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Effect of Aerobic Ageing at Elevated Temperatures on Dynamic Mechanical Properties of Certain Natural Rubber Vulcanisates

I.R. GOODCHILD* AND T.J. POND*#

Dynamic modulus and damping properties have been determined over a range of temperatures from five different natural rubber vulcanisates aerobically aged at elevated temperatures for periods up to 150 days. Overall, aerobic ageing is found to increase the modulus but decrease the damping of the vulcanisates. The rate of change of properties caused by ageing, though initially relatively small, may increase after longer times if antioxidant material becomes depleted. Results at longer ageing times indicate the modulus may, depending upon the vulcanisates' ingredients, pass through a maximum; however the modulus remains greater than for unaged material. Aerobic ageing is also shown to alter the glass transition temperature of a vulcanisate.

Like other elastomers, the mechanical properties of natural rubber may alter as a result of ageing. Aerobic ageing is normally caused by oxygen diffusing into the rubber and producing chemical reactions with the medium[1]. The oxidative reactions produce crosslinking, scission of the rubber chains and the attachment of polar oxygen-containing groups to the rubber molecules[2]. Such changes to the structure of the rubber can alter its modulus and damping.

The modulus of natural rubber vulcanisates aerobically aged at elevated temperatures has been studied by a number of workers[3–7]. The results have shown that, overall, the modulus tends to increase with aerobic ageing although not always in a monotonic manner. The majority of ageing studies have examined quasi-static stress-strain behaviour with only limited measurements of dynamic properties such as modulus and damping. Bjork and Stenburg[8] have conducted dynamic measurements on two natural rubber vulcanisates which show that the complex modulus and damping both increase after aerobic ageing at 100°C. Their finding that the damping increases with ageing is perhaps surprising since an increase in the modulus of the rubber might be expected to produce a fall in the damping of the material.

The purpose of the present study is to further investigate dynamic modulus and damping properties in aerobically aged natural rubber vulcanisates. The aerobic ageing conditions have been chosen to provide extensively oxidised samples in order to quantify and

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characterise the change in modulus and damping of these materials. The extent of the oxidation in the test samples studied is much greater than that present under lower operating temperatures and the measurements should not be considered representative of normal ambient conditions where the magnitude of the change in properties will be very much smaller.

EXPERIMENTAL

Natural rubber sheets were manufactured from five different compounds. The composition of each compound and the vulcanisation conditions are shown in *Table 1*. The rubber sheets (220 × 220 × 0.7 mm in thickness) were aged in circulating air ovens at a temperature of 90, 100, 125 or 150°C for various periods of time up to 150 days. After ageing, rectangular test pieces (35 × 10.6 × 0.7 mm) were cut from each sheet away from the edge, and the dynamic modulus (E') and damping ($\tan \delta$) were determined by using a dynamic mechanical thermal analyser (DMTA) test machine. The deformation geometry was dual cantilever.

All the measurements were conducted at a frequency of 1 Hz over a temperature range of –60°C to +30°C and at a strain of 0.002%. A very small test-strain is necessary to measure the modulus of highly oxidised rubber since the value of elongation at break is very small due to the glassy and brittle nature of this material. Dynamic test data were also obtained from unaged test pieces of each vulcanisate for comparison with the aged material. Tests were repeated to study the reproducibility of the data which was found to be good.

The weight of unreacted antioxidant (*Santoflex 6PPD*) and rubber-bound nitrogen were determined from test pieces of compound number five after various times of ageing at a temperature of 90°C. Antioxidant levels were determined by extracting each sample in acetone and analysing on a high performance liquid chromatography machine which for these experimental conditions has a low detection limit of 0.001%. The levels of rubber-bound nitrogen were measured using the Kjeldahl method in the usual manner.

RESULTS AND DISCUSSION

Results of dynamic mechanical testing are given in *Figures1–10*. Aerobic ageing increases the dynamic moduli of all the vulcanisates. A material which is extensively oxidised has a much greater modulus than an unaged material at temperatures greater than about –55°C as illustrated in *Figures 1* and *3*; however, the modulus at –60°C where the rubber behaves like glass is unaltered. The increase in the modulus of a particular vulcanisate is determined by the time and the temperature of ageing. The modulus may increase by a factor of about 100 when the rubber is extensively oxidised. In similar vulcanisates which undergo less extensive oxidation, such as a vulcanisate subjected to ageing temperatures approaching ambient, the increase in modulus is much smaller, typically of the order of 10% to 40%[7]. In extensively oxidised rubber as shown in *Figures 1* and *3*, the material is somewhat brittle and glass-like at ambient temperatures and the normal glass transition temperature is not apparent.

The rate of increase in the modulus of a vulcanisate is not constant with ageing time but typically is quite small during the initial ageing period. At longer ageing times the rate of change of the modulus tends to increase as illustrated in *Figures 3, 5* and *6*. Such a change

TABLE 1. COMPOSITION OF COMPOUNDS AND VULCANISATION CONDITIONS

Compound ingredients	Compound (p.p.h.r.)				
	1	2	3	4	5
Natural rubbers (SMR CV 60)	100	100	100	100	100
Zinc oxide	5	5	5	5	5
Stearic acid	2			2	2
Sulphur	2.5	0.7	0.7	2.5	1.0
CBS[1]	0.6			0.6	0.75
Santoflex 6PPD	3	3	3	3	3
Antilux 600	3	3	3	3	3
Carbon black N220			50	50	60
ZE[2]		1.0	1.0		
OBS[3]		1.7	1.7		
TBTD[4]		0.7	0.7		
Dutrex 729					18
Vulcanisation time (min)	11	12	8	9	8
Vulcanisation temperature (°C)	160	160	160	160	160

[1]N-cyclohexylbenzothiazole-2-sulphenamide

[2]**Zinc-2-e**thylhexanoate

[3]**N-oxy**diethylenebenzothiazole-2-sulphenamide

[4]Tetrabutylthiuram disulphide

in the modulus can be influenced by the presence of carbon black filler as well as curative ingredients as indicated in *Figure 6*. The ageing time required to reach the point when the modulus significantly increases becomes longer with a fall in the ageing temperature. For vulcanisate number 5 (*Figure 5*) such a point occurs after about 60 days ageing at a temperature of 90°C. From rheometry measurements it is estimated that this ageing condition is approximately equivalent to 700 years ageing at a temperature of 20°C. At long ageing times the modulus may fall according to the compound ingredients as shown in *Figure 6* but remains greater than unaged material. The significant change in the rate of increase of the modulus is believed to be caused by the depletion of antioxidant material within the test pieces.

A comparison of the concentration of unreacted antioxidant remaining in samples of compound five after ageing at 90°C (*Table 2*), with that of the modulus shown in *Figure 5* indicates that unreacted antioxidant levels have fallen to less than 0.001% after 60 days ageing. Such a small concentration of unreacted antioxidant is also present between 28 days and 60 days of ageing and alone could not be expected to provide effective protection of the rubber. Measurements of the nitrogen concentration, which remain unchanged, indicate that the antioxidants are not being significantly volatilised at the ageing

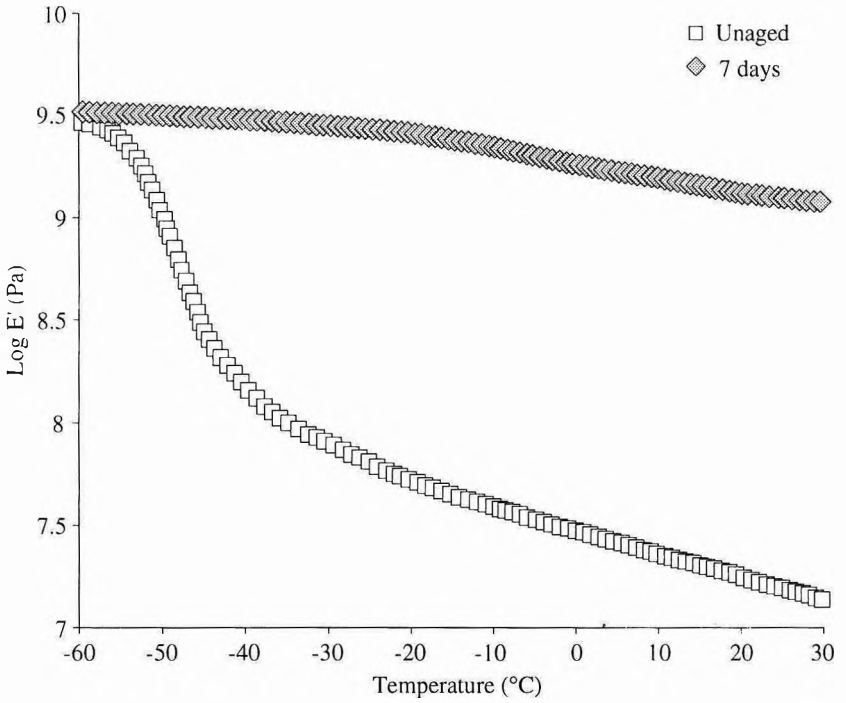

Figure 1. Data points showing the modulus of an unaged test piece and a test piece after ageing for 7 days at 150°C as a function of temperature (Compound 5).

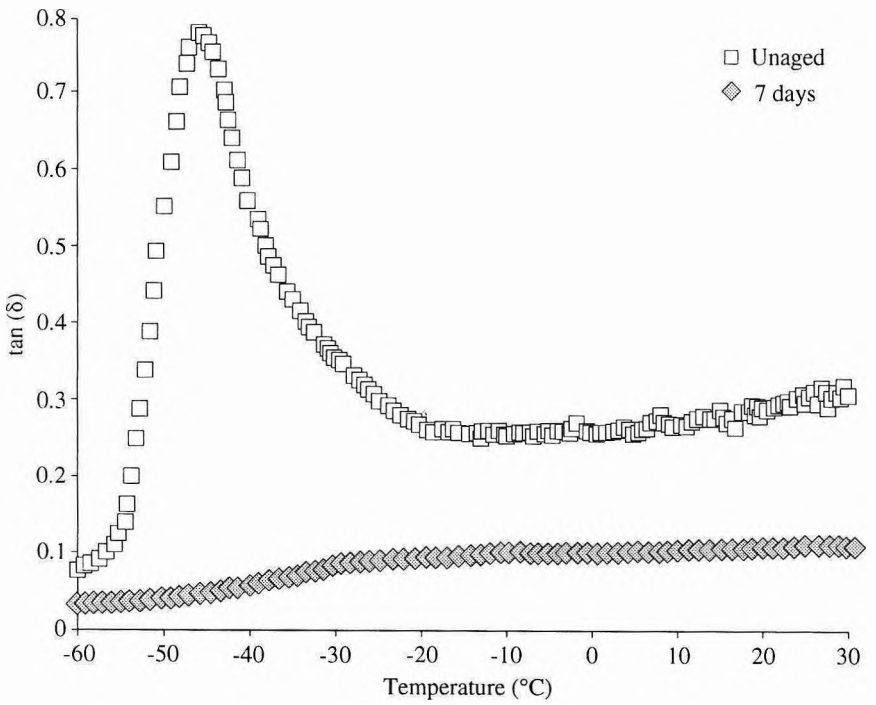

Figure 2. The damping (tan δ) of a test piece aged for 7 days at 150°C and an unaged test piece versus *temperature (Compound 5).*

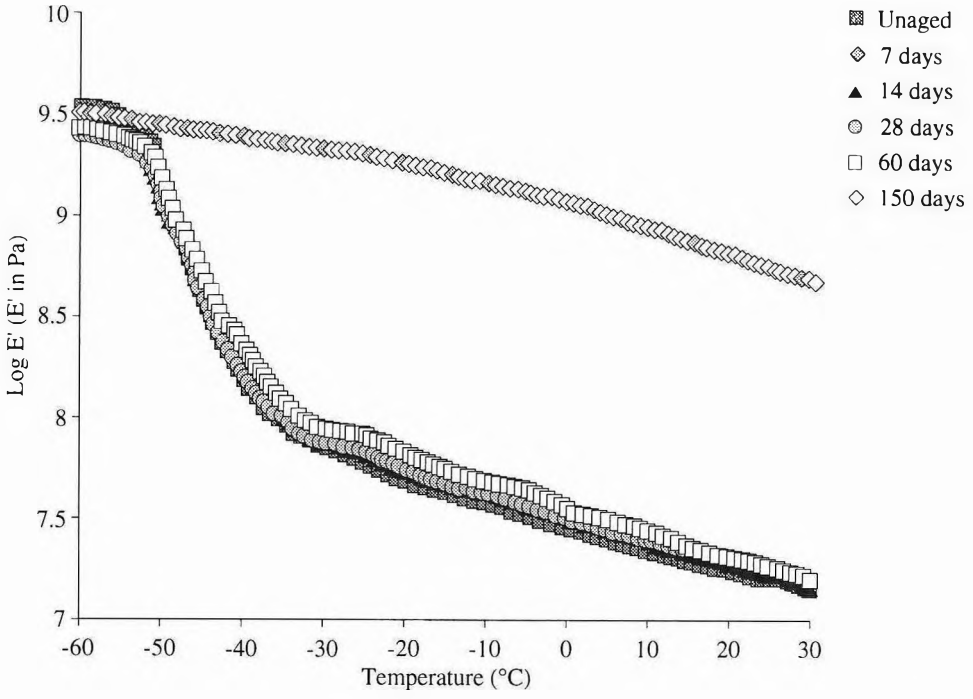

Figure 3. The moduli of test pieces aged for different times at a temperature of 90°C and an unaged test piece versus *temperature (Compound 5).*

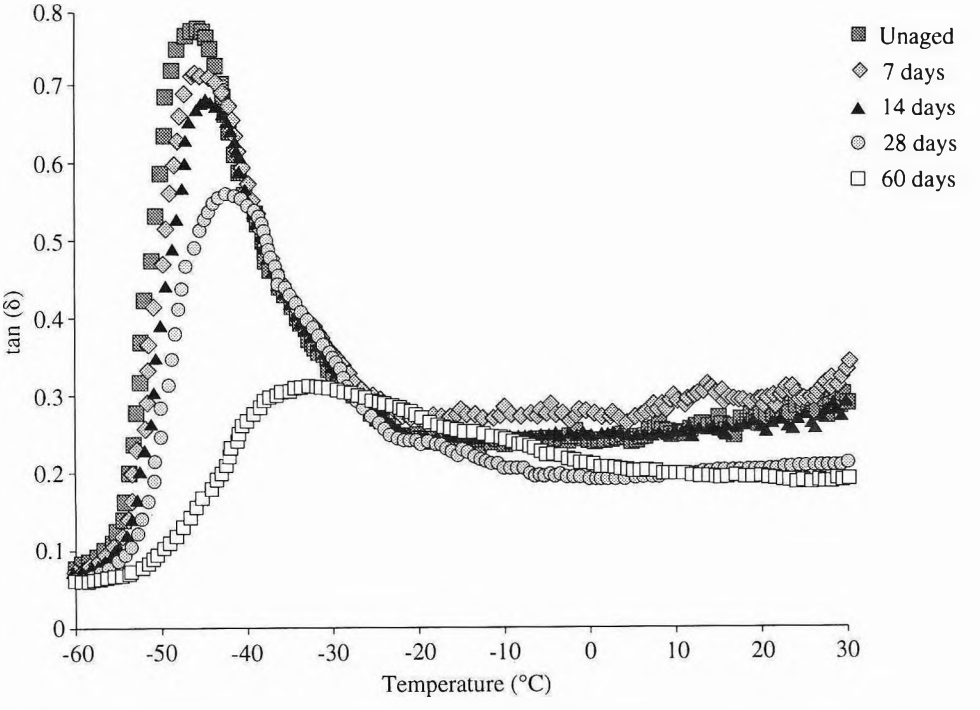

Figure 4. The damping of test pieces aged for different times at a temperature of 100°C and an unaged test piece versus *temperature (Compound 5).*

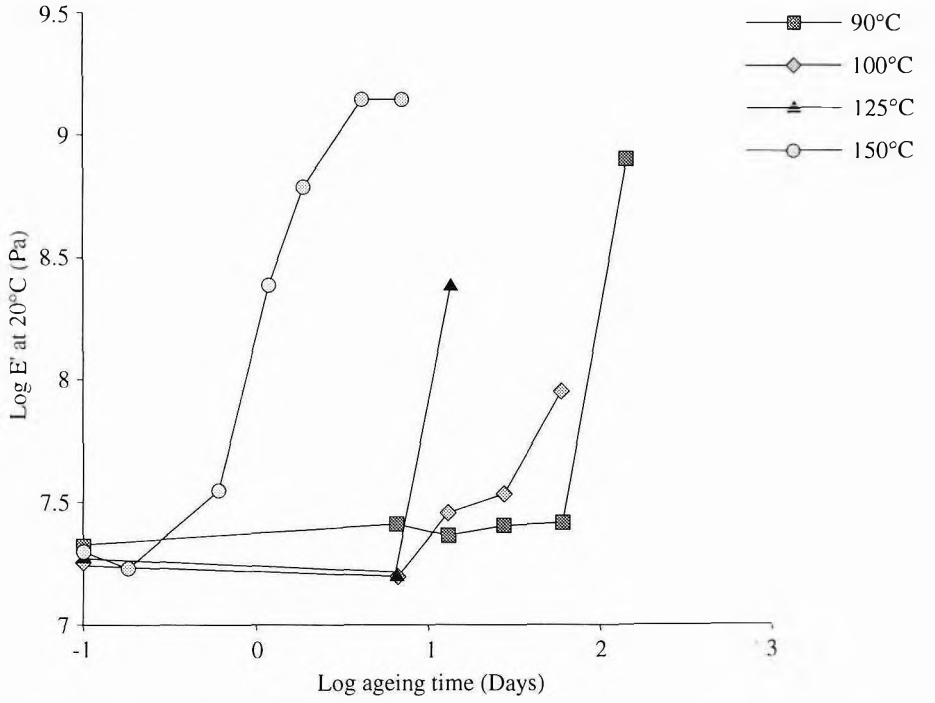

Figure 5. The moduli of test pieces at a temperature of 20°C after ageing at different temperatures between 90°C and 150°C versus *ageing time (Compound 5).*

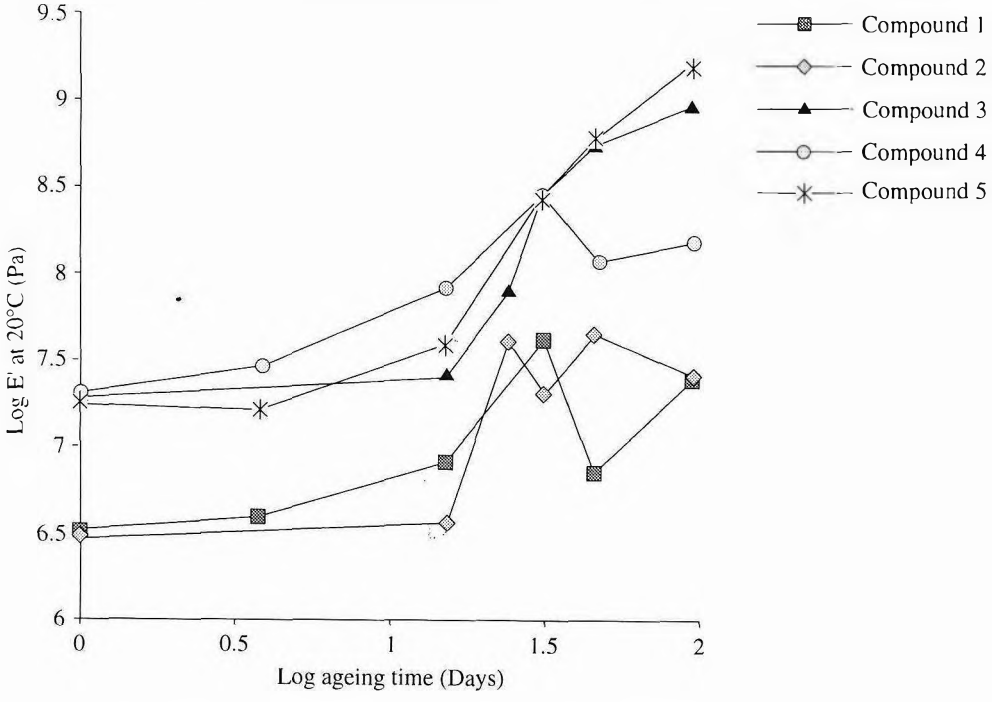

Figure 6. The moduli of test pieces at a temperature of 20°C versus *ageing time. Ageing temperature: 150°C (Compounds 1 to 5).*

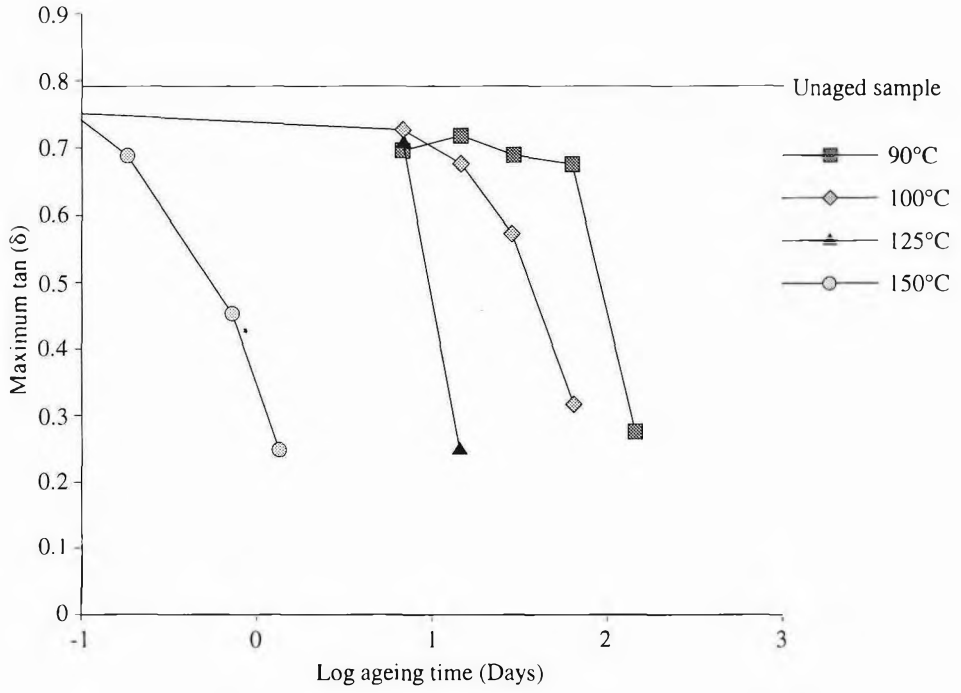

Figure 7. The maximum value of tan δ from test pieces at a temperature of 20°C after ageing at different temperatures between 90°C and 150°C versus *ageing time (Compound 5).*

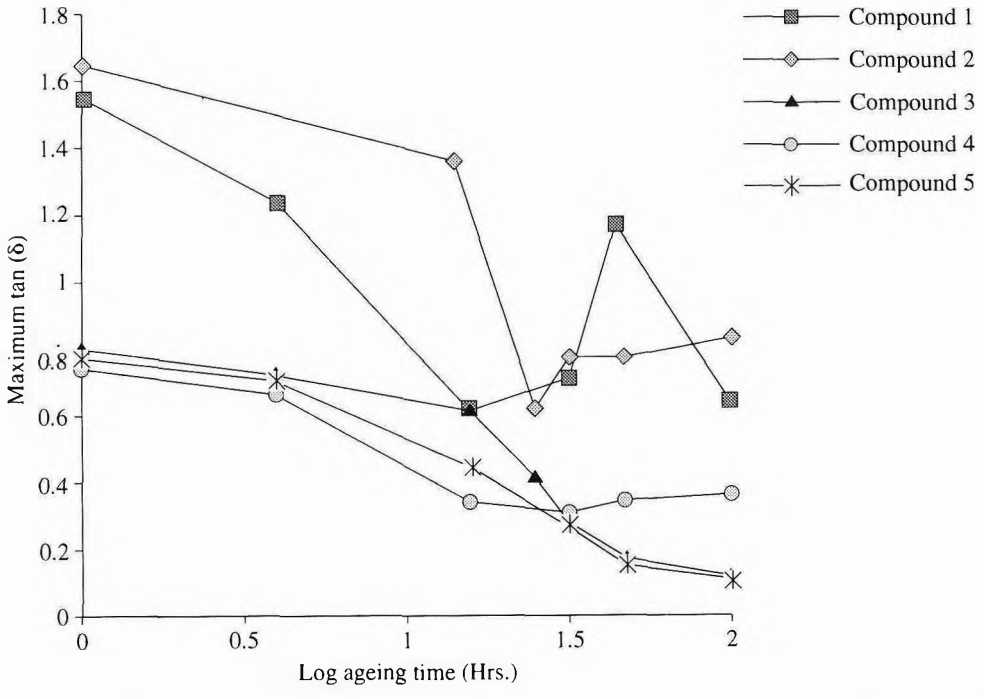

Figure 8. The maximum value of tan δ from test pieces at a temperature of 20°C versus *ageing time. Ageing temperature: 150°C (Compounds 1 to 5).*

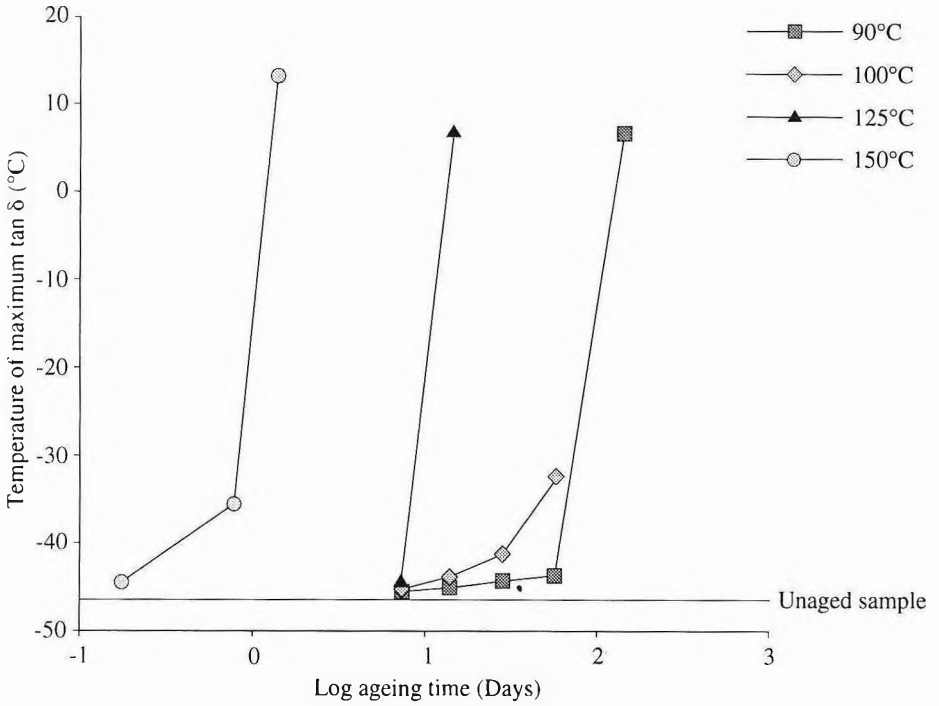

Figure 9. The temperature at which the maximum value of tan δ occurs from test pieces aged at different temperatures between 90°C and 150°C versus *ageing time (Compound 5).*

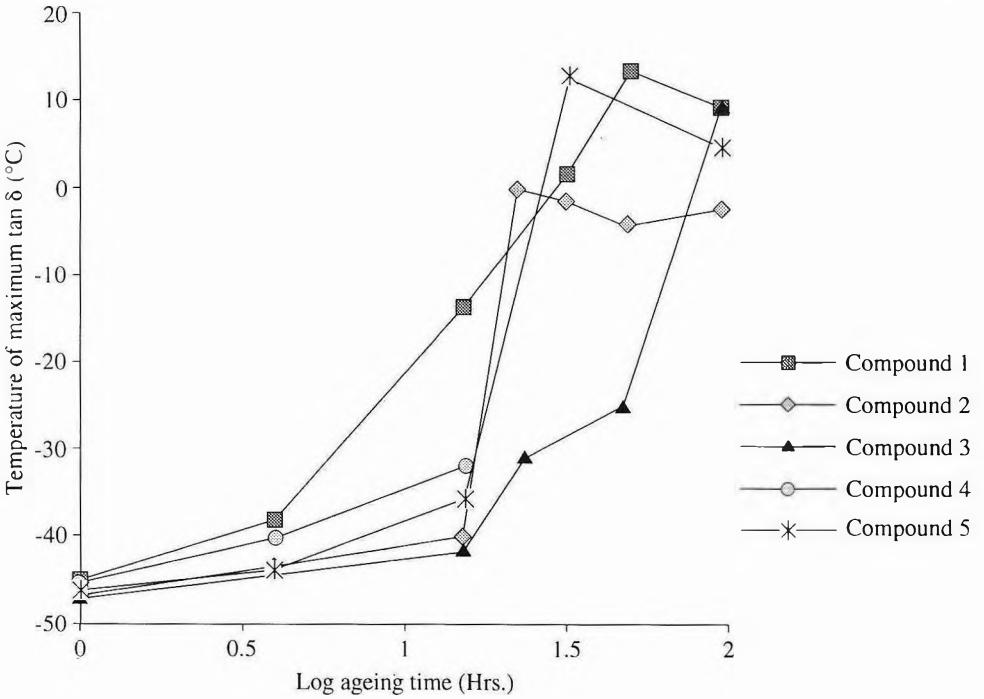

Figure 10. The temperature at which the maximum value of tan δ occurs versus *ageing time. Ageing temperature: 150°C (Compounds 1 to 5).*

TABLE 2. THE WEIGHT OF UNREACTED ANTIOXIDANT (*SANTOFLEX 6PPD*) AND NITROGEN IN TEST PIECES AEROBICALLY AGED FOR DIFFERENT TIMES AT A TEMPERATURE OF 90°C

Ageing time (days)	Compound 5 Antioxidant weight (%)	Nitrogen weight (%)
Unaged	0.948	0.32
7	0.136	0.32
14	0.016	0.32
28	< 0.001	0.32
60	< 0.001	0.33
150	< 0.001	0.33

The lower detection limit for the antioxidant is 0.001%

temperature of 90°C and that the antioxidant remains in the rubber, being converted to a reacted form. A reason why the modulus increases sharply after 60 days ageing and not just after 28 days when the unreacted antioxidant is virtually depleted could be that the reacted antioxidant material can continue to provide a measure of protection to the rubber even after the unreacted antioxidant is depleted[9].

Material which is extensively oxidised has a different damping characteristic from that of unaged material as illustrated in the damping profiles shown in *Figure 2*. The damping peak may be completely removed with ageing resulting in a flat curve with low tan δ values. The damping of aged material at ambient temperature can fall to about one third that of unaged material.

The temperature of the peak damping (tan δ) is termed the glass transition temperature. Ageing tends to lower the value of the damping peak and simultaneously raise the glass transition temperature as illustrated in *Figure 4*. The rate of fall of the peak damping value does not remain constant with ageing. The damping peak initially falls only slightly but is followed by a greater rate of fall at longer ageing times, as indicated by *Figures 7* and *8*. At longer ageing times the peak damping can rise as well as fall depending upon the compound ingredients. Such behaviour reflects that of the modulus characteristics. Ageing can raise the glass transition temperature of a vulcanisate to about 0°C or perhaps a little higher as illustrated in *Figures 9* and *10*. It is not possible accurately to determine the glass transition temperature in extensively aged vulcanisates due to the flat nature of the damping profile.

CONCLUSIONS

Aerobic ageing can significantly alter the dynamic modulus and damping of a vulcanisate. Ageing tends to increase the modulus and glass transition temperature of a vulcanisate but it decreases the damping. Depletion of antioxidant material in such vulcanisates by ageing is associated with increases in the rate of change of their properties. However, one notes that,

after depletion of antioxidant, reaction products remain. These appear to continue to give some protection.

ACKNOWLEDGEMENTS

Thanks are due to Dr D. Campbell and Mr P. Lewis for helpful discussion of chemical reactions.

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Distribution and Origin of Abnormal Groups in Natural Rubber

A.H. ENG[*#], JITLADDA TANGPAKDEE[**], S. KAWAHARA[**] AND Y. TANAKA[**]

Ester and aldehyde groups are found to remain in natural rubber even after purification by deproteinisation and acetone extraction. Rubber isolated from smaller latex particles in the serum fraction contains a lower level of ester group than that from larger latex particles. Both aldehyde and ester groups are found to have a similar distribution in fractionated natural rubbers of different molecular weights. The concentration of these groups decreases with decreasing molecular weight of the rubbers. This finding suggests that the aldehyde groups are not derived from oxidative degradation of natural rubber. A drastic reduction in aldehyde content of natural rubber after transesterification is observed showing that the aldehyde groups are derived from oxidative degradation of olefinic group of unsaturated fatty acids bonded to the rubber molecule.

The main component of natural rubber molecule is *cis* polyisoprene hydrocarbon. Structurally, this natural polymer is more complicated than its synthetic analogue due to the presence of a small quantity of non-rubber groups, normally referred to as abnormal groups, bonded to the main-chain molecule. These groups are believed to be of biological significance in the biosynthesis of rubber.

It is now generally accepted that crosslinking reactions of the abnormal groups are the major cause for the formation of branching in natural rubber[1]. These branching entities eventually lead to the formation of gel and the occurrence of storage hardening of natural rubber which distinguishes it from the synthetic *cis* polyisoprene. The formation of gel during storage of dry rubber may involve a mechanism which is different from that of microgel in latex because the former is accelerated under low humidity conditions while the latter occurs in the aqueous medium[1].

Despite many years of investigation carried out by various workers, the mechanism for the gel formation has yet to be conclusively explained[1]. Nevertheless, several abnormal groups have been reported to be present in the main-chain rubber molecule and these are summarised in *Table 1*.

Since the presence of abnormal groups other than ester and aldehyde in natural rubber molecule is only circumstantial, the aim of this work is to provide more information on the distribution and the possible origin of these groups in natural rubber.

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TABLE 1. ABNORMAL GROUPS IN NATURAL RUBBER

Groups	Fractions	Concentration/ mmol kg^{-1}	Method	Ref.
Fatty acids	Low Mw	10–20	^{13}C-NMR	2
Lactone	Gel	10–15	IR	3
Aldehyde	Whole	10–35	H_2NOH	4
		1.5–5.0	2,4-DNPH	5
Amine	Whole	20–35	HBr titration	6
Epoxide	Whole	45–75	HBr titration	6
		10–15	Degradation	7

2,4-DNPH: 2,4-dinitrophenylhydrazine

MATERIALS AND METHODS

Field latex of RRIM 600 clone was preserved in 0.7% ammonia for 5 days before deproteinisation was carried out. Pale crepe rubber was of commercial grade and used as received. Other reagents were of analytical grade and used without further purification.

Synthesis of 2,4-dinitrophenylhydrazine-acetaldehyde (DNPH-acetaldehyde) Derivative[8]

Approximately 1.25 g of DNPH was dissolved in 25 ml of methanol containing 1 ml of sulphuric acid and filtered. It was then added drop-wise into 5 ml of vigorously stirred methanol containing 0.28 g of acetaldehyde. The precipitate formed was filtered and purified by recrystallisation from 95% ethanol. It was dried under reduced pressure at room temperature to constant weight. The melting point of the hydrazone was found to be 147°C which is comparable to the literature value[9] of 146°C–147°C.

Determination of Molar Extinction Coefficient of DNPH-acetaldehyde Derivative

The characteristics of the UV absorption of DNPH-acetaldehyde derivative in tetrahydrofuran (THF) was studied with a Jasco U-best 30 double beam UV-Vis spectrometer. A series of the derivative solutions with concentration up to 5.8×10^{-5} mol dm^{-3} was scanned from 600 nm to 200 nm. The plot of absorbance of the derivative against its concentration produces a molar extinction coefficient of 2.16×10^4 $dm^3mol^{-1}cm^{-1}$ and λ_{max} value of 359 nm, both of which are close to the literature values of $2.13 \times 10^4 dm^3mol^{-1}cm^{-1}$ and 360 nm, respectively, for butanal derivative[10].

Determination of Aldehyde Content of Natural Rubber

This method involves the treatment of 5 ml of 4% toluene rubber solution in a glass tube with

5 ml of 1% DNPH in THF. The details have been described elsewhere[5].

Fourier Transform Infrared (FT-IR) Analysis of Ester Groups in Natural Rubber

Methyl stearate was used as a model compound for the FT-IR analysis of ester groups in the natural rubber. The absorbance of the carbonyl group of methyl stearate was measured in purified natural rubber obtained from serum fraction because this rubber was found to contain a very low level of ester groups as compared to that of cream and bottom fractions.

The rubber samples for FT-IR analysis were prepared by casting 0.6% of the rubber solutions in chloroform on a KBr disk placed on activated silica gel and drying under a stream of nitrogen gas to form a round transparent film of about 1.5 cm in diameter. The film was scanned with a Jasco 5300 FT-IR spectrometer at a resolution of 2 cm^{-1}. The spectrum obtained is the average of 300 scans.

The area ratio of peaks at 1738 cm^{-1} (C=O) to 1664 cm^{-1} (C=C), (A_{1738}/A_{1664}), was plotted against the concentration of the added ester groups in the rubber.

In the case of natural rubber containing unknown amount of ester groups, the FT-IR spectrum was obtained by the same procedure described above. Ester content was then obtained by substituting the area ratio of peaks at 1738 cm^{-1} to 1664 cm^{-1} into the following expression:

$$\text{Ester (mmol/kg)} = (A_{1738}/A_{1664}) / \text{Gradient of calibration curve} \quad ...1$$

Measurement of Gel Content

Rubber sample was allowed to dissolve in toluene at 0.3% w/v for one week in the dark. The toluene solution was then centrifuged at 13 000 r.p.m. (20 000 g) for 40 min. The sol and gel fractions were separated and the gel was dried under reduced pressure to constant weight. The percentage of gel fraction was calculated from the weight ratio of the gel fraction to the original sample.

Isolation of Different Fractions of Latex

Fresh latex of about 10% dry rubber content (DRC) was poured into a condom placed in a centrifugal tube of similar size. It was centrifuged at 13 000 r.p.m. (20 000 g) for 30 min at room temperature. Upon completion, it was immediately frozen in a freezer. The condom was then removed from the frozen latex. The cream, serum and bottom fractions were separated and precipitated into ethanol. The rubbers were purified by reprecipitation from hexane into ethanol.

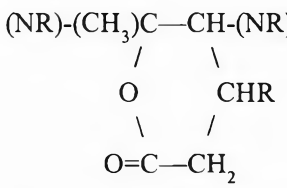
Other Measurements

Measurement of the molecular weight of rubber was carried out in toluene solution with a Wescan 231 Membrane Osmometer operating at 35°C using regenerated cellulose membrane. The accuracy of the instrument was tested by the use of three standard polystyrene samples of known molecular weight. The results showed that the osmometer could perform within a deviation of 5%. ^{13}C-NMR measurements were performed on deuterated chloroform solution of rubbers with tetramethylsilane (TMS) as an internal standard with a JEOL FX-200 NMR spectrometer operating at 50.1 MHz. The sample concentration and pulse interval were 10% w/v and 12 seconds, respectively.

RESULTS AND DISCUSSION

Ester Groups in Natural Rubber

The presence of ester groups in commercial natural rubber was first reported by Gregg and Macey[3]. However, they attributed the infrared band at 1738 cm^{-1} in the spectra of commercial rubber to the presence of lactone groups in the main-chain molecule as shown below:



where NR is the *cis* polyisoprene chain. However, ^{13}C-NMR studies on deproteinised natural rubber (DPNR) revealed that the ester groups in natural rubber are associated with the fatty acids which could be removed by transesterification with sodium methoxide[11]. On the other hand, if ester groups were of lactone origin, these groups would remain in the polymer chain even after treatment of the rubber with sodium methoxide.

Evidence for the presence of fatty acid ester groups in natural rubber is shown in *Figure 1* which shows ^{13}C-NMR spectra of whole fraction of DPNR and transesterified rubber obtained from fresh latex. Small signals at 14.0, 29.7 and 34.5 p.p.m. were assigned to terminal methyl ($-CH_3$), methylene ($-(CH2)_n-$) and methylene ($-O_2C\underline{C}H_2-$) carbons of long chain fatty acid, respectively. These signals disappeared after transesterification. Analysis of the methyl ester by ^1H-NMR revealed that it contains 20% unsaturated and 80% saturated fatty acids[11].

As shown in *Figure 2*, ester groups were found to remain in natural rubber even after extensive purification of latex with protease enzyme. *Alcalase 2.0T*, followed by successive washing with surfactant *via* centrifugation[12]. The DPNR showed a clear infrared band at 1738 cm^{-1}, which is a characteristic feature of carbonyl groups of fatty ester. Upon acetone extraction, about half of these groups remained in the polymer indicating that unbonded fatty acids were present in the purified rubber. The results on quantitative analyses of ester are shown in *Table 2*. The unextractable ester groups thus represent the level of bonded ester present in the rubber.

The ester content of natural rubber isolated from cream fraction, serum fraction and bottom fraction of centrifuged latex is indicated in *Table 3*. It is interesting to note that natural rubber from the serum fraction contained only 0.9 mmol/(kg rubber) of ester groups. This is much lower than those from cream and bottom fractions. Since the average diameter of rubber particles in the serum fraction has been shown to be about ten times smaller than that in the cream fraction[13], it is reasonable to assume that smaller particle size latex represents newly formed entity from which larger particle size latex is formed. Therefore, it is reasonable to predict that the esterification occurred after the rubber has been synthesised. This could account for the low ester content of rubber from smaller particle latex where only a small part of the rubber molecules had been esterified.

Distribution of Ester and Aldehyde Groups in Natural Rubber

The presence of aldehyde groups in natural rubber was proposed[4,5] because rubber-hydrazone was formed when the rubber was

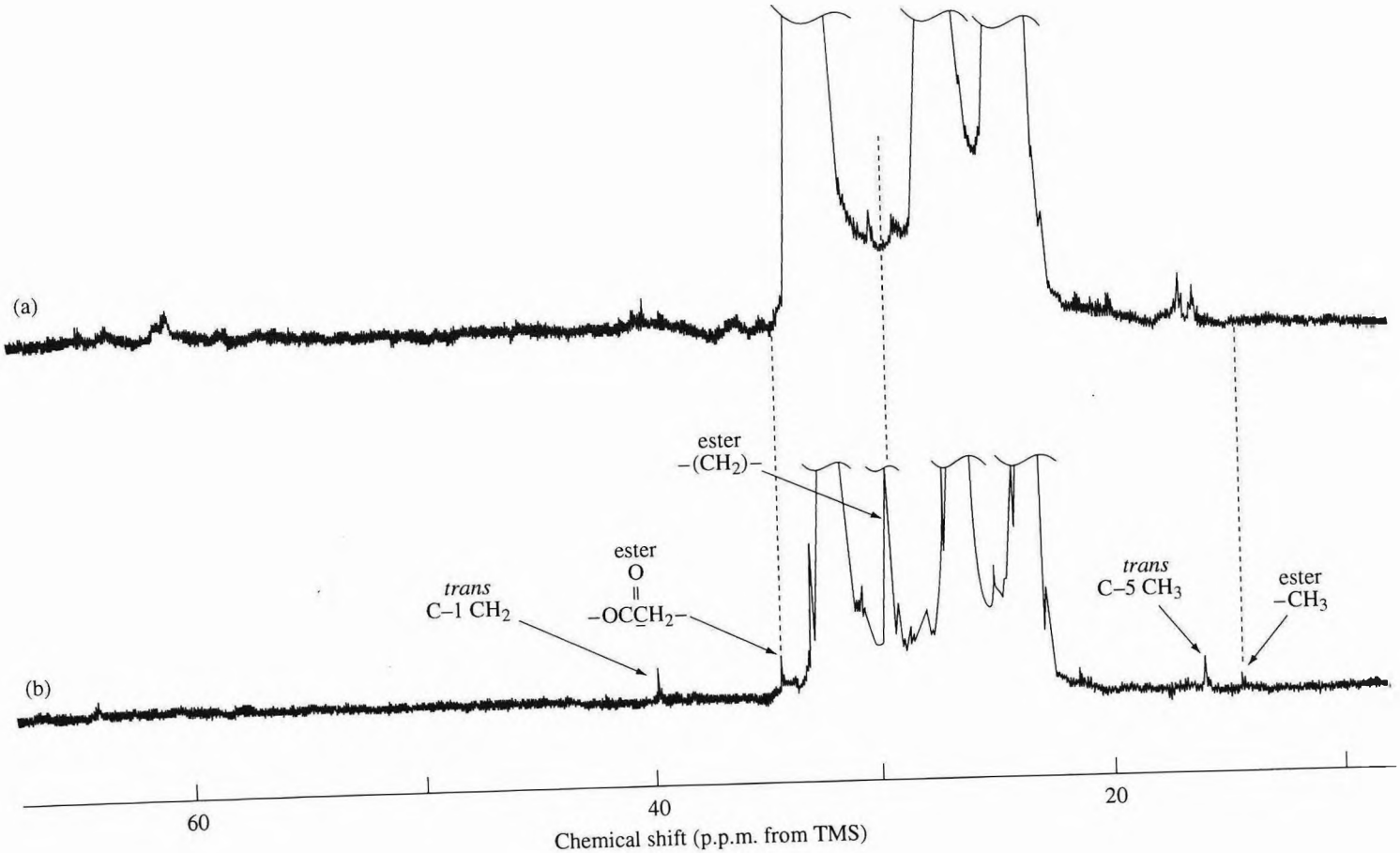


Figure 1. ^{13}C*-NMR spectra (a) transesterified natural rubber, and (b) deproteinised natural rubber.*

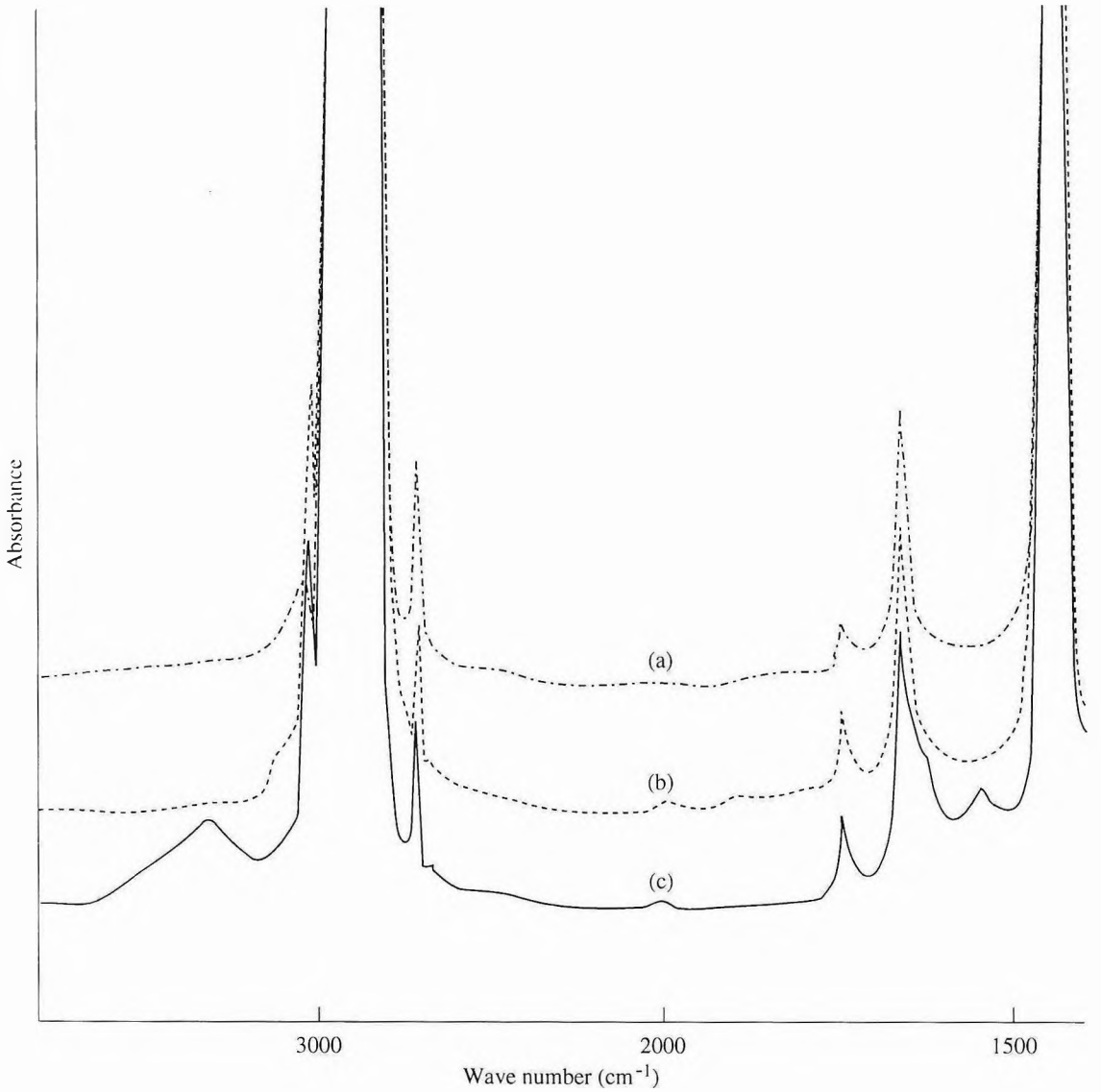


Figure 2. FT-IR Spectra (a) deproteinised and acetone extracted natural rubber (b) deproteinised natural rubber, and (c) control.

TABLE 2. ESTER CONTENT OF DIFFERENT NATURAL RUBBERS

Sample	Nitrogen content/% w/w	Ester content/mmol kg^{-1}
Control	0.30	18.0
DPNR	0.01	15.0
AE-DPNR	0.01	8.5

TABLE 3. ESTER CONTENT OF NATURAL RUBBER FROM CENTRIFUGED LATEX

Sample	Ester groups/ mmol $(kg\ NR)^{-1}$	Intrinsic viscosity
Cream fraction	7.8	7.1
Serum fraction	0.9	5.8
Bottom fraction	8.6	ND

ND: Not determined

treated with 2,4-dinitrophenylhydrazine (DNPH). The estimated level of aldehyde was in the region of 1.6–5.4 mmol/(kg rubber) and the λ_{max} value of 353 nm–357 nm for the derivative suggests the presence of non-conjugated aldehyde.

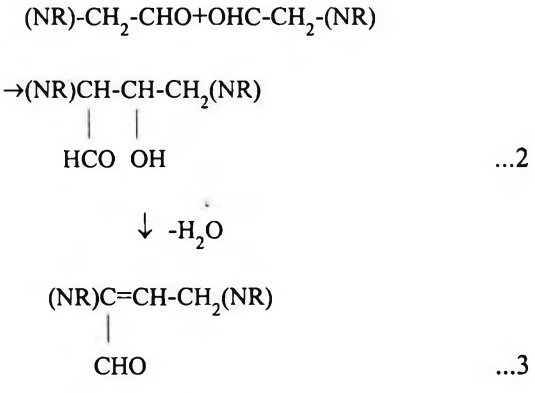
It has been demonstrated that the molecular weight of fractionated natural rubber before and after branching can be estimated by ^{13}C-NMR and membrane osmometry techniques, respectively[14]. These techniques were used in the present study to investigate the distribution of ester and aldehyde groups in fractionated natural rubbers. Thus, if two linear rubber molecules (molecular weight, *NR*) react to form a branched rubber molecule $[(NR)_2]$, the molecular weight of linear rubber molecule as analysed by ^{13}C-NMR will be *NR* and that analysed by osmometry will be $(NR)_2$.

Table 4 indicates that all fractions of the natural rubber contain an average of 1-2 ester groups and 0.2–0.4 aldehyde groups per linear rubber molecule based on two *trans* isoprene terminal units per chain[15]. The ester groups have been postulated to be located at the branching point of rubber molecule *via* association with phospholipid complex[14]. For branched rubber molecule, the aldehyde content increases with increasing molecular weight of the rubber indicating that the aldehyde groups are not auto-oxidative chain scission products of the natural rubber molecule because under these conditions, a reversed distribution order would be observed; *i.e.* low molecular weight rubber has high aldehyde content and *vice versa*. Since high molecular weight rubbers have been found to contain more branching points than that of low molecular weight rubbers[14], the distribution of aldehyde groups

TABLE 4. ALDEHYDE AND ESTER GROUPS IN FRACTIONATED NATURAL RUBBER

Fraction	$M_n \times 10^{-5}$		Ester group/chain		Aldehyde group/chain	
	Linear	Branched	Linear	Branched	Linear	Branched
1	1.8	10.9	1.4	8.5	0.2	1.2
2	1.7	8.7	1.3	6.7	0.2	1.1
3	1.4	6.1	2.1	2.1	0.3	1.1
4	0.4	0.7	1.4	2.4	0.2	0.4

found here is in accordance with the postulation that branching entities of natural rubber are derived from aldo-condensation of the aldehyde groups[16]. However, under these circumstances, high molecular weight branched rubber molecule should contain more than one aldehyde group because the formation of each branching point produces one aldehyde group in the rubber molecule as demonstrated in *Equation 2*:



Furthermore, under the proposed reaction scheme, a conjugated aldehyde group is expected to be present as shown in *Equation 3*, but this was not observed in the hydrazone derivative obtained in the present study.

Since the trend of the distribution of the aldehyde groups is similar to that of ester groups in natural rubber, there is a possibility that these groups are derived from oxidation of olefinic groups of unsaturated fatty acids bonded to natural rubber molecule. The oxidation of bonded unsaturated fatty acids would not cause a significant decrease in the molecular weight of the rubber. This process may proceed *via* an enzyme assisted mechanism. The presence of oxidising enzymes, *i.e.* oxidases in natural rubber latex has been reported[17]. Therefore, if the high molecular weight fractions represent old rubber molecules in the tree, it is not surprising to find that more olefinic groups of the bonded unsaturated fatty acids have been oxidised in the presence of the oxidases to form aldehyde groups as compared to those in low molecular weight fractions.

If the aldehyde groups originated from oxidation of bonded unsaturated fatty acids, the removal of the rubber ester groups will drastically reduce the aldehyde content of the rubber. *Table 5* shows the aldehyde content of commercial pale crepe and DPNR from high ammonia preserved field latex before and after transesterification[14]. These results indicate that substantial amount of the aldehyde groups could be removed *via* transesterification. The presence of higher residual aldehyde groups in the pale crepe rubber than those in DPNR after

TABLE 5. ALDEHYDE CONTENT OF TRANSESTERIFIED NATURAL RUBBERS

Sample	Aldehyde content/mmol kg^{-1}
Pale crepe	4.6
TE Pale crepe	2.2
DPNR	2.4
TE-DPNR	0.4
Synthetic polyisoprene	0.3

TE: transesterified

transesterification is probably due to oxidative degradation of the pale crepe rubber during the production and storage of the rubber. In the case of DPNR, the level of aldehyde groups after transesterification is much lower, indicating that the aldehyde groups are associated with the ester groups bonded to natural rubber molecule.

CONCLUSION

Ester groups are found to remain in natural rubber after deproteinisation and acetone extraction. Rubber isolated from smaller latex particles in the serum fraction contains a lower level of ester groups than that from larger latex particles in the cream fraction. Both aldehyde and ester groups are found to have a similar distribution in fractionated natural rubbers of different molecular weights. The concentration of these groups decreases with decreasing molecular weight. It is concluded that the aldehyde groups are not derived from oxidative degradation of natural rubber, but are derived from oxidative degradation of olefinic groups of unsaturated fatty acids bonded to natural rubber.

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J. nat. Rubb. Res., **12(1)**, 21–32

Changes to NR Latex Proteins on Processing the Latex to its Products

H. HASMA*# AND M.Y. AMIR-HASHIM*

Fresh NR latex contained two major proteins, 14 kD and 24 kD, associated with rubber particles, and a variety of acidic and basic proteins of molecular weights less than 14 kD to greater than 100 kD in the B- and C-serum fractions. Processing the latex to its products changed the composition of these proteins, more so with serum proteins than with rubber particle proteins. Serum proteins most affected were the very acidic and basic proteins of molecular weight greater than 14 kD and isoelectric point (pI) in the pH range of 3.5 to 4.6 and 7.0 to 9.5, respectively. The composition of these proteins was greatly influenced by the storage periods of high ammoniated (HA) latex concentrate, latex compounding ingredients and heating of the latex compound. Thus HA latex concentrate of more than 3-months-old, HA latex concentrate compounded with ZnO and prevulcanised latex contained mainly acidic proteins of pI in pH range of 4.6 to 6.0 and molecular weights less than 14 kD. A similar pattern was found in extractable proteins of NR examination gloves.

Studies on NR latex proteins commenced way back in the 1950s. The pace of research picked up after 1980s when it was established that soluble proteins extracted from latex products were linked to Type I hypersensitivity reaction in latex sensitised people[1–3]. Following this, numerous reports[4–8] on proteins in fresh latex, ammoniated latex and latex products, particularly examination gloves appeared. The focus was, however, on NR latex proteins that could bind to IgE antibodies which might result in an allergic reaction. Results from some of these studies showed that the protein composition changed on processing fresh latex to HA latex concentrate and to its products. With the exception of the study by Hasma[6], most of these are isolated studies, with no definite intention of systematically giving a clear picture of the changes on the latex proteins in processing the latex to its products. It is the objective of this study to add further information to the earlier work[6] besides presenting a clearer picture of the proteins in fresh NR latex and showing the effects of concentrating the latex, storing and compounding HA latex concentrate, and heating compounded latex. The composition of proteins extracted from examination gloves was also examined. This will enable latex researchers, producers and consumers to have a better understanding of the nature of proteins in NR latex and its products.

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MATERIALS AND METHODS

Isolation of NR Latex Proteins

Proteins from fresh latex. Fresh latex was centrifuged on a Beckman L8-70 ultra-centrifuge at 19 000 r.p.m. using rotor 21 for an hour. Rubber particles (RP), clear C-serum and bottom fraction were isolated. RP were redispersed in 0.7% ammonia twice before a final dispersion in 2% sodium dodecyl sulphate (SDS). At each step, RP dispersion was filtered to remove any coagulum, stored overnight and finally ultracentrifuged to separate the RP from the extracts. The second ammonia extract and SDS extract were collected, dialysed against distilled water for 3 days and freeze-dried.

The bottom fraction was freeze-thawed three times, centrifuged at 10 000 r.p.m. using rotor 21 for 45 min before obtaining the clear B-serum.

Proteins from HA latex concentrate. Clonal HA latex concentrates were prepared in the laboratory by centrifuging about 0.5% ammoniated field latex in a De Laval LRH 410-70A centrifugal latex separator. Latex concentrates of about 60% dry rubber content was further ammoniated to 0.7%. Commercial latex concentrates of mixed clones preserved with ammonia and other secondary preservatives were obtained from a few local producers.

HA latex concentrates were ultracentrifuged to separate the RP from the serum fraction as above. The clear HA serum fraction was isolated, dialysed and freeze-dried.

Proteins from compounded and prevulcanised latex. HA latex concentrates were compounded with sulphur vulcanising ingredients according to the formulations tabulated in *Table 1*, stored overnight and ultracentrifuged to obtain the clear serum fraction. The compounded latex serum was dialysed and freeze-dried.

A portion of the compounded latex was matured overnight and prevulcanised at 70°C for 2 h. After overnight storage, the pre-vulcanised latex was ultracentrifuged and its clear serum collected, dialysed and freeze-dried.

Extractable proteins from examination gloves. Pieces of gloves weighing 10 g were extracted with 50 ml of 0.01 M phosphate buffered saline solution at 35°C for 3 h. The extracts were filtered through Whatman filter paper No.1, dialysed and freeze-dried. The dried sample was redissolved in 1 ml water.

Polyacrylamide Gel Electrophoresis

The B- and C-sera of fresh latex were diluted 1:4 (v/v) with a solubilising buffer while the ammonia and SDS lyophilised extracts of RP were solubilised in the same solubilising buffer to a concentration of 4 mg/ml. The mixture was heated at 95°C for 4 min before being analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) following the procedure outlined in Bio-Rad Instruction Manual[9]. The SDS-PAGE was run for 35 min at a constant voltage of 200 volts (Mini Protean 11 Cell; Bio-Rad, Richmond, Calif., USA).

The freeze-dried samples from serum of HA latex concentrate, compounded latex and prevulcanised latex and from glove extracts were solubilised in the same manner as above. The analysis by SDS-PAGE was, however, in

TABLE 1. COMPOUNDING FORMULATIONS

Compounding formulation	F_1	F_2	F_3
60% HA latex concentrate	100[a]	100[a]	100[a]
50% Sulphur	1.0	1.0	1.0
50% Zinc diethyl dithiocarbamate	0.6	0.6	0.6
50% Zinc oxide	–	0.4	0.4
20% Potassium laurate	–	–	0.2
10% Potassium hydroxide	–	–	0.2

[a] Values in p.p.h.r.

accordance with the method described by Schagger and Jagow[10]. This method gave a better separation of low molecular weight proteins of 3.5 kD to 17 kD.

Isoelectric focusing (IEF) was carried out according to the LKB Instruction Note 2217[11] on a 0.5 mm polyacrylamide gel containing ampholine carrier ampholyte pre-blended to pH 3.5 to pH 9.5, purchased from LKB (Bromma, Sweden). This technique was only used to analyse the water soluble proteins since the water insoluble ones could not be resolved well under the present experimental conditions. About 20 µl of water soluble protein samples (4 mg/ml) were run at a constant power of 10 watts for 45 min with 1 M NaOH as cathode electrolyte and 1 M H_3PO_4 as anode electrolyte.

The SDS-PAGE and IEF gels were fixed, stained and destained according to the respective instruction manuals[9–11].

RESULTS AND DISCUSSION

Proteins in Fresh Latex

Figure 1 shows a wide range of proteins from 3 major fractions of fresh latex; the RP, the B- and C-serum as analysed by the SDS-PAGE. As reported[12], the 14 kD and 24 kD proteins formed the two major RP proteins extracted by ammonia while the 14 kD protein, identified as the rubber elongation factor by Dennis and Light[13], was the main RP protein extracted by SDS. The REF (*Hev b 1*) and the 24 kD protein (*Hev b 3*) have been shown to bind to the IgE antibodies of patients with *spina bifida* and latex allergy[14–16].

The B-serum clearly revealed protein bands of molecular weights less than 14, 14, 20, 22, 23, 30, 36 and 45 kD, in agreement with some of the B-serum proteins identified by Alenius[7]. The 20 kD prohevein has been demonstrated to exhibit high binding frequency to IgE antibodies of adult latex-allergic patients, suggesting it to be the major NR latex allergen[17]. However, the main allergenic epitope of prohevein is located at its N-terminus, which represents the 4.7 kD hevein molecule[17]. This makes hevein an important latex allergen. Hevein could be among those in the molecular weight region of less than 14 kD. The allergenicity of 30, 36 and 45 kD proteins have also been demonstrated[17].

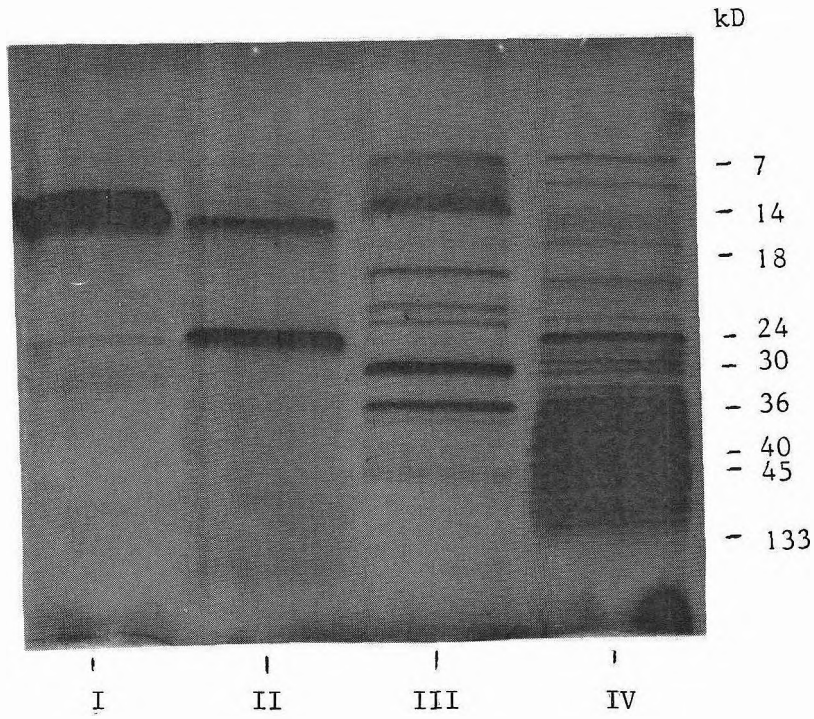

Figure 1. SDS-PAGE of fresh latex proteins.

I: SDS-extracted RP proteins
II: Ammonia-extracted RP proteins
III: B-serum proteins
IV: C-serum proteins

C-serum, however, contained a greater variety of proteins with molecular weights stretching from 7 kD to 133 kD. In fact a higher proportion of the C-serum proteins were of molecular weights greater than 36 kD, unlike the majority of the B-serum proteins which were of molecular weights less than 36 kD. The 27 kD C-serum protein has been found to be recognised characteristically by the IgE of latex-allergic children with *spina bifida* or other congenital anomalies and histories of multiple surgeries[18].

Analysis by IEF (*Figure 2*) showed that a high number of the ammonia-extractable RP proteins, the B- and C-serum proteins were acidic/anionic proteins with isoelectric points (pI) in the acidic pH regions of 3.5 to 6.0. The acidic proteins of the ammonia-extracted RP protein seemed to concentrate around pH 3.5

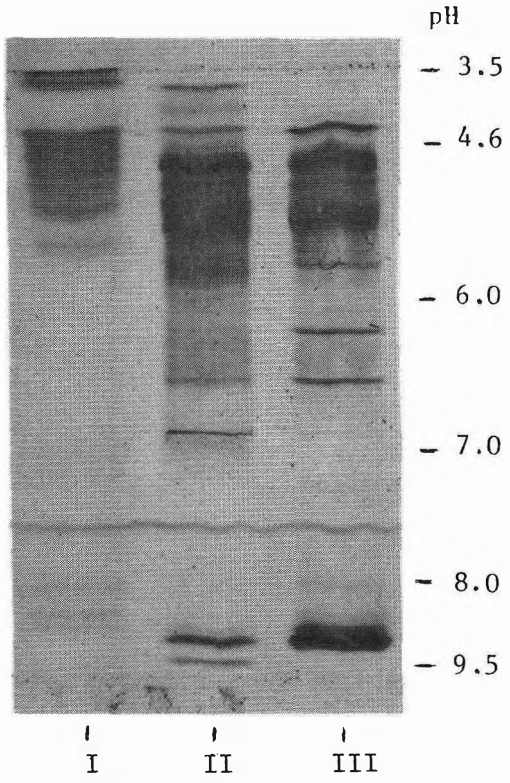

Figure 2. IEF of fresh latex proteins.

I: Ammonia-extracted RP proteins
II: C-serum proteins
III: B-serum proteins

to less than 6.0 while the B-serum proteins stretched from pH 4.6 to pH 6.8 and the C-serum proteins from pH 3.5 to pH 6.8. Protein bands were observed at pH 4.7 in B-serum and pH 4.55 in C-serum which could be hevein and α-globulin, respectively. However, based on the intensity of the two bands when compared to the rest, they did not represent the major B- and C-serum proteins of fresh latex. Hevein was reported to constitute 70% of the B-serum proteins while α-globulin was considered the highest concentrated protein in C-serum[19,20].

Unlike the acidic/anionic proteins, there were fewer number of basic/cationic proteins at pH 8

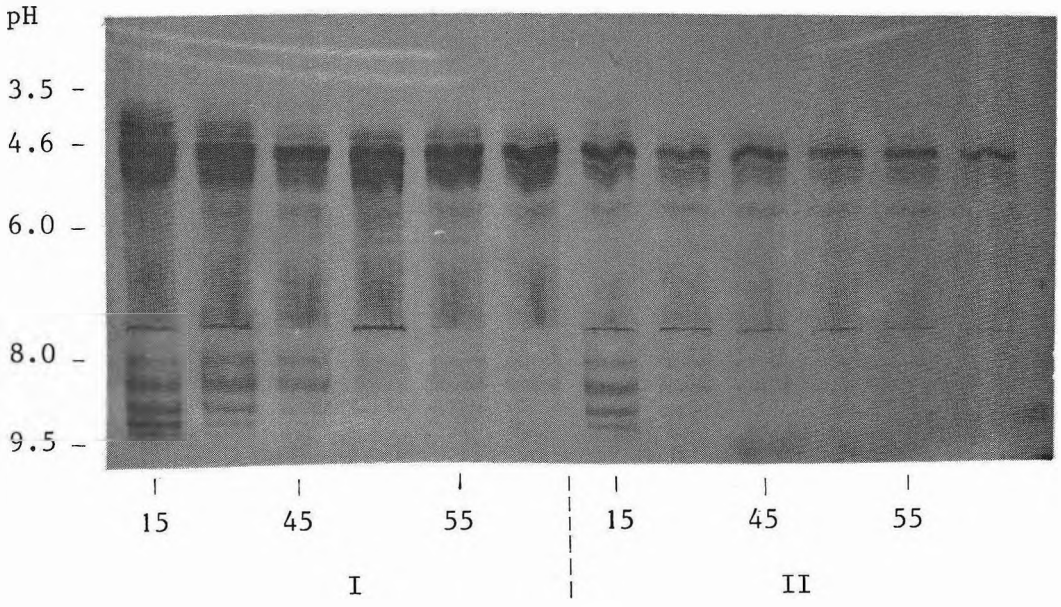

Figure 3. IEF of proteins from HA latex concentrate with (II) and without (I) secondary preservatives.

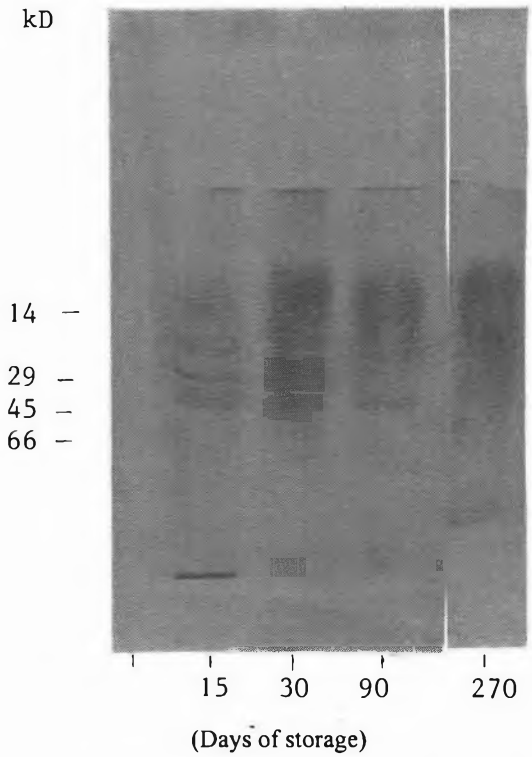

Figure 4. SDS-PAGE of proteins from HA latex concentrate.

to pH 9.5 in all the three latex fractions. Owing to the insolubility of the SDS-extractable RP protein in water, the protein was not analysed on the IEF. However, the above analysis showed that fresh NR latex contained a wide variety of proteins with pI stretching from very acidic pH of 3.5 to basic pH of 9.5 and molecular weights of less than 14 kD to greater than 100 kD.

Proteins in HA Latex Concentrate

The addition of ammonia and other preservatives such as tetramethyl thiuram disulphide-ZnO to field latex resulted in the bursting of the lutoids, releasing the B-serum content into the C-serum fraction. Consequently ammoniated latex contains only two fractions; the RP and the serum fraction. Centrifuging the latex to prepare latex concentrate resulted in not only the loss of proteins which are removed together with skim latex, but also changes to the composition of the proteins (*Figures 3* and *4*). These changes did not arise much from the RP proteins as similar proteins were extracted by ammonia and SDS from the RP of the latex concentrates of different storage times[12]. The differences were, however, in the serum proteins. HA latex concentrate of up to 15 days old contained a number of acidic and basic proteins as shown in *Figure 3*. The composition of these proteins changed on prolonged storage of the latex. The very acidic proteins of pI in the pH range of 3.5 to 4.6, and especially the basic ones greatly diminished after a month of storage, leaving the latex with mainly the acidic proteins of pI in the pH region of 4.6 to 6.0. The degradation rate was faster with the commercial HA latex concentrate which contained other secondary preservatives besides ammonia, than the laboratory prepared HA latex concentrate preserved with only ammonia. However, after 2–3 months of storage, serum of all HA latex concentrates contained mainly acidic proteins with a prominent band around pH 4.7 which is also the pI for hevein.

Likewise, changes were also shown by SDS-PAGE (*Figure 4*). A number of high (>14 kD) and low (<14 kD) molecular weight proteins were present in the serum of freshly prepared to 15 days old HA latex concentrates. After 15 days to a month of storage, the concentration of low molecular weight proteins in the latex concentrate increased. The level of high molecular weight proteins, however, diminised. On increasing the storage time further the amount of high molecular weight proteins became negligible such that older HA latex concentrate of more than 3-months-old contained only low molecular weight proteins of <14 kD.

Although latex concentrate serum did not contain the identified fresh latex allergenic proteins which are $\geqslant$ 14 kD, they could inhibit the binding of IgE to these proteins[21]. This indicates that the serum proteins of latex concentrate contained epitopes/allergenic determinants of fresh latex allergens. Thus degradation/hydrolysis of fresh latex proteins resulted in low molecular weight peptides with some of the allergenic determinants intact.

Proteins in Compounded and Prevulcanised Latex Serum

Compounding the HA latex concentrate with sulphur, zinc diethyl dithiocarbamate (ZDEC), zinc oxide (ZnO), potassium hydroxide (KOH) and potassium laurate affected the composition of the serum proteins (*Figure 5*). The effects

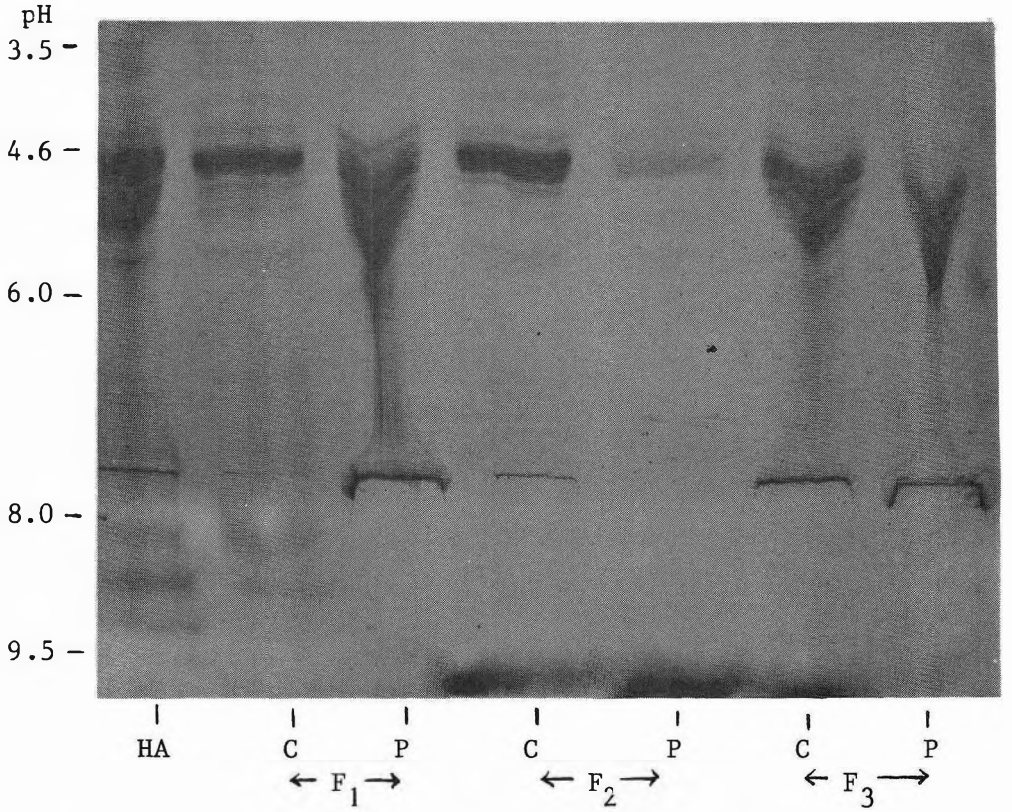

Figure 5. IEF of proteins from HA latex concentrate (HA), compounded latex (C) and prevulcanised latex (P) of different formulations (F).

were mostly on the basic proteins brought about by the presence of ZnO. When the latex concentrate was compounded with formulation 1 (*Table 1*) (in the absence of ZnO), the basic proteins were still present though at a lower concentration than originally present in the starting material of a month-old HA latex concentrate. These proteins were, however, rendered undetectable on incorporating ZnO in the presence or absence of KOH and potassium laurate. Heavy metals such as Zn are known to complex with proteins rendering it insoluble. Heating the compounded latex at 70°C for 2 h with or without ZnO, also resulted in the basic proteins being undetected. Furthermore, the very acidic proteins of pI in the pH range of 3.5 to 4.6 which were present in the serum of the compounded latex were denatured on heating the latex.

The difference in the serum proteins of the compounded latex as shown by the IEF (*Figure 5*) was not evident on SDS-PAGE (*Figure 6*). The serum of compounded latex of

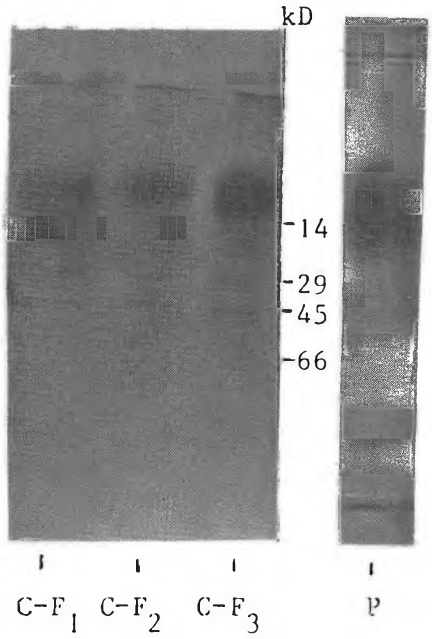

Figure 6. SDS-PAGE of proteins from compounded (C) prevulcanised (P) latex of different formulations (F).

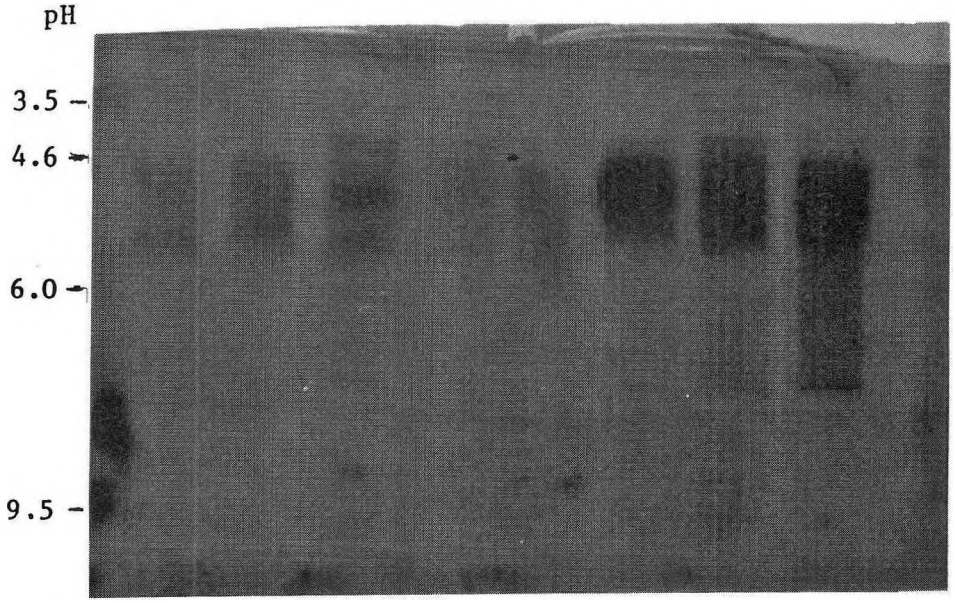

Figure 7. IEF of extractable proteins from commercial examination gloves.

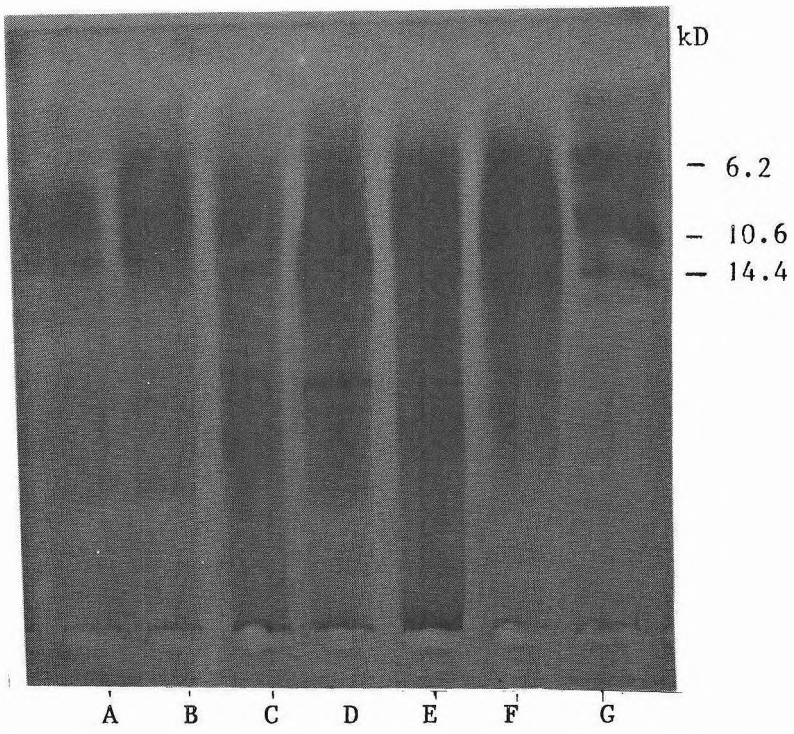

Figure 8. SDS-PAGE of extractable proteins from commercial examination gloves.

A-F: Extractable proteins from gloves
G: Low molecular weight markers

all the three formulations showed similar composition of proteins of molecular weights of <14, 29 and 45 kD. The prevulcanised latex, however, contained only the low molecular weight proteins of <14 kD. All the high molecular proteins of 29 kD and 45 kD could have been degraded on heating the HA latex concentrate at 70°C for 2 h. These proteins could include the very acidic proteins of pI in the pH range of 3.5 to 4.6 which were present in the three compounded latices but not in the prevulcanised latex. Consequently pre-vulcanised latex contained mainly acidic proteins (pH 4.6–pH 6.0) of molecular weight <14 kD.

Proteins Extractable from NR Examination Gloves

The IEF pattern of extractable proteins (EP) from the examination gloves (*Figure 7*) resembled more to that of the prevulcanised latex serum proteins, which formed the starting materials for most of NR latex gloves. The EP of about 20 gloves examined were mainly acidic proteins of pI in the pH range of 4.6 to 6.0. Basic proteins were only detected in one of the twenty gloves examined. This particular glove also contained a detectable level of high molecular weight proteins of greater than 14 kD besides a much higher concentration of low

molecular weight proteins of 6.2 kD to 14 kD (*Figure 8*). In fact the low molecular weight proteins of 6.2 kD to 14 kD formed the major proteins extracted from examination gloves (*Figure 8*). It could not be ruled out that hevein is not present in gloves extracts, as the present technique could not detect very low molecular weight peptides such as hevein.

CONCLUSION

Fresh latex contained a variety of acidic/ anionic and basic/cationic proteins with pI in the pH regions of 3.5 to 9.5 and molecular weights of less than 14 kD to greater than 100 kD. Adding ammonia and centrifuging the latex to HA latex concentrate resulted in a loss to the amount of proteins and changes to the composition of the proteins, particularly the serum proteins. The serum proteins most affected were the very acidic (pH 3.5–4.6) and basic (pH 8.0–9.5) proteins of molecular weights greater than 14 kD. The level of these proteins diminished on increasing the storage periods of the latex such that HA latex concentrate of more than 2–3 months old contained mainly acidic proteins (pH 4.6–6.0) of molecular weights less than 14 kD. Compounding the HA latex of less than a month old with sulphur vulcanising ingredients also affected the basic serum proteins. The effect was especially evident in the presence of ZnO but not in the presence of other normal sulphur vulcanising ingredients. Heating the compounded latex with or without ZnO, however, rendered the basic as well as the very acidic proteins undetectable. This explains the observation that prevulcanised latex comprises only acidic proteins of pI in the pH range of 4.6 to 6.0 and molecular weights of less than 14 kD. Similar protein composition was found in the extractable proteins of 20 brands of NR latex examination gloves.

Although gloves' extracts and sera of latex concentrate, compounded latex and prevulcanised latex do not contain the identified allergenic proteins of fresh latex, it is not unlikely that their peptides may still contain the relevant epitopes to enable them to elicit allergic reaction.

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Highly Purified Natural Rubber IV. Preparation and Characteristics of Gloves and Condoms

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Rubber gloves were produced using highly deproteinised natural rubber (HDPNR) latices with green strength comparable to that of gloves produced from a commercial high-ammonia natural rubber (HA) latex. These latices were prepared from HA latex by the method previously reported. The prevulcanisation of HDPNR latex gave characteristics which are slightly different from those of HA latex, although it did not affect the glove preparation process. The HDPNR gloves thus prepared (except chlorinated ones) satisfied the ASTM *specifications for examination gloves and were superior to the gloves from HA latex in terms of softness, clarity and low odour. Condoms also were prepared from two different types of HDPNR latex; one was the above mentioned HDPNR latex and another was a HDPNR latex prepared from a prevulcanised HA latex. The condoms from these HDPNR latices were superior to those from HA latex in terms of softness, transparency, water resistance and lack of odour.*

Ever since it was recognised that the residual extractable proteins in natural rubber (NR) latex products caused the Type 1 allergy, the development of low allergen products has been strongly requested for medical and hygienic rubber articles such as surgical gloves, examination gloves, condoms, *etc.*[1]

In the range of medical gloves, some hypo-allergenic gloves have been produced. The use of doubly centrifuged NR latex and techniques reducing extractable proteins such as leaching and chlorination have been employed in the commercial production of these gloves.

Recently some progress has been achieved in this area. First, the use of newly developed re-centrifuged prevulcanised NR latex that could achieve apparently lower level of the residual extractable proteins compared with previous techniques[2]. Second, the use of deproteinised NR (DPNR) latex. Production test on examination gloves was carried out using DPNR latex containing 0.06% - 0.1% nitrogen[3].

A new highly deproteinised NR (HDPNR) latex also was successfully developed whose nitrogen content was found to be less than 0.02%[4,5]. In the previous reports of this series,

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a HDPNR latex having excellent physical properties and processability was described and it was claimed that the HDPNR latex would be safer than conventional NR latex which was known to cause Type 1 allergy reaction because antibody was detected in the serum of animals sensitised with the latter but not with the former[6-8].

In this report, the results are presented for producing gloves and condoms from HDPNR latex having high green strength.

EXPERIMENTAL

Preparation of HDPNR Latex

HDPNR A. The commercial HA latex, diluted with water to 30% dry rubber content (DRC), was mixed with 0.02% (w/v) of a proteolytic enzyme (KP-3939, Kao Co.) and 1% (w/v) of a surfactant (KP-4401, Kao Co.), and the mixture was incubated for 24 h at room temperature under slow stirring. The reacted mixture was diluted with water to 10% DRC, then centrifuged (Alfa Laval) to obtain 60% DRC latex (A1). The washing procedure was repeated once more to obtain the latex A2. The concentration of ammonia in the latices was adjusted to 0.3% (w/v).

HDPNR B. The commercial HA latex was mixed with 0.5 p.h.r. accelerator, 1.0 p.h.r. sulphur, 1.0 p.h.r. ZnO and 0.5 p.h.r. antioxidant, and then prevulcanised for 15 h at 50°C. The prevulcanised latex was diluted with water to 30% DRC and then mixed with 0.02% (w/v) of the proteolytic enzyme (KP-3939, Kao Co.) and 1% (w/v) of the surfactant (KP-4401, Kao Co.), and the mixture was incubated for 24 h at room temperature under slow stirring. The reacted prevulcanised latex was washed twice using the same procedure as above. Also the ammonia concentration of the latex was adjusted to 0.3% (w/v).

Some properties of HDPNR latices prepared for the present work are shown in *Table 1*.

Compounding

The compound formulation for producing gloves consisted of 0.6 p.h.r. - 1.0 p.h.r. accelerator, 1.0 p.h.r. sulphur, 1.0 p.h.r. ZnO and 0.5 p.h.r. antioxidant.

TABLE 1. SOME PROPERTIES OF HDPNR LATICES

Kind of latex	A1	A2	B	C
N content (%)	0.030	0.013	0.014	0.30
Viscosity (cps)	60	65	60	100
Mechanical stability time (sec)	700	550	240	1 100
Gel fraction (%)	16	15	100	60
Green strength (MPa)	4.9	5.0	–	5.5

Note: 1. Latices A1, A2 and B (please see text)
2. Latex C is a commercial HA latex employed as a control.

Maturation of Latex Compound and Curing of Latex Film

The latex compound was stood at 30°C under slow stirring. After each maturing period, dry unvulcanised film was prepared from it by the method described in the previous paper[5]. The dry rubber film was vulcanised in a circulating hot-air oven at 100°C for 1 h.

Production of Gloves

The HDPNR gloves were produced in a coagulant dipping process as shown in *Figure 1*.

Production of Condom

Condoms were produced in a straight dipping process as shown in *Figure 2*. The thickness of condoms was adjusted to 0.06 mm.

Evaluation of Physical Properties

Viscosity and mechanical stability (MST) of latex concentrates were determined according to *JIS K6381-82*.

Gel fraction and green strength of HDPNR were determined by the method described in the previous paper[5].

Evaluation of physical properties such as tensile strength (TS), elongation-at-break (EB), modulus at 300% extension (M300), modulus at 500% extension (M500) and modulus at 800% extension (M800), and tear strength were carried out according to procedures set out by *JIS K6301-75, JIS T9107-92* and *JIS T9111-85*.

Total nitrogen content was determined by the Kjeldahl method.

Residual extractable protein content was determined by the Lowry microassay according to *ASTM D5712-95*.

Absorption of water and of toluene were determined after film specimens were soaked in either of the liquids for 24 h at 40°C.

Amount of $KMnO_4$ consumed by the extractable reducing ingredients ($KMnO_4$ value) was determined as follows: 1.5 g condom specimens were boiled with 150 ml water for 30 min. 150 ml test solution was prepared by addition of water to the above extracted solution. 10 ml 0.01 *N* $KMnO_4$ solution, 0.1 g KI and 5 drops of starch indicator were added into 10 ml test solution. The mixture was boiled for 3 min and was cooled. Then, it was titrated with 0.01 *N* $Na_2S_2O_3$ solution to determine the consumed amount of $KMnO_4$ solution.

The transparency of condoms was observed by visual comparison of each sample. The odour of condoms was judged by smelling each sample, after ageing for 7 days at 70°C.

Air-bursting test for condoms was carried out according to *ASTM D 3492-93*.

RESULTS AND DISCUSSION

Effect of Maturation of Latex Compound on Physical Properties of Vulcanisate

Tensile properties of products are frequently affected by the state of prevulcanisation of the latex compound. In this study, the effects of maturation time on physical properties of vulcanisates were investigated. In the case of HDPNR latex, modulus was found to be increased when the latex compound was

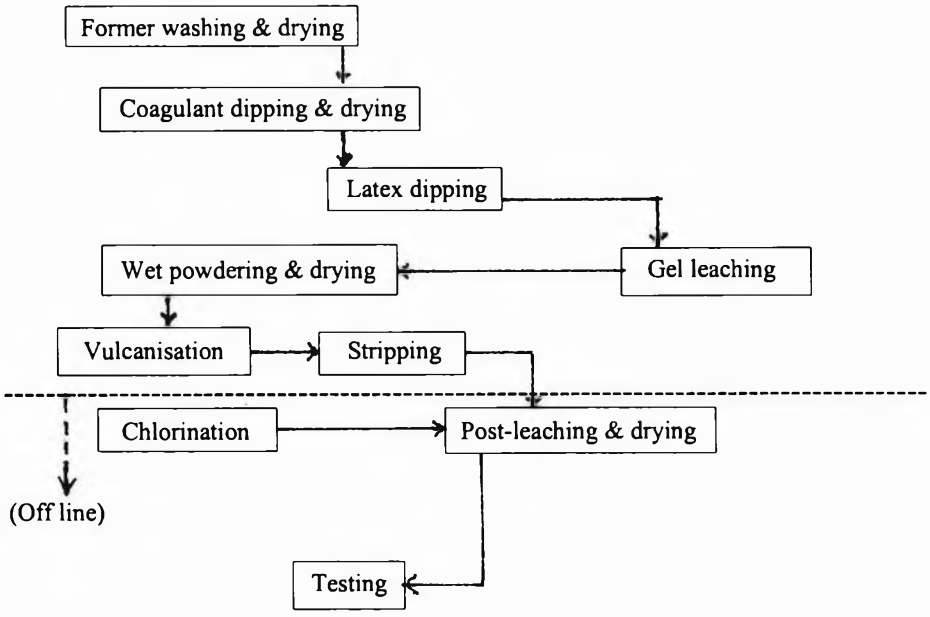

Figure 1. Process for the production of gloves.

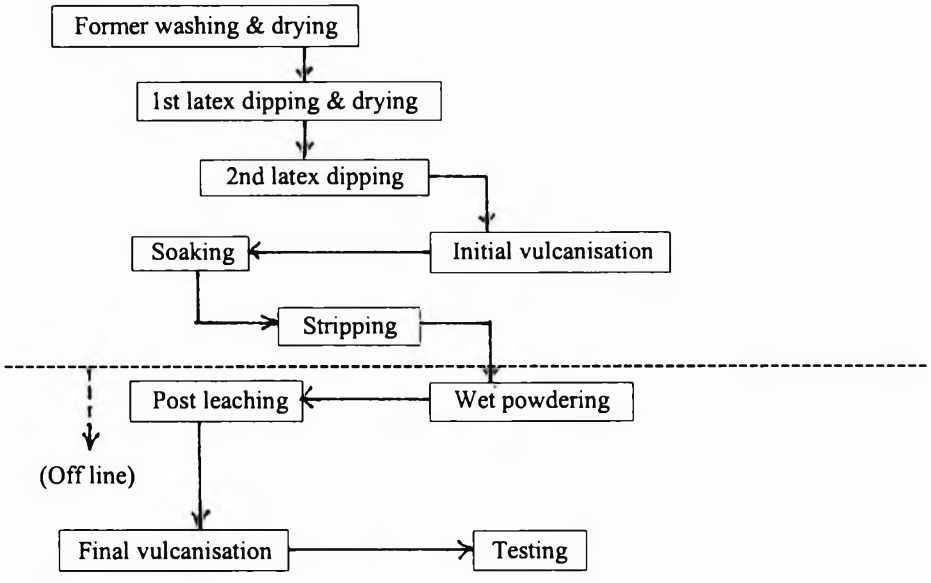

Figure 2. Process for the production of condoms.

matured longer whereas tensile strength was not so much affected. In contrast, in the case of commercial HA latex, tensile strength was found to be decreased as the maturation time was lengthened whereas modulus was less affected (*Figures 3* and *4*).

These results indicate that the prevulcanisation rate of HDPNR latex is slower than that of NR latex.

Properties of HDPNR Gloves

The tensile properties of the HDPNR gloves are as shown in *Table 2*. The tensile properties of all gloves (except chlorinated ones) were well beyond the minimum requirements of *ASTM* standards for examination gloves.

The chlorinated HDPNR gloves showed too poor physical properties after ageing. It is well known that chlorination causes physical deterioration of thin gloves and results in the gloves having poor physical properties particularly after high temperature ageing at 100°C for 22 h[9,10].

Furthermore, we consider that in HDPNR, the absence of bound protein exposes the rubber chains to attack by chlorine more easily. The experimental conditions for chlorination used in our work were conventional for examination gloves from HA latex but our results indicate that more careful control of the extent of chlorination may be necessary in the case of HDPNR gloves.

The extractable protein (EP) contents were found to be below the sensitivity limit of the Lowry method as shown in the bottom row of *Table 2*.

The HDPNR gloves were soft and comfortable to wear owing to their lower modulus. Further, HDPNR gloves had clear colour and less odour than the gloves of HA latex. The best gloves in terms of clarity and odour were those made from the A2 latex that was prepared by double washing after the incubation with the proteolytic enzyme.

Properties of HDPNR Condoms

The tensile properties of the HDPNR condoms were as shown in *Table 3* and the stress-strain curves for the vulcanised films were as shown in *Figure 5*.

Marked differences in physical properties between M300 and M500 were found on condoms produced from HDPNR latices and those from HA latex. The condoms from HDPNR latices were clearly softer than those from HA latex, showing about half the stress values.

Some other general properties were as shown in the lower columns of *Table 3*. The total nitrogen content of the condom produced from the latex B was the lowest. In the case of the latex B, as surplus chemicals were removed during centrifuging which was carried out after the latex compound was matured for prevulcanisation, the total nitrogen content of product was reduced to the lower level. The residual extractable protein contents of HDPNR condoms were below the sensitivity limit of the Lowry method.

Further, judging from $KMnO_4$ value, the HDPNR condoms seem to have contained less amount of reducing extractable ingredients. The HDPNR condoms proved to have high water resistance as shown by their relatively low water absorption which was related to removal

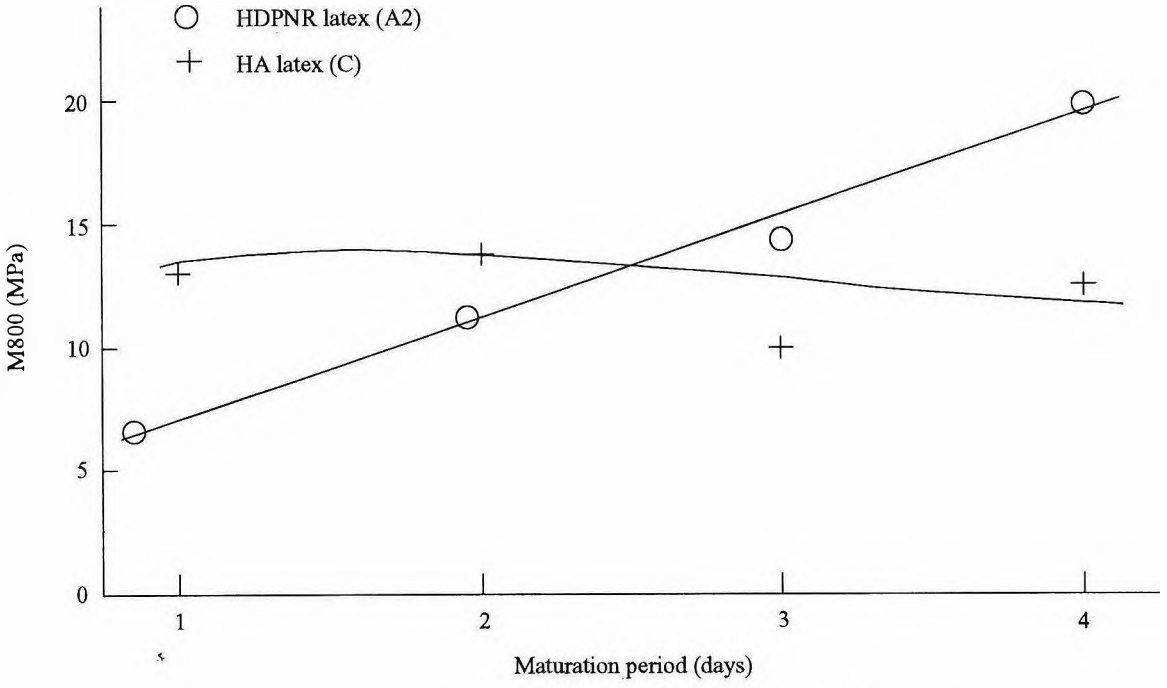

Figure 3. Maturation effect of latex compound on modulus of vulcanisate.

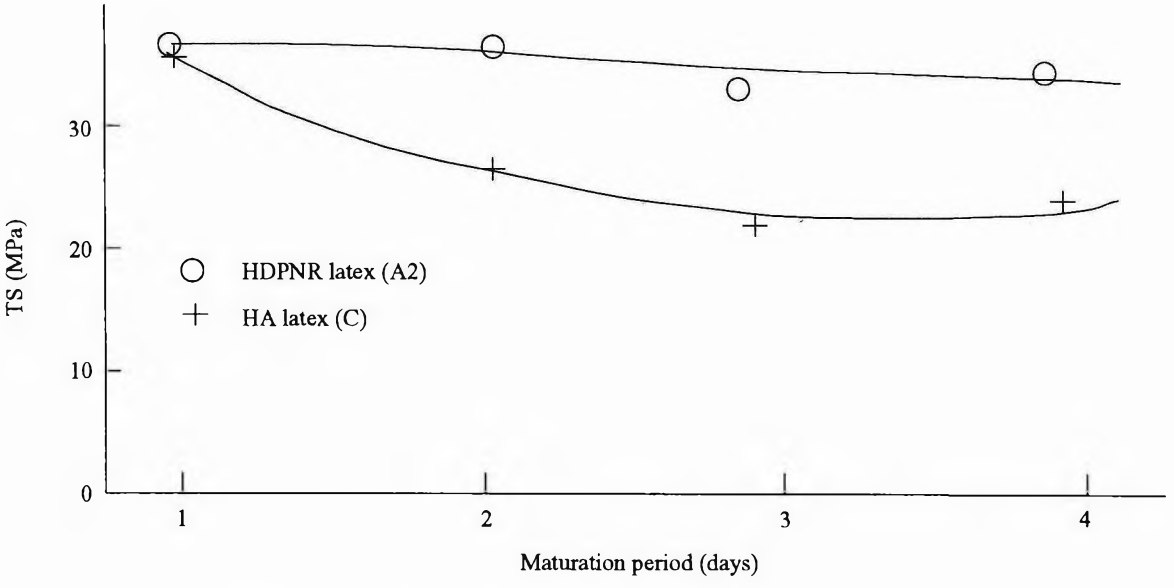

Figure 4. Maturation effect of latex compound on tensile strength of vulcanisate.

TABLE 2. TYPICAL PROPERTIES OF HDPNR GLOVES

No.	1	2	3	4	5
Specification					
Kind of latex	A1	A1	A1	A2	A2
Post-leaching	No	Yes	Yes	No	Yes
Powdering	s	s	cl	s	s
Colour	A little yellowish	A little yellowish	Yellow	Colourless	Colourless
Odour	Moderate	Moderate	Little	Little	Little
Unaged					
M500 (MPa)	2.38	2.40	2.18	1.69	1.99
TS (MPa)	28.8	30.7	30.6	33.2	33.3
EB (%)	895	908	917	999	964
Aged at 100°C for 22 h					
M500 (MPa)	1.85	1.73	0.89	1.31	1.43
TS (MPa)	29.7	29.0	6.4	21.5	33.3
EB (%)	969	972	1 063	1 043	964
EP (μ g/g)	<50	<50	<50	<50	<50

Note: 1. Latices A1 and A2 (please see text).
2. s: Corn starch; cl: Chlorination; EP: Extractable protein content
3. M500 for unaged gloves of HA latex is usually in 2.0 MPa to 2.4 MPa range
4. *ASTM* minimum requirements for TS and EB are 21 MPa and 700% before ageing and 16 MPa and 500% after ageing.

of hydrophilic ingredients including protein. The HDPNR condoms were more transparent and had less odour than the condoms of HA latex. The condom from the latex B was particularly transparent.

The stress-strain curves obtained in air-bursting test on condoms were as shown in *Figure 6*. The HDPNR condoms proved to have air bursting volume which was more consistent and larger than that of the condoms of HA latex.

CONCLUSION

HDPNR latices have good mechanical properties and processability for producing examination gloves in the coagulant dipping process; the gloves also have low levels of EP content as well as such advantages as less

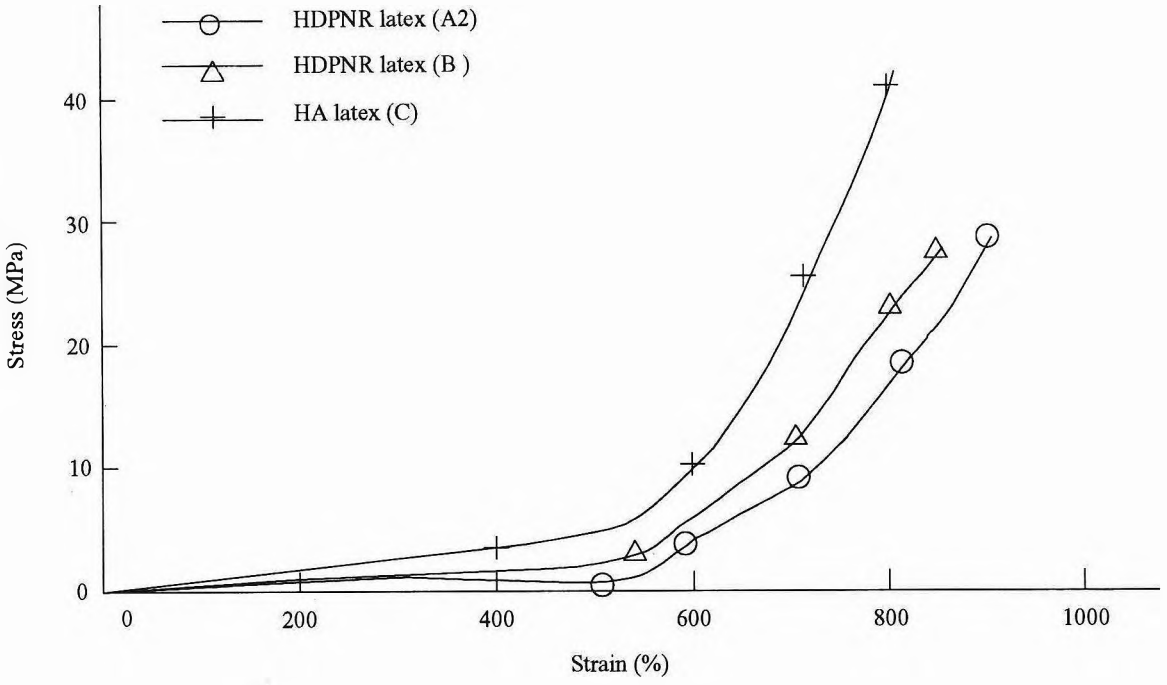

Figure 5. Stress-strain curves of vulcanised films.

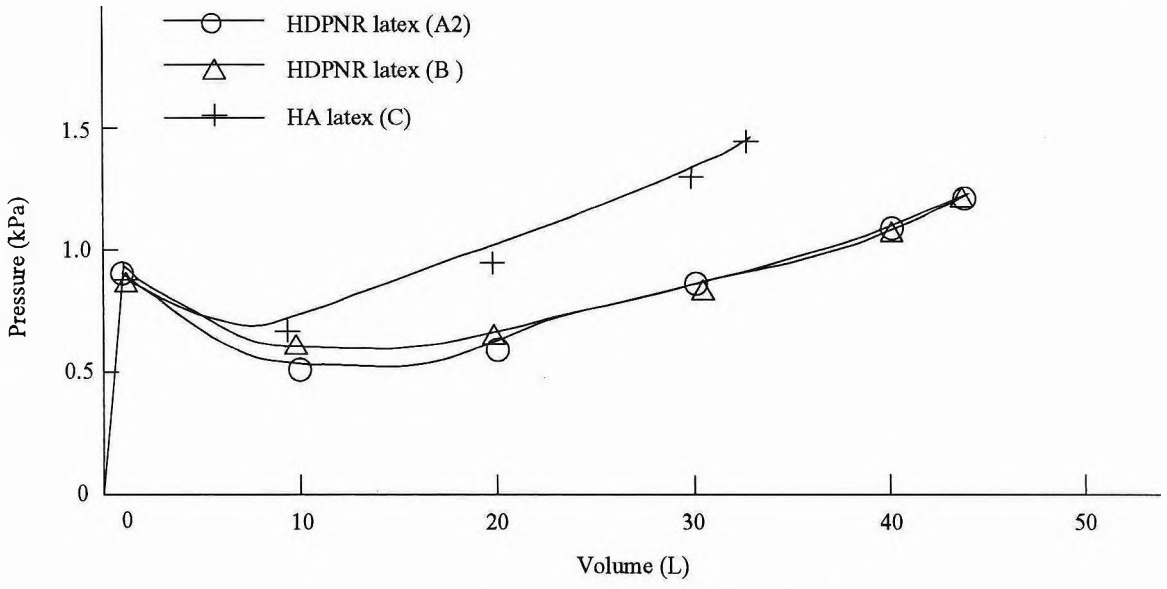

Figure 6. Air-bursting test curves of condoms.

TABLE 3. TYPICAL PROPERTIES OF HDPNR CONDOMS

Kind of latex	A2	B	C
Tensile properties			
Unaged			
M300 (MPa)	1.26	1.12	2.24
M500 (MPa)	1.90	2.24	6.71
TS (MPa)	34.0	32.8	36.0
EB (%)	933	875	863
Tear strength (kN/m)	45.0	62.5	63.3
Aged at 70°C for 10 days			
TS (MPa)	29.5	29.8	33.1
EB (%)	920	850	775
Total N content (%)	0.037	0.014	0.156
EP (μ g/g)	< 50	< 50	150
$KMnO_4$ value (ml)	0.25	0.50	0.80
Water absorption (%)	4.10	0.61	8.50
Toluene absorption (%)	414	431	393
Transparency	Good	Very good	Bad
Odour	Little	Little	Much

Note: Latices A2 and B --- (please see text)
Latex C is a commercial HA latex employed as a control.

odour, clarity in colour, and comfortable wearing feel. HDPNR latices are also good in mechanical properties and processability to produce condoms in the straight dipping process; the condoms also have low levels of EP content as well as advantages such as less odour, excellent transparency, softness, high water resistance and high burst volume. Especially, the HDPNR latex, gave the most transparent products among all latices evaluated.

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Effects of Integrating Trichoderma *and Fungicides on Control of White Root Disease of* Hevea *Rubber*

ISMAIL HASHIM*# AND B.H. CHEW*

The effects of integration of fungicide with Trichoderma koningii *on the control of white root disease of* Hevea *rubber were investigated. Control of white root disease with* Trichoderma *was erratic and not persistent. Similarly, the effects of* Trichoderma *on infected nursery plants drenched once with fungicides were also inconsistent. The addition of* Trichoderma *to plants in field plantings, which had been twice drenched with fungicides, did not improve control. In fact, addition of* Trichoderma *reduced control by propiconazole. Triadimefon and propiconazole were effective for the control of white root disease. The population of* Trichoderma *in soil following fungicide treatment was determined and its relationship with disease control discussed.*

White root disease of *Hevea* rubber, which is caused by the fungus, *Rigidoporus lignosus* Klotzsch, is still causing severe economic losses despite the existence of various recommendations and procedures to control the disease. Painting protective fungicides after exposing the tap and part of lateral roots is a procedure long adopted to manage root diseases of rubber[1]. As labour becomes more scarce and expensive, this procedure is difficult to be implemented. Subsequently, drenching of some fungicides was found effective and in Malaysia triadimefon and propiconazole were recommended[2,3]. Unfortunately drenching with these fungicides is effective only for young trees with mild infection. The cost of treatment is also expensive especially when a repeat drench is required.

Drenching of fungicide is popular, as the procedure is easy and fast. However, there is a need to reduce the cost of treatment and to improve control of more severe infection. Previously, the increase in population of *Trichoderma* following amendment of the soil around the plant with sulphur was associated to the mechanism of control of white root disease by sulphur since sulphur is not fungicidal to *R. lignosus*[4–6]. It has been shown that certain species of *Trichoderma* were antagonistic to various fungi[6] inclusive of *R. lignosus*[7,8]. *Trichoderma* has also been used in the field to control white root disease[9].

In the case of *Armillaria mellea* (Vahl.) Quel., *Trichoderma* was able to antagonise the pathogen which has been exposed to

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fumigants[10]. Integration of biological control with fungicides was effective against several diseases[11–13]. This paper reports on the effects of combinations of *Trichoderma* and fungicide treatments on white root disease.

MATERIALS AND METHODS

Laboratory Studies

Isolation of Trichoderma *from soil in a rubber area.* Soil samples were taken from around diseased plants and plants which two years previously, had been drenched with either tridemorph or propiconazole. The soil samples were plated on a *Trichoderma* selective medium[14] and incubated at room temperature in the dark. The colony of *Trichoderma* was isolated (and purified if necessary) and subsequently maintained on malt extract agar medium (MEA).

Evaluation of antagonism of Trichoderma *to* R. lignosus. The antagonism of *Trichoderma* to *R. lignosus* was tested by the paired dual culture technique. The degree of antagonism was visually assessed as described by Bell *et al.*[6]

The production of inhibitors by one isolate of *Trichoderma* was also determined. *Trichoderma* was inoculated on cellophane sheet placed on the surface of MEA in Petri plates. When the edge of the colony of *Trichoderma* nearly reached the edge of the plate, the cellophane sheet with the colony of *Trichoderma* was removed. The medium was then inoculated with *R. lignosus*. The radial growth of *R. lignosus* was periodically monitored.

Toxicity of fungicides to Trichoderma. The fungicides were added to MEA before autoclaving. The agar medium in Petri plates was then inoculated with a mycelial disk from a five-day-old culture of *Trichoderma* and was then incubated at room temperature. The diameter of the colony of *Trichoderma* was then measured. The fungicides evaluated were triadimefon (*Bayleton*), tridemorph (*Calixin*), propiconazole (*Tilt*), penconazole (*Topas*) and cyproconazole (*Alto*).

Population of indigenous Trichoderma *in fungicide drenched soil.* The experiment was carried out on about ten-year-old seedlings growing in a nursery at the RRIM Experiment Station, Sungai Buloh. As the plants were closely planted (1.2 m × 1.2 m), their sizes were equivalent to about four- to five-year-old plants growing in normal field planting. Plants infected by *R. lignosus* were selected by inspecting their collars for the presence of rhizomorphs after exposing part of the tree collars with a wooden spade to avoid injuring the roots. Ten randomly distributed plants were drenched with each fungicide by pouring one liter of aqueous solution of the fungicide into a shallow furrow dug around the tree collar. Sulphur powder was sprinkled around the plants and forked into the soil. Dazomet granules were applied into five holes dug in the soil around the plants and the treated soil around the plants was covered with plastic sheets. Soil samples were taken at periodic intervals (1, 2, 4 and 6 months) from three plants per treatment. Using a soil auger, the top 15 cm of soil was taken from three spots within 30 cm radius around the plants and the soil samples were pooled.

The population of *Trichoderma* in the soil was determined by the soil dilution plating technique using the *Trichoderma* selective medium of Elad *et al.*[14]

Survival of Trichoderma *preparation in fungicide treated soil.* Ten infected plants from the same nursery were treated with fungicides. For each fungicide treatment, five plants were also treated with *Supergro*; an organic fertiliser made from palm-oil-mill-effluent. *Trichoderma* cultures prepared as described below were applied to all the plants a day after the fungicide applications. Soil samples were taken from randomly selected plants and the population of *Trichoderma* was determined as described above.

Preparation of Trichoderma *for field application.* The rice bran-sawdust medium used for culturing *Trichoderma* was prepared by mixing 100 g of rice bran, 100 g of sawdust and 100 ml water in an autoclavable plastic bag, which was later sealed. The bags were autoclaved for 15 min and when cooled were inoculated by injecting 1 ml of a spore suspension of *Trichoderma*. The bags were covered with black cloth and were incubated at room temperature for about two weeks before being used.

The medium containing *Trichoderma* was used in the nursery and field trials by spreading the medium evenly in a shallow furrow dug around a rubber plant. The furrow was immediately covered with soil. Unless otherwise stated, *Trichoderma* was applied two months after fungicide drenching.

Experiment I: Control of Root Disease in Nursery Trials

The plants used in the nursery trials were the seedlings in the RRIM nursery at Sungai Buloh. The soil is sandy Sungai Buloh series. Infected plants were identified by carrying out collar inspection as described previously. The effects of treatments were assessed by determining the number of dead and living plants every six months.

Effects of Trichoderma. The effectiveness of *Trichoderma* cultures on control of root disease was assessed in two trials whereby in each trial, ten infected plants were treated with *Trichoderma* cultures and another ten plants were used as untreated control.

Effects of Trichoderma *and fungicides.* In a preliminary trial, the effects of time of application of *Trichoderma* on the control of root disease with fungicides was evaluated. In this trial, ten infected plants were selected and drenched with fungicides. *Trichoderma* cultures were applied either on the same day, one or two months after fungicide application.

In four subsequent trials, the *Trichoderma* cultures were applied at two months after fungicide applications. In each trial, ten plants were treated with fungicides while another ten plants were treated with fungicides and *Trichoderma*. The fungicides used in these trials were tridemorph, triadimefon and propiconazole.

Effects of number of application of fungicide and Trichoderma. In one trial, the effects of one or two applications of fungicides and *Trichoderma* were compared. The second application of fungicides and *Trichoderma* was given six months after the first application. In each trial, ten plants were treated with fungicides only and another ten plants were treated with fungicides and *Trichoderma*.

Experiment II. Control of Root Disease in Field Trials

The trials were carried out at several smallholdings at Labu, Negeri Sembilan. The

plants were about two years old at the commencement of the trials. Plants with light to moderately severe infection were identified by collar inspection as described above. Infected plants were drenched with aqueous solutions of fungicides (triadimefon or propiconazole) at one liter/tree. The concentrations of the fungicides were as stated in the text and tables. *Trichoderma* preparations were applied two months after the initial application of fungicide. A repeat application of fungicides was given one year after the first fungicide application.

The effects of fungicide and *Trichoderma* treatments were assessed every six months by determining whether the foliage of treated trees visually show the yellowing symptoms of being infected by *R. lignosus*, or the tree was dead.

Five trials were conducted in the field experiment. The number of plants used per trial varied (based on availability of infected plants) however, not less than ten plants were used per treatment per trial.

RESULTS

Population of *Trichoderma* Antagonistic to White Root Disease

About 37% of the *Trichoderma* isolates obtained from rubber soil showed good growth on malt extract medium and when paired with *R. lignosus*, these isolates overgrew *R. lignosus*. These isolates were considered as antagonistic to *R. lignosus*. About 26% of the isolates were less antagonistic. One antagonistic isolate was chosen for subsequent field trials and this isolate also produced inhibitors, which inhibited growth of *R. lignosus* in culture medium. This isolate was identified as *T. koningii* Oudem by the International Mycological Institute (IMI 363006).

Toxicity of Fungicides to *Trichoderma*

Triadimefon was less toxic to *Trichoderma* than tridemorph, propiconazole, penconazole and cyproconazole. At 10 mg/l, triadimefon inhibited radial growth by 10.6% while the % inhibition by the other fungicides was 71.6% for penconazole and 81.2%, 91.2% and 86.4% for tridemorph, propiconazole and cyproconazole, respectively. At 100 mg/l, triadimefon inhibited radial growth by 46.6% while the other fungicides totally inhibited growth.

Effects of Fungicides on Population of Indigenous *Trichoderma*

The population of indigenous *Trichoderma* was influenced by fungicide drenching. The population of *Trichoderma* in soils drenched with tridemorph and propiconazole was lower than in the untreated soil (*Table 1*). However, the differences were only significant for propiconazole at three and four months. Within the first four months, the population of *Trichoderma* in soils drenched with triadimefon was slightly lower in the first month, higher in the second and third month and lower in the fourth month as compared to the control. These differences were not significant. Dazomet and sulphur promoted population of *Trichoderma* but these differences were also not significant.

Survival of *Trichoderma* Cultures in Fungicide Treated Soil

In the trial whereby *Trichoderma* cultured in rice bran was applied to soil which had been drenched with various fungicides, the

TABLE 1. POPULATION OF INDIGENOUS *TRICHODERMA* IN SOILS DRENCHED WITH FUNGICIDES

Treatments (Quantity product/ plant)	Population of *Trichoderma* (cfu/g soils) at 1 mth	2 mth	3 mth	4 mth
No fungicide	502.2 (2.09)^{abcde}	344.4 (2.37)^{abcde}	273.3 (1.67)^{abcde}	720.0 (2.79)^{abc}
Tridemorph (10 ml)	455.5 (2.25)^{abcde}	193.3 (2.25)^{abcde}	191.1 (0.92)^{de}	215.6 (2.25)^{abcde}
Triadimefon (20 g)	413.3 (2.24)^{abcde}	900.0 (2.86)^{abc}	571.1 (2.51)^{abcde}	135.6 (1.54)^{abcde}
Propiconazole (20 ml)	206.7 (2.10)^{abcde}	262.2 (2.33)^{abcde}	182.2 (1.29)^{bcde}	46.7 (0.72)^{de}
Dazomet (20 g)	444.4 (1.04)^{bcde}	426.7 (1.83)^{abcde}	5 682.2 (3.00)^{ab}	4 911.0 (1.39)^{bcde}
Sulphur (200 g)	931.0 (2.68)^{abc}	474.1 (2.63)^{abc}	5 135.6 (3.34)^{a}	911.1 (2.84)^{abc}

Numbers in brackets are data transformed to log (x + 1)
Numbers with same letters are not significantly different at $p<0.05$ (LSD = 1.8195)

population of *Trichoderma* within the first six months after fungicide treatment is shown in *Table 2*. Within the first four months after fungicide treatment, the population of *Trichoderma* in soils treated with *Trichoderma* was significantly higher than in soils not treated with *Trichoderma*. In the majority of the treatments, the highest population of *Trichoderma* was recorded about two months after treatments and decreased thereafter and by the sixth month, the difference was not significant. Generally, the difference in the population of *Trichoderma* in soils treated with the organic fertiliser as compared to untreated soils was not significant. The mean populations were significantly different between treatments at $p<0.01$ (LSD = 0.313). The interaction between treatments, time and organic fertiliser was also significant.

Control of *R. lignosus* by Fungicides and *Trichoderma* in Nursery Trials

In the two trials on nursery plants, the *Trichoderma* isolate used had mild and non-persistent effect on control of white root disease. A summary of the results of the two trials indicated that the percentage number of trees treated with *Trichoderma* which were still alive at 12 and 24 months were 60% and 35%, respectively compared to 35% and 10%, respectively in the untreated control.

In the preliminary trial to ascertain a suitable period to apply *Trichoderma* after drenching of fungicide, the application of *Trichoderma* at one or two months after drenching of triadimefon or propiconazole was more effective in controlling the disease than applying *Trichoderma* immediately after fungicide drenching (*Table 3*). The number of plants living was higher in treatments where *Trichoderma* was applied at one or two months after fungicide treatments as compared to treatments where *Trichoderma* was applied on the same day with the fungicide. The difference was more obvious with propiconazole.

The survival of closely planted nursery plants following fungicide and *Trichoderma*

TABLE 2. SURVIVAL OF *TRICHODERMA* APPLIED TO SOIL DRENCHED WITH FUNGICIDES

Treatments (Product/plant)	*Trichoderma* population (cfu/g soil × 10^3) at									
	0 mth		1 mth		2 mth		4 mth		6 mth	
	P*	A*	P	A	P	A	P	A	P	A
Tridemorph (10 ml)	0.28	0.21	29.22	221.89	57.18	77.76	3.14	0.89	7.67	2.76
	$(0.11)^{d-l}$	$(0.07)^{o-p}$	$(1.24)^{c-j}$	$(1.39)^{b-l}$	$(1.66)^{a-d}$	$(1.17)^{c-k}$	$(0.45)^{k-p}$	$(0.27)^{d-g}$	$(0.84)^{f-n}$	$(0.44)^{k-p}$
Triadimefon (20 g)	0.08	0.16	33.00	4.34	118.00	136.00	28.22	1.57	4.73	7.69
	$(0.03)^{p}$	$(0.06)^{o-p}$	$(1.49)^{a-f}$	$(0.70)^{n-p}$	$(2.03)^{ab}$	$(2.09)^{ab}$	$(1.45)^{a-g}$	$(0.38)^{l-p}$	$(0.65)^{i-p}$	$(0.80)^{f-o}$
Propiconazole (20 ml)	0.46	0.07	12.24	9.25	290.00	42.78	17.78	1.06	2.91	9.87
	$(0.15)^{n-p}$	$(0.03)^{p}$	$(1.02)^{d-l}$	$(0.72)^{g-p}$	$(1.90)^{a-c}$	$(1.42)^{b-h}$	$(1.10)^{d-l}$	$(0.26)^{m-p}$	$(0.56)^{j-p}$	$(0.96)^{d-m}$
Dazomet (20 g)	0.46	0.22	54.73	6.18	94.44	22.92	10.00	2.93	7.16	10.22
	$(0.13)^{n-p}$	$(0.09)^{o-p}$	$(1.51)^{a-f}$	$(0.78)^{f-p}$	$(1.91)^{a-c}$	$(1.43)^{b-h}$	$(1.03)^{d-l}$	$(0.49)^{k-p}$	$(0.90)^{e-m}$	$(0.91)^{e-m}$
No fungicide	0.22	0.30	45.44	99.11	484.22	21.40	17.13	3.36	8.87	6.33
	$(0.07)^{o-p}$	$(0.11)^{n-p}$	$(1.64)^{a-e}$	$(1.64)^{a-e}$	$(2.20)^{a}$	$(1.13)^{d-k}$	$(1.02)^{d-l}$	$(0.74)^{g-p}$	$(0.99)^{d-m}$	$(0.80)^{f-o}$

*Organic fertiliser Supergro® applied (P) or not applied (A)

Numbers in brackets are data transformed to log (x + 1)

Numbers with same letters are not significantly different at $p< 0.05$ ($LSD_{0.05=0.748}$)

TABLE 3. INFLUENCE OF TIME OF *TRICHODERMA* AMENDMENT ON CONTROL OF WHITE ROOT DISEASE

Treatment	Tridemorph (10 ml/plant) Months				Triadimefon (20 g/plant) Months				Propiconazole (10 ml/plant) Months				Propiconazole (5 ml/plant) Months				Total mean
	12	24	36	Mean	12	24	36	Mean	12	24	36	Mean	12	24	36	Mean	
No *Trichoderma*	40	0	0	13.3[a]	50	30	30	36.7[a]	90	70	40	66.7[a]	70	30	0	33.3[a]	37.5[a]
Simultaneously	30	0	0	10.0[a]	80	40	30	50.0[ab]	20	10	0	10.0[b]	30	0	0	10.0[b]	20.0[a]
One month after fungicide drenching	40	20	20	26.7[b]	80	80	80	80.0[c]	70	40	40	50.0[a]	80	20	10	36.7[a]	48.4[b]
Two months after fungicide drenching	20	0	0	6.7[a]	90	50	50	63.3[bc]	90	90	40	73.3[a]	60	30	20	36.7[a]	45.0[ab]
LSD 0.05				11.04				24.24				24.24				22.83	9.79

Mean with same letters are not significantly different at $p < 0.05$

treatments varied with the type of fungicides, the trials and period after treatments (*Table 4*). The difference in percentage of living trees was not significant between fungicides. However better disease control was achieved with triadimefon and propiconazole as compared to tridemorph at 12 months and control was about similar at 24 and 36 months. Similarly, the difference in disease control between fungicide alone and fungicide plus *Trichoderma* was also not significant. The effectiveness of *Trichoderma* plus tridemorph or triadimefon varied between trials. At 12 months after treatment, *Trichoderma* plus tridemorph had higher control than tridemorph only in one trial but not in the other three trials. Similarly, mixture of *Trichoderma* and triadimefon had better control than triadimefon alone in two trials. The application of *Trichoderma* reduced root disease control by propiconazole in all the four trials. In addition, the effectiveness of control decreased significantly ($p<0.05$) with time as indicated by the lower number of living trees at 36 months compared to 12 months.

In the trial to determine the effects of multiple application of fungicides and *Trichoderma*, two application of tridemorph at six month interval failed to control root disease. However control was improved when *Trichoderma* was applied once or twice following two applications of tridemorph (*Table 5*). Two applications of triadimefon and *Trichoderma* produced better control than the other treatments. In the case of propiconazole, the application of *Trichoderma* reduced control by the fungicide.

Control of *R. lignosus* by Fungicides and *Trichoderma* in Field Plantings

Triadimefon and propiconazole were effective in controlling white root disease in field planted plants. The percentage number of living trees following triadimefon and propiconazole treatments was 89.3% and 92.7% respectively, at 36 months (*Table 6*). The inclusion of *Trichoderma* did not improve control. In fact, disease control was significantly lower in propiconazole plus *Trichoderma* treatment as compared to propiconazole alone at 36 months.

The addition of *Trichoderma* was however beneficial in reducing the presence of rhizomorphs of *R. lignosus* on the tree collars. The number of trees with rhizomorphs still occurring at their collars and tap roots were lower for trees treated with fungicide and *Trichoderma* as compared with fungicide only (*Table 7*). However the difference was not significant in most instances.

DISCUSSION

Increase in population of *Trichoderma* spp. in soils following chemical amendment has been associated to the control of several pathogens of rubber roots such as *R. lignosus*[15], *Ganoderma philippii*[16] and *Armillaria mellea*[10]. Lysis of mycelia of *R. lignosus* occurred when it touches mycelia of *Trichoderma*[7,8].

The current practice to control white root disease is to drench with fungicides. Normally, at least two drenches are given and this makes the treatment expensive. Moreover drenching of fungicides often fail to cure rubber trees severely infected by root disease[2]. Whether *Trichoderma* can contribute to the control of white root disease following chemical drenching is yet to be seen.

In this study, about 50% of the nursery plants treated with only *Trichoderma* survived at

TABLE 4. EFFECT OF FUNGICIDES AND *TRICHODERMA* ON SURVIVAL OF INFECTED NURSERY PLANTS

Treatments	Experiment	Survival of plants (%) 12 months +T	12 months –T*	24 months +T	24 months –T	36 months +T	36 months –T
Tridemorph (10 ml/plant)	1	80	40	80	20	80	10
	2	20	40	0	0	0	0
	3	10	0	10	0	10	0
	4	40	20	30	0	10	0
	Mean	37.5 (1.47)$^{a-c}$	25.0 (1.14)$^{a-d}$	30 (1.11)$^{a-d}$	5.0 (0.33)^{ef}	25 (1.00)$^{b-f}$	2.5 (0.26)^{f}
Triadimefon (20 g/plant)	1	90	60	70	0	50	10
	2	90	50	50	30	50	30
	3	10	10	0	0	0	0
	4	40	50	20	30	10	0
	Mean	57.5 (1.64)^{ab}	42.5 (1.56)^{ab}	35.0 (1.22)$^{a-d}$	15 (0.75)$^{c-f}$	27.5 (1.11)$^{a-e}$	10 (0.63)$^{d-f}$
Propiconazole (20 ml/plant)	1	60	90	10	70	0	70
	2	80	90	70	90	40	40
	3	70	80	30	80	30	20
	4	30	40	20	20	10	20
	Mean	60.0 (1.76)^{ab}	75 (1.86)^{a}	35.5 (1.43)$^{a-c}$	65 (1.76)^{ab}	20.0 (1.04)$^{b-f}$	37.5 (1.53)$^{a-c}$
		40.00 (1.04)^{y}		35.8 (1.11)^{xy}		24.6 (1.46)^{x}	

* +T, *Trichoderma* applied; –T, *Trichoderma* not applied

Numbers in brackets are data transformed to log (x + 1)

Numbers with same letters are not significantly different at $p < 0.05$

TABLE 5. EFFECT OF NUMBER OF APPLICATIONS OF FUNGICIDE AND *TRICHODERMA* ON CONTROL OF WHITE ROOT DISEASE

Treatment	Survival of plants (%) Tridemorph (10 ml/plant) Months				Triadimefon (20 g/plant) Months				Propiconazole (20 ml/plant) Months				Total mean
	15	24	36	Mean	15	24	36	Mean	15	24	36	Mean	
One application	0	0	0	0.0[a]	10	0	0	3.3[a]	80	80	20	60.0[a]	21.1[a]
One application + *Trichoderma* (1)*	10	10	10	10.0[a]	10	0	0	3.3[a]	70	30	30	43.4[a]	18.8[a]
Two applications	20	10	0	10.0[a]	40	30	20	30.0[b]	70	60	0	43.4[a]	27.8[a]
Two applications + *Trichoderma* (1)*	70	50	0	40.0[b]	40	40	30	36.7[b]	50	50	50	50.0[a]	42.2[b]
Two applications of fungicide + *Trichoderma* (2)*	80	80	60	73.3[c]	90	90	60	80.0[c]	30	30	20	26.7[a]	59.7[c]
LSD 0.05				28.0				12.4				36.1	13.7

**Trichoderma*, one (1) or two applications (2)
Twenty plants per treatment
Means with same alphabets are not significantly different at $p < 0.05$

TABLE 6. EFFECT OF *TRICHODERMA* ON CONTROL OF WHITE ROOT DISEASE BY FUNGICIDE IN FIELD PLANTING

Treatment	Experiment	Survival of plants (%)			
		24 months *Trichoderma*		36 months *Trichoderma*	
		+T*	−T	+T	−T
Triadimefon (20 g/plant)	1	88.9	100.0	88.9	100.0
	2	80.0	100.0	80.0	100.0
	3	85.7	75.0	71.4	75.0
	4	75.0	100.0	75.0	100.0
	5	100.0	71.4	100.0	71.4
	Mean	85.9 (1.94)^{ab}	89.3 (1.95)^{ab}	83.1 (1.95)^{ab}	89.3 (1.95)^{ab}
Propiconazole (10 ml/plant)	1	100.0	100.0	83.3	80.0
	2	75.0	100.0	75.0	100.0
	3	66.7	83.3	50.0	83.3
	4	75.0	100.0	75.0	100.0
	5	83.3	100.0	83.3	100.0
	Mean	80.0 (1.90)^{ab}	96.7 (1.99)^{a}	73.3 (1.86)^{c}	92.7 (1.97)^{ab}

Numbers in brackets are data transformed to (log x + 1)
Means with same letters are not significantly different at p = 0.05 (LSD = 0.0698)
*See *Figure 5*

TABLE 7. EFFECT OF *TRICHODERMA* AND FUNGICIDES ON RHIZOMORPHS OF *R. LIGNOSUS*

Treatment	Experiment	Number of trees (%) with rhizomorph 12 months +T	12 months −T*	18 months +T	18 months −T	24 months +T	24 months −T	36 months +T	36 months −T
Triadimefon	1	44.4	55.6	22.2	44.4	22.2	22.2	11.1	22.2
	2	0.0	0.0	50.0	25.0	25.0	0.0	0.0	25.0
	3	42.9	40.0	14.3	60.0	28.6	40.0	14.3	40.0
	4	0.0	0.0	50.0	20.0	0.0	0.0	0.0	0.0
	5	0.0	28.6	0.0	28.6	0.0	28.6	0.0	28.6
	6	–	50.0	44.4	33.3	55.6	66.7	55.6	66.7
	Mean	21.6 (0.823)^{b}	29.0 (1.091)^{ab}	30.2 (1.270)^{ab}	35.2 (1.531)^{a}	21.9 (1.001)^{ab}	26.3 (1.047)^{ab}	13.5 (0.670)^{b}	30.4 (1.283)^{ab}
Propiconazole	1	16.7	33.3	33.3	66.6	0.0	33.3	16.7	50.0
	2	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	3	16.7	16.7	50.0	16.7	50.0	16.7	66.7	16.7
	4	50.0	0.0	25.0	0.0	25.0	0.0	25.0	0.0
	5	100.0	0.0	0.0	0.0	50.0	0.0	0.0	0.0
	6	40.0	62.5	20.0	62.5	0.0	62.5	0.0	62.5
	Mean	42.8 (1.559)^{a}	18.8 (0.764)^{b}	21.4 (0.997)^{ab}	24.3 (0.813)^{b}	20.8 (0.805)^{b}	18.8 (0.769)^{b}	18.1 (0.749)^{b}	21.5 (0.793)^{b}

*See *Figure 5*

Numbers with same letters are not significantly different at $p < 0.05$

12 months after treatment, a percentage comparable to one drench of fungicides. Unfortunately the effect was not persistent as most of the treated trees died at 24 months or later. This indicates that *Trichoderma* isolate used could only delay the advancement of death of the trees but could not cure the disease. The better effect of *Trichoderma* at 12 months or earlier could be related to the population of *Trichoderma*. *Trichoderma* population was high especially within the first four months after soil application of *Trichoderma* cultures and the population decreased thereafter (*Table 2*). This indicates that the ability to maintain high population of *Trichoderma* is important in white root disease control. In fact better control of white root disease occurred when *Trichoderma* was applied twice in combinations with tridemorph or triadimefon as compared to two applications of these fungicides only (*Table 5*). The application of *Trichoderma* with sulphur would be useful as the population of *Trichoderma* is enhanced by sulphur[7].

The application of *Trichoderma*, two months after the nursery plants were drenched with fungicides produced inconsistent results (*Table 4*). In the case of tridemorph and triadimefon, control was higher when these fungicides were combined with *Trichoderma* in only some trials. The reason for the differences in the effectiveness of the combinations is unknown but it may be related to the influence of weather and different preparation of *Trichoderma* cultures on the population of *Trichoderma* in the soil. Similar results were obtained in the field trials whereby the addition of *Trichoderma* did not improve tree survival. On the contrary, the presence of *Trichoderma* lowered the survival of the trees treated with propiconazole (*Tables 4* and *6*). The reduced effectiveness was insignificant in the case of triadimefon but significant for propiconazole. The reduced effectiveness could be due to the interaction of the fungicides with the organic matter used as carriers for *Trichoderma*. Proper choice of carriers of the biocontrol organism is necessary if fungicide is to be integrated with biocontrol so as to avoid deactivation of the fungicides by the carriers of the biocontrol organism.

In the field trial, originally only one application of fungicide was planned. However, after one year, as an important proportion of the trees treated with fungicides and/or *Trichoderma* were still not cured and tree death cannot be tolerated, a second drenching was performed. As the result more than 80% of the treated trees still survived three years after treatment. With the good effects of two drenching of fungicides on disease control, the contributory effects of *Trichoderma* was not seen.

The fungicide drenching was more effective in the trial on field plants where the soil is loamier as compared to the nursery trial where the soil is very sandy. Soil structure may influence the effectiveness of fungicides when applied by soil drenching. To date, there are no available reports on the influence of soil structure on the control of white root disease by drenching of fungicide.

Trichoderma has been used in Indonesia for the control of white root disease[9]. In the current study, *Trichoderma* delayed death of infected trees. Further work is necessary to explore its potential especially on means to maintain high population for a prolong period. The results presented here indicated that there was no benefit of integrating *Trichoderma* with fungicide application. In the literature,

integrated control using *Trichoderma* and fungicides was more successful with pathogens such as *Rhizoctonia, Fusarium* and *Sclerotium*[11-13]. Nevertheless *Trichoderma* successfully colonised *A. mellea*, another rhizomorph producing fungus only after *A. mellea* was weakened by fumigants[10]. In this study, the fungicides were applied only to the narrow regions around the bases of trees. Rhizomorphs of *R. lignosus* on lateral roots and progressing to the base of the trees may not be sufficiently exposed to the fungicide to allow parasitisation by *Trichoderma*. The ability of *Trichoderma* to parasitise rhizomorphs of *R. lignosus* has yet to be shown as to date only *in vitro* antagonism and lysis of mycelia were indicated. In addition to *Trichoderma* other microorganisms should be investigated. Several of these organisms have also been identified[8].

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J. nat. Rubb. Res., **12(1)**, 58–66

Studies on Epidemiology of Pink Disease and the Effect of Temperature on Mycelial Growth of Corticium salmonicolor *of* Hevea *Rubber*

M.H. SHAMSURI*#, M. OMAR** AND D. NAPI*

The effects of temperature on mycelial growth and the relationship between rainfall, temperature and disease incidence were studied. The optimum temperature for the growth of the C. salmonicolor was *28° C. The minimum and maximum temperatures for fungal growth were 5° C and 40° C, respectively. In the field there was an exponential reduction of basidiospores caught after rainfall. Studies carried out for 22 months in the infected field showed that basidiospores played an important role in the incidence of pink disease.*

Corticium salmonicolor (Berk. & Br.) is synonymous with *Pellicularia salmonicolor* (Berk. & Br.) Dastur, *Botryobasidium salmonicolor* (Berk. & Br.) Venkatarayan, *Phanerochaete salmonicolor* (Berk. & Br.) Julich[1]. This fungus cause damage to important economic crops such as *Hevea*[2], *Eucalyptus*[3], apples[4–5] and cocoa[6].

Pink disease is presumably transmitted by basidiospores and conidia[1]. Christians *et al.*[7] showed that basidiospores play an important role in the incidence of pink disease in cocoa. Previous studies had shown that environmental factors such as rainfall is important in basidiospore release[8–9]. The severity of infection depended on the pattern of rainfall[10]. In Eucalyptus plantations in India, the disease was not effective in areas receiving less than 200 cm of rainfall per year[3]. Christians *et al.*[7] observed that *C. salmonicolor* in the infected cocoa branch was stimulated by sporadic rain while heavy and regular rainfall induced the formation of corticium stage of the fungus which can cause new infection. Temperature also plays an important role in pink disease development. In Himachal Pradesh, India, the maximum incidence and development of pink disease on apple was observed during the month of July–August with average atmospheric temperature of 26°C and 27°C[11].

There are only few reports on the biological aspects of the fungus and the epidemiology of the disease eventhough the disease is present in most rubber growing countries. This paper describes the effects of temperature on mycelial growth, the relationship between rainfall with basidiospore release and disease incidence.

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MATERIALS AND METHODS

Isolates of *C. salmonicolor*

The isolates used in the study were obtained from rubber collected from various states in West Malaysia. Isolate Cort. 1 was from Kedah, Cort. 2 and Cort. 6 from Selangor, Cort. 3 and Cort. 4 from Pahang while Cort. 5 was from Perak. The isolates were maintained on malt extract agar (MEA) at 21°C under fluorescent light.

Effect of Temperature on Mycelial Growth

Inoculum disks of six mm diameter from the margin of 5-day-old cultures were placed invertedly on MEA in 9 cm petri dishes and were incubated in light at different temperatures (5°C, 10°C, 21°C, 23°C, 25°C, 28°C, 31°C, 35°C and 40°C). There were five replicates per treatment. The diameter of each colony was measured after incubation for four days. Mean colony diameter of different isolates was determined after substracting the initial diameter of inoculum plug.

Epidemiology

The experiment was conducted in a three-and-a-half-year-old rubber plantation (PB 260 clone) in Tanjung Malim, Perak. A Burkard spore trap was placed about 3.15 m from an infected tree with disease lesion about 3 m from the ground. The orifice of the spore trap which is about 2.2 m from the ground faced the infected tree. The volume of air drawn through the trap was 10 l/min (0.6 m^3/h). All the pink disease infected trees (except the tree being examined) located within 30 m radius were treated with Calixin Ready-mixed®. This was to minimise interference from basidiospores from the surrounding infected trees.

The drum containing Melinex tape, thinly coated with a mixture of phenol-wax-vaseline (1:19:180; w/w/w) was changed every seven days at 12.00 noon. The Melinex tape was cut into seven strips, each representing a day of trapping of spores. The number of spores per hour was estimated by counting spores after the strips was marked using a sharp knife with the aid of perspex block provided by Burkard. Basidiospores were stained with trypan blue before counting under a microscope (*Figure 1*).

An automatic weather station was used to record rainfall. The experiment was conducted for 28 days.

In another trial at another estate in Tanjung Malim, Perak, a Burkard spore trap was placed in the centre of a block (56 ha) of 5-year-old trees (PB 260 clone). The orifice was 3.5 m from the ground and the trap was allowed to swing freely. One hundred trees including the pink disease infected trees were selected within 30 m radius from the spore trap. The trees were monitored weekly for new pink disease incidence (proportion of trees with symptoms of pink disease in relation to the total number of observed trees). Spore trapping was carried out for nearly two years (22 months) from 1992 to 1993. The same method of counting of spores on the Melinex tape mentioned above was used.

Temperature and relative humidity were recorded using a thermohygrograph (Saito, Japan) and the rainfall data were obtained from the management of the estate.

RESULTS

Effect of Temperature on Mycelial Growth

The optimum temperature for mycelial growth of *C. salmonicolor* isolates was 28°C.

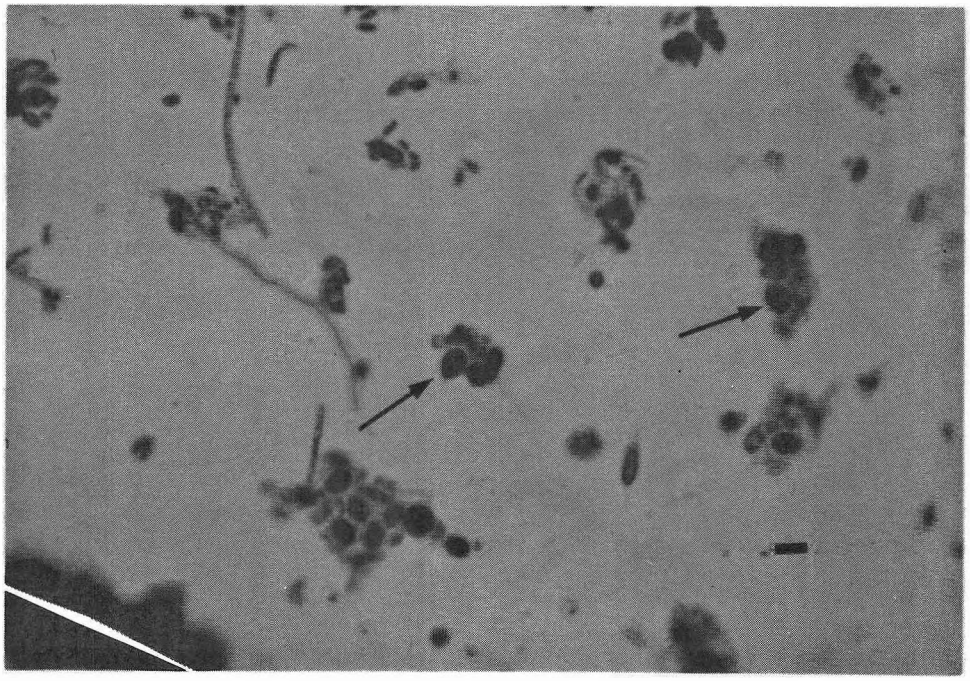

Figure 1. Basidiospores on Melinex tape from spore trap (1 bar = 10 μm).

At 5°C and 40°C, the growth was negligible as these were the minimum and maximum temperatures for the growth of the fungus, respectively. The measurable growth was within a range of 10°C–35°C (*Table 1a*). At 28°C, the growth of isolate Cort.1 was significantly slower than other isolates (*Table 1b*).

Epidemiology

Rainfall was recorded for 22 days out of the 28 days trial period. More basidiospores were caught during rainfall (*Figure 2*) and the number of basidiospores released, decreased exponentially after rainfall as shown in the transformed model below:

$$Y' = 4.103 - 0.21X \quad \ldots 1$$

where Y' is the Ln Y whereby Y is the number of basidiospores caught per hour
X is the duration during (x= 0 h) or after rainfall (x= 1, 2, 3.... 15 h)

Basidiospores were caught even 15 hours after rainfall.

Studies carried out for 22 months had shown that under the rubber canopy, only minimal fluctuations in the minimum and maximum temperatures were observed. The minimum and maximum temperatures were between 21°C–25°C and 28°C–33°C, respectively. In 1992 and 1993, high percentage rainy days occurred

TABLE 1. GROWTH OF *C. SALMONICOLOR* ON MALT EXTRACT AGAR (MEA)

a) GROWTH AT DIFFERENT TEMPERATURES

Temperature	Mean colony diameter (cm)					
	Cort.1	Cort.2	Cort.3	Cort.4	Cort.5	Cort.6
5° C	0 a[1]	0 a	0 a	0 a	0 a	0 a
10° C	0.30 c	0.57 d	0.42 d	0.56 c	0.44 c	0.37 d
21° C	4.35 b	5.66 b	5.95 b	5.45 b	4.95 b	5.25 b
23° C	5.37 e	6.17 f	6.17 f	5.99 e	5.47 d	5.91 e
25° C	5.61 f	6.90 g	6.84 g	6.68 f	6.53 e	6.33 f
28° C	6.07 g	7.46 h	7.05 h	7.23 g	7.17 f	7.33 g
31° C	4.41 b	4.80 c	5.13 c	5.41 b	5.05 b	3.28 c
35° C	0.20 d	0.43 e	0.26 e	0.25 d	0.53 c	0.38 d
40° C	0 a	0 a	0 a	0 a	0 a	0 a

b) GROWTH AT 28° C

Isolate	Mean colonly diameter (cm)
Cort.1	6.07 a[1]
Cort.2	7.46 e
Cort.3	7.05 b
Cort.4	7.23 cd
Cort.5	7.17 bc
Cort.6	7.33 de

[1]Mean on the same column followed by the same letter are not significantly different at 5% level of probability by Duncan's New Multiple Range Test.

in April–May and November–December. The month of June, 1992 was dry (< 20% rainy days). The monthly exposure of high relative humidity (RH ≥90%) under the rubber canopy was between 10 h–20 h (*Figure 3*).

Generally, the concentration of basidiospores in the air increased from March, 1992 to December, 1993. High concentration of basidiospores were caught in March and November, 1992. In 1993, high concentration of basidiospores occurred from March to July and November to December. In general, the incidence of disease increased with the increase in concentration of basidiospores in the air as indicated by the increased incidence from March, 1992 until December, 1993 especially during March to July 1992, November to January 1993, June–July and October to December, 1993. No new disease incidence was observed during July–September, 1992 and

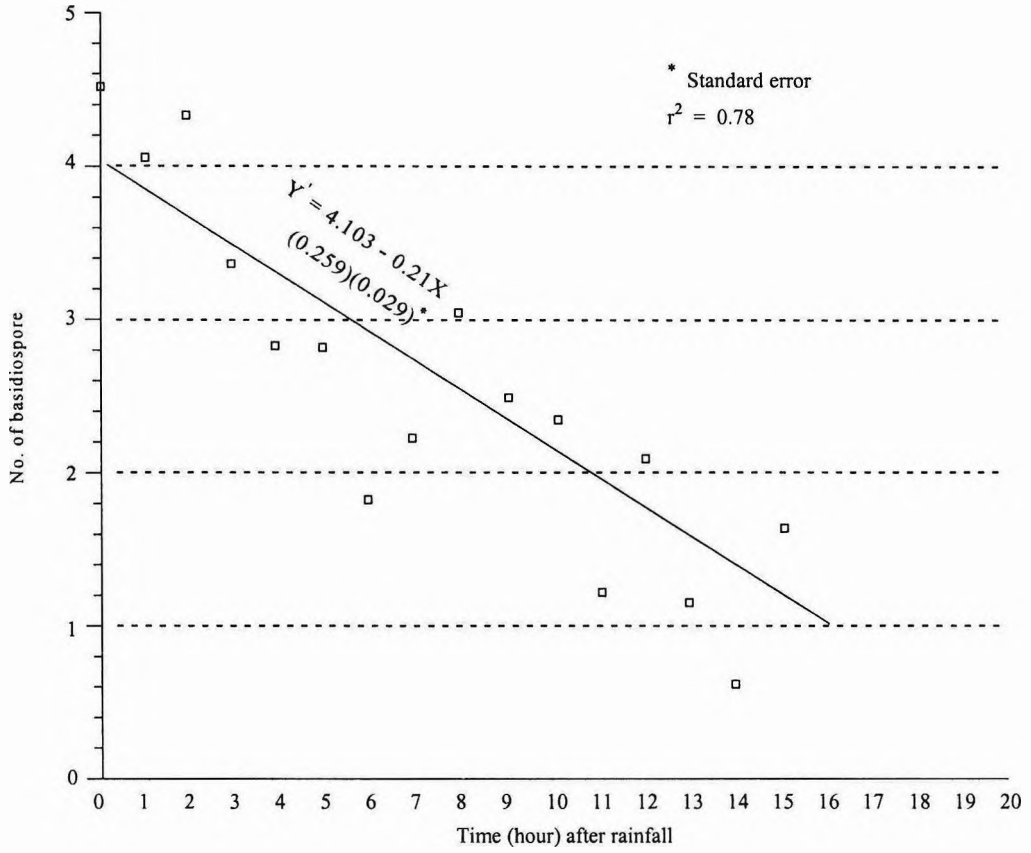

Figure 2. Regression line between time (during and after rain) and number of basidiospores per h.

1993. However, in the dry month (June 1992), new disease incidence was observed (*Figure 3*).

The relationship between disease incidence with basidiospores in the air, rainy days, and maximum temperature can be presented by the model:

$$Y = 30.2444 + \underset{(2.25)^{*}}{0.0702SP} + \underset{(1.73)}{0.0927PERRD(-1)}$$

$$\underset{(-1.41)}{-0.8233MT} + \underset{(5.00)}{0.496TREND} \qquad ...2$$

$r^2 = 0.735$

where Y is the disease incidence
SP is the spore
$PERRD(-1)$ is the rainfall in the previous month
MT is the maximum temperature
$TREND$ is the trend

* the number in the bracket represents t-test.

Time Series Analysis showed that basidiospores in the air was significantly [5% (t>2.07)] related with pink disease incidence. However, there is a tendency that the other

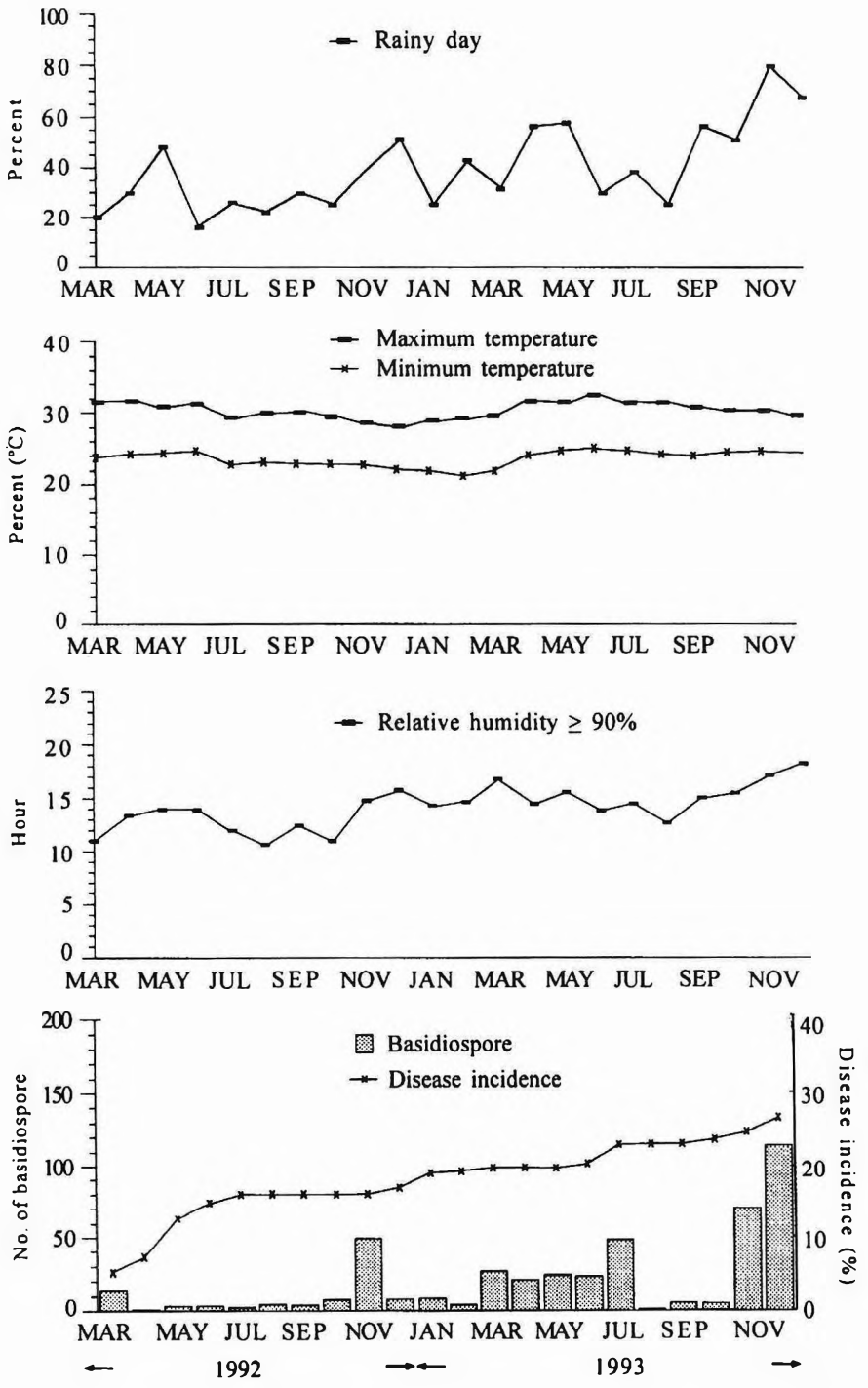

Figure 3. Profile of monthly average of number of basidiospores, disease incidence and weather measurements.

two variables, maximum temperature and rainfall in the previous month, contributed to the incidence of pink disease since the two variables are significant at 20% (t>1.32) and 10% (t>1.72) respectively with pink disease incidence. Provided all the variables are constant, increase of temperature will decrease pink disease incidence. However, an increase in rainy days of previous month will increase the incidence of pink disease. The other parameter, relative humidity, more or equal to 90% (RH ≥90%) was not significant.

DISCUSSION

There are three cardinal temperatures which affect the mycelial growth of a fungus *i.e.* the minimum, maximum and optimum temperatures[12]. Since the minimum and maximum temperatures for growth of *C. salmonicolor* isolates obtained from Malaysia were 5°C and 40°C, respectively while the optimum temperature was 28°C, the fungus is in the Mesofil group. Verma and Munjal[11] stated that 25°C was the best temperature for growth of *C. salmonicolor* and no growth was observed at 5°C. Luz[13] stated that the optimum temperature for *C. salmonicolor* isolate from cocoa in Brazil was between 23°C–26°C, and 35°C completely inhibited fungal growth. This study showed that the optimum temperature for mycelial growth of *C. salmonicolor* in Malaysia is higher than in other countries.

As the growth at temperatures between 5°C–10°C and 35°C–40°C were not evaluated, the 'minimum' and 'maximum' growth temperatures for *C. salmonicolor* may be within those figures. However, the minimum and maximum temperature for the growth of the fungus may not be less than 5°C or more than 40°C.

The release of basidiospores of *C. salmonicolor* in cocoa was during and after rainfall and lasted 13.5 h after rainfall[7-8]. Many basidiospores of *C. salmonicolor* from the pink disease lesion in the rubber plantations were also released during rainfall period and reduced exponentially after rainfall. Basidiospores were still released 15 h after rainfall since the basidiospores could be caught during that particular hour. This indicated that high inoculum density of basidiospores was available during and immediately after rainfall. This may explain why the incidence of pink disease normally occur during rainfall period.

The temperature under the canopy of rubber trees in Malaysia was between 21°C–33°C. This range of temperatures is suitable for the growth of the pink disease fungus as the optimum temperature for growth was 28°C. This temperature is also suitable for the germination of basidiospores which was within 18°C–32°C[14]. Christians *et al.*[7] mentioned that even during the dry season, development of pink disease symptoms can still be observed in cocoa plantations. This observation can also be seen in the rubber plantations whereby new incidences of pink disease were observed in June 1992. Eventhough the month of June was considered a dry month, there were still sporadic rains during the month. The role of sporadic rain on pink disease in cocoa trees had been mentioned by Christians *et al.*[7] The sporadic rain probably not only stimulated active infection but also inactive infections (dormant) in cocoa. Similarly, this may occur in rubber trees as well thus creating new disease incidence. The rainy days which occur during

the previous months (May) also provide condusive environment for the growth of the fungus. Hilton[2] mentioned that few months were needed for the pathogen to kill the stem of a rubber tree and a longer time was required during dry weather. Although, the disease can develop during dry period, more pink disease occurred during the wet period.

It has been reported that high relative humidity due to intercultivation or heavy weed growth created ideal micro-climatic conditions for the development of pink disease in Eucalyptus[3]. High relative humidity is always present under rubber canopy (RH ≥90%) whether on dry or wet months. However, it was observed that high relative humidity (RH ≥90%) was not a critical factor governing incidence of pink disease in rubber as compared to rainfall.

Harrison *et al.*[15] has used spore trapping as a guide to initiate control of potato early blight caused by *Alternaria solani*. The use of spore trapping to determine the presence of basidiospores as an early indicator to initiate control will be advantageous as the corticium stage which produces the basidiospores was the most destructive[16]. However, the practise is not practical for small rubber planters.

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ERRATA

Effect of Interstock on Dry Matter Production and Growth Analysis of *Hevea brasiliensis* (Muell. Arg.)

[*J. nat. Rubb. Res., Volume 11(4), page 278.*]

The y-axis in **Figure 8** should be **Specific leaf area (cm^2/g)**:

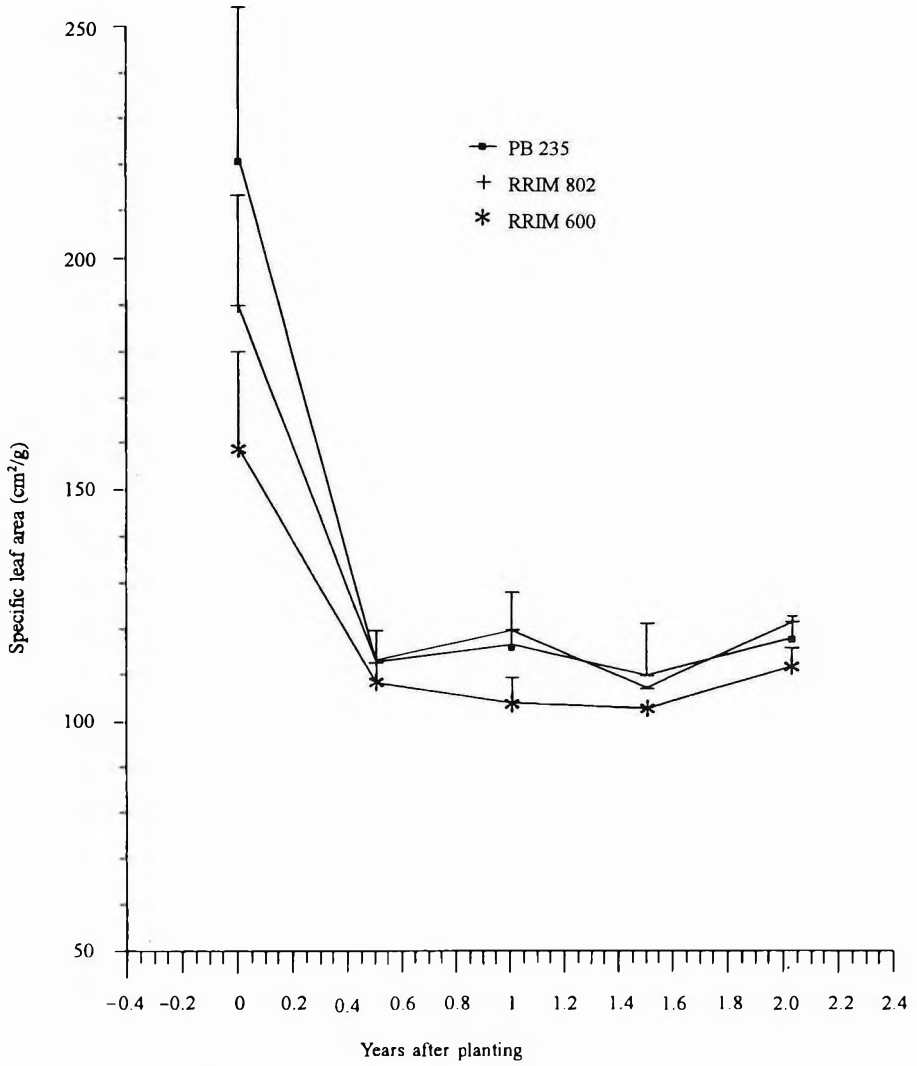

Figure 8. Specific leaf area of three scion clones from 0 to 2 years after planting. (Each point represents the mean of 6 interstocks +SD of the mean; some SDs are smaller than the symbols representing each point.)

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