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# *Proteins of Natural Rubber Latex Concentrate*

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*Proteins of high ammoniated latex concentrate were distributed in two main fractions: the rubber fraction and the serum fraction. The rubber fraction contained primarily the 14 kDa protein, with a minor one at 24 kDa, both of which were extractable by ammonia, sodium dodecyl sulphate and chloroform-methanol. The serum proteins comprised the 14, 24, 29, 36 and 45 kDa proteins and one of molecular weight greater than 100 kDa. These proteins originated from the B- and C-sera of fresh latex and the rubber particles.*

Fresh natural latex derived from *Hevea brasiliensis* contains about 0.95% proteins of which 27.2% is in the rubber fraction, 47.5% in the serum fraction and 25.3% in the bottom fraction[1]. In the process of producing centrifuged natural rubber (NR) latex concentrate from which most of the latex articles are fabricated, some of these proteins are lost in the skim latex. Those remaining in the latex concentrate have long been associated with some of the properties of the latex and its products. The mechanical stability of high ammoniated (HA) latex concentrate, for example, is thought to be influenced by the proteins as well as the lipids[2,3]. Proteins have also been shown to influence the stress-strain and modulus of the vulcanised latex concentrate film[4]. Recently proteins in NR latex gloves have been linked to some allergic reactions in human beings[5,6,7].

Although proteins in NR latex have been extensively studied by a number of workers[8-11], their investigations have been mostly confined to the B- and C-sera proteins of fresh latex. Of late, some investigations[12,13,14] have begun on proteins associated with rubber particles (RP), but till now very little attention has been given to the study of proteins in NR latex concentrate. In view of the increasing importance of proteins in this area, the present work was undertaken to investigate the composition of proteins in HA latex concentrate, both adsorbed on the RP and dispersed in the serum fraction.

## MATERIALS AND METHODS

### Materials

Fresh latex was obtained from mature unstimulated trees. The HA latex concentrate was prepared by centrifuging about 0.5% ammoniated field latex in a De Laval LRH 410-70A centrifugal latex separator. This gave latex concentrate of about 60% dry rubber content which was further ammoniated to 0.7%.

Dialysis tubing of 12 000 molecular weight cut-off and all the chemicals for electrophoresis including the SDS molecular weight markers were purchased from Sigma Chemical Company.

### Isolation of Serum Proteins from HA Latex Concentrate

The HA latex concentrate was ultracentrifuged on a Beckman L8-70 ultracentrifuge at 19 000 r.p.m. for about 1 h using rotor 21. The latex fractionated into two layers; the rubber phase and the serum phase with some sludge at the bottom at the centrifuge tube. The milky top layer of the

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serum phase was carefully sucked out and discarded while the clear serum fraction was siphoned using a long needled syringe. The latter fraction was then dialysed against water in the cold for two to three days with frequent changes of fresh water. The dialysed materials were freeze-dried and their weight determined.

**Extraction of Proteins from Rubber Particles**

Rubber particles (RP) were isolated from fresh latex or HA latex concentrate by ultracentrifugation as above. The isolated RP were rinsed with water two to three times before dispersing them in water, followed by ammoniation to 0.7%, or dispersing them in 2% sodium dodecyl sulphate (SDS). The dispersed RP were stirred for some time and left to stand in the cold overnight. The mixture was then ultracentrifuged and the clear ammonia and SDS extracts were collected as described in the isolation of serum above. The extracts were dialysed against water for two to three days before the dialysed materials were freeze-dried.

In the extraction of proteins from the RP by chloroform-methanol, the isolated RP were first dispersed in a minimum amount of water, filtered through a muslin cloth and the suspension added drop-wise to five volumes of continuously-stirred chloroform-methanol (2:1, v/v) mixture. The extracts, separated from the rubber coagulum, were washed with 0.6% sodium chloride and the resulting mixture separated into three layers on standing in the cold overnight. Any glycoproteins would appear in the top aqueous layer, according to Hamaguchi and Cleve[15]. This fraction was dialysed against water for two to three days, and then freeze-dried. The proteolipids occurred mainly in the interfacial layer, with some in the chloroform layer as reported previously[12]. In this study, the proteolipids were isolated from the interfacial layer by precipitation with acetone and the precipitate dried at room temperature.

**Polyacrylamide Gel Electrophoresis**

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a linear gradient gel of 8.5% to 17.5% acrylamide with a stacking gel of 3% acrylamide, following the procedure outlined by Boschetti *et al*[16]. Electrophoresis was carried out with 10 $\mu$l of sample (40 mg/ml) at a constant current of 30 mA for 5-6 h.

Isoelectric focusing (IEF) was carried out according to the LKB Instruction Note 2217[17] on a 0.5 mm polyacrylamide gel containing ampholine carrier ampholyte pre-blended to pH 3.5 to 9.5, purchased from LKB (Bromma, Sweden). About 10 $\mu$l of sample (20 mg/ml) dissolved in 1% glycine was run at a constant power of 25 watts for 45 min with 1*M* sodium hydroxide as cathode electrolyte and 1*M* phosphoric acid as anode electrolyte.

The SDS-PAGE and IEF gels were fixed for 1 h, stained overnight and de-stained several times in solutions prepared according to the LKB Application Note 306[18].

**Determination of Sugar and Nitrogen**

The glycoprotein fraction was first hydrolysed[19] with 2*N* hydrochloric acid for 2 h and the sugar liberated in the hydrolysate was determined colorimetrically by the Dubois method[20]. The nitrogen content was determined by the Kjeldahl method[21].

## RESULTS AND DISCUSSION

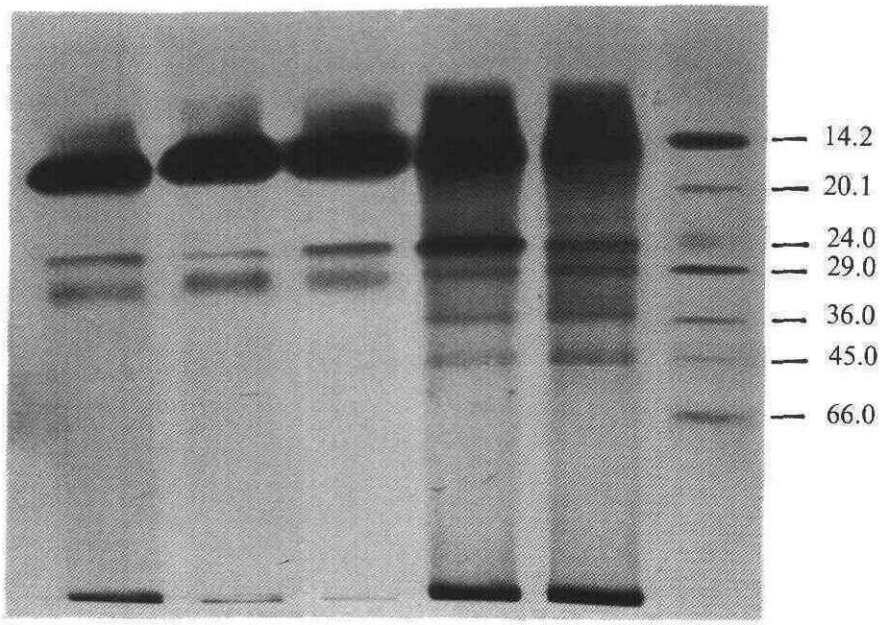
Proteins of HA latex concentrate are distributed between two main fractions; the serum fraction and the rubber fraction.

***Proteins in the Serum Fraction***

Freeze-drying a dialysed sample of HA latex concentrate serum yielded a brownish powdery material which constituted about 0.51% of the rubber and which contained 12% nitrogen. SDS-PAGE of the sample showed a protein band of a slightly lower

mobility than the 14.2 kDa protein marker with some undefined protein bands around the same region, four clear bands at 24, 29, 36 and 45 kDa and another band of very high molecular weight at the boundary between the stacking and separating gels *(Figure 1)*. Some, or all, of these proteins must derive from the B- and C-sera of fresh latex and the rubber particles. The B-serum was incidentally released from the lutoids which burst in ammonia. Previous reports of paper, starch and polyacrylamide gel electrophoresis of B- and C-sera of fresh latex[22,23,24] however showed that there are more protein bands than seen here on SDS-PAGE of the proteins in serum from HA latex. This almost certainly results in part from variations in the methods of analysis. Previous separations were based on differences in both the charge and size of the proteins while the present analysis with SDS-PAGE is based on differences in size or molecular weight of SDS-denatured proteins.

Further analysis on IEF polyacrylamide gel showed that serum from HA latex concentrate indeed contained fewer proteins than the B- and C-sera proteins of fresh latex *(Figure 2)*. The serum proteins of




A. SDS-extracted proteins of RP from 30-day-old RRIM 729
B. Proteolipid fraction of RP from 35-day-old RRIM 600
C. Glycoprotein fraction of RP from 44-day-old PR 255
D. Ammonia-extracted proteins of RP from 48-day-old RRIM 701
E. Serum proteins of 48-day-old RRIM 701 concentrate
F. Molecular weight markers (kDa)

*Figure 1. SDS-PAGE of proteins from the rubber and serum fractions of HA latex concentrates.*

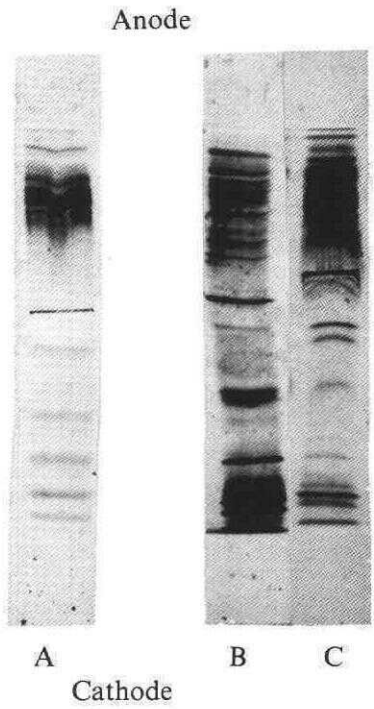



*Figure 2. IEF of serum proteins of HA latex concentrate (A) and the proteins from the B- and C-sera of fresh latex (B and C).*

latex concentrate were found to focus mainly in the acidic region with fewer bands in the basic region. This is in accordance with the IEF pattern of the proteins from latex serum prepared by Morales *et al.*[25], by precipitating the rubber in the concentrate with acetic acid. The B- and C-sera proteins of fresh latex however showed a significantly higher number of anionic and cationic protein bands. Thus, in the production of the latex concentrate, a number of proteins may have been lost in the skim latex and sludge, or by denaturation in ammonia as shown by Archer and Sekhar[22].

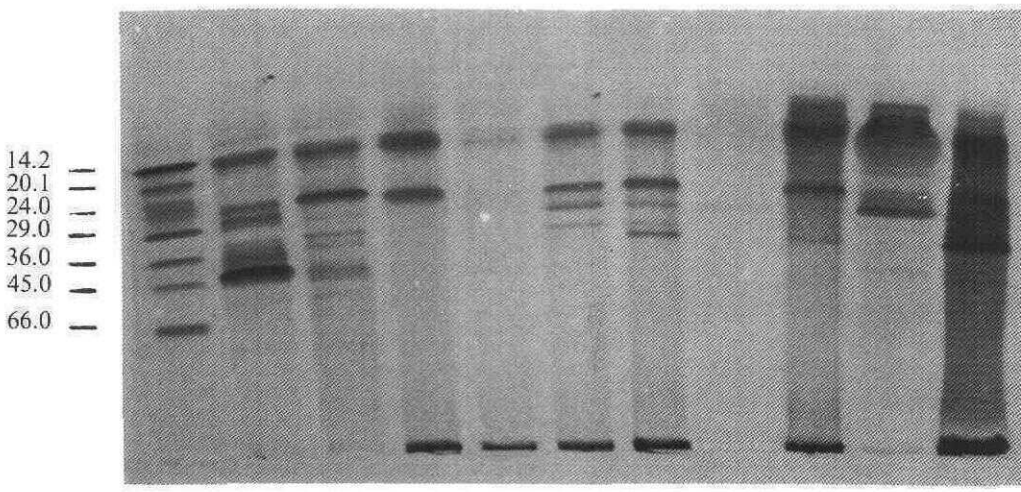
**Proteins in the Rubber Fraction**

The proteins associated with the RP could be extracted by ammonia solution, SDS or chloroform-methanol. Extraction by ammonia resembled the process occurring in the storage of latex concentrate and the proteins so extracted are possibly similar to the peripheral proteins of cellular and other membranes, which are known to be easily extracted by changing the pH of the medium[26] e.g. by addition of ammonia[27]. The removal of integral membrane proteins, however, requires detergents and organic solvents[26].

**Proteins Extracted by Ammonia**

Dispersing the isolated RP of HA latex concentrate in water followed by ammoniation to 0.7%, resulted in an extraction of about 0.1% (gramme per 100 g rubber) of compounds with a nitrogen content of 11%. SDS-PAGE of the extracts showed a protein band at a slightly lower mobility than the 14.2 kDa protein marker, with a few more unresolved bands around the same region, four clear protein bands at 24, 29, 36 and 45 kDa, and another band at the boundary between the stacking and

the separating gels *(Figure 1)*. This was in fact similar to the pattern found with serum from HA latex concentrate except for the 14 kDa and 24 kDa proteins, which appeared more concentrated in the ammonia extracts than in the serum. These two proteins probably originated from the RP, while the rest were very likely the proteins of the serum occluded in the rubber during ultracentrifugation. To check this, RP from fresh latex were dispersed both in water and 0.7% ammonia overnight before analysis. The water extracts were found to contain most of the serum proteins, including the 24 kDa protein, but in a lesser amount than the major 45 kDa protein *(Figure 3)*; a 14 kDa protein was also detected. The ammonia extracts, on the other hand, contained mainly the 14 kDa and 24 kDa proteins, besides some serum proteins *(Figure 3)*. In fact, when the RP were re-extracted with ammonia for a second and third time, the 14 kDa and 24 kDa proteins were still present *(Figure 3)*. The 14 kDa protein of the ammonia extracts however ran slightly more slowly than the 14 kDa protein in the water extracts when the mixtures were re-analysed on 15% polyacrylamide gel *(Figure 4)*. The 14 kDa protein in the ammonia extract might have a molecular weight of 14.6 kDa, which is




A. Molecular weight markers (kDa)

B. Proteins from RP of RRIM 730 fresh latex dispersed in water

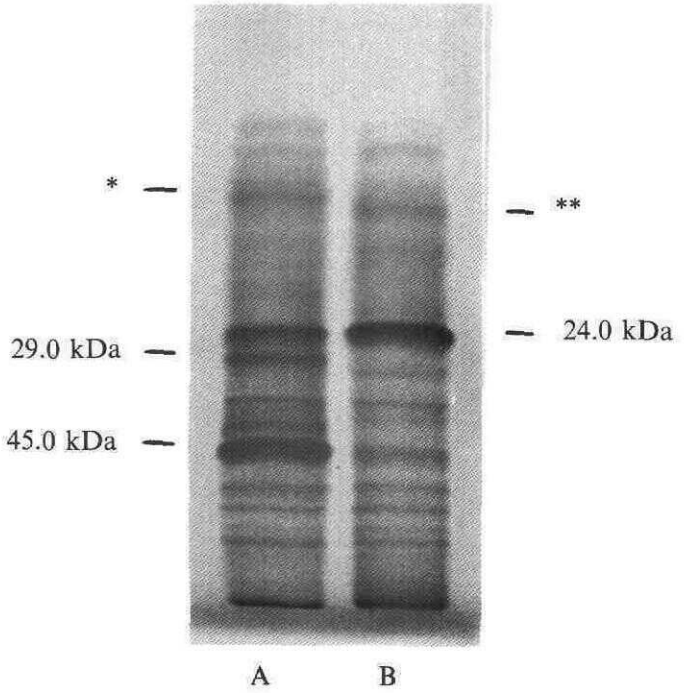
C,D,E. Proteins in the first (C), second (D) and third (E), ammonia extracts of RP from RRIM 730 fresh latex

F,G. Water-soluble (F) and water-insoluble (G) proteins in the ammonia extracts of RP from 4-day-old RRIM 729 latex concentrate

H,I. Water-soluble (H) and water-insoluble (I) proteins in the ammonia extracts of RP from 70-day-old RRIM 729 latex concentrate

J,K. Water-soluble (J) and water-insoluble (K) serum proteins of 24-day-old RRIM 729 latex concentrate

*Figure 3. SDS-PAGE of proteins in the ammonia extracts of RP from fresh latex and HA latex concentrate and proteins in the serum fraction of the latex concentrate.*




*and** distinguish the two proteins around 14.0 kDa.

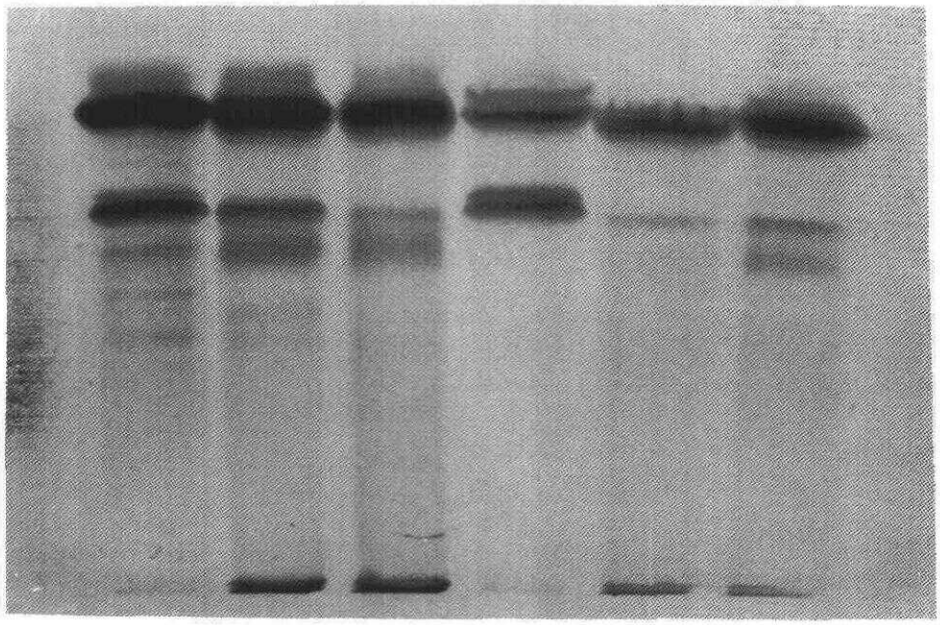
*Figure 4. SDS-PAGE of proteins in the water extracts (A) and ammonia extracts (B) of RP from RRIM 730 fresh latex.*

the molecular weight of the protein shown by Dennis and Light[13] to occur on the RP, but not in the C-serum of fresh *H. brasiliensis* latex. Thus, ammonia extracted some proteins from the RP besides removing some serum proteins.

It was expected that as the RP were dispersed and stored in ammonia, as in latex concentrate, the 14 kDa and 24 kDa proteins would be progressively removed. The two proteins were however still detectable from the seventy-day-old RP *(Figure 3)*, implying the incomplete or slow extraction of the proteins by ammonia.

**Proteins Extracted by SDS and Chloroform-Methanol**

A 2% SDS solution was found to extract some membrane components amounting to about 0.3% of the rubber and which contained 10% nitrogen. The extracts were expected to contain some SDS as not all the surfactant could be removed on dialysis. Analysis of the extracts showed one very prominent protein band at 14 kDa, which may again be the 14.6 kDa protein, and two less prominent bands at 24 kDa and 29 kDa *(Figure 1)*. The band at 29 kDa, however, occurred mainly in the extracts of the RP of HA latex concentrate. The SDS extracts of RP from fresh latex did not show this protein band well *(Figure 5)*. A similar extraction of serum-free RP of *H. brasiliensis* latex by Dennis and Light[13] showed a minor 24 kDa protein and a major 14.6 kDa protein [which they termed, the rubber elongation factor (REF)]; no 29 kDa protein was mentioned. So the 29 kDa protein must be derived from the serum fraction of HA latex concentrate, which had earlier been shown to contain this protein *(Figure 1)*. The SDS




A,B,C. SDS-extracted proteins of RP from fresh latex of RRIM 730 (A), and 4-day-old (B) and 60-day-old (C) latex concentrate of RRIM 729

D,E,F. The glycoprotein fraction of RP from fresh latex of PR 225 (D), 10-day-old RRIM 729 (E) and 44-day-old RRIM 701 (F) latex concentrates.

*Figure 5. SDS-PAGE of SDS-extracted proteins and the glycoprotein fraction of RP from fresh latex and HA latex concentrate.*

extracts of the RP of HA latex concentrates from RRIM 804, RRIM 600, RRIM 729 and RRIM 730, which had been stored for four to sixty days, showed a similar pattern of three protein bands.

A more selective extraction of the RP proteins can be obtained with chloroform-methanol. Washing such extracts of cell membranes with sodium chloride gives a group of proteins at the interface between the chloroform and aqueous layers, which is classified by Folch and Lees[28] as proteolipids. The group of proteins occurring in the aqueous phase from such a procedure are glycoproteins[15].

The SDS-PAGE analysis of the proteolipids from the RP of HA latex concentrate showed a similar pattern to the SDS extracts, with a major band at 14 kDa and two minor ones at 24 kDa and 29 kDa *(Figure 1)*. The pattern however differed from that in the earlier report on proteolipids of RP from fresh unammoniated latex[12]. Proteolipids of such RP gave only two bands, at 14.3 kDa and 24 kDa, as shown in *Figure 6*. As with the SDS extracts, the 29 kDa protein originated from the serum. *Figure 6* demonstrates the insignificant difference in the nature of proteolipids from latex concentrates of different clones and ages.

The amount of proteolipids from the RP of fresh latex of six clones varied from 0.37% to 0.57% of the rubber, while RP matured in ammonia solution gave about

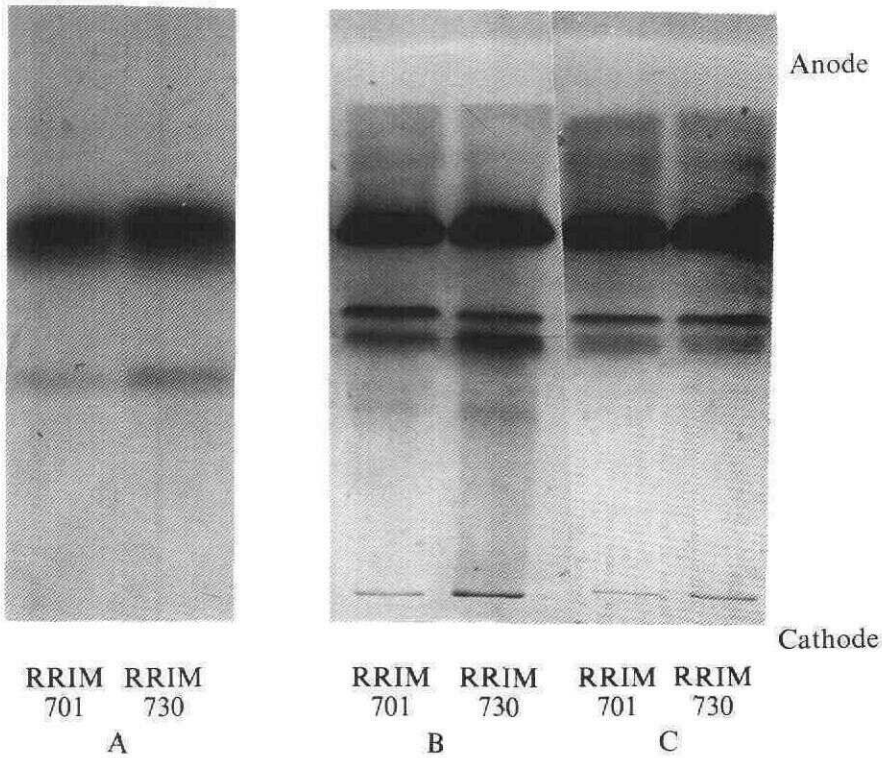



*Figure 6. SDS-PAGE of proteolipids of RP from fresh latex (A), and 4-day-old (B) and 35-day-old (C) HA latex concentrates of RRIM 701 and RRIM 730, respectively.*

0.40% proteolipids *(Table 1)*. Thus the levels of proteolipids from fresh and matured RP were similar. This is not unusual, since integral membrane proteins are not expected to be easily removed by ammonia. The nature of the proteolipids associated with the RP dispersed in 0.7% ammonia of pH 10.0 might however differ from the proteolipids of fresh RP dispersed in C-serum of pH 6.9. This possible difference was not investigated.

As with the SDS extracts and the proteolipid fraction, the glycoprotein frac-

TABLE 1. PROTEOLIPID CONTENT OF RUBBER PARTICLES (RP) FROM FRESH LATEX AND HA LATEX CONCENTRATE OF SIX CLONES

| Clone | Amount of proteolipid (% dry weight of rubber) | |
|---|---|---|
| | RP of fresh latex | RP of 1-month-old HA latex concentrate |
| RRIM 730 | 0.39 | 0.39 |
| RRIM 701 | 0.54 | 0.42 |
| PB 28/59 | 0.53 | 0.40 |
| RRIM 600 | 0.57 | - |
| PR 255 | 0.37 | 0.43 |
| RRIM 804 | 0.54 | - |

tion from the RP of HA latex concentrate also gave one major protein band at 14 kDa, and two minor ones at 24 kDa and 29 kDa *(Figure 1)*. Again, the 29 kDa protein proved to be serum protein, based on its absence in the glycoprotein fraction of fresh RP *(Figure 5)*. Although SDS-PAGE did not reveal marked differences in the patterns of the proteins in the glycoprotein fraction from the few clones studied (RRIM 730, RRIM 701, RRIM 729, RRIM 600 and PR 255), the amount of material extracted by chloroform-methanol differed significantly with the nature of the latex. Extraction from fresh RP gave about 0.01% to 0.05% (gramme per 100 g of rubber) of materials dissolved in the aqueous fraction *(Table 2)*. The same level was obtained from the RP of HA latex concentrate of less than ten days old. Rubber particles from older latex concentrate, however, gave a much higher level of materials of about 0.23% to 0.35%. It was not ascertained why there were less materials in the glycoprotein fraction from RP of fresh latex than from RP matured in ammonia for more than ten days.

The expected presence of sugar in the glycoprotein fraction was tested for by the Dubois method[20]. The sugar was found to constitute about 1% of the materials in the glycoprotein fraction *(Table 2)*. Preliminary analysis on gas-liquid chromatography showed the glycoprotein fraction to contain only two hexoses, glucose and an unknown sugar, which did not correspond to other sugars tested: *i.e.* arabinose, xylose, galactose, mannose and fucose which are generally known to occur in glycoproteins of biological membranes. Due to very low sugar content associated with the proteins in the glycoprotein fraction of the natural rubber particles, these proteins could not be referred to as glycoproteins, normally found in the membranes of cells and organelles.

Thus the major protein associated with the RP is of molecular weight *ca.* 14 kDa besides the minor 24 kDa protein. The 14 kDa protein is almost certainly the same as the 14.6 kDa REF protein of Dennis and Light[13]. Although these proteins are extractable by different extracting media, namely ammonia, SDS and chloroform-methanol, the current experimental evidence could not implicitly classify them to be different. Furthermore, Dennis and Light[13] showed that there is only one major 14.6 kDa protein and one minor 24 kDa protein in the surface of the rubber particles. Thus, the 14 kDa and the 24 kDa proteins extracted by ammonia and chloroform-methanol may be the same as the ones extracted by SDS.

TABLE 2. AMOUNT OF MATERIAL IN THE GLYCOPROTEIN FRACTION (FOLCH METHOD) FROM RUBBER PARTICLES OF FRESH LATEX AND HA LATEX CONCENTRATES OF SIX CLONES

| Clone | Amount of glycoprotein fraction (% dry weight of rubber) | | Sugar content of glycoprotein fraction of matured RP[b] (% dry weight of materials in the glycoprotein fraction |
|---|---|---|---|
| | Fresh RP[a] | Matured RP[b] | |
| RRIM 730 | 0.02 | 0.35 | - |
| RRIM 701 | 0.04 | 0.25 | 1.6 |
| PB 28/59 | 0.01 | 0 24 | 0 9 |
| RRIM 600 | 0.01 | 0 29 | 0.9 |
| PR 255 | 0.05 | 0.32 | 1.1 |
| RRIM 804 | 0.02 | 0 23 | 1.8 |

[a] RP from fresh latex

[b] RP from 30- to 45-day-old HA latex concentrate

## CONCLUSION

Proteins of HA latex concentrate were fractionated by SDS-PAGE into six serum proteins (strictly, polypeptides) and two RP membrane proteins The serum proteins had molecular weights of 14, 24, 29, 36 and 45 kDa and another protein of molecular weight greater than 100 kDa They form the group of proteins which are most likely to be present in aqueous extracts of latex products It is to be expected that the water soluble proteins *(Figure 3)* are the most readily leached out

The proteins associated with the RP are mainly the 14 kDa protein, with a minor 24 kDa protein These were partially extractable with ammonia solution and thus presumed to be loosely bound to the RP membrane A greater proportion was tightly bound to the RP and required detergent (SDS), or organic solvent (chloroform-methanol) for its extraction Such proteins would be expected to remain in the leached latex products Thus, any influence of proteins on the properties of latex products would be largely attributable to these proteins

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