of low molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 188 MHz.

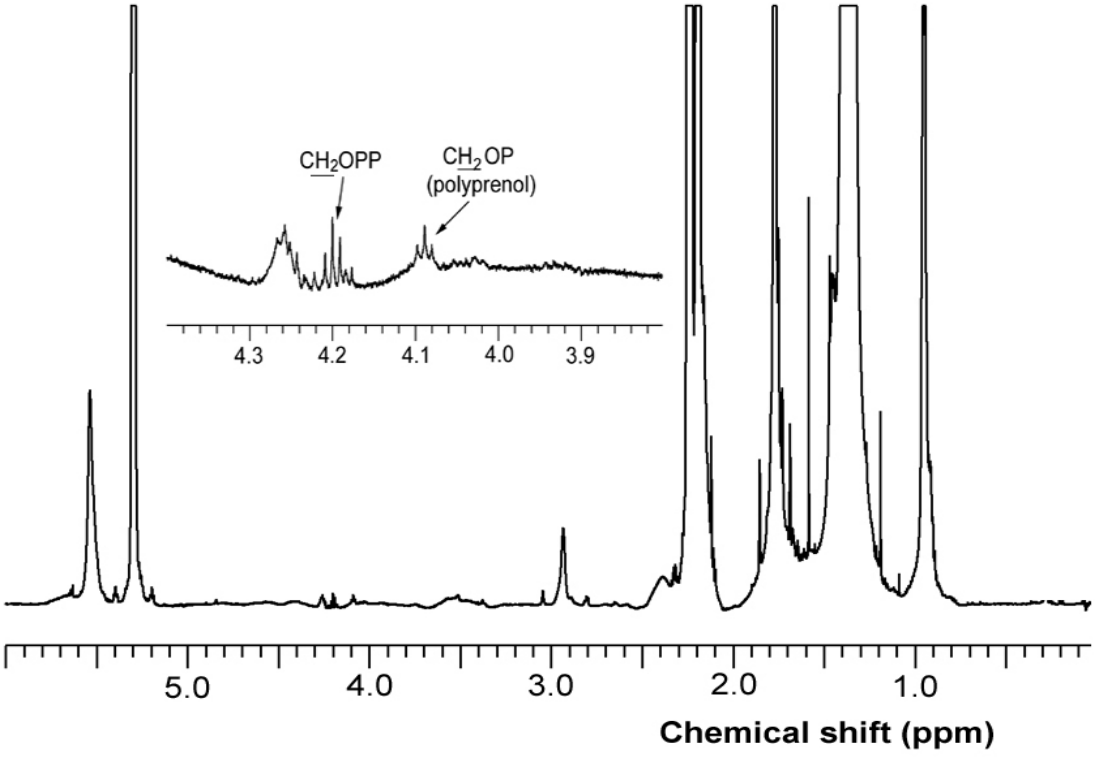


Figure 3.20 ^{1}H-NMR spectrum of low molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 750 MHz.

Figure 3.20 represents the ^{1}H-NMR spectrum of low molecular-weight DPNR fraction. This rubber fraction showed signals with chemical shifts similar to those of high molecular-weight one. The proton signals due to the linked and/or mixed fatty acid fatty acid ester groups appeared at 0.95, 1.37 and 2.94 ppm, which are assigned to the methyl, long chain methylene protons and methylene protons next to double bond, respectively. A pronounced triplet signal was observed around at 4.09 ppm, which is assignable to the methylene protons linked to phosphate group of polyprenyl phosphates, C$\underline{H}_2$OP. It is very interesting to observe a triplet signal at 4.20 ppm and singlet signal at 5.50 ppm. These two signals were presumed to be C$\underline{H}_2$OPP and C$\underline{H}$OC=O in phospholipid, respectively. The relative intensity of these two signals against C-1 methylene proton signal was higher than that of the high molecular-weight rubber fraction, supporting the assumption that these signals were derived from terminal groups. The above findings suggest that the phospholipid is one of constituents in DPNR and present in the form of both free and linked to the rubber molecule. This phospholipid was expected to consist of two moles of long-chain fatty acid ester and nitrogenous groups as well as hydroxyl group.

3.3.4 ^{31}P-NMR spectroscopy of fractionated acetone-extracted DPNR

The presence of 1 to 2 phosphorus atoms per rubber chain was found by chemical analysis [61]. However, there was no direct evidence showing the presence of phosphorus atom linked to rubber chain. Accordingly, it is useful to confirm the presence of phosphorus atom by ^{31}P-NMR analysis. The ^{31}P-NMR spectra of high and low molecular-weight rubber fractions are shown in **Figures 3.21** and **3.22**, respectively. The clear phosphorus signals of the high and low molecular-weight rubber fractions were observed at 1.53 and 1.47 ppm, respectively, upfield shift from H_3PO_4. These two signals showed very broad half line-width of about 420 Hz, while that obtained from PC and PA was about 26 and 120 Hz, respectively. The appearance of broad half line-width of the ^{31}P signal suggests that phospholipids are linked and/or associated to high molecular-weight molecules, e.g. rubber molecule. The presence of phosphorus signal both in the high and low molecular-weight fraction is confirming evidence showing the presence of phospholipids in rubber molecules.

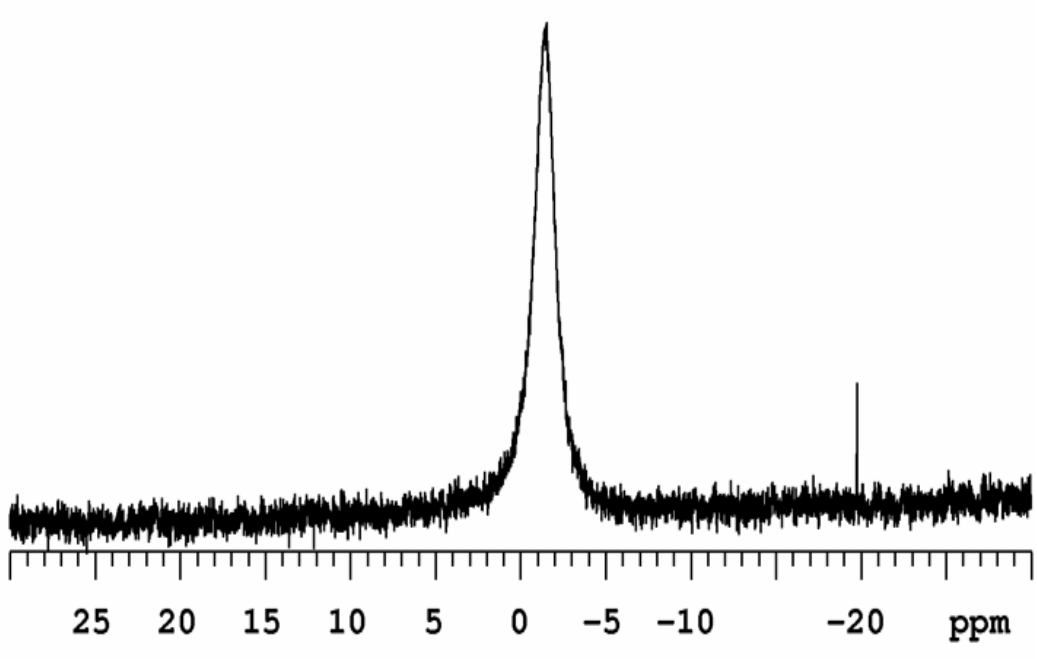


Figure 3.21 ^{31}P-NMR spectrum of high molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 303 MHz.

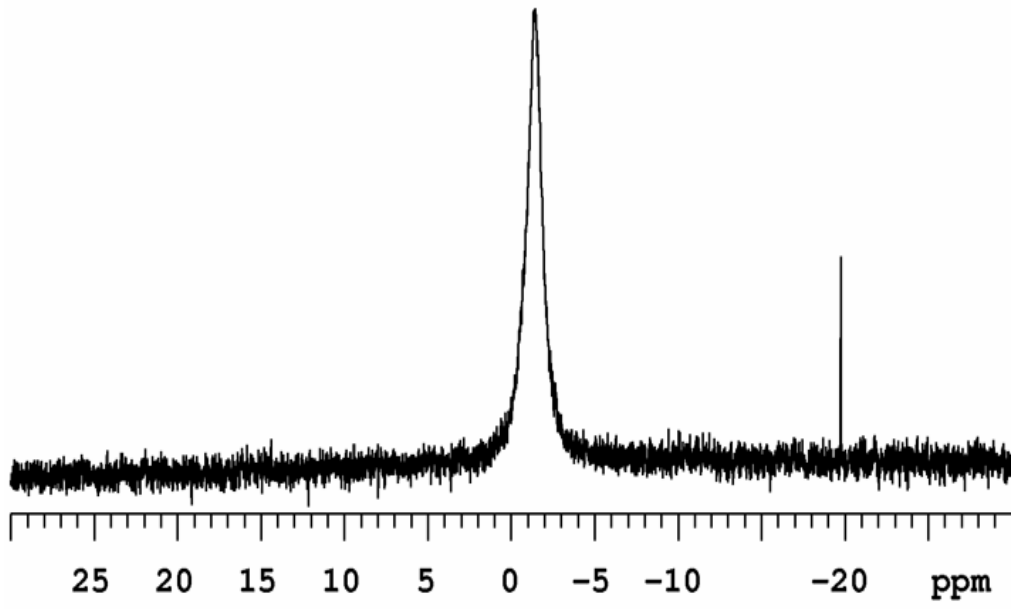
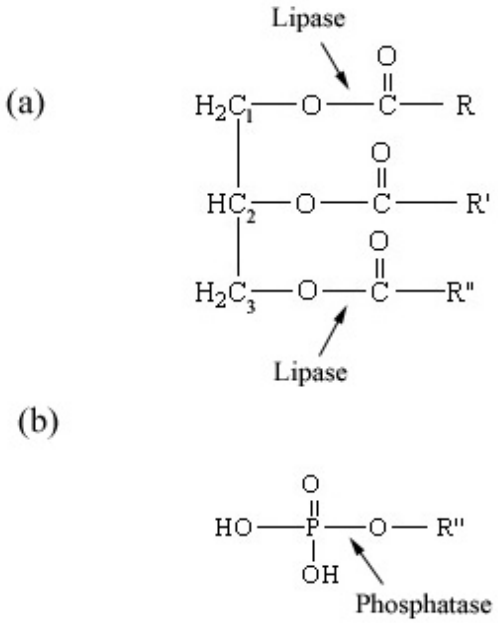


Figure 3.22 ^{31}P-NMR spectrum of low molecular-weight rubber fraction obtained from GPC fractionation, measured in C_6D_6 at 303 MHz.

3.4 Structural Characterization of α-Terminal Group of Natural Rubber -Decomposition of Branch-points by Enzymatic Treatment-

The presence of triacylglycerol and/or phospholipid at the chain-end of NR was assumed based on a series of supporting evidence. This can be confirmed by the structural change of rubber chain after treatment with lipase, phosphatase and phospholipase. These enzymes have high efficiency to hydrolyze selectively triacylglycerol, monophosphate ester and phospholipid, respectively. NR latex is known to be covered with lipid and protein layers [29]. Direct reaction of these enzymes on the rubber particles was difficult due to the presence of protein layer on the outside of the rubber particles. Consequently, it was necessary to remove the proteins by enzymatic deproteinization before the reaction with these enzymes. Thus, the effect of lipase, phosphatase and phospholipase was analyzed on DPNR latex.

3.4.1 Selective decomposition of phospholipids by lipase and phosphatase



Scheme 3.1 Schematic representation of selective decomposition position of (a) lipase on the acylglycerol and (b) phosphatase on monophosphate ester group.

It is known that lipase (triacylglycerol acylhydrolase or triacylglycerol lipase) is able to hydrolyze selectively the ester linkage of tri-, di- and monoglyceride at C1 and C3 positions in triacylglycerol compounds, while phosphatase decomposes monophosphate ester linkage, respectively, as shown in **Scheme 3.1**. However, it is very important to investigate the efficiency of these two enzymes over phospholipid molecules, such as PC and PA, as models of phospholipids in NR assuming the linkage at the α-terminal. The result obtained from enzymatic treatments would give useful information on the structure of phospholipid presumed to be at the rubber chain-end.

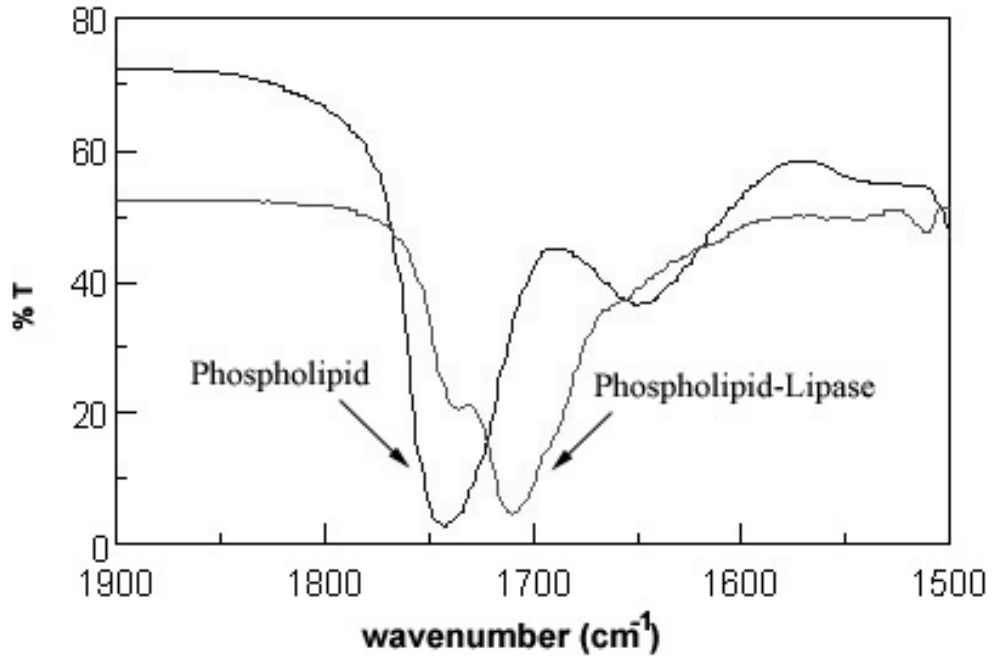


Figure 3.23 FTIR spectra of L-α-phosphatidylcholine before and after lipase treatment.

Figure 3.23 shows the FTIR spectra of phospholipid before and after treatment with lipase. In the spectra it can be observed clearly that the C=O stretching band of fatty acid ester in PC shifted from 1743 cm^{-1} to 1710 cm^{-1} with residual small band at 1743 cm^{-1} after reaction with lipase. This indicates that lipase treatment removed most parts of the fatty acid ester groups, but not completely. As mentioned above, lipase decomposes fatty acid esters at the C1 and C3 of triacylglycerol. The shift of C=O band after lipase treatment clearly indicates that lipase has a capability to decompose

fatty acid ester at C1 position of phospholipid molecule, and liberates free fatty acid group.

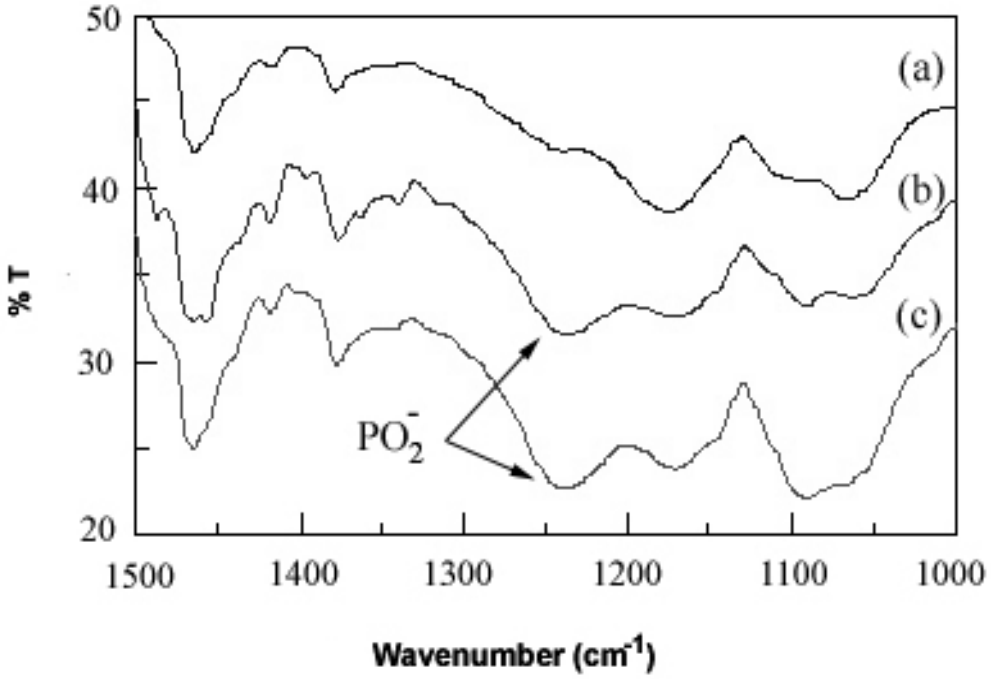


Figure 3.24 FTIR spectra of (a) L-α-phosphatidic acid treated with phosphatase, (b) L-α-phosphatidylcholine treated with phosphatase and (c) L-α-phosphatidic acid monosidium salt.

Figure 3.24 shows the FTIR spectra of two kinds of phospholipids i.e. PC and PA treated with phosphatase. These phospholipids are expected to give information about the difference due to the polar head group of phospholipid. By considering the fact that phosphatase decomposes monophosphate, it is expected that the phosphate group in PC was intact, whereas that of PA was almost removed after phosphatase treatment. The absorption band at 1240 cm^{-1} was assigned to PO^-_2 asymmetric stretching of phospholipid bilayer [108, 109]. In the spectrum of PC, the PO^-_2 band remained even after phosphatase treatment, while it disappeared when PA was treated with phosphatase. This indicates that phosphatase has an efficiency to decompose only monophosphate ester linkage of PA and liberates as phosphoric acid, while phosphate ester group in PC cannot be removed by phosphatase treatment. In addition to the decomposition of monophosphate linkage in PA, phosphatase has possibility to

hydrolyze the phosphate group of polyprenyl phosphate ($-CH_2OPO_3^{2-}$) and polyprenyl diphosphate ($-CH_2OP_2O_6^{3-}$) in rubber chain due to the presence of monophosphate linkage.

3.4.2 Effect of lipase concentration on decomposition of fatty acid ester groups by lipase treatment

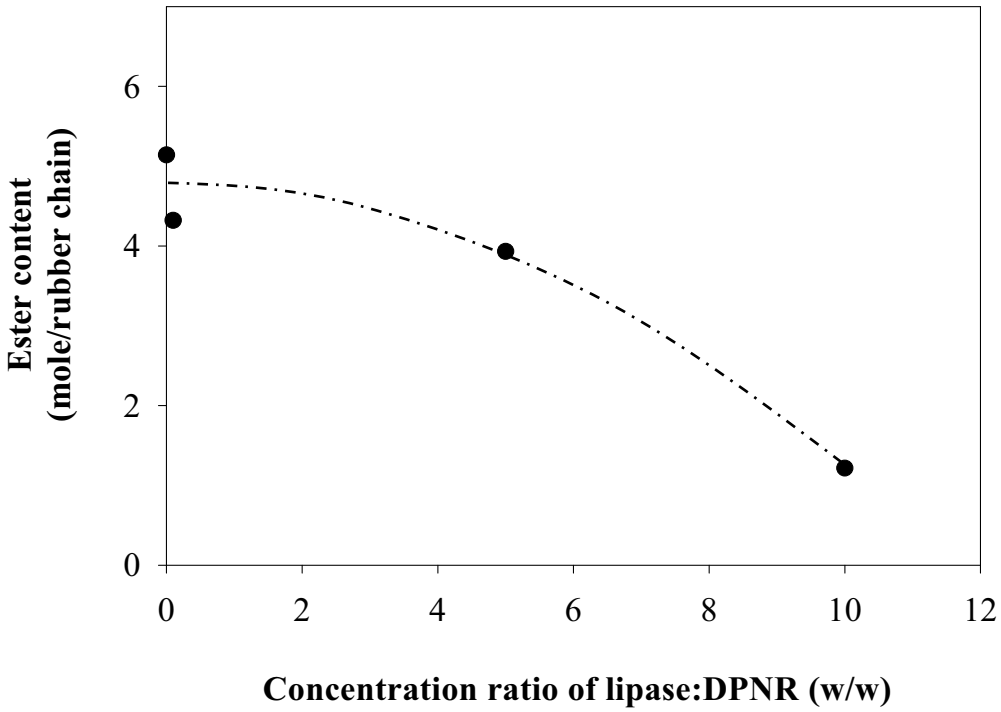


Figure 3.25 Content of long-chain fatty acid ester in DPNR treated with lipase at different lipase concentrations.

Figure 3.25 illustrates the relationship between the content of long-chain fatty acid ester groups in the rubber from DPNR latex against the lipase concentration from 1:1 to 10:1 by weight against the rubber weight, after incubation at 37°C from 0 to 196 hr. Here, the measurement of fatty acid ester content was carried out after extraction of lipase treated rubber with acetone to remove free fatty acids and glycerides present as a mixture. AE-DPNR contained long-chain fatty acid ester groups ca ~ 5.0 moles/ rubber chain. It decreased gradually to ca ~ 1.5 moles/ rubber chain with increasing the lipase concentration. Here, the ester content per rubber chain was calculated by the use of the $\overline{M}_n$ values of rubber. The $\overline{M}_n$ values used for

this calculation were obtained by GPC using polyisoprene standards, which represent the whole molecule including the branch-points. In this experiment, it was necessary to use a large amount of crude lipase up to 10 times of rubber weight to decompose acylglycerol. This may be due to the low activity of crude lipase. As mentioned above, lipase can decompose fatty acid esters at the C1 and C3 of acylglycerol compound. The decrease of ester content after lipase treatment indicates clearly the presence of acylglycerol compound in NR. However, it is remarkable that the ester groups were not removed completely even after the treatment of DPNR latex with extremely high lipase concentration. This can be explained by considering the fact that fatty acid ester at the C2 position of acylglycerol cannot be decomposed by lipase treatment. This is direct evidence showing the presence of acylglycerol compound in NR.

3.4.3 Determination of appropriate reaction condition for lipase treatment

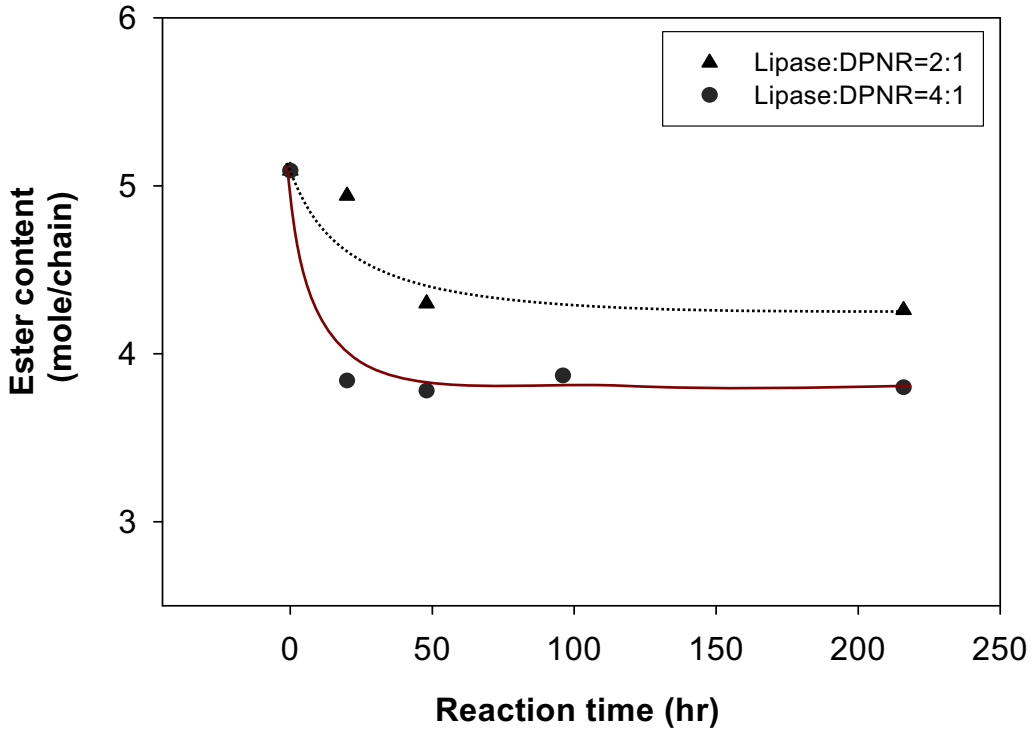


Figure 3.26 Content of long-chain fatty acid ester in DPNR treated with lipase at pH 7.2 and 37°C.

The appropriate reaction time for lipase treatment of DPNR was determined for the reaction with pH 7.2 at 37°C using the ratio of lipase to DPNR of 2:1 and 4:1 by weight, as shown in **Figure 3.26**. At the lower lipase concentrations the ester content in DPNR attained equilibrium similar to that obtained under the high lipase concentration after 20 hr. Under these reaction conditions, the content of fatty acid ester groups in DPNR decreased significantly from ca ~ 5.0 to 4.3 moles/rubber chain reaching an equilibrium value after the reaction time of 20 hr. This indicates that the reaction of 20 hr is enough to decompose the ester groups by the addition of lipase into DPNR latex in a ratio of 2:1 against the rubber by weight. Similarly, in the case of lipase concentration of 4:1 against the rubber weight, the ester content in the lipase treated DPNR reached an equilibrium value of ca ~ 3.8 moles/chain after 20 hr of reaction time. It is remarkable that two-third of long-chain fatty acid ester remains even after reaching equilibrium by lipase treatment, whereas the ester group removed perfectly after chemical treatment such as transesterification and saponification [61]. The presence of residual fatty acid ester groups in lipase treated DPNR implies that NR contains a fatty acid ester group other than triglyceride type. In view of the fact that all the simple fatty acid esters and fatty acids can be extracted from NR by acetone after enzymatic treatment, the residual fatty acid esters are presumed to be linked to rubber chain or polar lipids such as phospholipids and glycolipids, which are insoluble in acetone.

The presence of glycolipids in NR, however, can be neglected because of the absence of ^{13}C- and ^{1}H-NMR signals characteristic of saccharides. That is to say, the residual fatty acid esters are presumed to be included in a functional group linking to rubber chain, presumably at the α-terminal, and/or free phospholipids, which cannot be extracted completely with acetone even after the treatment with lipase. In higher plants, it is common that polyprenols are present as fatty acid ester [110]. It was reported that the α-terminal of rubbers from leaves of sunflower (*Helianthus annuus*) [111] and *Lactarius* mushroom (*Lactarius volemus*) [60] is esterified with fatty acids. However, in the case of NR, the presence of fatty acid esterified at the α-terminal can be neglected by the fact that AE-DPNR and lipase treated DPNR showed no ^{13}C-NMR signal due to C-4 methylene carbon of the α-terminal isoprene unit linked to ester group, which resonates at 60.9 ppm in polyprenol esters [60, 111].

3.4.4 Effect of lipase and phosphatase on decomposition of branch-points

If the treatment of lipase decomposes the branch-points of DPNR, it should be accompanied by decreasing in molecular weight and degree of branching as well as Huggins' *k'* constant.

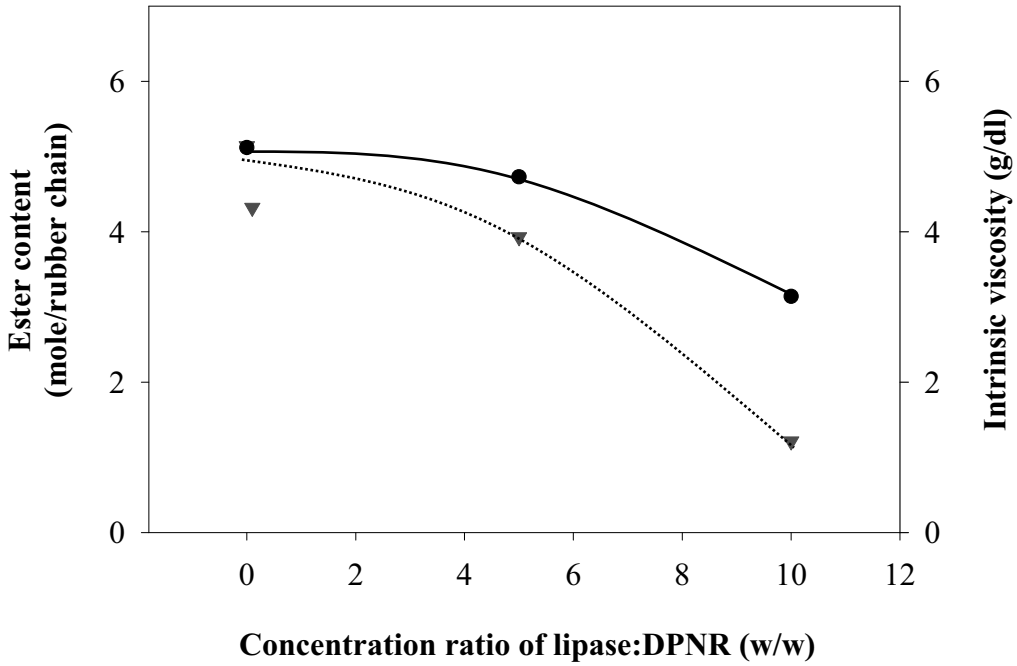


Figure 3.27 Intrinsic viscosity and content of long-chain fatty acid ester in DPNR after lipase treatment at 37°C with pH 7.2 for 20 hr.

Figure 3.27 shows the relationship between intrinsic viscosity of the lipase treated DPNR and the concentration of lipase, together with the long-chain fatty acid ester content in DPNR. The intrinsic viscosity of the lipase treated DPNR decreased from ca ~ 5.1 to ca ~ 3.3 g/dl with increasing the concentration of lipase. In proportion to the decrease in the intrinsic viscosity, the ester content decreased gradually from ca ~ 5.1 to 1.6 moles of ester groups per rubber molecule. Accordingly, it means that about 5 moles of ester groups per rubber molecule in AE-DPNR reduced to about two moles per molecule after lipase treatment. It is noteworthy that the presence of about two ester groups per single chain, which means

a chain constituting branching, was confirmed by ^{13}C-NMR measurement of fractionated DPNR from FL-latex [62]. In the present experiment, AE-DPNR and lipase treated DPNR showed the ^{13}C-NMR signal due to the methylene carbon next to carboxyl group of fatty acid ester, OC=O$\underline{C}$H$_2$, at 34.6 ppm. The relative intensity of this signal was about twice of the C-1 methylene carbon signal from *trans*-1,4 isoprene units, which was confirmed to be two units per single chain [63]. These findings are strong supporting evidence to deduce that NR molecules are partially linked together by a functional group containing glyceride backbone such as acylglycerol and/or phospholipid to form the branch-points.

The decrease of ester content by lipase treatment was accompanied by the decrease in molecular weight. In the case of phospholipid, the hydrolysis of C1 ester group will contribute to reduce the ester content, by considering the active site of lipase. Meanwhile, the decrease in molecular-weight of DPNR should be the decomposition of branch-points. Consequently, it can be deduced that the removal of long-chain fatty acid ester group at the C1 in the phospholipids by lipase treatment partly destroys the micelle structure and results in the decomposition of branch-points.

Table 3.4 Structural characteristics of DPNR, TE-DPNR, lipase and phosphatase treated DPNR after acetone extraction

Characteristics	AE-DPNR	TE-DPNR	Lipase treated DPNR (10:1)	Phosphatase treated DPNR (10:1)
Ester content (mmol/kg rubber)	31.3 (5.09)*	~0	16.2 (1.59)	18.5 (2.14)
[η]	5.12	3.08	3.75	3.47
k'	0.47	0.43	0.23	0.35
$\overline{M}_v$ **(×10^5)**	7.92	3.87	5.10	4.12
$\overline{M}_n$ **(× 10^5)**	1.93	1.18	0.97	1.16
$\overline{M}_w$ **(× 10^5)**	4.34	2.71	3.27	3.64
$\overline{M}_w$**/** $\overline{M}_n$	3.84	3.94	3.04	3.58

Note: The value in parentheses is the ester content in mol/rubber single chain

Table 3.4 shows the content of long-chain fatty acid ester, intrinsic viscosity [η], and molecular weight of lipase treated DPNR compared with AE-DPNR and TE-DPNR as well as phosphatase treated DPNR at the concentration ratio 10:1 against the rubber weight. The [η] value of the rubber from lipase treated DPNR latex was lower than that of AE-DPNR, while higher than that of TE-DPNR. It is known that the Huggins' constant, *k*', is a qualitative indicator of the presence of long-chain branching. For a given polymer, the *k'* value is nearly independent of molecular weight and MWD and it increases in proportion to the quantity of branching in the polymer chain [88, 112]. It is remarkable that the *k'* value of DPNR decreased from 0.47 to 0.23 after treatment of DPNR latex with lipase. This indicates that branch-points in the lipase-treated DPNR were entirely decomposed to form linear molecules after lipase treatment. In addition, the lipase-treated DPNR showed lower $\overline{M}_n$ and $\overline{M}_w$ values than

those of the untreated AE-DPNR and lower polydispersity index, $\overline{M}_w/\overline{M}_n$. The decrease in MW is strong supporting evidence that the fatty acid ester group at the C1 of acylglycerol and/or phospholipid participates in the branching formation of NR, expecting by micelle formation.

As discussed above, phosphatase hydrolyzes the monophosphate ester linkage, which is included in a phospholipid containing hydroxyl group as polar headgroup i.e. PA. It is remarkable that the phosphatase treatment decreased the molecular weight as well as the [η] value of DPNR, although these values were higher than those of TE-DPNR and lipase treated DPNR. The decreases of these values indicate the presence of monophosphate linkage in the branch-points of DPNR molecules, expecting by hydroxyl group *via* hydrogen bonding. In other words, the phosphatase treatment of DPNR may result in the scission of rubber chain from the branch-points. It is not difficult to expect the presence of phosphate group at the α-terminal in rubber molecule. The chain elongation of rubber molecule is believed to proceed by the addition of isopentenyl diphosphate to polyisoprenyl diphosphate [20]. If polyisoprenyl phosphate formed by hydrolysis of polyisoprenyl diphosphate stabilized in latex, it can be the origin of this phosphate linkage in NR molecule.

It is interesting to observe the decrease in the content of long-chain fatty acid ester in DPNR after phosphatase treatment from ca ~ 5.1 to ca ~ 2.1 moles per rubber chain. Ordinarily, however, it is difficult to expect that phosphatase decomposes phospholipids, because phosphatase cannot hydrolyze the diphosphate ester as mentioned above. Most of free phospholipids are partly soluble either in acetone, methanol or toluene due to its amphiphilic character and cannot be separated completely from rubber by ordinary precipitation due to the formation of micelle structure. However, it is possible to remove almost all free phospholipids in DPNR by dissolving into toluene containing small amounts of ethanol followed by precipitation into methanol. The detailed mechanism of removing phospholipids from DPNR will be discussed later. In usual treatment, therefore, phospholipids separated from rubber molecules by phosphatase treatment can remain in DPNR. This ester group mixed into DPNR can be counted as the ester content. However, if the branch-points contain diacylglycerol-3-phosphate or phosphatidic acid, the simplest phosphoglyceride, it can

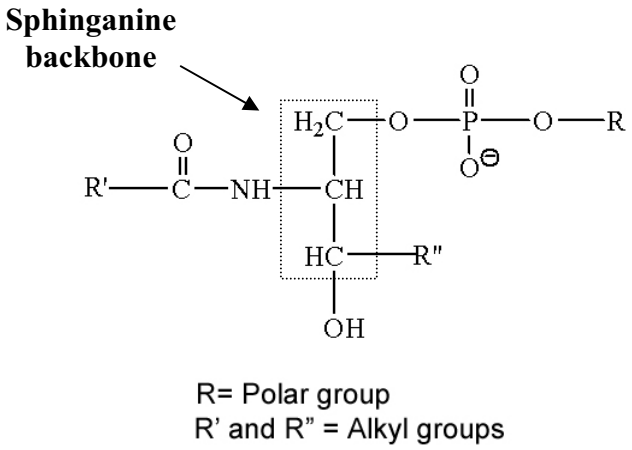
be hydrolyzed by phosphatase to give acetone soluble product and contributed to reduction in ester content.

3.4.5 ^{1}H- and ^{13}C-NMR measurements of DPNR after lipase and phosphatase treatments

It was disclosed that the α-terminal group of naturally occurring polyisoprene, such as rubbers from *Lactarius* mushroom, Sunflower and Goldenrod, is normally hydroxyl or ester groups [57]. However, it was confirmed that these terminal groups are absent in case of rubber from *H. brasiliensis* by ^{1}H- and ^{13}C-NMR studies [57]. In order to disclose the peculiarity of NR, high-frequency NMR measurements corresponding to 750 MHz in the case of ^{1}H-NMR was applied to the characterization of α-terminal group in NR after enzymatic treatment.

The ^{13}C-NMR spectrum of DPNR is shown in **Figure 3.28**. The signals at 63.7 ppm was assignable to the methine carbon of glyceride structure of phospholipid, while that at 64.7 ppm corresponded to methylene carbons of glyceride structure of phospholipid linked to carboxyl group of phospholipid or the methylene carbon next to mono- or diphosphate group. In addition, a small signal resonating at 69.2 ppm was assigned to the methine carbon linked to hydroxyl group of phospholipid molecule containing hydroxyl group such as phosphosphingolipid [106]. It is accepted that different kinds of phospholipid give the different resonances of carbon atom linked to polar groups distinguishable each other. The ^{13}C-NMR signals of DPNR showed three signals in 50-75 ppm region indicating the presence of different phospholipids in NR. The ^{13}C-NMR signals appeared at 63.7 and 64.7 ppm are expected to be the carbon atom corresponding to ordinary phospholipid such as PC or PA. On the other hand, a small signal at 69.2 ppm is expected to be derived from the methylene protons next to hydroxyl group of complex phospholipid such as phosphosphingolipid, even the chemical shift value was slightly different from previous report resonating at 70.7 ppm in $CDCl_3$ at 50°C [106]. These findings make it possible to postulate the presence of at least two kinds of phospholipid, simple phospholipid and complex phospholipid contating nitrogeneous and hydroxyl group in rubber molecule. A typical example of

phospholipid containing nitrogeneous and hydroxyl group is phosphosphingolipid as shown is **Scheme 3.2**.



Scheme 3.2 Chemical structure of phosphosphingolipid.

It has been confirmed that NR contains two *trans*-isoprene units per single rubber molecule [63]. The total relative intensity of these three signals was roughly comparable to the C1 methylene signal due to *trans*-***trans*** and ω-***trans*** units, appearing at 40.3 ppm. This suggests the presence of about two moles of phospholipid per single chain.

The presence of phospholipids was further confirmed by ^{1}H-NMR measurement as illustrated in **Figure 3.29**. A sharp peak appeared at 3.49 ppm corresponded to the methyl proton next to nitrogen of choline head group of phospholipid. In addition, two small multiplet signals appeared at 3.92 and 4.04 ppm are expected to be derived from the non-equivalent methylene protons linking to phosphate group, C$\underline{H}_2$OP, in glyceride structure of phospholipid containing nitrogenous compound and hydroxyl group such as phosphosphingolipid. It is remarkable that signals due to phosphate and diphosphate groups were detected in this spectrum in addition to those from phospholipid molecules. A sharp triplet signal at 4.09 ppm having the coupling constant of 6.5 Hz was assignable to C-4 methylene protons of *cis*-isoprene unit next to phosphate group, C$\underline{H}_2$OP. In addition, a small triplet signal appeared at 4.22 ppm was assignable to methylene proton linked to diphosphate group, C$\underline{H}_2$OPP, although the chemical shifts of these signals were

different from synthetic polyprenyl diphosphates. It was found that the methylene protons linked to phosphate and diphosphate groups of phosphorylated betulaprenol-18 showed broad triplet signals at 4.41 and 4.46 ppm with coupling constant of 6.6 Hz, measured in $CDCl_3$ and at 27°C [105]. The difference of this chemical shift is expected to be due to the difference of measuring conditions and structure of the protecting group of phosphate. These findings suggest the presence at least three phosphate functional groups at the α-terminal in NR i.e. monophosphate, diphosphate and phospholipid groups.

It is interesting that in this spectrum the relative intensity of phospholipid:monophosphate was 2:1 indicating the presence of two moles of phospholipid and one mole of monophosphate groups. This observation is in good agreement with that from ^{13}C-NMR showing the presence of two moles of phospholipid. Based on this finding it is possible to assume that rubber molecule is functionalized with mono- or diphosphate groups. The presence of monophosphate terminal is postulated to due to the hydrolysis of diphosphate group because of the less stability of polyisoprenyl diphosphate compounds.

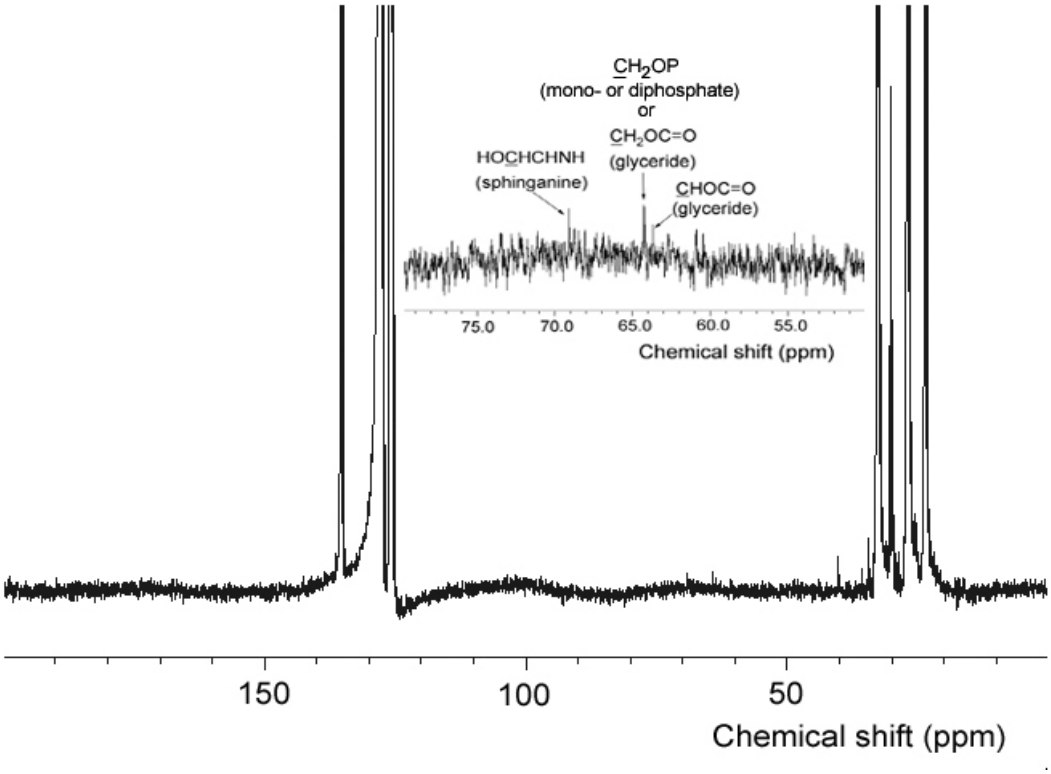


Figure 3.28 ^{13}C-NMR spectrum of DPNR measured in C_6D_6 at 188 MHz.

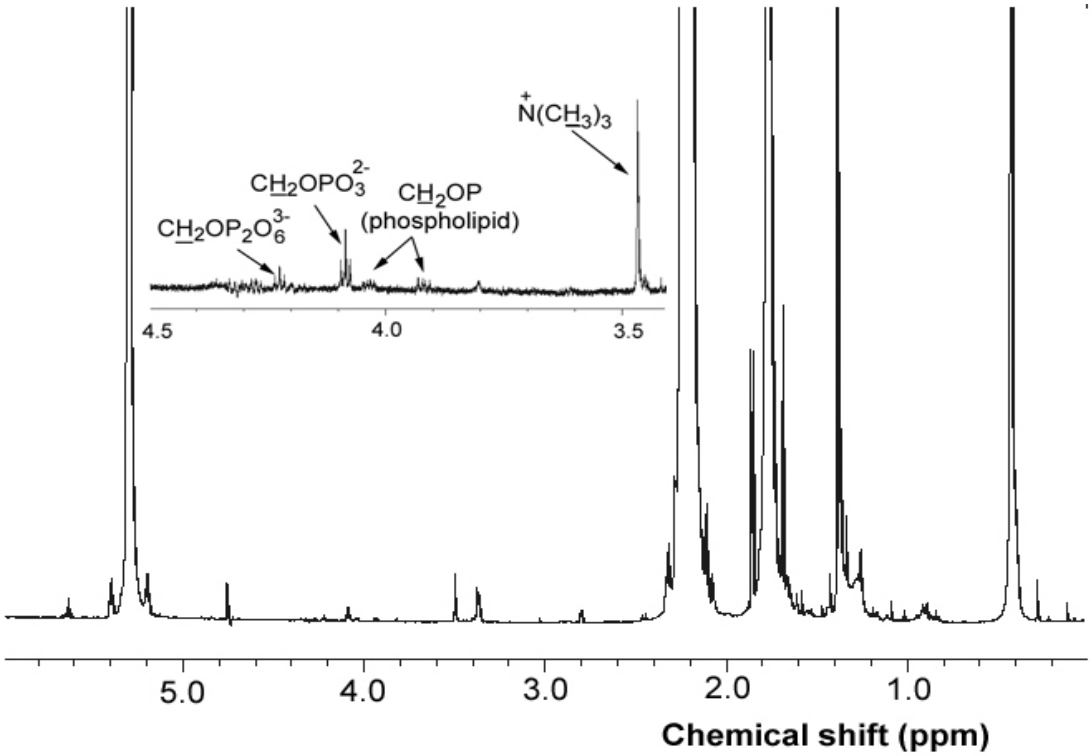
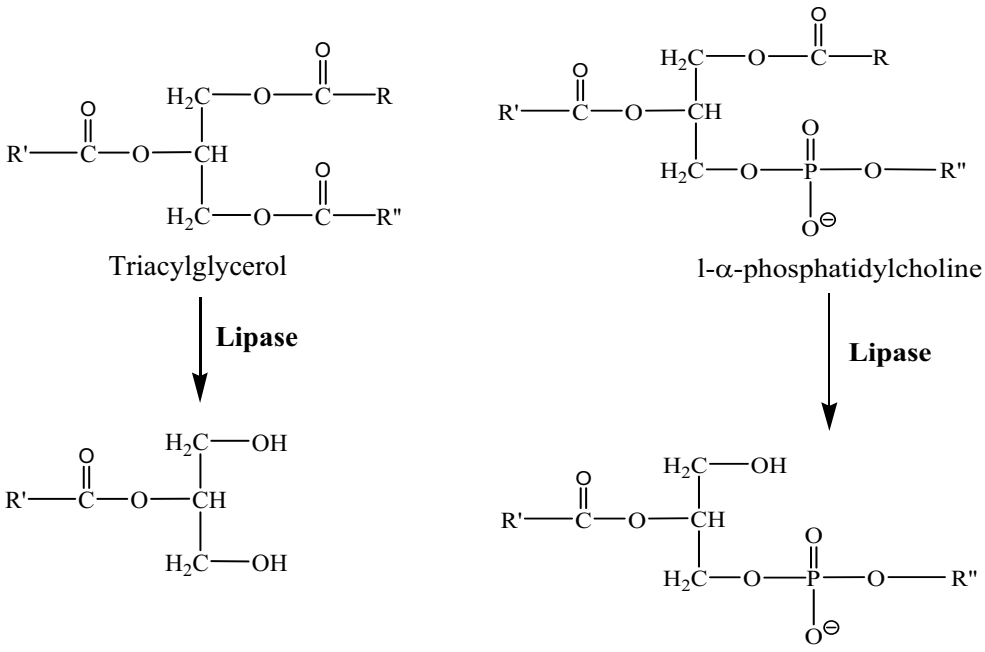


Figure 3.29 ^{1}H-NMR spectrum of DPNR measured in C_6D_6 at 750 MHz.

The proposed structure of acylglycerol and phospholipid i.e. PC after lipase treatment is shown in **Scheme 3.3**.



Scheme 3.3 Proposed structures of triacylglycerol and phospholipid after lipase treatment.

The ^{13}C- and ^{1}H-NMR spectra of lipase treated DPNR are illustrated in **Figures 3.30** and **3.31**, respectively. It can be seen from **Figure 3.30** that the methyl carbon of long-chain fatty acid ester resonating at 14.0 ppm as well as the methine and methylene carbons linked to carboxyl group of phospholipid, which is usually appeared at 63.7 and 64.7 ppm, respectively, disappeared after lipase treatment. In addition, the relative intensity of long-chain methylene carbon signal of fatty acid ester appearing at 29.9 ppm against methylene carbon backbone drastically decreased after lipase treatment compared to DPNR (cf. **Figure 3.28)**. As mentioned above, lipase can decompose selectively fatty acid ester groups of acylglycerol at C1 and C3 positions and of phospholipid at C1 position. The decomposition of acylglycerol structure after lipase treatment is supporting evidence demonstrates the presence of acylglycerol compound in NR. The decomposed glycerol and fatty acid compounds then can be removed by extraction with acetone. However, it is remarkable that the phospholipids or acylglycerol compound cannot be removed completely by considering the residual of long-chain fatty acid esters of about 2 moles/chain after lipase treatment, even the carboxyl carbon signal was absent in ^{13}C-NMR spectrum. The absent of the ^{13}C-NMR signal of carboxyl group might be due to the presence of very small amount of carboxyl compound comparing to the high molecular-weight rubber molecule.

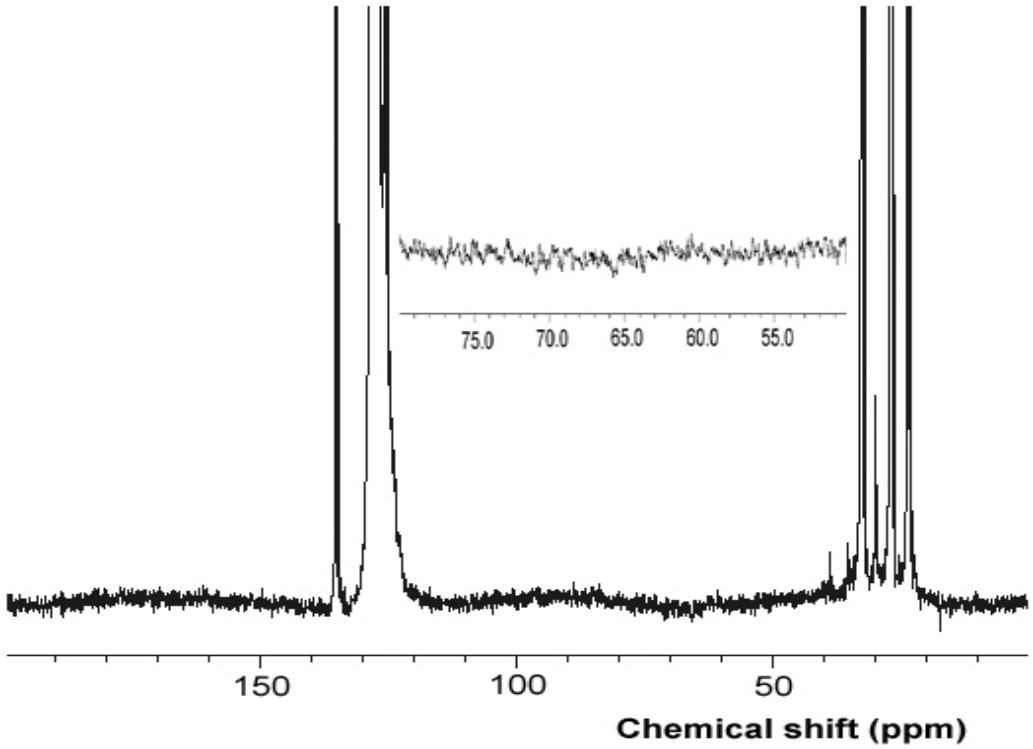


Figure 3.30 ^{13}C-NMR spectrum of DPNR after lipase treatment measured at 188 MHz.

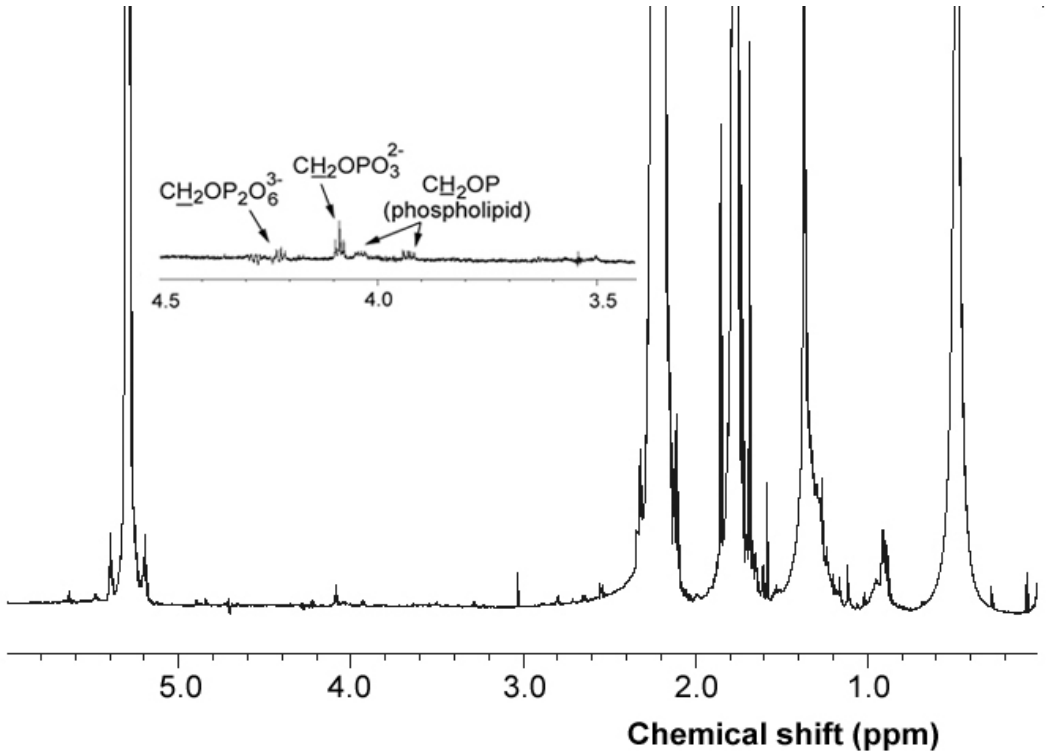


Figure 3.31 ^{1}H-NMR spectrum of DPNR after lipase treatment measured at 750 MHz.

It is remarkable that lipase treated DPNR showed the decrease of relative intensity of the phospholipid signals resonating at 3.93 and 4.04 ppm compared to untreated DPNR (cf. **Figure 3.29**). In addition, the signal due to methyl protons next to nitrogen atom of choline headgroup resonating at 3.49 ppm disappeared after lipase treatment. This demonstrates the presence of acylglycerol compounds in rubber molecule. However, the C4 methylene protons linked to monophosphate and diphosphate were observed at 4.08 and 4.22 ppm, respectively, as shown in **Figure 3.31**. This indicates clearly that phosphate and diphosphate groups were remained even after treatment with lipase. Namely, the acylglycerol compound containing one to three fatty acid ester groups was not directly linked to isoprene unit of rubber molecule, i.e. it may be present in NR as a mixture or aggregated one.

The ^{13}C-NMR spectrum of DPNR treated with phosphatase is shown in **Figure 3.32**. After phosphatase treatment, DPNR showed small signals around 60-70 ppm similar to the untreated DPNR. A small signals resonated at 64.3 ppm was assignable to the methylene carbon next to the carboxyl group of glyceride structure of phospholipid or mono- or diphosphate groups, while the signal appeared at 69.2 ppm was the methine carbon next to the hydroxyl group of phospholipid containing hydroxyl and nitrogeneous groups [106]. This indicates the presence of acylglycerol compound or phospholipids as a component of the α-terminal of rubber chain. Similarly, the ^{1}H-NMR spectrum of phosphatase treated DPNR showed proton signals similar to that of untreated DPNR as shown in **Figure 3.33** (cf. **Figure 3.29)**. The double doublet signals at 3.93 and 4.04 ppm corresponded to the methylene protons linked to phosphate group of a complex phospholipid containing hydroxyl and nitrogenous compounds, while a sharp triplet signal at 4.09 ppm is assignable to the C4 methylene protons of *cis*-isoprene unit next to the phosphate group. The methylene protons linked to the diphosphate group appeared as a triplet signal at 4.22 ppm. The presence of phosphate signals (CH_2OP) in DPNR even after phosphatase treatment points out that the rubber chain has no direct linkage with phosphate group as an ordinary monophosphate ester. Thus, the phosphate group present in the α-terminal is not simple monophosphate and postulated to be a modified one.

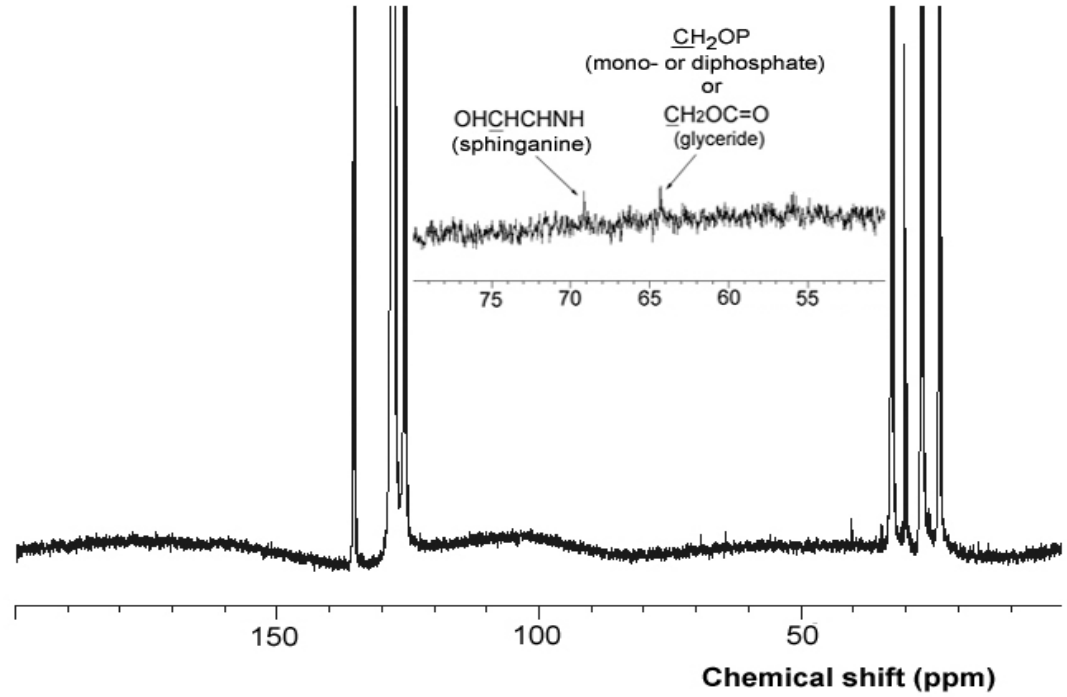


Figure 3.32 ^{13}C-NMR spectrum of DPNR after phosphatase treatment measured at 750 MHz.

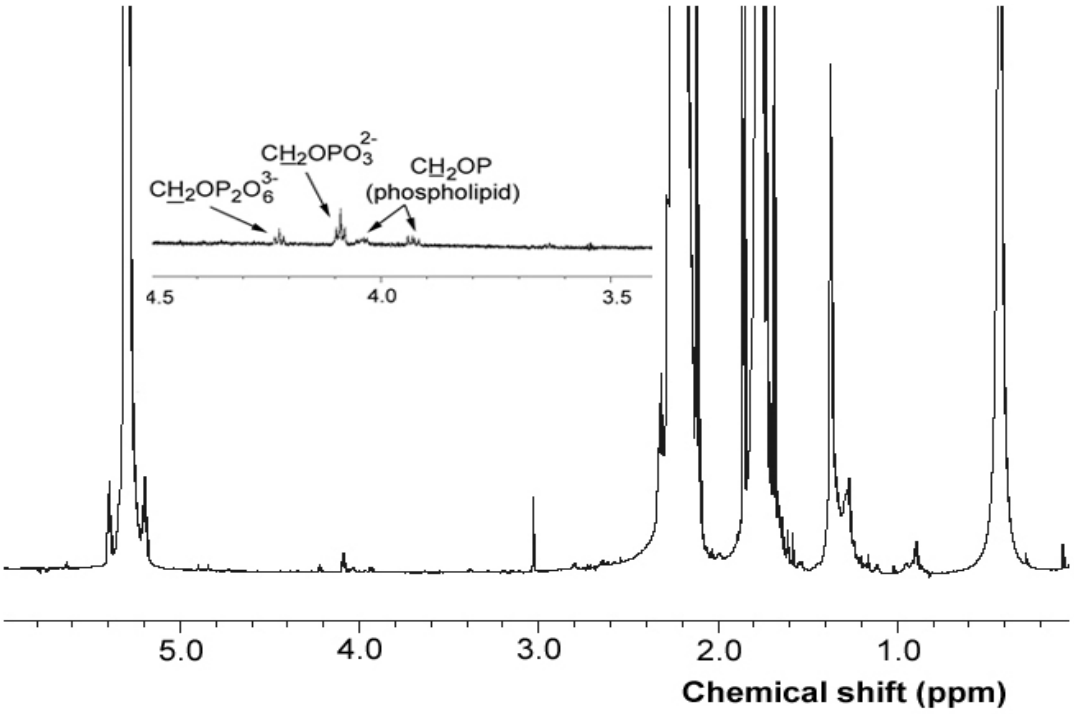


Figure 3.33 ^{1}H-NMR spectrum of DPNR after phosphatase treatment measured at 750 MHz.

Figure 3.34 and **3.35** show the ^{13}C- and ^{1}H-NMR spectra, respectively, of lipase and phosphatase treated DPNR compared to DPNR in the α-terminal regions. The absence of phospholipid signals was observed in the ^{13}C-NMR spectrum of DPNR after the treatment of DPNR latex with lipase, while there was no significant change in the case of phosphatase treated DPNR. The presence of acylglycerol compound or phospholipid in rubber molecule is confirming evidence showing that the decomposition of acylglycerol structure after lipase treatment.

Figure 3.35 provides supporting evidence on the structure of α-terminal group. The ^{1}H-NMR spectrum of lipase treated DPNR showed disappearance of phospholipid signals at about 3.49 ppm, whereas no change was observed for mono- and diphosphate signals. This clearly indicates that mono- and diphosphate groups are directly linked to rubber chain at the α-terminal group, while phospholipids are present in the rubber as a mixture or associated with the α-terminal group. Similarly, phosphatase treatment also gave no significant change in the ^{1}H-NMR spectrum, except for the disappearance of phospholipid signal at 3.49 ppm., By considering the fact that phosphatase decomposes monophosphate ester linkage as mentioned above, the presence of monophosphate signal after phosphatase treatment clearly illustrates that the functional group at rubber chain end or α-terminal group is not a simple monophosphate group, but modified at the hydroxyl group in phosphate molecule.

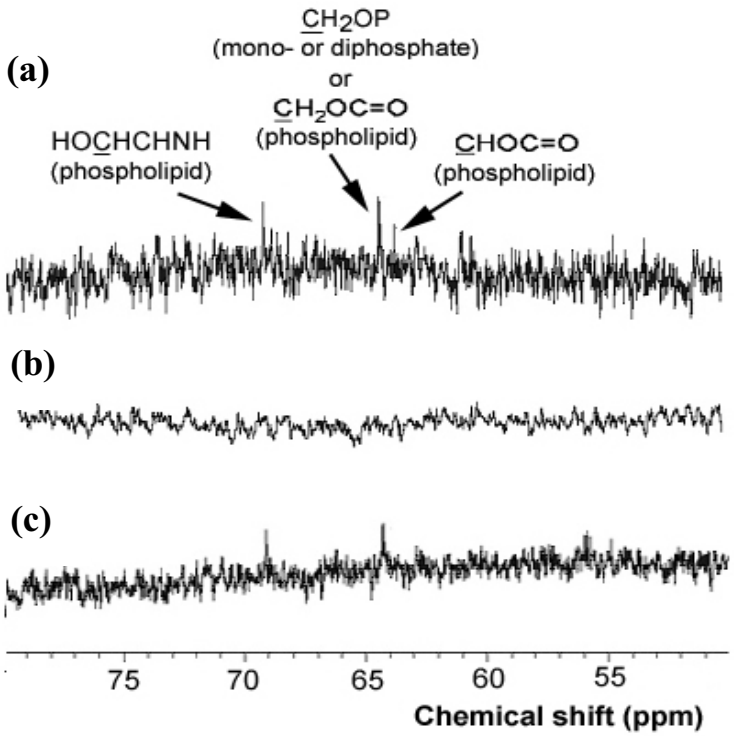


Figure 3.34 ^{13}C-NMR spectra of (a) DPNR, (b) lipase treated DPNR and (c) phosphatase treated DPNR after acetone extraction measured in C_6D_6 at 188 MHz.

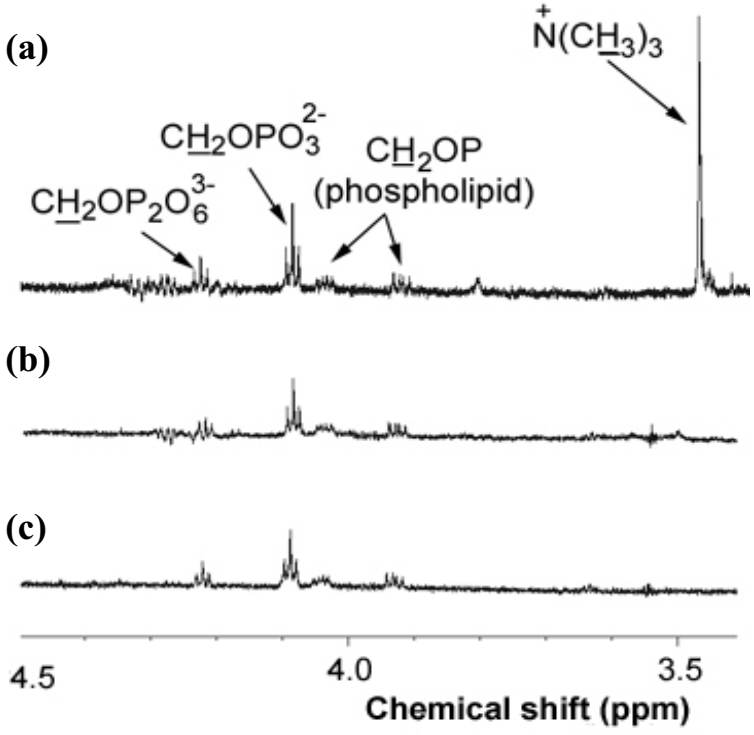
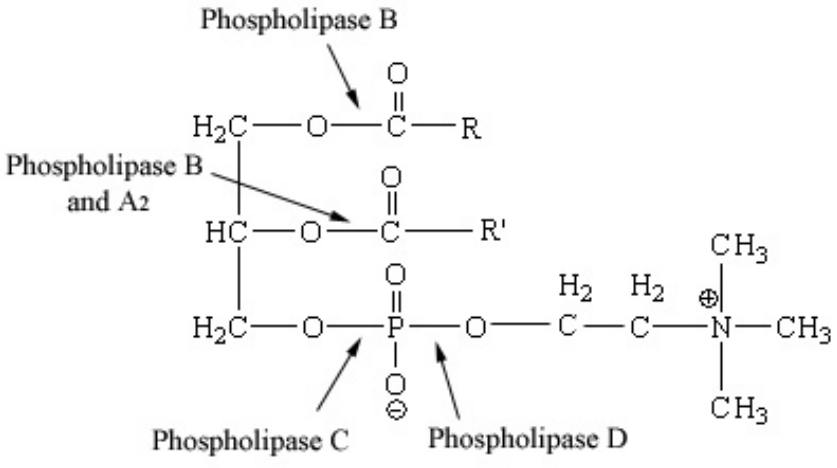


Figure 3.35 ^{1}H-NMR spectra of (a) DPNR, (b) lipase treated DPNR and (c) phosphatase treated DPNR after acetone extraction measured in C_6D_6 at 750 MHz.

3.4.6 Effect of phospholipases on the decomposition of branch-points



Scheme 3.4 Chemical structure of L-α-phosphatidylcholine and reaction site for phospholipase decomposition.

The reaction site of phospholipid that can be hydrolyzed by phospholipases is shown in **Scheme 3.4**. It is well known that phospholipase A_2 hydrolyzes l-α-phosphatidylcholine to give l-α-lyso-phosphatidylcholine and fatty acids, while phospholipase B produces glycerol phosphorylcholine. Water-soluble organic phosphorus and choline are liberated by the treatment of l-α-phosphatidylcholine with phospholipases C and D, respectively.

Figure 3.36 shows the content of long-chain fatty acid ester determined for AE-DPNR after the treatment of DPNR latex with phospholipases A_2, B, C and D. It is clear that all the phospholipases used here decreased the ester content of DPNR, more effectively with increasing the concentration of phospholipases.

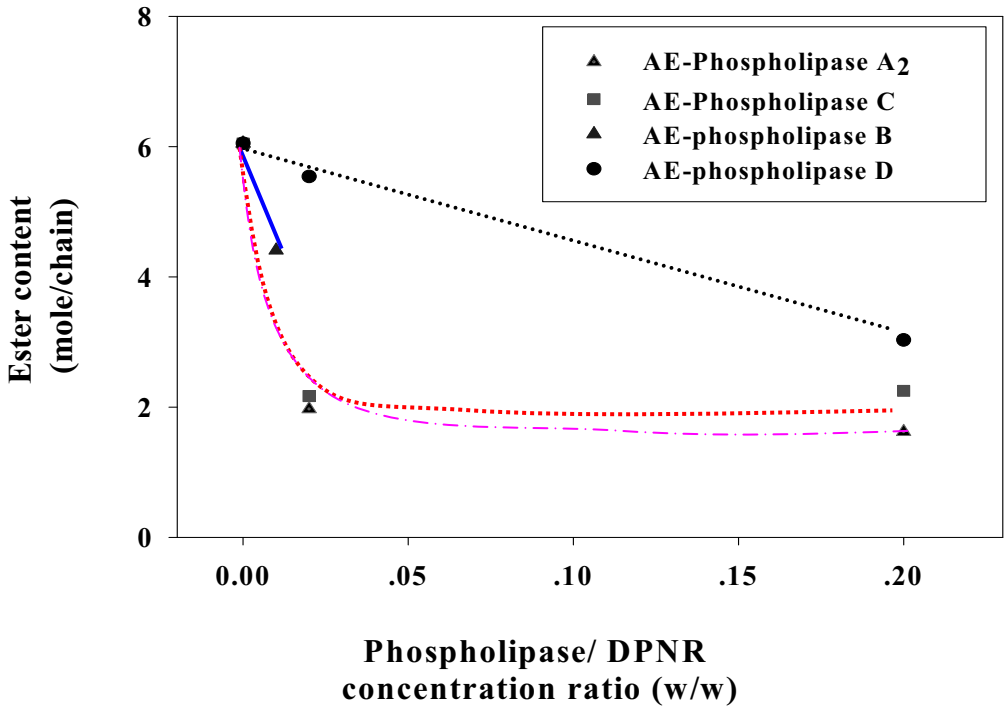


Figure 3.36 Content of long-chain fatty acid ester of acetone-extracted DPNR treated with phospholipases at different phospholipase concentration.

Table 3.5 tabulates the content of long-chain fatty acid ester after treatment with phospholipases, together with molecular weight and [η] values. Phospholipase A_2 can decompose the ester linkage only at the C2 position of glyceride structure (cf. **Scheme 3.4**), while phospholipase B is effective to decompose fatty acid ester linking at both C1 and C2 positions. The phosphate linked to glyceride backbone at the C3 position and choline group in PC can be removed by treatment with phospholipases C and D, respectively. The fatty acid ester content of DPNR decreased significantly from 6.1 to 1.6, 2.2, and 3.0 moles per rubber molecule by treatment with phospholipases A_2, C, and D, respectively, at phospholipases concentration ratio of 1:5 against rubber weight. On the other hand, the treatment with phospholipase B showed a slightly decrease to 4.4 moles per rubber molecule at phospholipase:DPNR of 1:100 by weight. This might be due to the use of less amount of phospholipase B.

It is remarkable that the treatment with phospholipases A_2, B, C and D decreased the content of long-chain fatty acid in DPNR. Ordinarily, it is difficult to

expect the decrease of fatty acid ester content by treatment with phospholipases C and D, because they hydrolyze only phosphate and choline linkages, respectively, in a phospholipid, and cannot decompose fatty acid ester linkages in it. The decrease of fatty acid ester content can be interpreted by considering the solubility of lipids after treatment with phospholipases C and D. The hydrolysis of polar group in the phospholipid will decompose a micelle structure, which is presumed to be branch-points of DPNR formed at the α-terminal. This assumption is the same as that mentioned above for the decomposition of branch-points by lipase treatment.

The treatment of DPNR latex with phospholipases A_2, B and C decreased significantly M_n and [η] values to about one third to one half from the original values, whereas no change was observed in the case of treatment with phospholipase D. This supports the idea that most parts of branch-points are originating from fatty acid groups at the C1, C2 and phosphate group at the C3 in phospholipid molecule by the formation of a micelle structure. It is remarkable that the treatment of DPNR latex with phospholipase D substantially decreased the ester content, while only slight decreases was observed in the molecular weight and [η] values were observed. This suggests that choline group in the phospholipid was not participated in branching formation.

It is remarkable in **Table 3.5** that the *k'* value of DPNR is higher than those of DPNR treated with phospholipases B and C, while that of DPNR treated with phospholipase D is almost the same as the original DPNR. Taking into account the decreases in molecular weight and [η] values, it is clear that some parts of branch-points of DPNR were decomposed by treatment with phospholipases B and C, while phospholipase D did not decompose the branch-points. It is interesting to compare the effect of phospholipases C and D with phosphatase on the branch-points and ester content. The reaction of phospholipases C and D is restricted to the phosphate and choline linkages in phospholipids, whereas phosphatase, that is phosphoric monoester hydrolase, hydrolyzes monophosphate ester. Therefore, it is reasonable to assume that phosphatase decomposes the linkage between the phospholipids such as phosphatidic acid *via* hydrogen bonding, while phospholipases C and D decomposes micelle structure of phospholipids.

Table 3.5 Structural characteristics of phospholipases treated DPNR after acetone extraction.

Characteristic	AE-DPNR	Concentration ratio of phospholipase:DPNR (w/w)						
		A_2		B	C		D	
		1:50	1:5	1:100	1:50	1:5	1:50	1:5
Ester content (mole/chain)	31.3 (6.05)*	34.9 (1.97)	25.5 (1.62)	28.8 (4.41)	21.9 (2.17)	21.2 (2.25)	30.4 (5.54)	17.7 (3.03)
$\overline{M}_n$ ($\times 10^5$)	1.93	0.56	0.63	1.53	0.99	1.06	1.82	1.72
$\overline{M}_w$ ($\times 10^5$)	4.34	4.29	5.21	3.88	5.22	4.41	8.35	7.40
$\overline{M}_w/\overline{M}_n$	3.84	7.62	8.19	2.52	4.43	4.94	4.60	4.31
$[\eta]$	5.12	-	-	3.33	3.25	3.40	4.28	4.00
$\overline{M}_v$ ($\times 10^5$)	7.92	-	-	4.32	4.17	4.45	6.15	5.59
k'	0.47	-	-	0.19	0.25	0.29	0.42	0.47

Note: The value in parentheses is the ester content in mol/chain unit

It is established that NR is composed of long-chain branched molecules [71, 72]. It is also well known that NR shows the bimodal MWD with high and low molecular-weight peaks from 1.0×10^6 to 2.5×10^6 and from 1.0×10^5 to 2.0×10^5, respectively [79, 113, 114]. The bimodal MWD was confirmed not to be due to long-chain branching based on the fact that it remains unchanged even after decomposition of all the branch-points by deproteinization followed by transesterification [61].

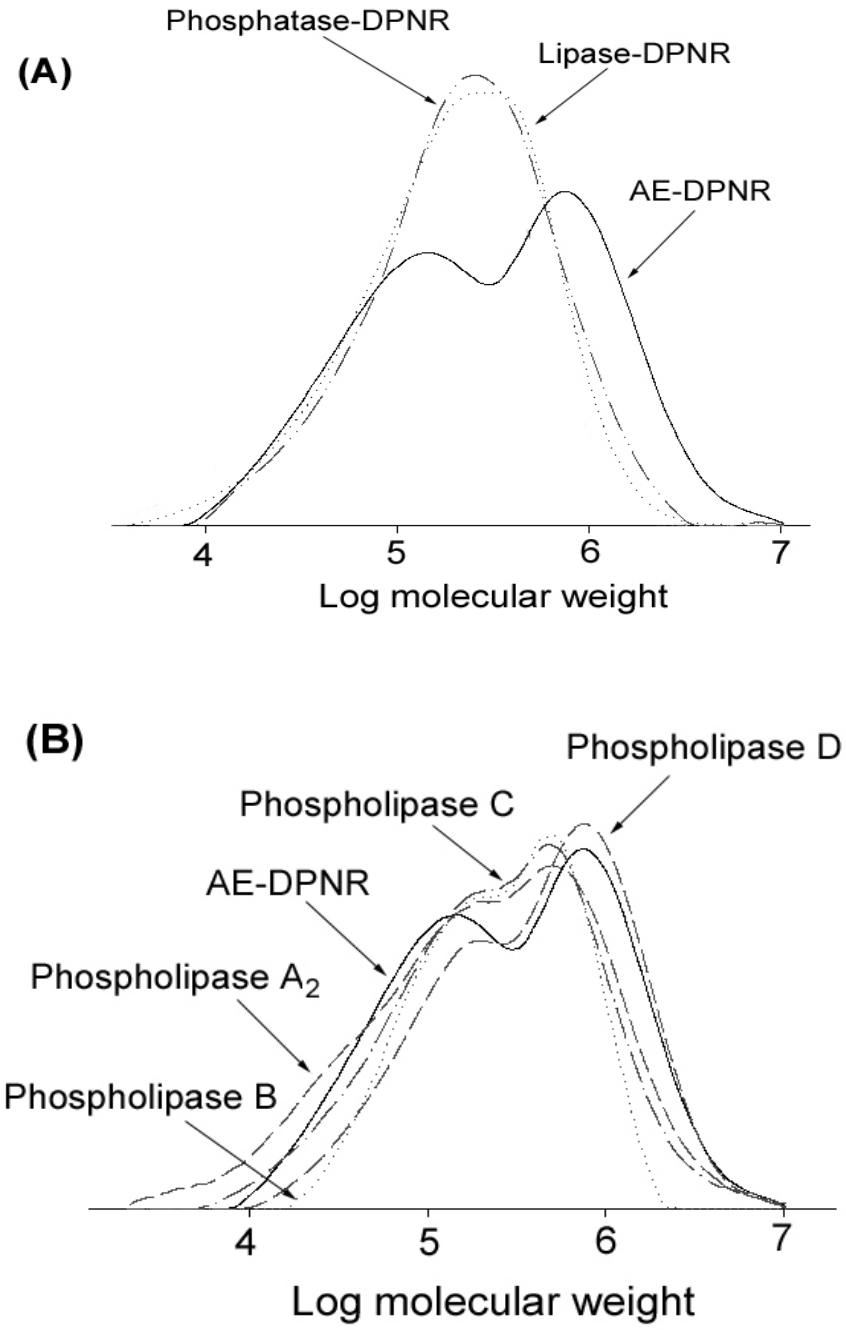


Figure 3.37 Molecular-weight distribution of acetone-extracted DPNR before and after treatment with (a) lipase and phosphatase and (b) phospholipases.

Figure 3.37 shows MWD of DPNR, after treatment with lipase, phosphatase, and phospholipases A_2, B, C and D. The bimodal MWD of DPNR was not changed in the case of treatment with phospholipase D. On the other hand, the treatment with phospholipases A_2, B, and C resulted in the apparent shift of the high molecular-weight fraction peak to low molecular-weight (cf. **Figure 3.37b**). This tendency is more clearly observed in the case of treatment with lipase and phosphatase (cf. **Figure 3.37a**). The high molecular-weight peak clearly shifted to low molecular-weight and overlapped with the peak of low molecular-weight fraction. Apparent change of MWD by the treatment with phosphatase indicates the presence of the monophosphate ester compounds in DPNR, in addition to phospholipids. This assumption can be supported by the fact that it cannot be decomposed with phospholipases C. The shift of high molecular-weight fraction peak to lower one after lipase and phosphatase treatment is presumed to be due to the decomposition of branched-point to form linear molecules. These findings are strong supporting evidence showing that branching formation is participated by fatty acid ester group in phospholipid and phosphate group in monophosphate ester compound.

Table 3.6 Effect of treatment of lipase, phosphatase and phospholipases on the structure of DPNR

Enzyme	Reaction position	Activity for lipids		Change of DPNR			
		Triglycerol	Phospholipid	MW[*a]	MWD[*b]	Ester content[*c]	k' [*d]
Lipase	$C_1 + C_3$	+	C_1 only	0.64	Uni	16.2 (1.6)	0.23
Phosphatase	C–O-P	–	–	0.52	Uni	18.5 (2.1)	0.35
Phospholipase A_2	C_1	+	+	-	Bi	25.5 (1.6)	-
B	$C_1 + C_2$	+	+	0.55	Bi	21.9 (4.4)	0.19
C	O–P-O	–	+	0.56	Bi	21.2 (2.2)	0.29
D	O-P–OX	–	+	0.71	Bi	17.7 (3.0)	0.47

[*a] Decrease of $\bar{M}_v$ given by % against DPNR (DPNR: $\bar{M}_v = 7.92 \times 10^5$)

[*b] Bi and Uni: Bimodal and unimodal molecular weight distribution

[*c] Ester content mmol/kg rubber and mol/rubber molecule in parentheses (DPNR: 31.3 (6.1))

[*d] Huggins' constant (DPNR: $k' = 0.47$)

Table 3.6 summarizes the structural change of DPNR treated with lipase, phosphatase and phospholipases, in connection with the reaction site of these enzymes for triacylglycerol and phospholipid. In the above discussion, it was postulated that the α-terminal of rubber molecule is composed of both mono and diphosphate groups. Diphosphate groups present in NR cannot be removed by phosphatase by considering the fact that phosphatase has an efficiency to decompose only monophosphate ester linkage. It is interesting that the monophosphate group present in DPNR cannot be decomposed by treatment with phosphatase. This suggests that the phosphate group in DPNR is not simple phosphate, but having modified structure.

The formation of branch-points is presumed to be originated from the micelle formation and hydrogen bonding of phospholipids and/or phosphate group in DPNR. Here, it is necessary to consider a linkage to hold together two or more rubber chains at the α-terminal. Formation of oligomeric phosphate can be considered, although no report can be found for oligomerization of phosphates. In this case, however, it is difficult to expect the linkage between the presumed phospholipids and rubber chain. It was confirmed by ^{13}C-NMR analysis that even the high molecular-weight fraction fractionated by GPC using chloroform contained the long-chain ester group, as discussed in the above section. This indicates that the presumed phospholipids are mostly linked or associated to rubber chain. Another possibility is the formation of ionic bonds between the α-terminal group, expecting a phosphate group and phospholipids. It was found that Mg^{2+} ion in latex acts to form branch-points during storage of FL-latex in the presence of ammonia. If Mg^{2+} ion links the phosphates at the α-terminal group of DPNR and phospholipids, a single rubber chain can contain phospholipid and phosphate groups. This assumption is advantageous for the explanation of reasons why a single rubber chain contains about two ester groups per chain.

3.4.7 ^{13}C- and ^{1}H-NMR measurements of DPNR after phospholipase treatment

The ^{13}C- and ^{1}H-NMR spectra of DPNR after phospholipase B treatment are shown in **Figures 3.38** and **3.39**, respectively. As mentioned above, phospholipase B

is known to be a kind of enzyme which can decompose fatty acid ester group at the C1 and C2 positions of phospholipid molecule. The ^{13}C-NMR spectrum of DPNR treated with phosphalipase B showed a signal due to the methylene carbon linked to carboxyl group and/or the methylene carbon next to phosphate group of monophosphate group at 64.2 ppm, while the signal at 63.7 ppm, assignable to the methine carbon of glyceride backbone, disappeared. This indicates that some fatty acid ester groups in the phospholipids were decomposed by phospholipase B, although the removal was not perfect due to the low concentration of phospholipase B. It is remarkable that the ^{1}H-NMR spectrum of DPNR showed insignificant change after treatment with phospholipase B. It shows the small signals at 3.92 and 4.04 ppm due to the non-equivalent methylene protons linked to the phosphate group of phospholipid as well as the C-4 mehylene proton linked to mono- and diphosphate groups at 4.09 and 4.22 ppm, respectively. However, the signal due to the methyl proton linked to nitrogen of choline head group disappeared after phospholipase B treatment. This indicates that the removal of fatty acid ester groups by phospholipase B results in the decomposition of phospholipid associated to the α-terminal phosphate group.

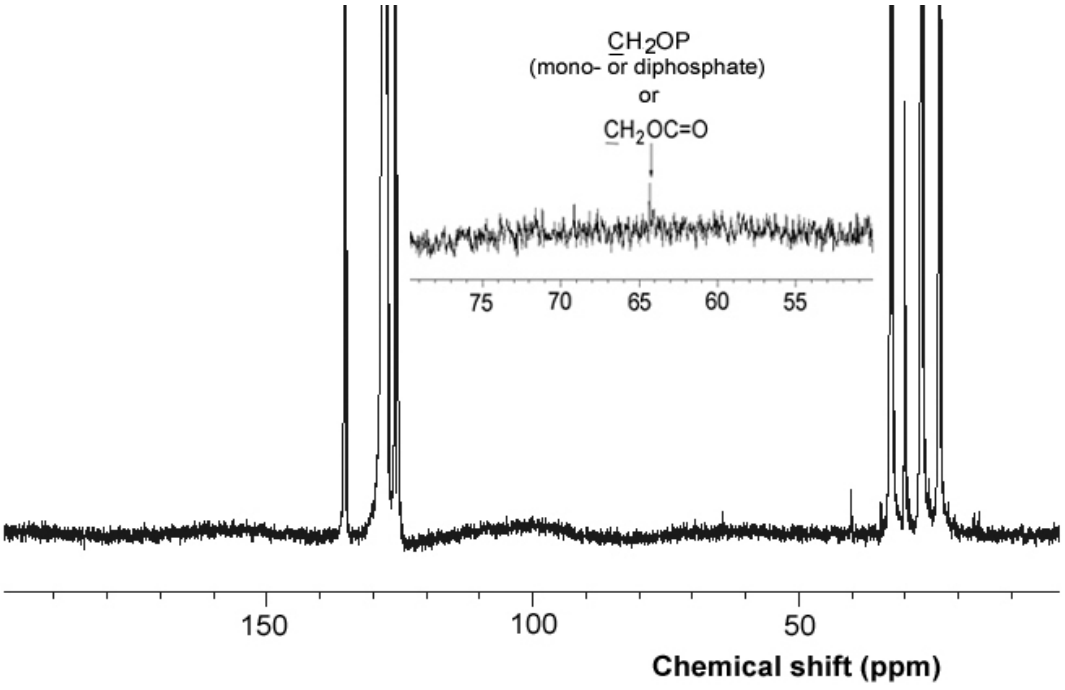


Figure 3.38 ^{13}C-NMR spectrum of DPNR after phospholipase B treatment, measured at 188 MHz in C_6D_6.

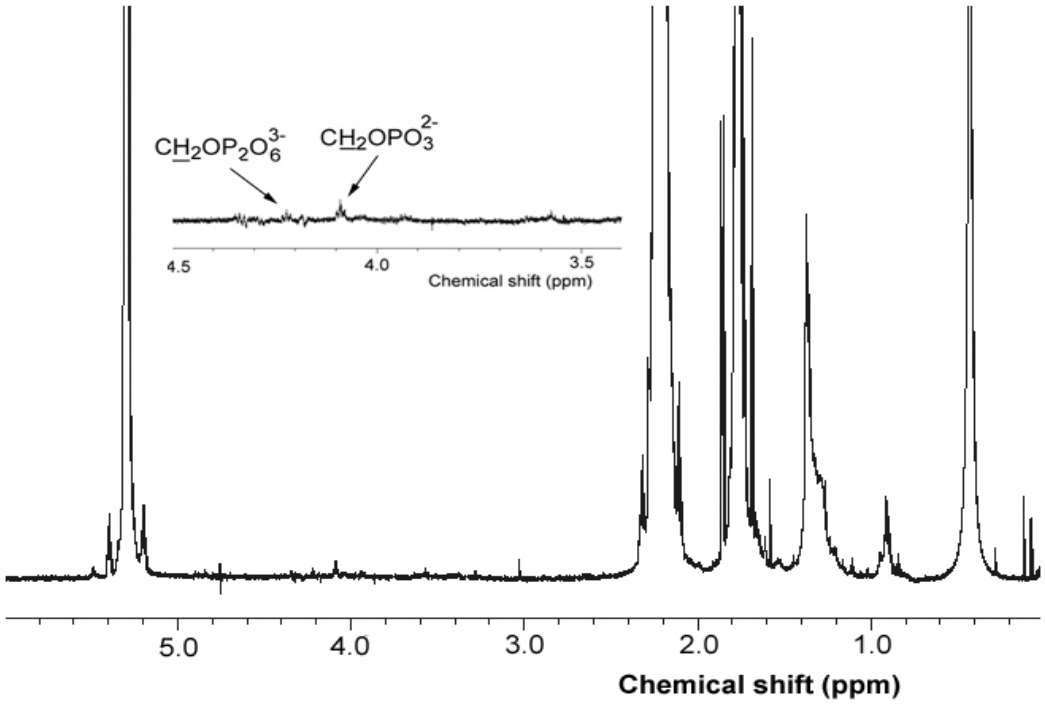


Figure 3.39 ^{1}H-NMR spectrum of DPNR after phospholipase B treatment, measured at 750 MHz in C_6D_6.

It is obvious that phospholipase C decomposes phosphate ester bond linked to glyceride backbone of phospholipid and form primary alcohol (-CH_2OH) at the C3 position. The existance of this hydroxyl group in glyceride structure was confirmed by the presence of small ^{13}C-NMR signal due to methylene carbon next to hydroxyl group appearing at 59.6 ppm, as shown in **Figure 3.40**. It is interesting that the methine carbon linked to hydroxyl group in phospholipid resonating at 69.2 ppm was absent in DPNR rubber after phospholipase C treatment. It can be deduced from this result that decomposition of phosphate group enhances the solubility of phospholipid by the loss of some interactions between phosphate groups. Then, decomposed phospholipid molecules were removed by acetone extraction. The removal of phospholipids was further confirmed by ^{1}H-NMR analysis. However, the small signal at 64.7 ppm still remained after phospholipase C treatment. As mentioned thus far, the signal at 64.7 ppm can be assigned to the signal corresponding to phospholipid and/or also methylene carbon next to phosphate group of mono- or diphosphate. The presence of this signal after the removal phospholipid by phospholipase C treatment indicates the presence of mono- or diphosphate group at the α-terminal.

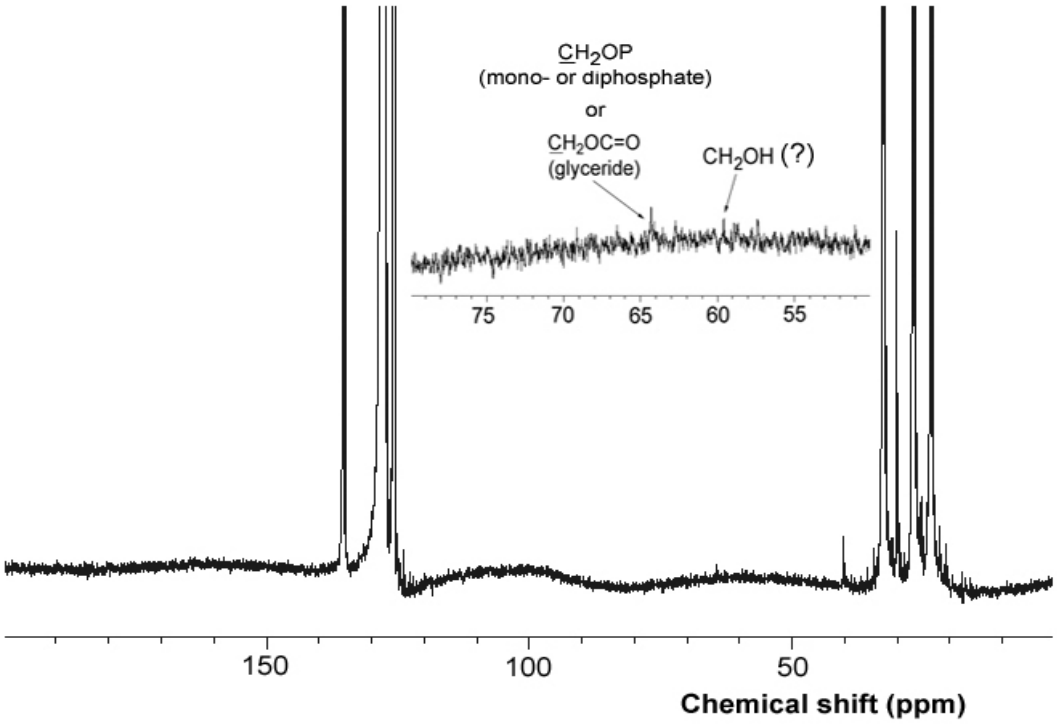


Figure 3.40 ^{13}C-NMR spectrum of DPNR after phospholipase C treatment at 188 MHz in C_6D_6

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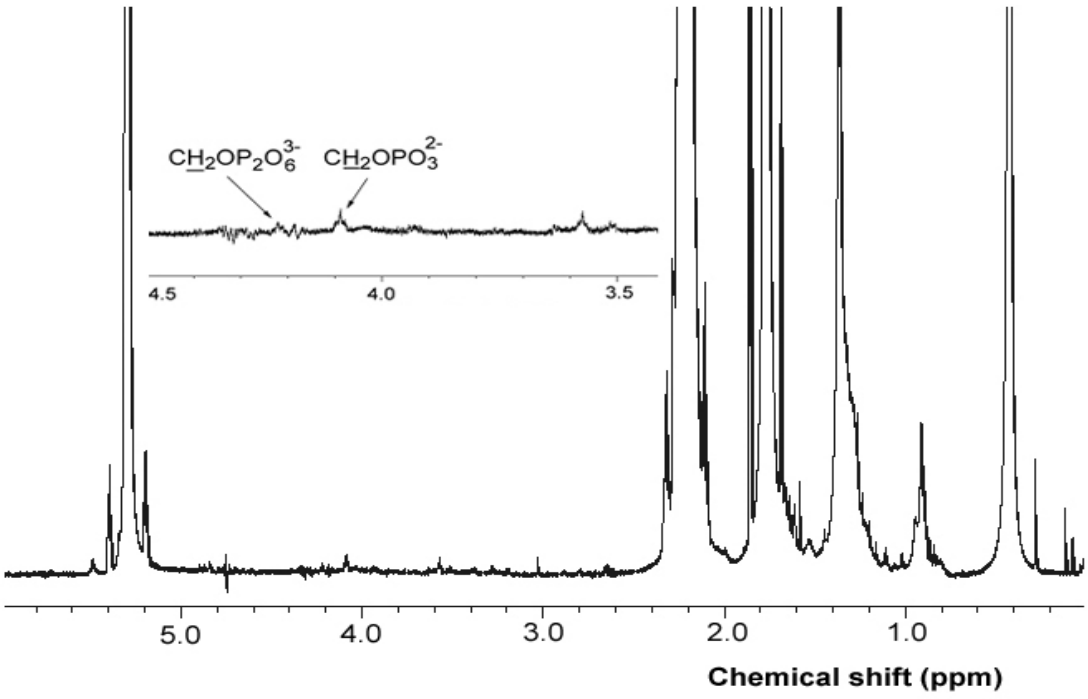


Figure 3.41 ^{1}H-NMR spectrum of DPNR after phospholipase C treatment at 750 MHz in C_6D_6.

The ^{1}H-NMR spectrum of DPNR after phospholipase C treatment is shown in **Figure 3.41**. The phospholipase C treatment resulted in the removal of signals at 3.92 and 4.04 ppm, which were assignable to the non-equivalent methylene protons linked to phosphate group of phospholipid, while it remained the signals due to the methylene protons linked to mono- and diphosphate groups. These findings support the idea mentioned above that phospholipid was not directly linked to rubber chain-end at the α-terminal group as mentioned above. The α-terminal group of rubber molecule is then expected to link directly to mono- or diphosphate groups. The phospholipids existing as free molecules are postulated to play an important role on the branching formation of NR. The branching formed by phospholipids will be discussed afterward.

Figure 3.42 and 3.43 show the ^{13}C- and ^{1}H-NMR spectra of phospholipase B and C treated DPNR comparing with the untreated AE-DPNR**,** respectively. The absence of phospholipid signals in DPNR treated with phospholipase B and C, both in ^{13}C- and ^{1}H-NMR spectra, clearly indicate the decomposition of phospholipids in rubber molecule. However, the signal due to the mono- and diphosphate groups still remained even after phospholipase B and C treatment. This clearly demonstrates that rubber molecule is linked to mono- and diphosphate groups at the α-terminal group, while phospholipids present only as a mixture and participated into the branching formation, expecting *via* micelle formation.

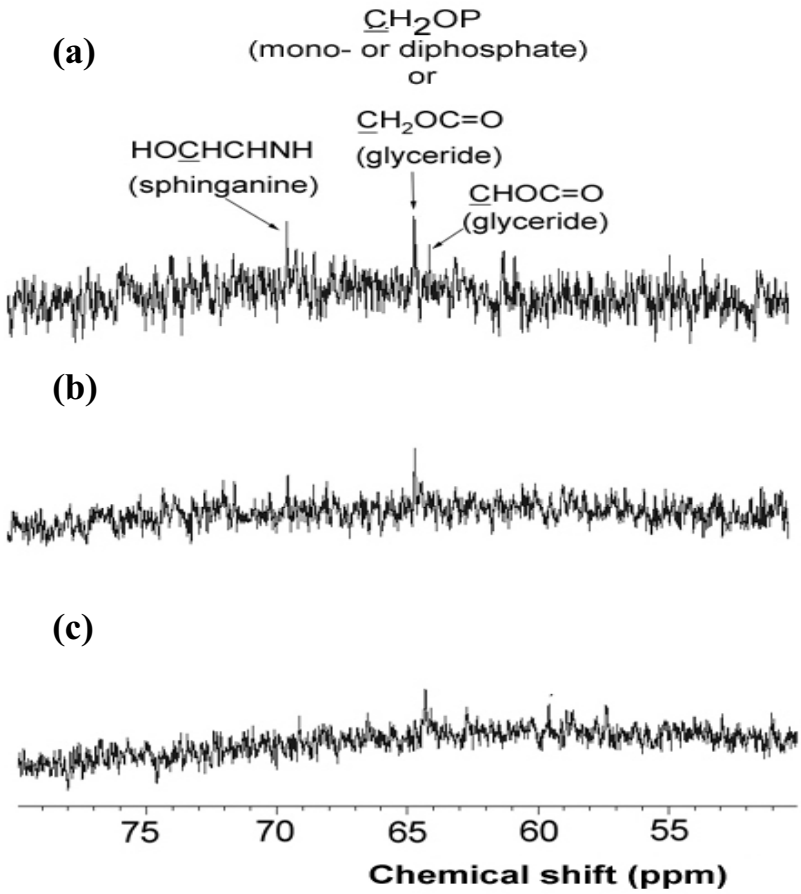


Figure 3.42 ^{13}C-NMR spectra of (a) DPNR, (b) phospholipase B treated DPNR and (c) phospholipase C treated DPNR after acetone extraction measured in C_6D_6 at 188 MHz.

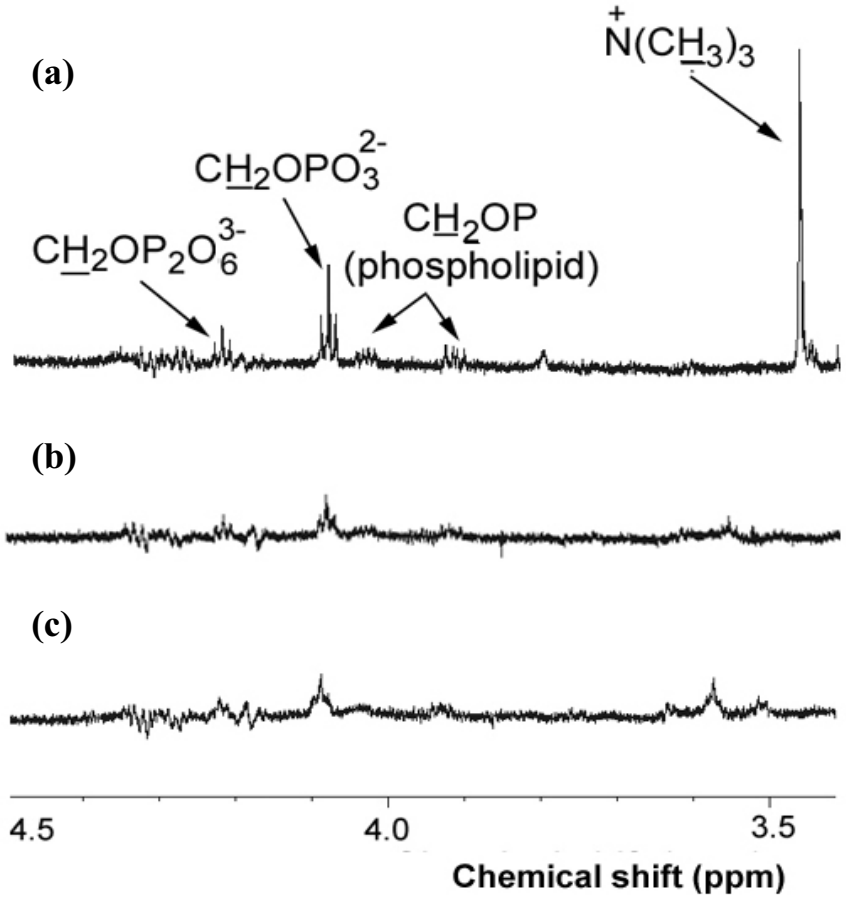


Figure 3.43 ^{1}H-NMR spectra of (a) DPNR, (b) phospholipase B treated DPNR and (c) phospholipase C treated DPNR after acetone extraction measured in C_6D_6 at 750 MHz.

3.4.8 Analysis of phospholipids in natural rubber by chemical treatment

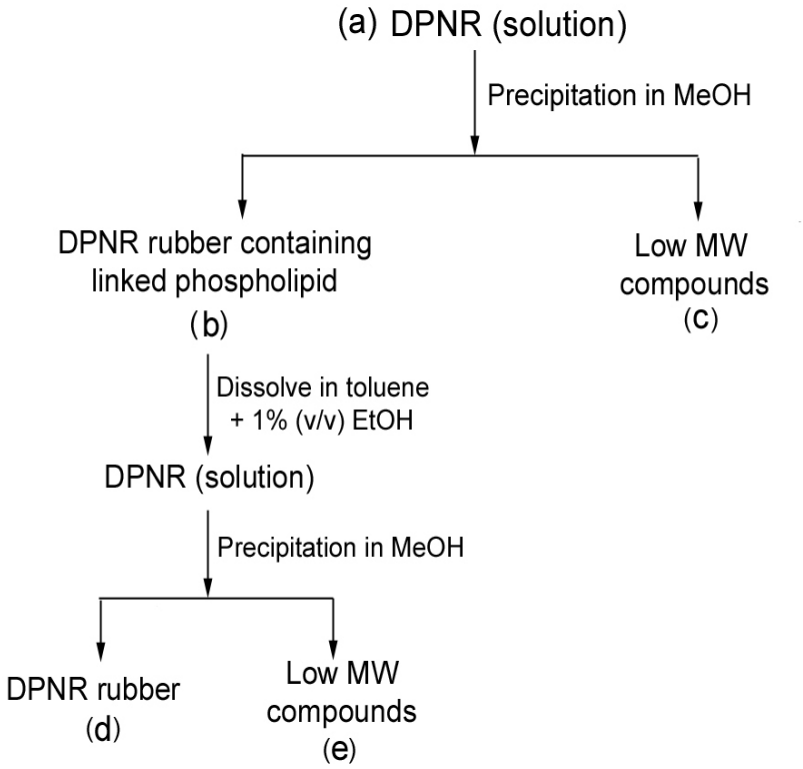
As mentioned above, the results of ^{1}H- and ^{13}C-NMR analysis of DPNR showed the presence of monophosphate and diphosphate groups as well as phospholipids. These functional groups still remained in DPNR even after treatment with lipase, phosphatase and phospholipases. This implies existence of some strong interactions among phosphate groups and phospholipid. At present, it can be postulated that phospholipid linked to phosphate group at the α-terminal *via* hydrogen bonding. The detailed analyses by chemical treatment of branching formation by hydrogen bonding will provide supporting evidence for this assumption. The presumed hydrogen bonding between phospholipids and/or phosphate groups is

expected to be decomposed by the addition of small amount of polar solvent such as ethanol into DPNR in toluene solution, leading to the dissociation of aggregated phospholipids into individual molecules. The dissociated phospholipid molecules then are expected to be separated from DPNR by precipitation of the rubber solution into excess amounts of methanol. The free phospholipid was then expected to change the conformation to micellar structure in methanol, as illustrated in **Table 3.7**. However, some dissociated phospholipids were expected to change the conformation to micellar strcuture in methanol [115] and might not be removed completely by ordinary precipitation. Thus, the sucessive re-precipitation process of rubber solution containing 1% (v/v) ethanol into methanol is one of the possibility to remove phospholipids from rubber chain.. The separation procedure carried out in this experiment is shown in **Scheme 3.5**.

Table 3.7 Solubility of phospholipids in polar and non polar-solvent as well as in rubber fraction

Fractions	**Single phospholipid molecule**	**Aggreated phospholipids**
Rubber phase	-	-
Toluene	-	- (inverse micelle)
Methanol	-	- (micelle)
Toluene/methanol	++	+

- denotes low solubility.
+ denotes partial soluble.
++ denotes high solubility.



Scheme 3.5 Decomposition of hydrogen bonding in DPNR in toluene solution by addition of 1% (v/v) ethanol.

The ^{13}C-NMR spectrum of low molecular-weight rubber fraction obtained from the toluene/methanol fraction of re-precipitation of DPNR solution containing 1% (v/v) ethanol, (d), is shown in **Figure 3.44**. The signals appeared at 64.3 and 71.1 ppm are assigned to the methylene carbons of glyceride structure linked to carboxyl and phosphate groups, respectively. The methylene carbon next to hydroxyl group of sphinganine structure of phosphosphingolipid appeared at 69.14 ppm. The presence of phospholipid signals in ^{13}C-NMR spectrum even after the addition of ethanol indicates that phospholipid micelles cannot be decompose perfectly. It has been reported that divalent metal cation such as Ca^{2+} and Mg^{2+} interact to negatively charged phospholipids such as diacyl phosphatidylserines (PS), diacylphosphatidyl glycerol (PG) and cardiolipin (CL) as well as of lipids with the PA head group [116]. Ca^{2+} induces the decompostion of hydrogen bonding of the phosphate groups of bovine brain PS [117]. The binding behavior of Mg^{2+} to PS bilayers was found to be similar to that of Ca^{2+} [118]. Based on these studies, it can be deduced that the residual

phospholipids might be linked to phosphate group at the α-terminal of rubber chain by ionic linkage such as Mg^{2+} ions. At present, it is postulated that the α-terminal of rubber molecule is composed of the terminal isoprene units linked directly to monophosphate and diphosphate groups, which might be linked to phospholipids *via* H-bonding or Mg^{2+} ions.

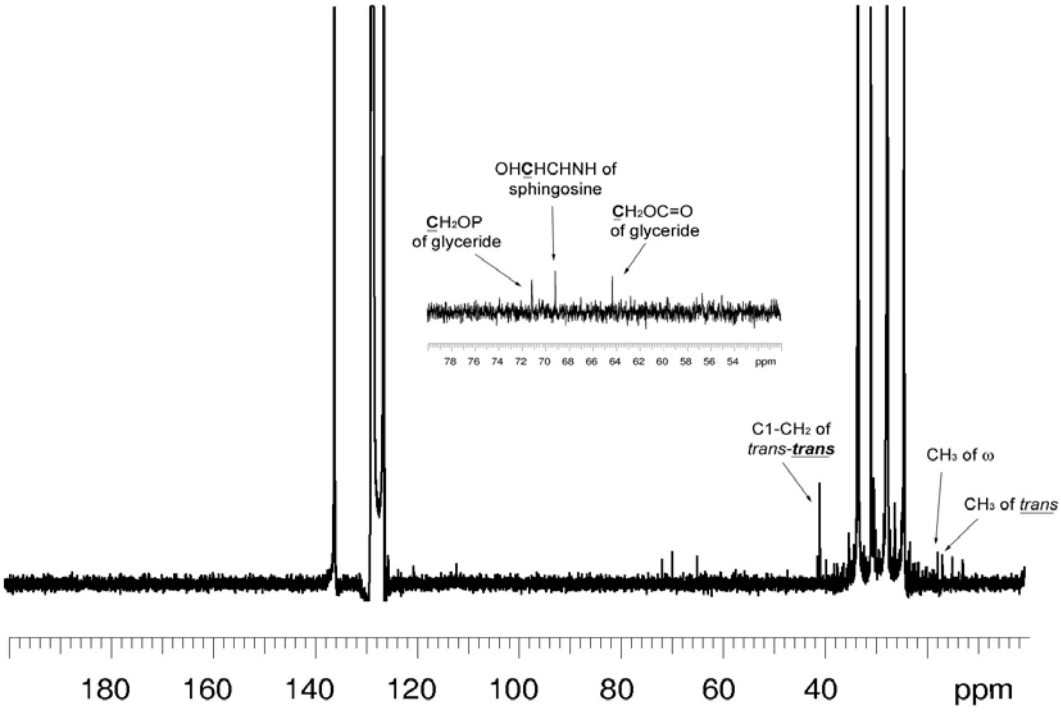


Figure 3.44 ^{13}C-NMR spectrum of low molecular-weight DPNR obtained from re-precipitation of DPNR solution including 1% (v/v) ethanol, measured at 188 MHz in C_6D_6.

If the hydrogen bonding is the origin of branching formation in NR, the addition of ethanol into rubber solution should lead to the removal of aggregated phospholipids as to free phospholipid molecules, which are expected to be separatable from rubber by GPC. DPNR was dissolved in toluene solution containing 1% (v/v) ethanol and precipitated into excess amount of methanol. The low molecular-weight compound in toluene/methanol fraction, (e, cf. **Scheme 3.5**) was subjected to GPC analysis using low exclusion limit GPC column using chloroform containing 1% (v/v) ethanol as eluent. The GPC curve of this low molecular-weight fraction was compared

with that prepared without the addition of 1% ethanol (v/v) in toluene solution (c, cf. **Scheme 3.5**) together with that of high purity PC as a phospholipid standard are shown in **Figure 3.45**.

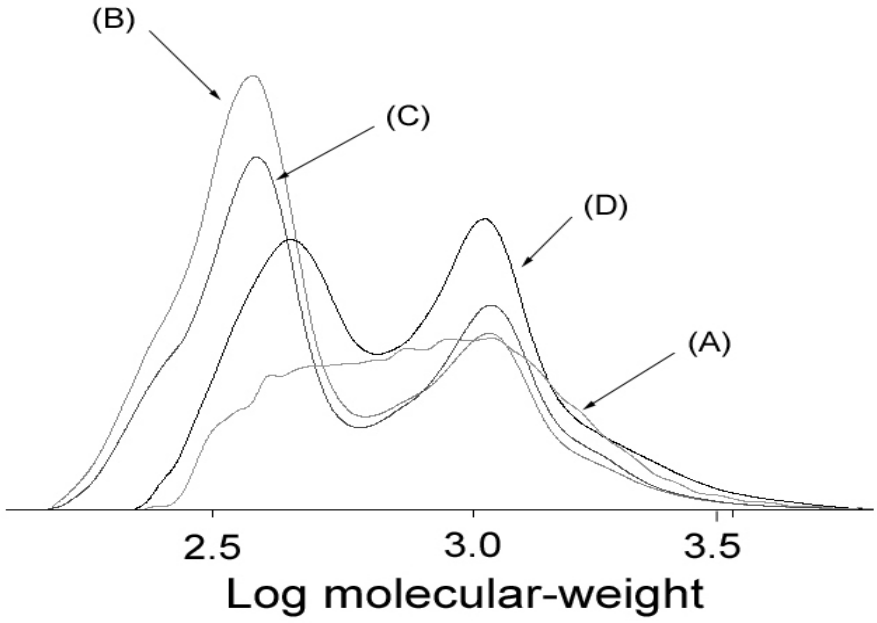


Figure 3.45 Molecular-weight distribution of (A) L-α-phosphatidylcholine and (B-D) low molecular-weight compound in toluene/methanol fraction obtained from precipitation of DPNR solution: (B) precipitation from toluene solution, (C) precipitation from toluene solution containing 1% (v/v) ethanol, and (D) re-precipitation from toluene solution containing 1% (v/v) ethanol.

PC showed a broad distribution in the molecular weight region 5.0×10^2 to 7.0×10^3, while the low molecular-weight compound gave a clear bimodal distribution in a similar molecular weight region as PC. The broad molecular distribution of PC is due to the aggregation of PC, partly decomposed by ethanol in chloroform. Contrary to PC, the bimodal MWD was observed for all the low molecular-weight compounds even after the addition of ethanol in toluene solution. In this GPC, the $\overline{M}_n$ value of the high molecular-weight fraction was esimated to be 1.1×10^2, while that of low molecular-weight one was about 5.0×10^2. The high molecular-weight fraction in this bimodal distribution is postulated to be derived from the small aggregation or association of phospholipid molecules, by considering the molecular weight of high molecular-weight fraction. It is remarkable that the peak-top value of the high

molecular-weight fraction in this bimodal MWD was about 1.1×10^2, which corresponds to two phospholipid molecules linked together. The highest molecular-weight in the high molecular-weight fraction was about 5×10^3, expecting to be derived from the formation of inverse micelle structure of five to ten phospholipid molecules. This assumption is based on the fact that phospholipid can form inverse micelle structure in organic solvent.

It is remarkable that low molecular-weight compound showed almost the same MWD with or without the addition of 1% (v/v) ethanol into DPNR toluene solution as can be seen in (C) and (B) in **Figure 3.45**, respectively. This clearly indicates that free phospholipids and small aggregated one can be removed from DPNR by GPC in chloroform without the addition of ethanol. DPNR was recovered after precipitation with toluene solution into methanol and re-precipitated again from toluene solution containing 1% (v/v) ethanol. The low molecular-weight compounds obtained from this re-precipitation process (D) showed the bimodal MWD rich in high molecular-weight fraction. This low molecular-weight compound corresponds to linked or associated phospholipids to rubber chain after removal of free phospholipids by precipitation. The low molecular-weight compound obtained from precipitation of rubber solution containing 1% (v/v) ethanol (C) showed the MWD rich in low molecular-weight fraction, while that of rubber obtained from re-precipitation (D) was shifted to higher molecular-weight fraction. As mentioned above, the low molecular-weight fraction corresponds to single phospholipid molecules, while that of higher one is related to the small aggregated phospholipids. The increase in the high molecular-weight fraction in (D) is expected due to the decrease of low-molecular weight phospholipids after the removal of some non-linked phospholipids. The linked phospholipids in NR were partly decomposed into small aggregated molecules by the addition of ethanol and eluted as the high molecular-weight fraction. This should be strong supporting evidence demonstrating that phospholipids are mainly linked or associated to rubber molecules by hydrogen bonding. Nevertheless, it is clear that the association of phospholipid cannot be decomposed completely to single molecule by the addition of ethanol due to the presence of high molecular-weight fraction. It can be still associated with at least two molecules based on the molecular weight of high molecular-weight fraction.

Figure 3.46a and 3.46b shows the ^{1}H-NMR spectra of low molecular-weight compound obtained from precipitation of DPNR solution and that containing 1% (v/v) ethanol into excess amount of methanol, respectively. The ^{1}H-NMR signals appeared at 2.89 and 5.50 ppm are assignable to the methylene proton next to double bond ($C\underline{H}_2C{=}C$) and $C\underline{H}{=}CH$ of fatty acid group, respectively. The sharp signal resonating at 3.50 ppm is assigned to methyl proton next to nitrogen of choline head group of PC and also methine proton next to hydroxyl group ($OHC\underline{H}CHN$) of phospholipid containing hydroxyl and nitrogenous compounds. In addition, the methylene proton next to nitrogen of choline group in phosphosphingolipid was clearly observed at 3.62 ppm. Methylene protons linked to phosphate group of PC and PA were observed at 4.34 and 4.43 ppm, respectively. A non-equivalent methylene protons linked to phosphate group of phosphosphingolipid were appeared at 3.93 and 4.02 ppm. A sharp signal appearing at 5.78 ppm was expected to be be derived from the methine proton linked to carboxyl group of phospholipid.

These results indicates clearly that the low molecular-weight compound extracted from DPNR rubber is composed of phospholipids. The phospholipids present in NR are postulated to compose of nitrogen and hydroxyl groups, based on the assignment of phospholipid model compound.

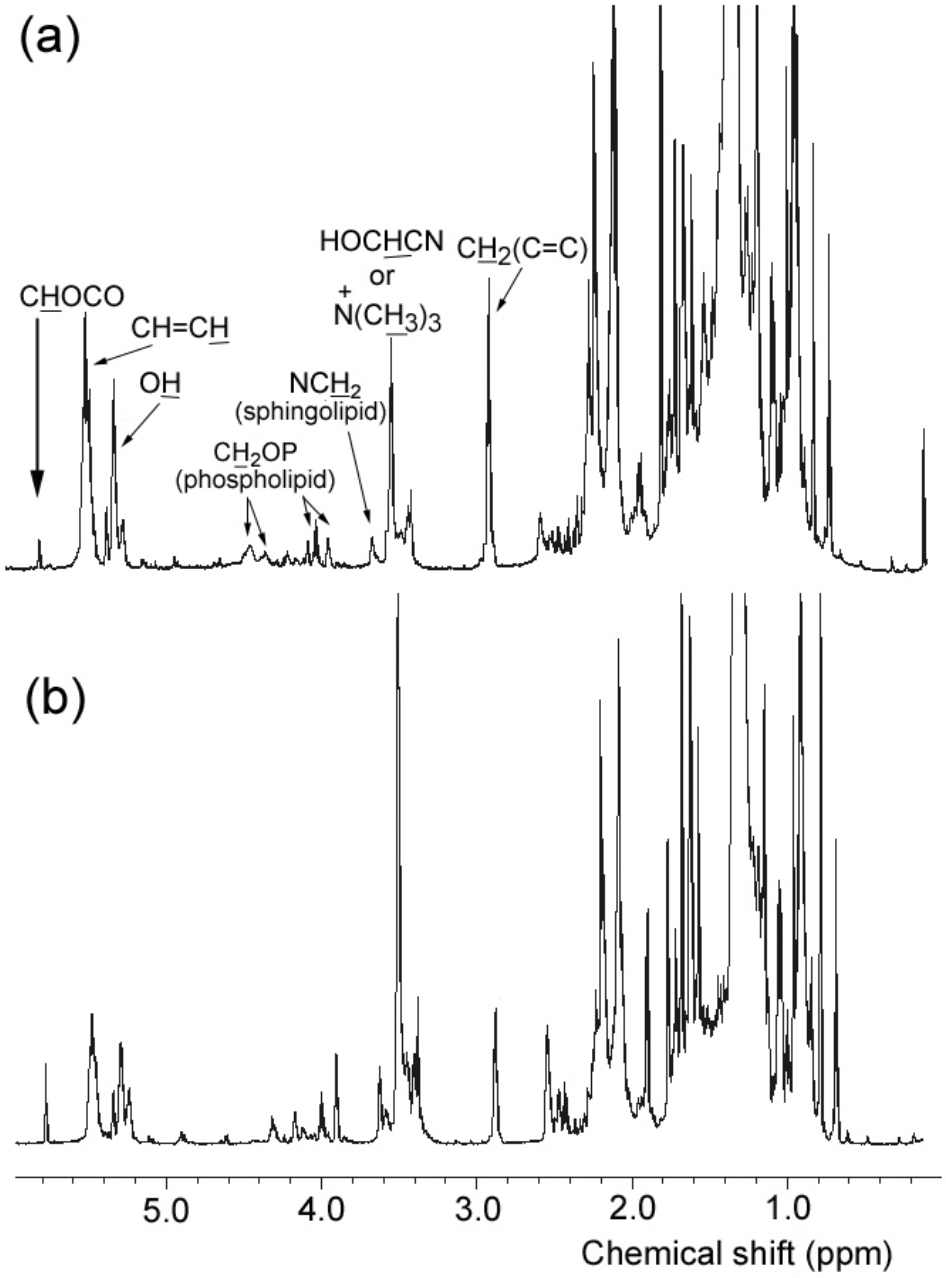


Figure 3.46 ^{1}H-NMR spectrum at 750 MHzof low molecular-weight compound obtained from precipitation of DPNR solution (a) and DPNR solution containing 1% (v/v) ethanol measured (b) at 750 MHz in C_6D_6 at 50°C.

3.4.9 Determination of phosphorus content in natural rubber by solid-state ^{31}P-NMR analysis

The presence of phosphate group in mono, diphosphate and phospholipid was confirmed by solid-state ^{31}P-NMR measurement. As shown in **Figure 3.47,** the ^{31}P-NMR spectrum of AE-DPNR showed a broad signal resonating at 1.6 ppm. The ^{31}P-NMR assignment of phospholipid model compounds indicates that the phosphate group in PC and PA resonate at 2.13 and 0.83 ppm, down field shifted from H_3PO_4 as reference signal, respectively. The ^{31}P-NMR signal of phosphorylated buteraprenol-18 was resonated at 1.56 and 8.38 ppm for monophosphate and diphosphate, respectively. The presence of broad ^{31}P-NMR signal of DPNR is expected to be derived from the overlapping of mono and diphosphate signal. The chemical shift of the ^{31}P-NMR signal observed for DPNR indicates the evidence of phosphate group in NR.

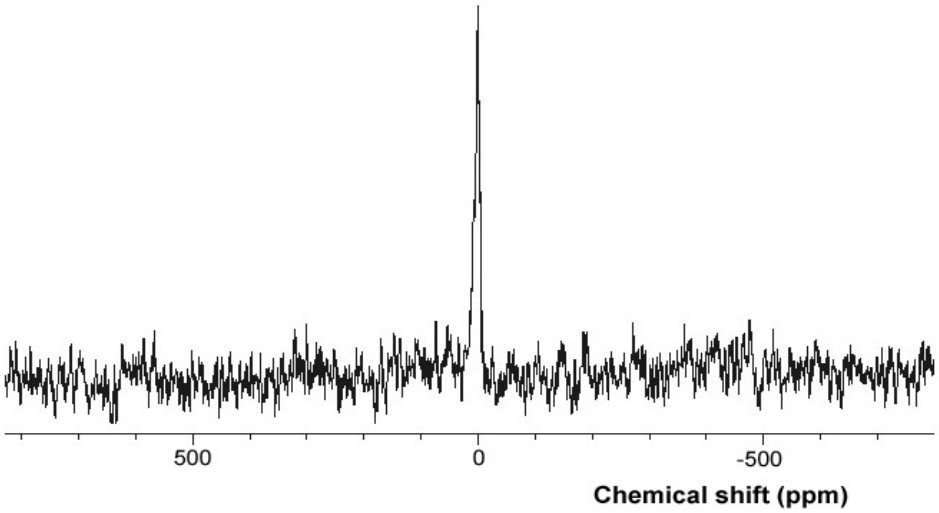
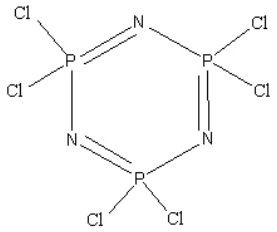


Figure 3.47 Solid-state ^{31}P-NMR spectrum of acetone-extracted DPNR measured at 121.6 MHz.

Quantitative anylysis of the amount of phosphorus atom present in DPNR was carried out by solid-state ^{31}P-NMR measurment using hexachlorocyclophosphazine, composing of three phosphorus atoms per molecule, as an internal standard. The chemical structure of hexachlorocyclophosphazine is shown in **Scheme 3.6**. Hexachlorocyclophosphazine was added as an internal standard of quantitative analysis into AE-DPNR in toluene solution to make the molar ratio of the α-terminal

to phosphorus atom to 1:3 and dried at room temperature. The ^{31}P-NMR spectrum of AE-DPNR mixed with hexachlorocyclophosphazine is shown in **Figure 3.48**.



Scheme 3.6 Chemical structure of hexachlorocyclophosphazine.

The ^{31}P signal due to hexachlorocyclophosphazine resonated at 18.0 ppm, while that of AE-DPNR appeared around at 1.8 ppm. The relative intensity of AE-DPNR against hexachlorocyclophosphazine indicated about the presence of 2.5 moles phosphorus per single rubber chain. This value is in good agreement with that of relative intensity of C$\underline{H_2}$OP of phospholipids obtained from ^{1}H-NMR. This implies that rubber chain might be associated with about two phospholipid molecules at the α-terminal group.

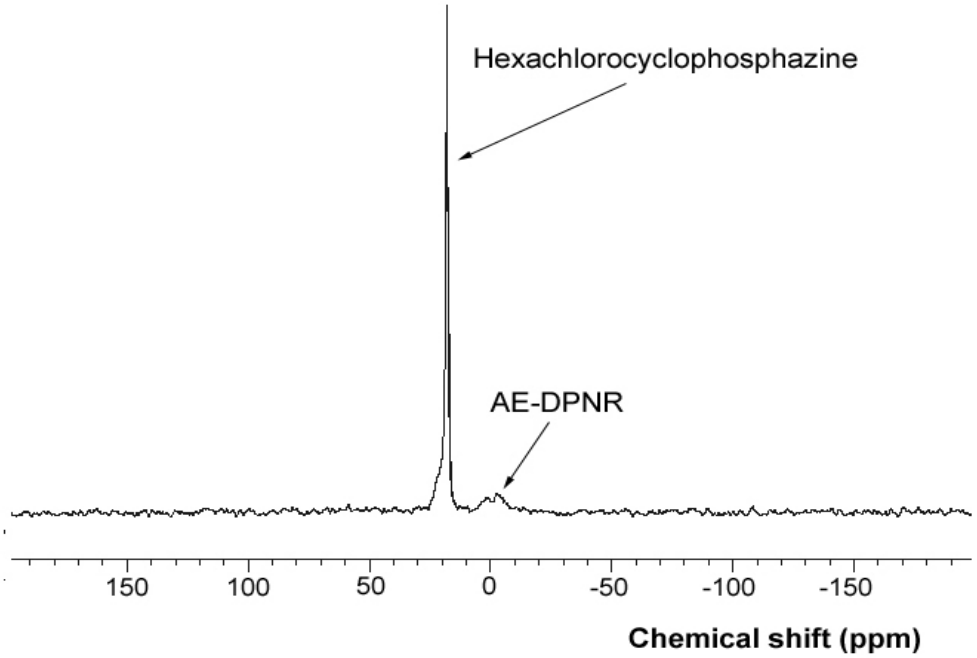


Figure 3.48 Solid-state ^{31}P-NMR spectrum of acetone-extracted DPNR mixed with hexachlorocyclophosphazine measured at 121.6 MHz.

3.4.10 Branching and gel formations in natural rubber by phospholipid association

It has been reported the gel phase in NR is composed of two types of crosslinking points; one is made up mainly by hydrogen bonding of proteins and the other is concerned with phospholipid [86]. The hydrogen bonding concerened with proteins can be decomposed after deproteinization of latex or by the addition of small amounts of polar solvent to toluene solution [80], while the latter branch-points are broken after transesterification. It was reported that deproteinization of HA-latex leads to a slight change in MWD [80]. Nevertheless, transesterification results in a significant decrease in both $\overline{M}_w$ and $\overline{M}_n$ values. The bimodal MWD was retained even after solubilization of gel fraction, the distribution rich in the peak at the low molecular-wieght region [80]. However, at present, the mechanism of branching formation caused by phospholipids has not been clarified yet.

It has been postulated that the morphology of lipid aggregates depends on the total phospholipid concentration, the ratio of the constituents, and the temperature [119, 120]. Most phospholipids, due to their amphiphilic nature, resist solubilization in polar as well as nonpolar solvents by the formation of liposomes or reverse micelles, respectively [121]. It has been reported that fatty acid chain carbon atoms of dipalmitoyl lecithin form micelles in chloroform and bilayers in deuterium oxide [122]. The long-chain acyl groups in triacylglycerol are extremely water insoluble, exhibit interfacial properties in water. Thus, they orient at air-water interfaces with the polar glyceryl portion interacting with water [123]. In small unilamellar PC vesicles, triolein (TO) has a finite solubility and a preferred orientation with the carboxyl groups at the aqueous interface [124]. Similar result was also observed for TO in multilamellar PC [125].

Two major classes of phosphosphingolipids are found in nature, phosphocholine derivatives of ceramides, known trivially as sphingomyelins (cf. **Scheme 3.2**), and glycosylated derivatives of inositol phosphoceramides, known as phyotoglycolipids. The amide proton in phosphosphingolipid has been reported to be mostly involved in intermolecular hydrogen bondings that link neighboring phospholipids through bridging water molecules. On the contrary, in the absence of

water, the NH group is participated in an intramolecular hydrogen bonding that restricts the mobility of the phosphate group [126]. Complete hydration leads to an extension of the head group as water molecules bind to the phosphate and NH groups *via* hydrogen bondings. Based on these results, it can be proposed that branch-points in NR are predominantly formed by hydrogen bonding between phospholipid polar groups. Minor effect due to the ionic crosslinking between negatively charged phospholipids with divalent metal cations constituent can be a subject of considerable interest, since this process has been implicated in many membranes associated events [116].

The effect of hydrogen bonding and Mg^{2+} ions on branching formation deriving from phospholipids was analyzed by the addition of 1% (v/v) ethanol and 5% (w/w) diammonium hydrogenphosphate (DAHP) into DPNR solution and latex, respectively. The MWD of DPNR in toluene solution treated with ethanol and that of DPNR latex treated with DAHP is shown in **Figure 3.49a** and **3.49b**, respectively. AE-DPNR showed the bimodal MWD rich in the high molecular-weight fraction, transesterification leads to the decomposition of branch-points resulting in the decrease in the molecular weight, as shown in **Table 3.8**, similar to the previous work [80]. It was presumed that the increase in the low molecular-weight fraction in the bimodal MWD after transesterification is derived from the decomposition of branch-points to form linear molecules. It is interesting that the addition of small amount of ethanol into DPNR solution led to the decrease in molecular-weight and narrower MWD comparable to TE-DPNR. It can be deduced that the addition of ethanol into DPNR solution resulted in the decomposition of branch-points originated by hydrogen bonding between phospholipid molecules to form linear molecules. On the contrary, the addition of DAHP into DPNR latex caused only a slight decrease in the molecular weight, although the MWD was comparable to DPNR. This indicates that Mg^{2+} ions have less effect on branching formation than hydrogen bonding. This supports the idea mentioned above that branching formation in DPNR is mainly derived by hydrogen bonding of phospholipids associated to phosphate group at the α-terminal of rubber molecules. It is remarkable that the molecular weight of DPNR after the addition of 1% (v/v) ethanol decreased about one-half comparable to that of TE-DPNR, while it reduced insignificantly after DAHP treatment. This is supporting

evidence showing that branch-points in DPNR is mainly originated by hydrogen bonding. At present, however, it is difficult to neglect the ionic bonding for branching formation, since there is no supporting evidence to show the effectiveness of DAHP to remove Mg^{2+} ions which are lined to the the α-terminal in DPNR latex. The effect of Mg^{2+} will be discussed in section 3.6.

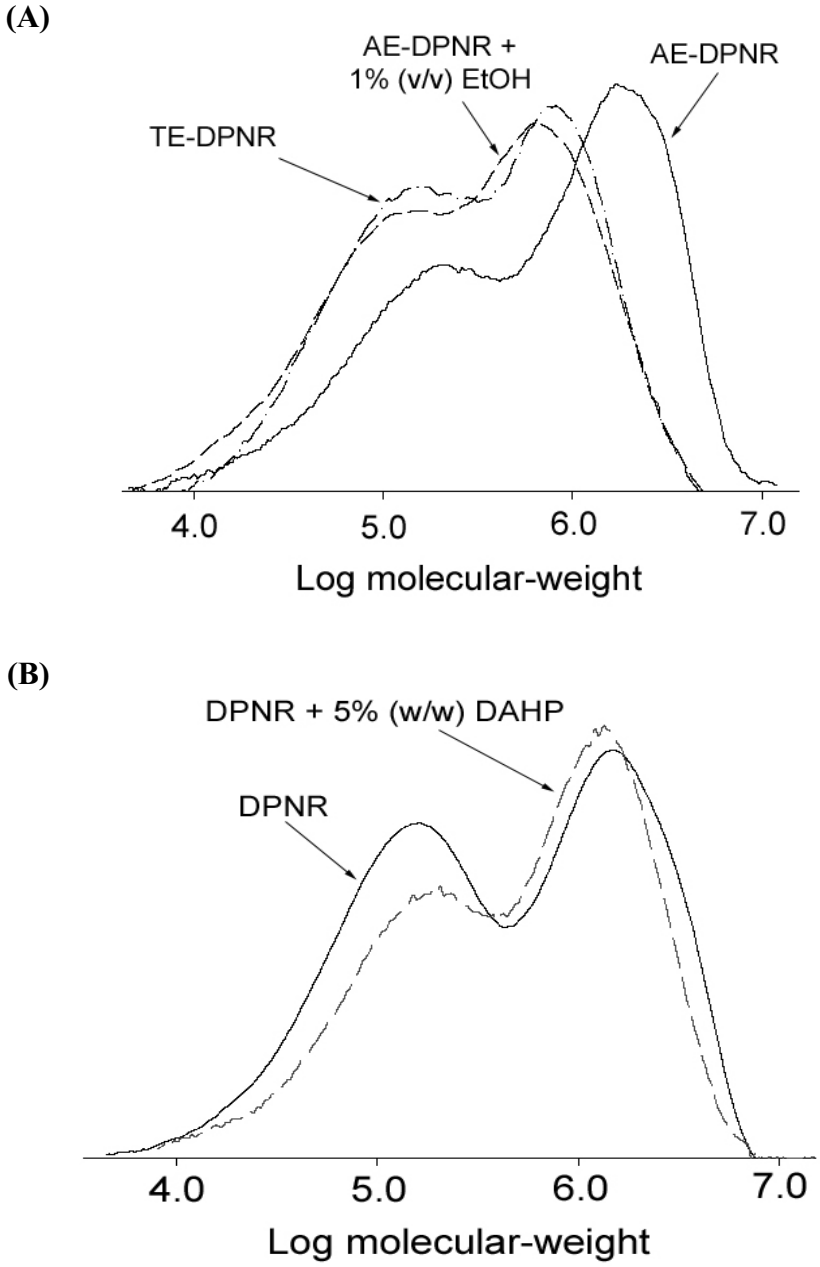


Figure 3.49 Molecular-weight distribution of (A) acetone-extracted DPNR, transesterified DPNR and 1% ethanol in toluene solution compared to (B) DPNR-latex treated with 5% (w/w) diammonium hydrogenphosphate (DAHP).

Table 3.8 Molecular weight of (A) DPNR solution after treated with 1% (v/v) ethanol, compared with DPNR and transesterifed DPNR, (B) DPNR latex after treated with 5% (w/w) diammonium hydrogen phosphate (DAHP)

	Sample	$\overline{M}_n$	$\overline{M}_w$	$\overline{M}_w/\overline{M}_n$
(A) Solution	AE-DPNR	1.80×10^5	1.33×10^6	7.41
	AE-DPNR + 1% EtOH	1.01×10^5	5.64×10^5	5.57
	TE-DPNR	1.04×10^5	4.02×10^5	3.88
(B) Latex	DPNR	1.30×10^5	7.86×10^5	6.05
	DPNR + 5% $(NH_4)_2HPO_4$	1.26×10^5	6.31×10^5	5.01

Infrared spectroscopy has been the method of choice for studying intermolecular interactions by hyogen bondings, because the vibrational modes of the donors and acceptor groups are sensitive to this interaction leading to the change in a vibrational characteristic [116]. The difference in vibrational frequencies can be used to observe direct or indirect hydration or dehydration effects, intra- or extramolecular binding of hydroxyl or amino groups as well as cation mediated changed at the bilayer surface [127]. The vibrational modes mostly studied in diacylphospholipids are the phosphate vibrational bands and the carboxyl vibrations of the fatty acid groups. Hydrogen bonding to the oxygen atoms leads to a weakening of the vibrational force constants and therefore to a decrease in frequency. In addition, sphingolipids or other lipids containing amide groups, the amide I band is sensitive to hydrogen bonding and to electrostatic interactions [127]. The sensitivity of the phosphate vibrations of phospholipid to hydration was described a long time ago. It has been reported that the asymmetric PO_2^- vibrational band is extremely sensitive to hydration, because of the shift of its frequency from 1250 for dry to 1230 cm^{-1} in a hydrated bilayer has been observed [128].

Based on these studies, it is possible to confirm the formation of hydrogen bonding due to phospholipids in DPNR by FTIR spectroscopy. The low molecular-weight compound obtained from the reprecipitation of DPNR in toluene solution including 1% (v/v) ethanol, which mentioned above, was subjected to FTIR

measurment together with PC as standard reference. **Figures 3.50** and **3.51** show the FTIR spectra of low molecular-weight compounds from DPNR with and without the addition of ethanol, respectively. As mentioned above, the C=O and O-P-O stretching bands provide an important clue to differentiation of the different phospholipid conformations. Normally, phospholipid shows two C=O stretching bands centering at 1738-1742 and 1724-1729 cm^{-1}. The presence of two C=O peaks is expected to be due to the different degree of hydration and/or hydrogen bonding to C=O [129]. Recently, it was reported that phosphotidylenanthiolamine bilayer (PE) gave three C=O signals centering at 1742, 1728 and 1714 cm^{-1}. The latter band was derived from hydrogen bonding of C=O of PE bilayer [130]. The O-P-O asymmetric stretching of hydrated phospholipid bilayer is usually observed at 1230 cm^{-1}, while dried or anhydrous lipid always appeares at 30 cm^{-1} higher frequency [131].

It can be seen in **Figure 3.50** that the low molecular-weight compounds from DPNR and that of DPNR treated with 1% (v/v) ethanol in toluene give FTIR spectra similar to that obtained from PC. This indicates that phospholipids are a component of rubber molecules in terms of both linked and free molecules. It is remarkable that the new band at 1713 cm^{-1} was observed in both DPNR and DPNR treated with toluene including 1% (v/v) ethanol, even the intensity of latter case was lower. This small band clearly indicates the presence of hydrogen bonding between phospholipid molecules. However, the relative intensity of 1713 cm^{-1} band in low molecular-weight compounds from DPNR treated with toluene containing 1% (v/v) ethanol was lower than that without ethanol. This indicates that hydrogen bonding between phospholipid molecules in NR can be decomposed by ethanol. The presence of hydrogen bonding between phospholipids was further confirmed by the O-P-O stretching band. The low molecular-weight compounds from DPNR showed the O-P-O asymmetric stretching at 1219 and 1240 cm^{-1}, while the former stretching was absent in both PC and low molecular-weight compound obtained from DPNR solution including 1% (v/v) ethanol. This result strongly suggestes that phospholipids in DPNR rubber aggregate or link together *via* hydrogen bonding through phosphate and carboxyl groups.

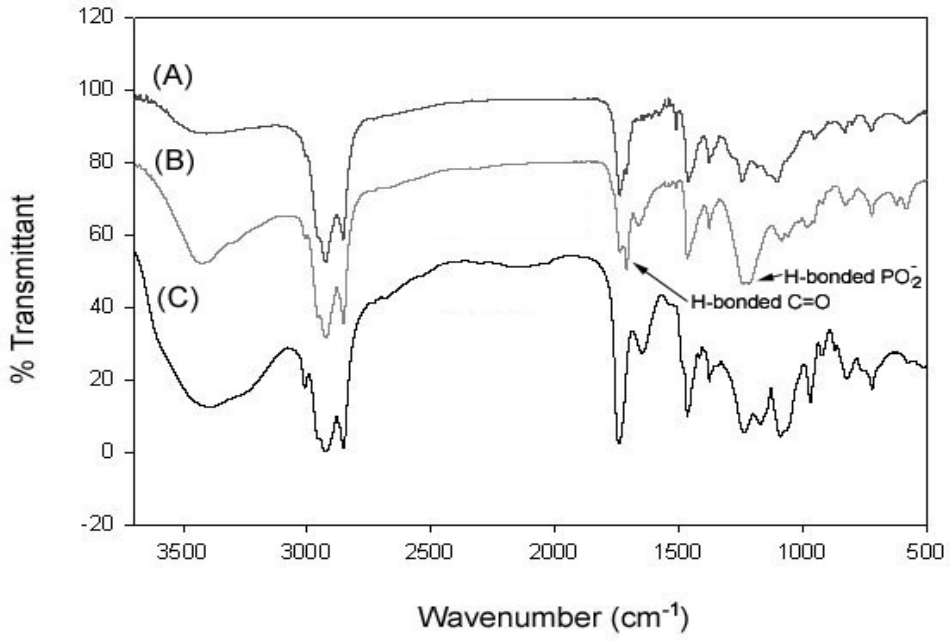


Figure 3.50 FTIR spectra of low molecular-weight compounds extracted from (A) DPNR solution containing 1% (v/v) ethanol, (B) DPNR solution, and (C) l-α-phosphatidylcholine (PC).

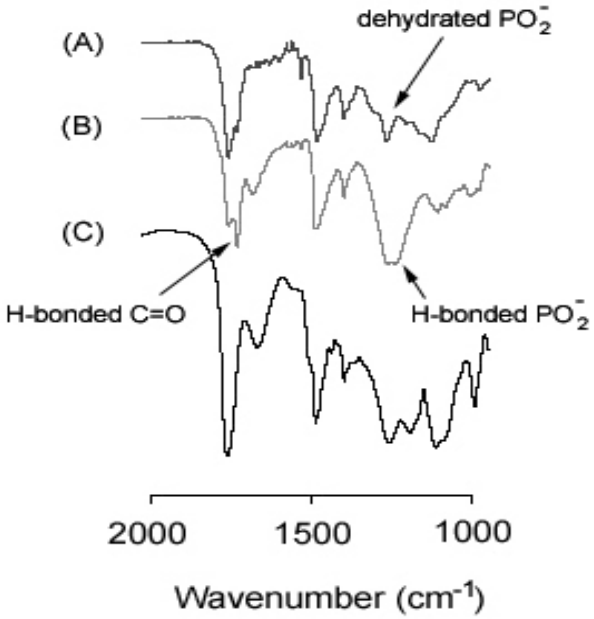


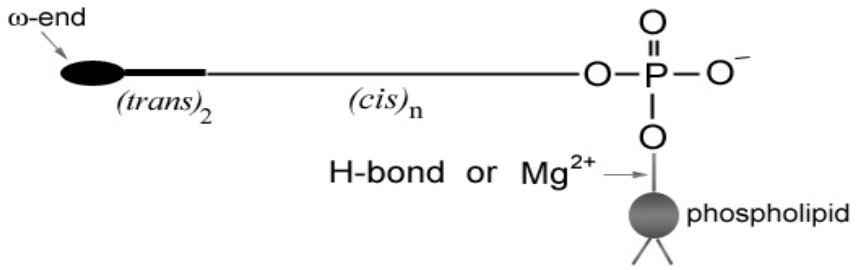
Figure 3.51 FTIR spectra of low molecular-weight compound extracted from (A) DPNR solution including 1% (v/v) ethanol, (B) DPNR solution, and (C) l-α-phosphatidylcholine (PC) ranging between 1000-2000 cm^{-1}.

Based on the above findings, it can be concluded that the α-terminal of NR molecule consists of two kinds of functional group, i.e. monophosphate and diphosphate groups, which is linked with phospholipid *via* hydrogen bonding as a predominant linkage and some is linked *via* ionic linkage.

Branch-points are originated from phospholipids which are associated to rubber chains and/or free molecules. The phospholipids are associated together by the formation of micelle structure mainly *via* hydrogen bonding between polar group in phospholipids molecules. The addition of small amount of EtOH into toluene solution leads to the decomposition of H-boding to form linear rubber chains.

The proposed structure of the α-terminal group of NR molecule and branching formation at terminal group are shown in **Figures 3.52** and **3.53**, respectively.

(a)



(b)

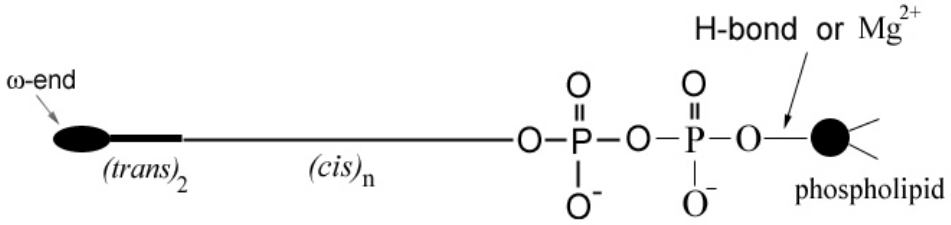


Figure 3.52 Proposed structure of α-terminal group of NR.

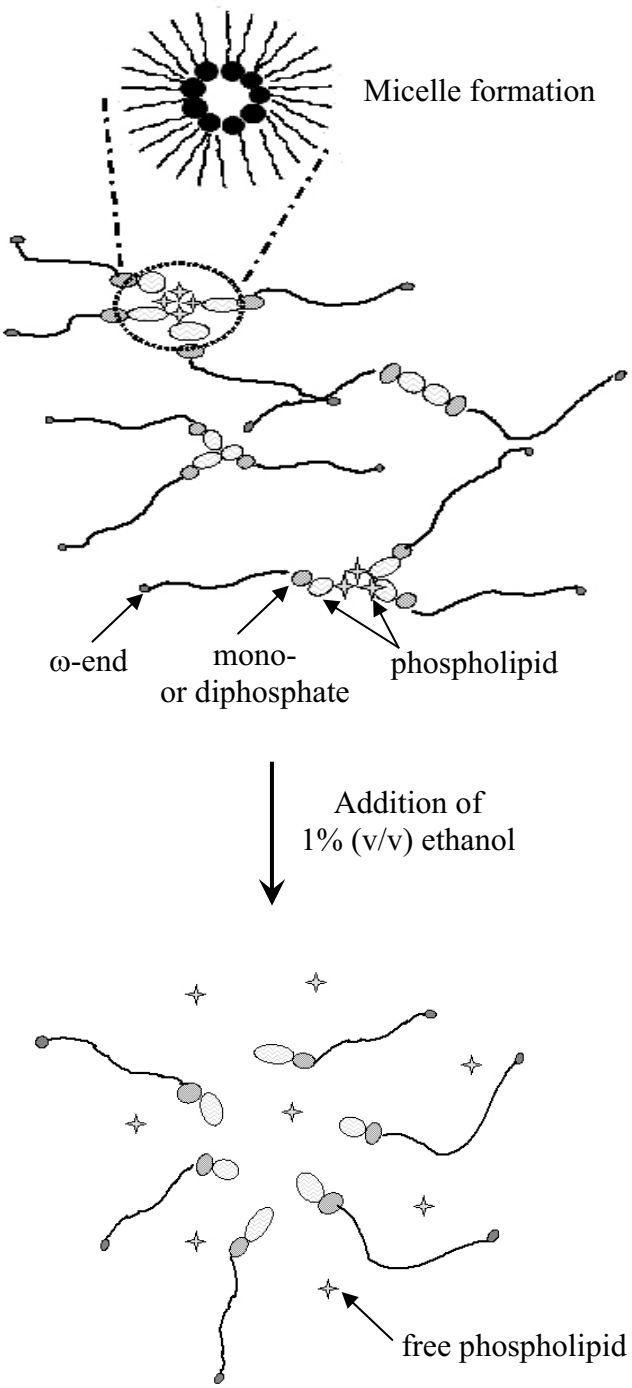


Figure 3.53 Proposed structure of branch-points and decomposition of branch-points in NR.

3.5 Gel Formation in Natural Rubber Latex: Effect of $(NH_4)_2HPO_4$ and TMTD/ZnO Additives

3.5.1 Effect of TMTD/ZnO on gel formation in commercial HA-latex

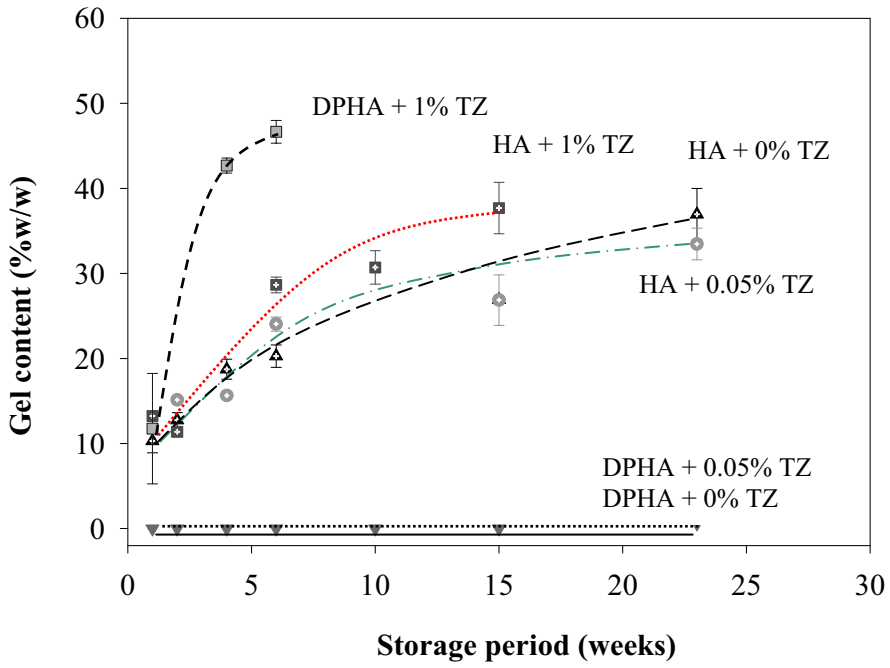


Figure 3.54 Relationship between the storage period and gel content in commercial HA- and DPHA-latices with and without TMTD/ZnO.

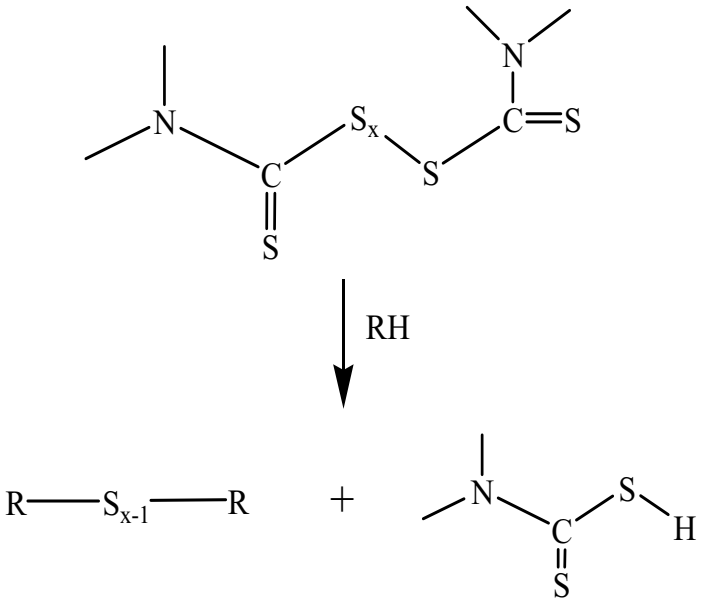
The effect of TMTD/ZnO on the gel formation in HA-latex was investigated by the addition of 0.05 and 1.0% (w/v) TMTD/ZnO into the commercial HA-latex, which was treated with $(NH_4)_2HPO_4$, and compared with the deproteinized HA-latex from this latex (DPHA-latex). **Figure 3.54** shows the gel content of rubber from commercial HA- and DPHA-latices during storage for 23 weeks after the addition of TMTD/ZnO. The gel content of the commercial HA-latex increased gradually from 10 to 35% within 23 weeks without the addition of TMTD/ZnO. The gel content of commercial HA-latex increased with increasing TMTD/ZnO concentration from 0 to 1.0% (w/v). In **Figure 3.54** both the untreated commercial HA-latex and that including 0.05% (w/v) TMTD/ZnO showed a similar tendency to increase the gel content, indicating that this concentration was too weak to affect the gel formation. The

increase in TMTD/ZnO concentration to 1.0% (w/v) resulted in a rapid increase in the gel content to as high as 30% for the commercial HA-latex after 6 weeks.

It was observed that both the DPNR-latex and that treated with 0.05% (w/v) TMTD/ZnO showed very small amounts of gel content, showing an insignificant change in the gel content during storage period from ca. 0.5% of the initial stage to less than 2% after storage for 23 weeks. However, with the addition of 1.0% (w/v) TMTD/ZnO, a rapid increase in gel content was observed in the case of DPHA-latex. The gel content of DPHA-latex was greatly increased from 10 to 40% after 3 weeks and further increased to 47 % within 6 weeks. This indicates that the addition of TMTD/ZnO could accelerate gel formation in both in commercial HA- and DPHA-latices within 6 weeks, except when a low concentration of TMTD/ZnO was used.

3.5.2 Effect of proteins on the acceleration reaction of TMTD/ZnO

It was reported that the removal of proteins by deproteinization of HA-latex reduced the gel content due to the decomposition of branch points originating from proteins [86]. It is interesting to note that the gel content of the DPHA-latex was higher than that of the commercial HA-latex after the addition of 1.0% (w/v) TMTD/ZnO. This indicates that the gel formation in the commercial HA-latex in the presence of TMTD/ZnO proceeds mainly by the reaction between polyisoprene chain and sulfur atoms in TMTD molecule as expected by Kruger and McGill [132], Coleman et al. [133] and Dogadkin and Shershnev [134], where proteins slightly act to reduce the rate of reaction. Previous studies suggested the formation of polysulfidic accelerator species *via* a radical mechanism [133, 134], which will react with rubber chain to form rubber-bound intermediate or pendent groups. The presumed crosslink formation in rubber by TMTD is illustated in **Scheme 3.7**.



Here RH refers rubber hydrocarbon.

Scheme 3.7 Presumed crosslinking mechanism of NR promoted by TMTD.

As shown in **Table 3.9**, the gel content of rubber from the commercial HA-latex after storage for 15 weeks was almost constant even after deproteinization. The decomposition of proteins was confirmed by the reduction of nitrogen content from 0.15 to 0.024% and 0.17% to 0.23% in the case of with and without the addition of 0.05% (w/v) TMTD/ZnO, respectively. This is additional evidence to support the proposition that proteins show no important role in the gel formation in commercial HA-latex. In addition, this indicates clearly that the gel phase formed in the commercial HA-latex is not so-called soft gel, which has been presumed to form *via* proteins at this stage.

Table 3.9 Change of gel and nitrogen contents of rubber from commercial HA-latex with and without TMTD/ZnO after deproteinization

Sample*[a]	Gel content (%w/w)	N content (% w/w)
HA + 0%TZ	26.9	0.15
DP [HA + 0%TZ]	26.2	0.024
HA + 0. 05%TZ	29.0	0.17
DP [HA + 0.05%TZ]	28.7	0.023

* Samples were kept at room temperature for 15 weeks before measurement.
DP [HA + 0% TZ] means deproteinization HA-latex containing TMTD/ZnO

Table 3.10 shows the gel content determined by the addition of 1.0% (v/v) ethanol into toluene observed for the rubber from commercial HA- and DPHA-latices. It was reported that the gel fraction in ordinary NR is so-called soft-gel, which can be solubilized by the addition of polar solvent such as ethanol into the rubber solution [65, 80]. It is clear that the gel content of commercial HA-latex after storage for 23 weeks, with and without the addition of 0.05% (w/v) TMTD/ZnO, decreased from ca 35% to ca 20% by the addition of 1.0% (v/v) ethanol into the rubber solution. On the other hand, the DPHA-latex after storage of 15 weeks showed almost the same gel content of about 25% and 28% whether toluene or toluene containing 1% (v/v) ethanol were used for determination of gel content. This indicates that the gel fraction in the DPHA-latex produced from the commercial HA-latex is not soft-gel.

Table 3.10 Gel content of rubber from commercial HA- and deproteinized HA-latices, DP[HA-latex] determined with toluene and toluene including 1.0% (v/v) ethanol

Sample*[a]	Gel content (%w/w)*[d]
HA + 0% TZ*[b]	34.8 (19.1)
HA + 0.05% TZ*[b]	34.8 (20.8)
DP [HA + 0% TZ]*[c]	25.7 (24.8)
DP [HA + 0.05% TZ] *[c]	28.2 (29.2)

*[a] DP [HA + 0.05% TZ] means deproteinization HA-latex containing 0.05% w/v TMTD/ZnO.

*[b] Samples were stored at room temperature for 23 weeks before measurement.

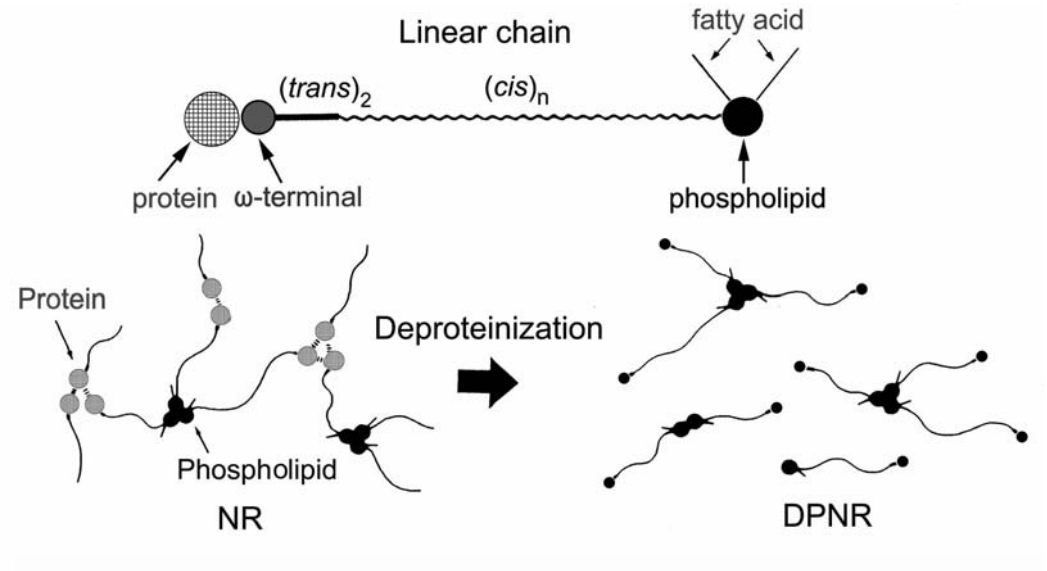
*[c] Samples were stored at room temperature for 15 weeks before measurement.

*[d] Value in parentheses is the gel content determined with toluene containing 1.0% (v/v) ethanol.

3.5.3 Branching and gel formations in commercial HA-latex

As discussed above, the gel content of commercial HA-latex after storage for 15 weeks was about 26% even after deproteinization, while it increased to *ca* 35% after storage for 23 weeks. However, the gel content decreased to *ca* 20% by the addition of 1.0% (v/v) ethanol (cf. **Tables 3.9** and **3.10**). These results suggest that the gel fraction in commercial HA-latex after long storage contains soft-gel partly formed by hydrogen bonding *via* proteins and phospholipids with ω-terminating and α-terminating end-group, as well as hard-gel formed by chemical crosslinking reactions. This finding supports the idea that the gel phase in the commercial HA-latex is composed of at least two types of crosslinks. The first type is crosslinks made up by hydrogen bonding *via* proteins and functional terminal group, which can be decomposed by the addition of small amounts of ethanol into the rubber solution or deproteinization and transesterification, respectively. Although the structure and mechanism of crosslink formation by the latter have not been clarified yet, it may be possible to assume the formation of crosslinks *via* ionic linkage and/or association of

phosphate group as illustrated in **Scheme 3.8**. The second type is chemical branch-points formed by additives present in commercial HA-latex, i.e. TMTD/ZnO or $(NH_4)_2HPO_4$.



Scheme 3.8 Presumed structure of gel phase in NR (ω- and α-terminals stand for initiating and terminating chain-end groups, respectively. DPNR represents deproteinized NR).

3.5.4 Effect of TMTD/ZnO and $(NH_4)_2HPO_4$ on gel formation in fresh latex preserved with NH_4OH

In general, the gel fraction in commercial HA-latex during storage can be formed by the following crosslinking reactions:

1) Chemical crosslinks by C-C and C-O bonds between rubber chains

2) Ionic crosslinks by di- or trivalent metal ions existing in latex

3) Crosslinks by hydrogen bonding.

The first one is mostly the reaction of polyisoprene chain, although very small amounts of functional groups in NR termed as abnormal groups such as aldehyde, lactone and epoxide [82, 83, 135] are presumed to cause storage hardening in the case of solid NR. On the other hand, the second and third mechanisms are reactions *via* some functional groups on the rubber chain. The presence of functional groups at both chain-ends of NR has been confirmed by structural studies [65]. It was also clarified

that the crosslinks composed of proteins can be removed by deproteinization and those contained phospholipids can be decomposed by transesterification. Saponification of NR decomposes the proteins as well as the phospholipids.

3.5.5 Synergistic effect of TMTD/ZnO and $(NH_4)_2HPO_4$ on the gel formation of commercial HA-latex

For the preparation of commercial HA-latex, FL-latex is normally treated with $(NH_4)_2HPO_4$ to reduce Mg^{2+} ions and is preserved with NH_4OH. The effect of $(NH_4)_2HPO_4$ on the gel formation in ammonia preserved FL-latex (PRD-FL-latex) was analyzed at various $(NH_2)_2HPO_4$ concentrations during storage for 7 weeks, as shown in **Figure 3.55**.

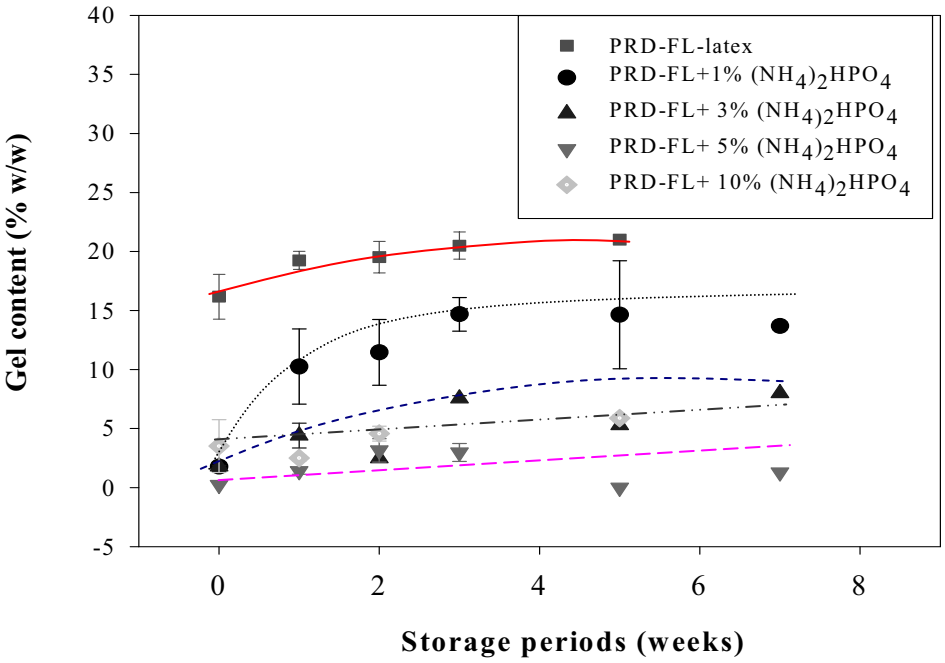


Figure 3.55 Relationship between the storage period and gel content of FL-latex preserved with 0.6% $(NH_4)OH$ with the addition of $(NH_4)_2HPO_4$.

It is clear that the gel content of PRD-FL-latex decreased from 15% to less than 4% after $(NH_4)_2HPO_4$ treatment. The minimum gel content of about 0.5% was observed at the $(NH_4)_2HPO_4$ concentration of 5%. However, as $(NH_4)_2HPO_4$ increased to 10% (w/v) the gel content further increased. It is remarkable that the PRD-FL-latex

including $(NH_4)_2HPO_4$ showed a tendency to increasing gel content as the storage period increased. This indicates clearly that the excess amounts of $(NH_4)_2HPO_4$ promote crosslink formation in the ammonia preserved FL-latex and also in commercial HA-latex, in which $(NH_4)_2HPO_4$ is added in order to remove Mg^{2+} ions, during long storage.

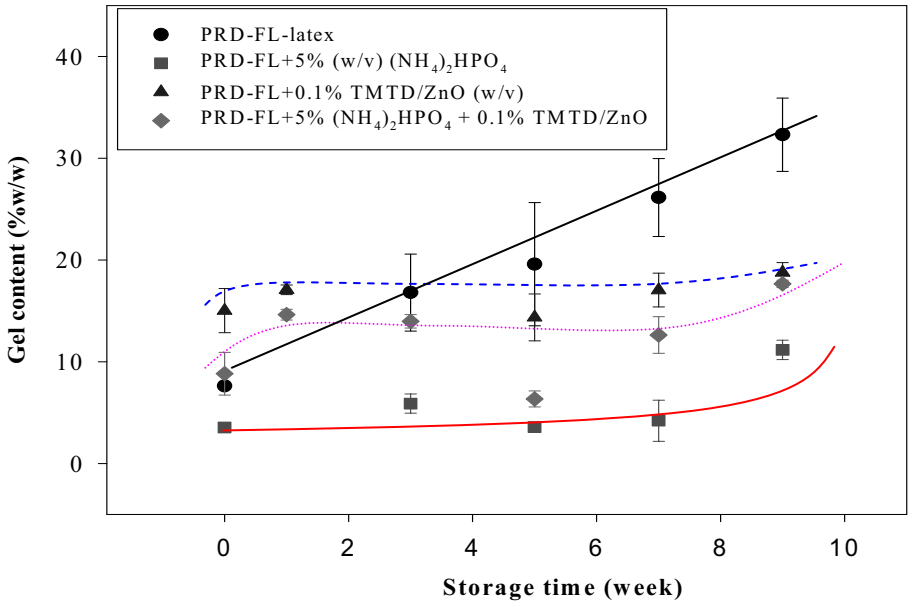


Figure 3.56 Relationship between the storage period and gel content of FL-latex preserved with 0.6% NH_4OH with the addition of TMTD/ZnO and $(NH_4)_2HPO_4$.

The synergistic effect of TMTD/ZnO and excess amounts of $(NH_4)_2HPO_4$ on the gel formation of PRD-FL-latex during long time storage was studied. As shown in **Figure 3.56**, the gel content of the rubber from the PRD-FL-latex gradually increased from 5% to 30% after 9 weeks. The addition of 5% (w/v) $(NH_4)_2HPO_4$ into the PRD-FL-latex decreased the gel content from 5% to 3% and prevented the increase in gel content until 7 weeks; after that it increased to about 10%. The drastic reduction in the gel content of the PRD-FL-latex after the addition of 5% (w/v) $(NH_4)_2HPO_4$ indicates that the gel formation mechanism should be concerned with ionic crosslinks formed by Mg^{2+} ions. This finding implies that divalent or trivalent metal ions in NR latex, presumably Mg^{2+} ions, are responsible for the formation of crosslinking in PRD-FL-latex. However, the gel fraction of FL-latex treated with $(NH_4)_2HPO_4$

showed a tendency to increase after storage longer than 9 weeks. This suggests that another mechanism participates slowly to form crosslinks during long time storage.

In **Figure 3.56**, it is also observed that the addition of 0.1% (w/v) TMTD/ZnO into the PED-FL-latex resulted in an increase in gel formation at the initial stage from 5% to 15% and further increased to 18% within 1 week, followed by a leveling off. The addition of 0.1% (w/v) TMTD/ZnO into the PRD-FL-latex treated with 5% (w/v) $(NH_4)_2HPO_4$ caused the increment of gel content in comparison to in the case of latex without TMTD/ZnO. The tendency to increase of the gel content in the PRD-FL-latex treated with 0.1% (w/v) TMTD/ZnO was almost the same as of that treated with both 0.1% (w/v) TMTD/ZnO and 5% w/v $(NH_4)_2HPO_4$, although the gel content of the former was higher than the latter. This result supports the idea that some parts of the crosslinking are generated by TMTD/ZnO and $(NH_4)_2HPO_4$ in the commercial HA-latex, which includes TMTD/ZnO and $(NH_4)_2HPO_4$. Here, TMTD/ZnO accelerates the gel formation at the initial stage of storage, while $(NH_4)_2HPO_4$ activates the crosslink formation for long time storage. This is clear evidence showing that excess amounts of Mg^{2+}, TMTD/ZnO and $(NH_4)_2HPO_4$ can play an important role in gel formation in commercial HA-latex during long time storage.

3.6 Gel Formation in Natural Rubber Latex: Effect of Magnesium Ions

3.6.1 Effect of $(NH_4)_2SO_4$ on the gel and branching formations of FL- and commercial HA-latices

FL-latex contains about 6% non-rubber components including mono-, di- and trivalent metal ions with an amount greater than 200 ppm, depending on season and clone of rubber tree. If divalent or trivalent metal atoms such as Mg^{2+} and Fe^{3+} ions linked to rubber chains by ionic linkage, these metals can form ionic crosslink points. The presence of a phospholipid group in a rubber chain was presumed by chemical and structural analyses of NR [61, 86]. Metal ions are expected to link to rubber molecules through the phosphoric ester linkage, which cannot be decomposed by ordinary $(NH_4)_2HPO_4$ treatment during the production process of concentrated latex. This idea suggests the existence of some ions in rubber chains that form ionic bonds. The substitution of these metal ions with a univalent ion such as ammonium ion is expected to decompose the ionic crosslinks according to the following equation.

$$R\text{-}PO_4^-\text{-}Mg^{2+}\text{-}PO_4^-\text{-}R \quad + \quad (NH_4)_2SO_4 \longrightarrow 2\ R\text{-}PO^-_4\ NH_4^+ \quad + \quad MgSO_4$$

The effect of substitution with a univalent ion on the decomposition of presumed ionic crosslinks was analyzed based on the change of gel content before and after the addition of 0.05-1.00% w/v $(NH_4)_2SO_4$ into FL- and commercial HA-latices. **Figure 3.57** shows the relationship between the gel content of rubber from FL-latex and the concentration of $(NH_4)_2SO_4$. The gel content was ca. 2.5% independent of the concentration of $(NH_4)_2SO_4$. On the other hand, the gel content of rubber from commercial HA-latex apparently decreased from 6 % to ca. 2.5 % and leveled off at 0.10% w/v of $(NH_4)_2SO_4$, as shown in **Figure 3.58**.

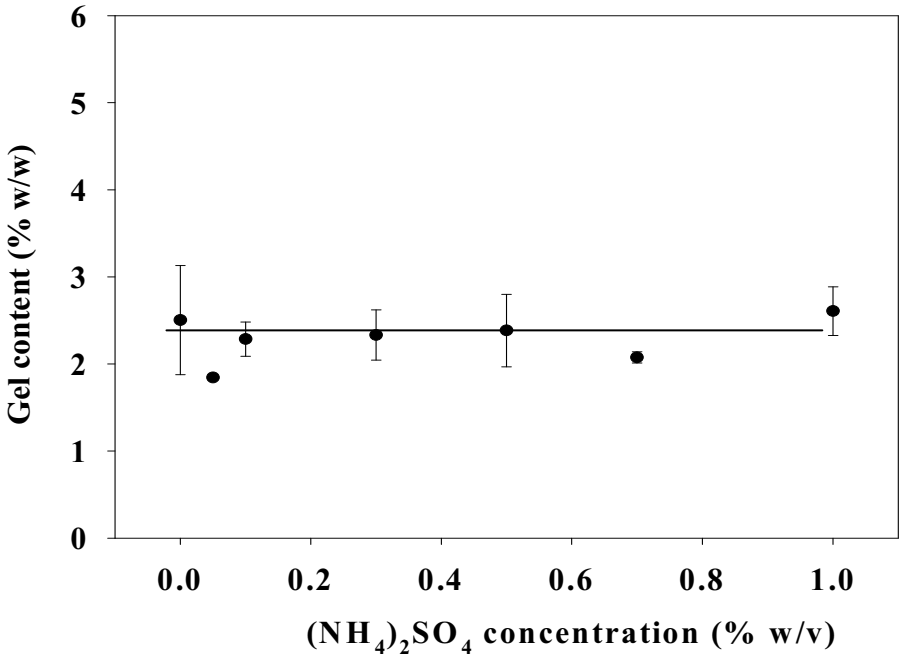


Figure 3.57 Gel content of rubber from FL-latex treated with $(NH_4)_2SO_4$ at 37°C for 40 hr.

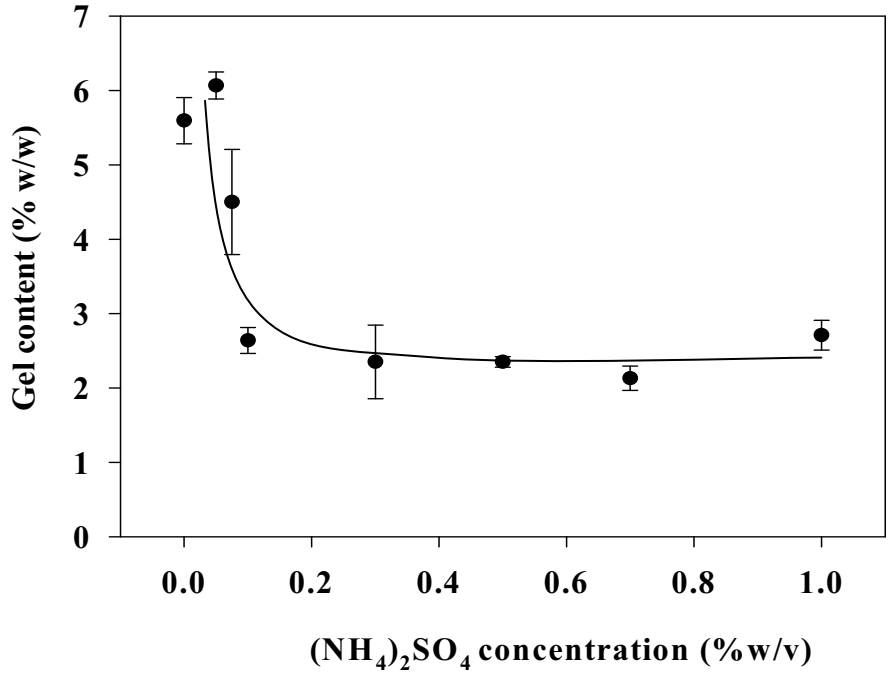


Figure 3.58 Gel content of rubber from HA-latex treated with $(NH_4)_2SO_4$ at 37°C for 40 hr.

It is remarkable that the gel content of commercial HA-latex after the addition of $(NH_4)_2SO_4$ is almost the same as that observed in the case of FL-latex. This finding is strong supporting evidence to show the occurrence of gel formation by ionic bonds or dissociation of phosphate groups by $(NH_4)_2SO_4$. The ionic crosslink formation by Mg^{2+} is more likely by the fact that Mg^{2+} is the most common divalent ion in FL-latex which can be removed with the treatment of $(NH_4)_2SO_4$ or $(NH_4)_2HPO_4$. It was reported that the gel content reduced to almost 0% after enzymatic deproteinization followed by transesterification with sodium methoxide in solution [80]. This suggests that the residual gel fraction in the $(NH_4)_2SO_4$ treated commercial HA-latex and in FL-latex may be derived from the hydrogen bonding of proteins and functional terminal groups in rubber chain as reported by Tangpakdee and Tanaka [80].

The gel content of the rubber from commercial HA-latex is known to increase to higher than 50% by increasing the period of storage [80]. The effect of $(NH_4)_2SO_4$ on the gel formation was analyzed in long-storage commercial HA-latex containing about 50% gel and compared to that of FL- and commercial HA-latices as shown in **Figure 3.59**.

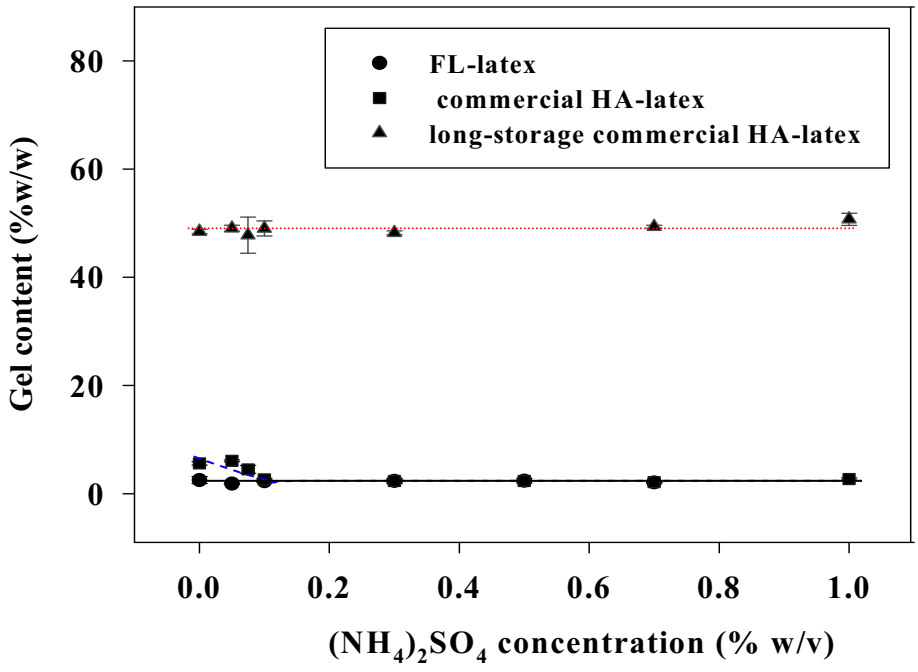


Figure 3.59 Gel content of rubber from long-storage HA-latex treated with $(NH_4)_2SO_4$ at 37°C for 40 hr.

It is interesting that long-storage commercial HA-latex showed no decrease in the gel content with the addition of $(NH_4)_2SO_4$. It is noteworthy that the gel phase in long-storage commercial HA-latex was not solubilized by the treatment of latex with a proteolytic enzyme or extraction with toluene containing 1.0% (v/v) ethanol, as mentioned in the previous part. These findings indicate that most of the gel fraction in long-storage commercial HA-latex is not simply derived from ionic crosslinks or hydrogen bonding of the terminal group in a rubber chain *via* proteins.

3.6.2 Molecular weight of FL- and commercial HA-latices after $(NH_4)_2SO_4$ treatment

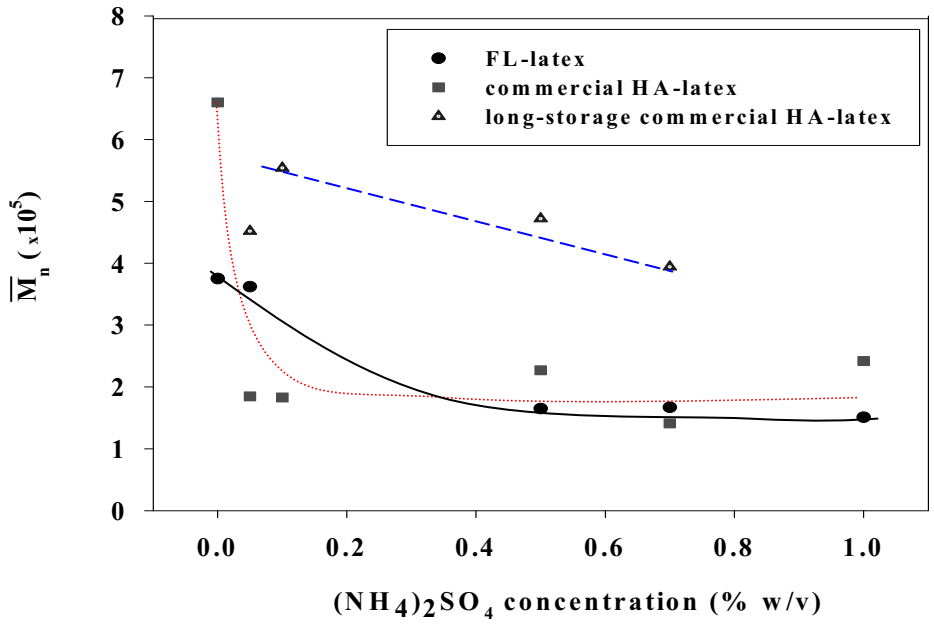


Figure 3.60 Number-average molecular-weight ($\overline{M}_n$) of sol fraction in rubber from FL-latex and commercial HA-latex treated with $(NH_4)_2SO_4$.

Figure 3.60 shows the number-average molecular-weight ($\overline{M}_n$) of the toluene soluble fraction of rubber from FL-, commercial HA-lattices and long-storage commercial HA-latex containing 50% gel after $(NH_4)_2SO_4$ treatment. It is clearly observed that the $\overline{M}_n$ value decreased upon increasing the concentration of $(NH_4)_2SO_4$. The striking reduction in the $\overline{M}_n$ value was observed for the commercial HA-latex from 6.5×10^5 to 2×10^5 after the addition of 0.1% (w/v) $(NH_4)_2SO$, and then leveled

off to a value similar to FL-latex. The $\overline{M}_n$ value of rubber from the long-storage commercial HA-latex apparently decreased with an increasing $(NH_4)_2SO_4$ concentration, but it was higher than the rubbers obtained from FL- and commercial HA-latices. This tendency, observed in commercial HA-latex, is in good agreement with that of the gel content, as shown in **Figure 3.58**. The decrease in the gel content and $\overline{M}_n$ value of a sol fraction of the rubber from commercial HA-latex indicates that Mg^{2+} ions play an important role on the formation of branch-points derived by ionic crosslink between rubber chains.

However, the ionic crosslinks are presumed to act to form long-chain branching in FL-latex and comprise only a small part in the gel phase of long-storage commercial HA-latex that contains the gel fraction of 50%. This assumption is supported by the fact that the $\overline{M}_n$ value of the commercial HA- and long-storage commercial HA-latices is higher than that of FL-latex. These findings are conclusive evidence showing the presence of other crosslink points in commercial HA-latex derived from additives, which are commonly added into FL-latex. The detail of these chemical crosslinks will be discussed in next section.

3.6.3 Mg^{2+} content of FL- and commercial HA-latices after $(NH_4)_2SO_4$ treatment

The Mg^{2+} content of rubbers obtained from commercial HA- and long storage commercial HA-latices was presumed to be similar for all commercially available rubbers. It is interesting to note that the gel content of rubbers from long-storage HA-latex was very high and did not decrease after $(NH_4)_2SO_4$ treatment, as shown in **Figure 3.59**. Thus, the Mg^{2+} content of the long storage commercial HA-latex was determined. The content of Mg^{2+} ions in FL-latex and long-storage commercial HA-latex was analyzed after centrifugation of the latices added $(NH_4)_2SO_4$, as illustrated in **Figure 3.61**.

The Mg^{2+} content in FL-latex was as high as 0.035%, while that of commercial HA-latex was about 0.0025%. This difference might be due to the treatment of FL-latex with $(NH_4)_2HPO_4$ before centrifugation to remove the excess Mg^{2+} ions as sludge for the preparation of commercial HA-latex. Thus, the Mg^{2+} content of

commercial HA-latex was lower than that of FL-latex. It is remarkable that the Mg^{2+} content in FL-latex was almost constant independent of the treatment of $(NH_4)_2SO_4$, while that of commercial HA-latex showed a significant decrease from 0.0025% to about 0.0008-0.0012% and leveled off at the concentration of 0.1% (w/v) $(NH_4)_2SO_4$. This result is expected to be due to the different solubility between $MgSO_4$ and $MgHPO_4$; the former is soluble in water, while the latter is insoluble. The resulting $MgSO_4$ could not be removed completely from rubber phase by centrifugation during Mg^{2+} content determination, contrary to $MgHPO_4$, which was removed completely after centrifugation.

All the above findings suggest that most of the Mg^{2+} ions present in FL-latex do not participate in the gel formation. On the other hand, a part of the Mg^{2+} ions in commercial HA-latex are presumed to take part in the gel formation, which was almost removed by $(NH_4)_2SO_4$ treatment (cf. **Figure 3.58**).

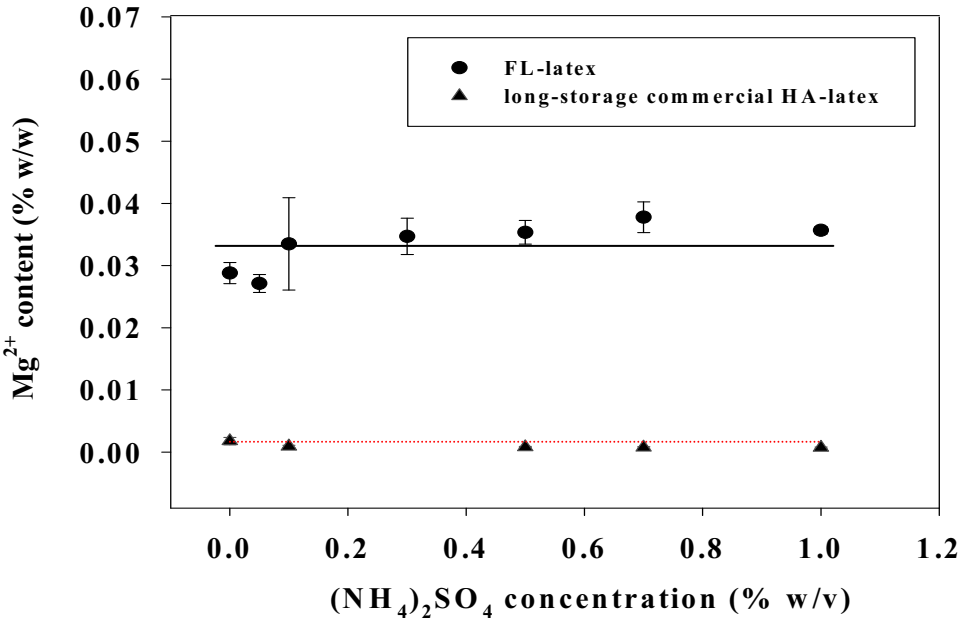


Figure 3.61 Mg^{2+} content of rubber from FL-latex and long-storage commercial HA-latex treated with $(NH_4)_2SO_4$.

3.6.4 Effect of storage time on gel formation of FL- and commercial HA-latices

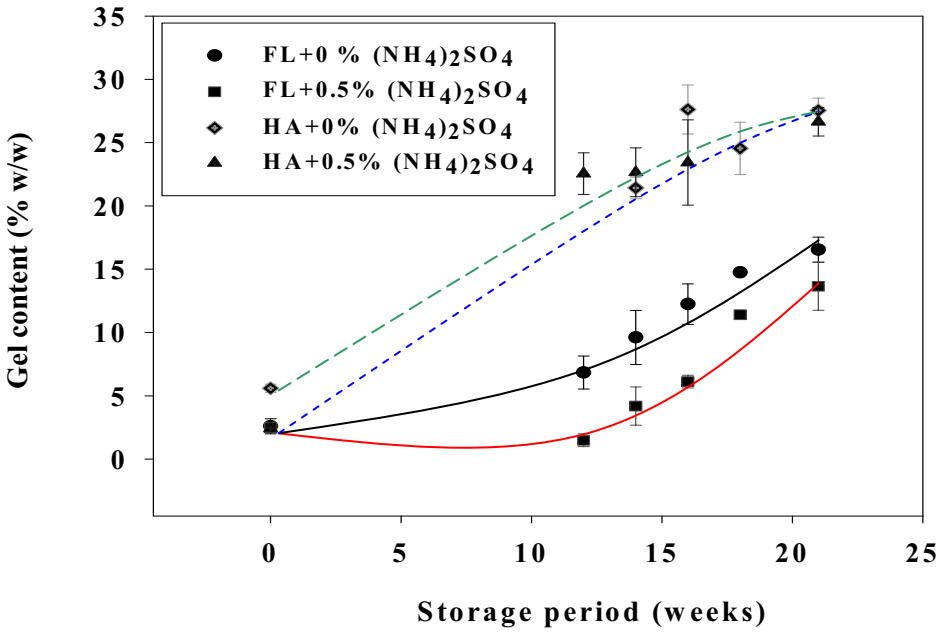


Figure 3.62 Relationship between gel content and storage period of FL- and commercial HA-latices with and without $(NH_4)_2SO_4$ treatment.

The effect of storage period on the gel formation was investigated for FL-latex and commercial HA-latex as shown in **Figure 3.62**. It is interesting that both latices showed an increasing in gel content with an increase in storage period, both with and without the addition of 0.5% (w/v) $(NH_4)_2SO_4$. The gel content of FL-latex increased slowly from 2.5 to 6% in 12 weeks, after that increased rapidly to 15% within 22 weeks. A similar tendency was observed for FL-latex treated with 0.5% (w/v) $(NH_4)_2SO_4$. The gel content of FL-latex treated with 0.5% (w/v) $(NH_4)_2SO_4$ increased to 12% after 22 weeks. It is clear that the addition of 0.5% (w/v) $(NH_4)_2SO_4$ slowed down the gel formation in FL-latex by removing Mg^{2+} ions, although other mechanisms, such as the crosslink formation by hydrogen bonding and chemical crosslinks, are a predominant factor for the increase in gel fraction during prolonged storage. On the other hand, a rapid increase in the gel content was observed for commercial HA-latex with and without the addition of 0.5% (w/v) $(NH_4)_2SO_4$. This result supports the idea that hydrogen bonding *via* proteins and chemical crosslinks

play a predominant role in the gel formation of commercial HA-latex rather than ionic crosslink *via* Mg^{2+} ions.

The presence of TMTD/ZnO, which is normally added as preservatives of NR latex, was also found to cause gel formation for prolonged storage of latex even in deproteinized latex as mentioned previously. Based on the above results, it can be concluded that there are at least three kinds of crosslinks in NR latex, i.e. derived by ionic linkages, hydrogen bonding *via* proteins and phospholipid, and covalent bonding by chemical crosslinks from additives.

CHAPTER IV
CONCLUSION

Free lipids in deproteinized natural rubber (DPNR) were removed by extraction with acetone. The residual fatty acid ester groups were presumed to be derived from phospholipids. Mixed phospholipids were separated by GPC using chloroform as eluent. The high molecular-weight rubber fraction was composed of rubber molecules containing linked phospholipids. The ^{1}H-, ^{13}C- and ^{31}P-NMR of high molecular-weight rubber fractions showed the signals corresponding to monophosphate, diphosphate and acylgylcerol compound containing nitrogenous group. This was confirmed by the analysis of phospholipid model compounds. The relative intensity of carbonyl carbon signal indicated the presence of two phospholipid molecules per rubber chain.

The presence of acylglycerol and/or phospholipid at rubber chain-end was confirmed by the structural change of rubber chain after treatment with lipase, phosphatase and phospholipases A_2, B, C and D. The content of fatty acid ester groups and molecular weight of DPNR rubber drastically decreased after lipase and phosphatase treatments. The removal of long-chain fatty acid ester group linked at C1 in the phospholipid by lipase treatment and phosphoric ester groups by phosphatase treatment resulted in the decrease in molecular weight and decomposition of branch-points. The decrease in the molecular weight and Huggins' constant, *k'*, indicated the presence of acylglycerol compound and phosphoric monoester linkage at rubber chain-end or α-terminal group, which form branch-points. The decomposition of branch-points was confirmed by molecular-weight distribution, changing from typical bimodal distribution to unimodal one by shifting the peak of high molecular-weight fraction to low molecular-weight. The content of long-chain fatty acid ester in DPNR rubber decreased after treatment with phospholipases A_2, B, C and D. The treatment of DPNR latex with phospholipases A_2, B and C decreased the molecular weight significantly, whereas no change was observed for the treatment with

phospholipase D. This supported the idea that most parts of branch-points are originating from fatty acid groups at C1, C2 and phosphate group at C3 in phospholipid molecule by the formation of a micelle structure. DPNR treated with these phospholipases showed the residual phosphates by ^{13}C- and ^{1}H-NMR analyses, showing the presence of mono- and diphosphate groups at the α-terminal. These phosphate group were not able to be removed by phosphatase treatment suggesting that the phosphate groups in DPNR are not simple phosphate, but having modified or protecting groups.

The formation of branch-points in DPNR was postulated to be due to micelle formation *via* hydrogen bonding or ionic linkages between phospholipids and phosphate groups. This assumption was confirmed by the analysis of GPC using chloroform containing small amounts of ethanol as eluent as well as NMR analysis study. The addition of ethanol into DPNR solution in toluene resulted in the decomposition of branch-points originating from hydrogen bonding between phospholipid and/or phospholipids to form free phospholipid molecules and was able to be separated from rubber by re-precipitation in methanol. The small signals corresponding to phospholipid remained in ^{13}C-NMR spectrum of low molecular-weight DPNR after the addition of ethanol and re-precipitation. This can be deduced that the residual phospholipids might be linked to mono- or diphosphate groups at the α-terminal of rubber chain by ionic linkage such as Mg^{2+} ions. Quantitaive analysis of solid-state ^{31}P-NMR indicates the presence of two to three phosphorus atoms per rubber chain. Based on the above results, it can be concluded that α-terminal of rubber is composed of mono- and diphosphate group which is linked to phospholipid by hydrogen bonding or ionic linkage. The low molecular-weight compounds corresponding to phospholipids were obtained from toluene/methanol fraction of DPNR precipitated from toluene solution into methanol, which showed bimodal molecular-weight distribution corresponding to micelle structure of phospholipids. The effect of hydrogen bonding and Mg^{2+} ions on branching formation derived from phospholipids was analyzed by the addition of 1% (v/v) ethanol and 5% (w/w) diammonium hydrogenphosphate (DAHP) into DPNR solution and latex, respectively. The addition of ethanol into DPNR solution resulted in the decomposition of branch-points originated by hydrogen bonding between

phospholipid molecules to form linear molecules leading to the decrease in molecular-weight and narrower MWD comparable to TE-DPNR. The addition of DAHP into DPNR latex caused only a slight decrease in the molecular weight. This indicates that Mg^{2+} ions have less effect on branching formation than hydrogen bonding. These are supporting evidences showing that branch-points in DPNR are mainly originated by hydrogen bonding.

The addition of TMTD/ZnO into the ammonia preserved FL-latex (PRD-FL-latex) treated with DAHP caused an increase of gel fraction in comparison with no TMTD/ZnO. This indicates that excess amounts of Mg^{2+}, TMTD/ZnO and DAHP play an important role in gel formation in commercial high ammonia-latex (HA-latex) during long time storage. TMTD/ZnO accelerates the gel after formation at the initial period, while DAHP activates the crosslink formation at long time storage.

Addition of ammonium sulfate into commercial HA-latex, which was prepared from FL-latex treated with DAHP before centrifugation to remove Mg^{2+} ions, resulted in an apparent decrease in the gel content comparable to that of FL-latex, while an insignificant effect was observed for long-storage commercial HA-latex containing the gel fraction as high as 50%. This indicates that ionic bonds take part in the gel formation in commercial HA-latex, but are not predominant in long-storage commercial HA-latex. The gel content of FL-latex and commercial HA-latex increased with increasing the period of storage even in the presence of ammonium sulfate. These findings indicate that the gel formation in commercial HA-latex during prolonged storage is derived partly by Mg^{2+} ion, but predominantly by hydrogen bonding and chemical crosslinks.

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2. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y., "Gel formation in natural rubber latex: 1. Effect of Magnesium ion". Rubber. Chem. Technol., 76, 1185 (2003)
3. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y., "Relationship between particle size and molecular weight of natural rubber from *Hevea brasiliensis*" in preparation
4. Tarachiwin, L., Sakdapipanich, J. Tanaka, Y., Fukuda, I., Ute, K. and Kitayama, T. "Structural characterization of terminal groups in natural rubber-Part 1. Structural characteristic of α-terminal group of GPC fractionated natural rubber" in preparation
5. Tarachiwin, L., Sakdapipanich, J. Tanaka, Y., Fukuda, I., Ute, K. and Kitayama, T. "Structural characterization of terminal groups in natural rubber-Part 2. ^{1}H-, ^{13}C- and ^{31}P-NMR analysis of phospholipids as a model of structural characterization of *Hevea* rubber" in preparation
6. Tarachiwin, L., Sakdapipanich, J. Tanaka, Y., Fukuda, I., Ute, K. and Kitayama, T. "Structural characterization of terminal groups in natural rubber-Part 3. Decomposition of branch-points by enzymatic treatment" in preparation
7. Tarachiwin, L., Sakdapipanich, J. Tanaka, Y., Fukuda, I., Ute, K. and Kitayama, T. "Structural characterization of α-terminal of natural rubber. Decomposition of branch-points by chemical treatment" in preparation

LIST OF REVIEW

1. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y. “Formation and structure of long-chain branching in natural rubber” in preparation

LIST OF PROCEEDING PRESENTATIONS

1. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y., "Relationship between particle size and molecular weight of natural rubber from *Hevea brasiliensis*". 27th Congress on Science and Technology of Thailand, October 2001, Songkla, Thailand.
2. Tarachiwin, L., Sakdapipanich, J., Tanaka, Y., Fukuda, I. and Ute, K., "Characterization of terminating end of natural rubber using phospholipid as a model compound". 9th International Symposium on Elastomers, April 2003, Kyoto, Japan
3. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y. "Gel formation in natural rubber. Effect of Additives and Mg^{2+}. 29th Congress on Science and Technology of Thailand, October 2003, Khon kaen, Thailand.
4. Tarachiwin, L., Sakdapipanich, J. and Tanaka, Y. "Structural characterization of natural rubber. Decomposition of branch-points by enzymatic treatment" 16th Elastomer Symposium, December 2003, Tokyo, Japan

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