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Distribution of Proteins between the Fractions of Hevea *Latex Separated by Ultracentrifugation*

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Fresh latex was ultracentrifuged under refrigerated conditions and three major fractions were recovered: the rubber fraction, serum fraction and bottom fraction. The proteins of the purified fractions were precipitated, redissolved in sodium hydroxide and assayed by a modified polymetric method. The total protein content of eighteen latex samples (protein of B and C sera as standards) was 0.95% of which 27.2% was in the rubber fraction, 47.5% in the serum fraction and 25.3% in the bottom fraction. Using bovine serum albumin as a standard, the total protein content was 1.57% and the corresponding figures for the rubber fraction, serum fraction and bottom fraction were 26.0%, 45.8% and 28.2% respectively.

The classical procedure of fractionating fresh *Hevea* latex by ultracentrifugation was developed by Cook and Sekhar[1] and later extended by Moir[2] who introduced more physiologically acceptable measures such as the collection of latex in chilled containers (0°C–4°C) and the use of pre-chilled rotors for ultracentrifugation. Fresh latex, when ultracentrifuged at approximately 59 000 g, can be separated into three major fractions *viz.* a top rubber fraction, a particulate C serum and a dense bottom fraction[1,2]. A yellow orange layer containing the Frey-Wyssling particles[3] in latex is separated immediately above the C serum but for the purpose of the present work it is considered as a minor fraction.

Cook and Sekhar[1] presented an approximate distribution of N in rubber, serum and bottom fraction as 34%, 35% and 31% respectively. However, these figures were for total N in those fractions, which also included the non-protein N. Archer and McMullen[4] estimated the total protein in latex as 1% of which about 20% was adsorbed on the rubber surface and about 66% dissolved in the C serum leaving the remainder (14%) in the bottom fraction. Later, Archer *et al.*[5], reported 1% as the total protein content of fresh latex of which about 20% was in the rubber fraction and 20% in the bottom fraction, with the remaining 60% dissolved in the serum. However, no systematic estimation of the proteins in the ultracentrifugally separated fractions of *Hevea* latex has been described. In view of the discrepancies in the results presented earlier[4,5] work was undertaken to investigate the distribution of proteins in latex to obtain reliable results which could be used in future investigations on *Hevea* proteins.

EXPERIMENTAL

Ultracentrifugation and Recovery of the Fractions of Latex

Latex was collected from mature trees of RRIM 501, RRIM 600 and Tjir 1 at the RRIM Experiment Station. The collection and ultracentrifugation were under refrigerated conditions as described by Moir[2], using SPINCO rotor 40 tubes of approximately 12.9 ml capacity. The resulting fractionation of latex was essentially as described previously[2]. *Zones 1* and *2*

were recovered as the rubber fraction, *Zones 3, 4* and *5* as the serum fraction and *Zones 6–11* as the bottom fraction.

Rubber Fraction

This was briefly washed by dispersing in deionised water (2 ml per millilitre latex) and centrifugation. The protein from the rubber surface was dissolved off by suspending overnight in a buffer containing 0.5% Triton X-100 in 2% KCl adjusted to pH 7 (2 ml buffer per millilitre latex was used) followed by centrifugation. The proteins were first precipitated with trichloro acetic acid and then redissolved in NaOH. Aliquots were diluted ten times with water and the colour was developed and read at 660 nm, following a modification of Lowry *et al.*[6] as described by Litwack[7]. All protein estimations in this and in the other fractions were carried out in triplicate and the mean value was used.

Serum Fraction

The washing from the rubber fraction was combined with the serum fraction and the Triton X-100/KCl buffer (2 ml per millilitre latex) was mixed and centrifuged. A small amount of rubber which is present in these fractions[8] separated centripetally. The proteins from the aqueous phase were precipitated with trichloro acetic acid (0.5 ml per millilitre latex) and redissolved in 5 ml of 0.5*M* NaOH. After twenty times diluting the aliquots the proteins were estimated as mentioned above.

Bottom Fraction

Proteins from this fraction were extracted in the Triton/KCl buffer (1.5 ml per millilitre latex) by repeatedly freezing and thawing and ultrasonication for 1 min at 20 KHZ followed by centrifugation. Trichloro acetic acid was added (1.5 ml per millilitre latex) to precipitate some of the proteins which were recovered by centrifugation, dissolved in 5 ml of 0.5*M* NaOH and estimated as above.

The major soluble protein in the bottom fraction *viz.* hevein and the related protein pseudo-hevein were soluble in trichloro acetic acid[9] and remained dissolved in the supernatant from the trichloro acetic acid precipitation step. They were precipitated by adding one volume of 10% (weight/volume) phosphotungstic acid to the supernatant. These proteins were redissolved in 5 ml of 0.5*M* NaOH and estimated as mentioned above. The proteins estimated in the bottom fraction were the proteins in the B serum plus the proteins from the lutoid membrane.

Standard Reference Graphs for Estimation of Proteins

The proteins in the B and C sera differed considerably as judged by their electrophoretic mobility[10] and it was considered desirable to use standard reference graphs of these proteins to estimate the proteins in the latex fractions. The proteins from B serum (prepared as described previously[11]) and C serum were isolated by precipitation with ammonium sulphate followed by desalting in a dialysis tubing of a controlled pore size ('SPECTRAPOR' grade 3, manufactured by Spectrum Medical Laboratories Inc., California, U.S.A.) to minimise the loss through diffusion of hevein and pseudo-hevein[9]. The desalted, freeze-dried proteins were redissolved in 0.1*M* NaOH and protein estimation was carried out as above to produce standard graphs. To compare the results between the *Hevea* proteins as the reference and bovine serum albumin, a standard reference graph was also prepared from the latter (obtained from SIGMA Chemicals, U.S.A.).

RESULTS AND DISCUSSION

Several methods are available for the assay of proteins. The simplest method is the determination of N by the micro-kjeldahl method and multiplying the percentage N by 6.25. In this case, the non-protein N must be removed before carrying out the determination. In the usual practice, the non-protein N is removed by dialysis and the non-diffusible material is recovered for the protein estimation.

The peptide bonds in a protein molecule absorb at 185 nm[12] and one of the methods of measuring the protein concentration is by spectrometry at 205 nm[13] where most proteins have a high extinction coefficient. In most biological preparations however, there are materials other than protein which also absorb at 205 nm (e.g. free amino acids, nucleotides, *etc.*), which have to be removed for an accurate protein assay by spectrometry.

Among the colorimetric methods, one of the earliest and still most widely used method is by Lowry *et al.*[6] where the Folin-Ciocalteu phenol reagent is reduced by the copper treated protein giving a blue colour. The colour yield of this reaction is considered to arise mainly from tyrosine, phenylalanine and tryptophan and to some extent from the sequence of certain amino acids bearing functional side groups such as arginine, glutamic acid and histidine. The method is claimed to be very sensitive and there is negligible interference from several non-protein substances[7].

Recently, Bradford[14] recommended the binding of Coomassie Brilliant Blue G-250 to the protein in a protein denaturing solvent and reading the absorption of the protein dye complex at 595 nanometres. However, later workers[15,16] reported this method to be of limited analytical value since it showed a wide variability in its sensitivity to various proteins.

For the present work, any method which involves dialysis is unsuitable especially in the case of B serum (or extract of the bottom fraction) which contains a large proportion of hevein and pseudo-hevein. Although the loss of these proteins can be minimised by using a controlled pore size dialysis tubing (see above) the procedure is tedious and unsuitable for routine estimations, and was therefore used only for preparing a standard reference graph as described above. The Lowry method[6] as described by Litwack[7] was chosen in the present work for the above reasons.

The standard reference graphs of B and C sera proteins are shown in *Figure 1* which also includes a standard reference

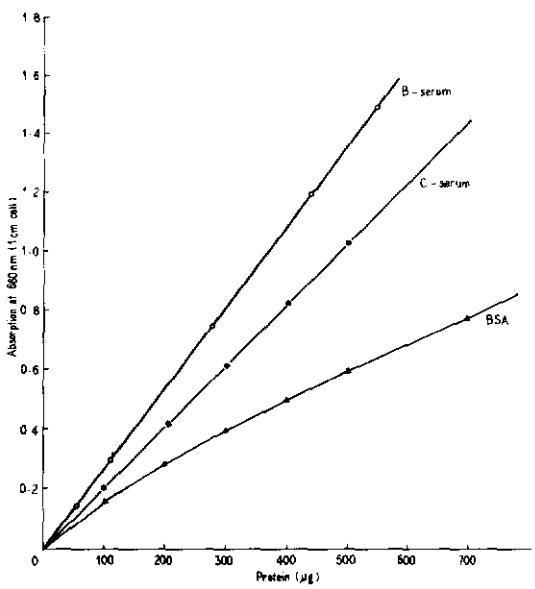

Figure 1. Standard reference graphs prepared from B and C sera proteins and bovine serum albumin.

graph of bovine serum albumin. A linear relationship was obtained between the absorption at 660 nm and the concentration of protein upto 500 μg protein from the B and C sera. For an accurate estimation, the protein extracts from the latex fractions were appropriately diluted before the colour development so that the protein concentration was well below 500 microgramme. For the estimation of proteins in the rubber and the serum fractions, the standard reference graph of the C serum proteins was used. The bottom fraction proteins were estimated using the standard graph for the B serum proteins.

Trichloro Acetic Acid-Soluble Proteins in Bottom Fraction

The proteins in the rubber and in the serum fractions were completely precipitated with trichloro acetic acid. (Addition of phosphotungstic acid to the supernatant from the trichloro acetic acid precipitation gave a small quantity of a further precipitate which was found to be non-protein material.) The proteins in the bottom fraction extract, however, had to be precipitated in two stages as described above. Assuming that the lutoid membranes did not have trichloro acetic acid -soluble proteins, the phosphotungstic acid precipitate contained only hevein and pseudo-hevein. Estimates of these two proteins as a percentage of the total proteins in the whole bottom fraction are presented in *Table 1*.

In *Table 1*, the mean figure 39.4% shows that hevein + pseudo-hevein together make less than 40% of the total protein in the bottom fraction. It has been reported[17] that about 70% of the *water soluble* protein in the bottom fraction was hevein + pseudo-hevein. This figure obviously did not take into account the protein in the lutoid membrane which makes a substantial contribution (approximately 44%) to the total protein in the bottom fraction. It would appear from these results that the bottom fraction has 44% membrane proteins from the lutoids, 39.4% hevein + pseudo-hevein and 16.6% basic proteins. (If the membrane proteins were not included, the percentage figure for hevein + pseudo-hevein would increase to approximately 70%).

Distribution of Proteins in Latex

The figures for the distribution of proteins between the ultracentrifuged fractions of latex were calculated using different reference standards. In *Table 2*, proteins from the B and C sera were used as the standards. For the eighteen latex samples analysed (six from each of the three clones), the mean figure for the total protein in latex was 0.95 g per 100 ml (0.95%) which compares well with the previously published figure of 1%[4,5]. The distribution of proteins over the rubber fraction, serum fraction and bottom fraction was 27.2%, 47.5% and 25.3% respectively.

A much higher mean figure (1.57 g per 100 ml) was obtained when the standard graph of bovine serum albumin was used. The distribution figures also altered with somewhat less protein in the rubber (26.0%) and in the serum (45.8%) and more in the bottom fraction (28.2%) *(Table 3)*.

The previous figures[4,5] had been based partly on data for the protein content of freeze-dried C serum[18] and the total N content of rubber coagulum to which a correction was applied to allow for the choline, *etc.*, content of the phospholipid in the rubber fraction[19].

TABLE 1. ESTIMATION OF TRICHLORO ACETIC ACID-SOLUBLE PROTEINS IN BOTTOM FRACTION

Latex Sample	Protein (mg) Trichloro acetic acid—precipitate	Protein (mg) Trichloro acetic acid—soluble	Bottom fraction	Trichloro acetic acid—soluble (%)
1	15.1	9.9	25.0	39.6
2	15.8	10.0	25.8	38.7
3	17.8	11.5	29.3	39.2
4	17.9	12.2	30.1	40.5
5	19.0	11.6	30.6	37.9
6	17.2	10.2	27.4	37.2
7	19.3	11.4	30.7	37.1
8	17.7	12.3	30.0	41.0
9	18.8	12.8	31.6	40.5
10	18.4	12.5	30.9	40.4
11	20.1	14.1	34.2	41.2
12	20.6	14.6	35.2	41.4
13	17.3	10.8	28.1	38.4
14	18.8	11.5	30.3	37.9
15	20.7	14.1	34.8	40.5
16	20.0	13.9	33.9	41.0
17	21.0	12.8	33.8	37.8
18	22.2	14.1	36.3	38.8
Mean	18.8	12.2	31.0	39.4
Standard error	0.43	0.35	0.76	0.34

The data obtained for the present work were based on certain assumptions. It was for example, assumed that all the contaminating serum proteins were removed by washing the rubber fraction with water and also that all the proteins from the rubber fraction and in the bottom fraction were dissolved in the Triton/KCl buffer. Thus, the major source of error in this type of work is in isolating the proteins from the fractions to be analysed rather than in the analysis techniques themselves. However, within limitations, the protein distribution figures in *Table 2* (using the latex proteins as the standards) should be considered reliable and may be used for future investigations.

The variation in the total protein content between samples of latex is not unexpected. It may be a clonal variation or day-to-day variation but this could not be ascertained in the present work. The eighteen samples of latex were from random collections but care was taken to ensure that they were not collected either during the wintering period or during the rainy season. They represented normal samples collected by the S/2.d/2 100% tapping procedure.

TABLE 2. DISTRIBUTION OF PROTEINS BETWEEN THE MAJOR FRACTIONS OF LATEX

Latex sample	Rubber fraction[a] (mg)	Serum fraction[b] (mg)	Bottom fraction[c] (mg)	Total in latex	Percentage in latex (g protein in 100 ml latex)
1	33.4 (28.7)	57.8 (49.7)	25.0 (21.5)	116.2	0.90
2	31.7 (28.4)	54.1 (48.5)	25.8 (23.1)	111.6	0.86
3	34.0 (28.0)	58.3 (47.9)	29.3 (24.1)	121.6	0.94
4	31.4 (27.9)	50.9 (45.3)	30.1 (26.8)	112.4	0.87
5	36.7 (29.6)	56.8 (45.8)	30.6 (24.6)	124.1	0.96
6	30.8 (26.3)	59.1 (50.3)	27.4 (23.4)	117.3	0.91
7	33.8 (27.7)	57.6 (47.2)	30.7 (25.1)	122.1	0.94
8	32.6 (27.0)	58.2 (48.2)	30.0 (24.8)	120.8	0.93
9	35.2 (28.2)	58.1 (46.5)	31.6 (25.3)	124.9	0.97
10	32.8 (27.1)	57.3 (47.4)	30.9 (25.5)	121.0	0.94
11	34.4 (27.0)	59.0 (46.2)	34.2 (26.8)	127.6	0.99
12	34.5 (26.7)	59.6 (46.1)	35.2 (27.2)	129.3	1.00
13	32.6 (27.3)	58.5 (49.1)	28.1 (23.6)	119.2	0.92
14	33.5 (27.4)	58.3 (47.7)	30.3 (24.8)	122.1	0.94
15	34.9 (26.7)	60.9 (46.6)	34.8 (26.7)	130.6	1.01
16	32.2 (27.0)	53.1 (44.5)	33.9 (28.4)	119.4	0.92
17	34.3 (26.1)	63.3 (48.2)	33.8 (25.7)	131.4	1.02
18	29.7 (22.2)	67.9 (50.7)	36.3 (27.1)	133.9	1.04
Mean	33.3 (27.2)	58.3 (47.5)	31.0 (25.3)	122.5	0.95
Standard error	0.40(0.36)	0.87(0.40)	0.76(0.41)	1.48	0.01

[a]Calculated from the standard reference graph of C serum proteins.

[b]Calculated from the standard reference graph of the C serum proteins and include the proteins in the aqueous washing of the rubber fraction.

[c]Refer to the proteins in the trichloro acetic acid precipitate + the phosphotungstic acid precipitate and were calculated from the standard reference graph of the B serum proteins.

Figures within brackets are percentage values.

TABLE 3. RESULTS RECALCULATED FROM TABLE 2 USING THE STANDARD REFERENCE GRAPH OF BOVINE SERUM ALBUMIN

Latex Sample	Rubber fraction (mg)	Serum fraction (mg)	Bottom fraction (mg)	Total in latex (mg)	Percentage in latex (g protein in 100 ml latex)
1	52.7 (27.5)	92.5 (48.4)	46.2 (24.1)	191.4	1.48
2	50.5 (27.3)	86.5 (46.8)	47.7 (25.9)	184.7	1.43
3	53.7 (26.7)	93.3 (46.2)	54.2 (27.1)	201.2	1.56
4	49.6 (26.5)	81.4 (43.5)	56.0 (30.0)	187.0	1.45
5	58.0 (28.2)	90.9 (44.2)	56.6 (27.6)	205.5	1.59
6	48.6 (25.1)	94.6 (48.8)	50.6 (26.1)	193.8	1.50
7	53.7 (27.1)	87.3 (44.1)	56.8 (28.8)	197.8	1.53
8	51.8 (25.9)	93.1 (46.4)	55.5 (27.7)	200.4	1.55
9	56.0 (27.0)	92.9 (44.8)	58.4 (28.2)	207.3	1.60
10	52.1 (25.9)	91.7 (45.6)	57.1 (28.5)	200.9	1.56
11	54.7 (25.7)	95.0 (44.6)	63.2 (29.7)	212.9	1.65
12	55.0 (25.5)	95.3 (44.2)	65.1 (30.3)	215.4	1.67
13	51.8 (26.3)	93.6 (47.4)	52.0 (26.3)	197.4	1.53
14	52.9 (26.1)	93.3 (46.2)	56.0 (27.7)	202.2	1.57
15	55.5 (25.6)	97.4 (44.8)	64.4 (29.6)	217.3	1.68
16	51.2 (25.7)	84.9 (42.7)	62.7 (31.6)	198.8	1.54
17	54.5 (24.9)	101.3 (46.4)	62.5 (28.7)	218.3	1.69
18	47.2 (21.1)	108.6 (48.8)	67.1 (30.1)	222.9	1.72
Mean	52.8 (26.0)	93.0 (45.8)	57.3 (28.2)	203.1	1.57
Standard error	0.64 (0.35)	1.43 (0.43)	1.41 (0.44)	2.56	0.02

Figures within brackets are percentage values.

A comparison between the corresponding figures in *Tables 2* and *3* shows that a protein assay is a relative measurement and the results depend upon the reference protein used. In the case of *Hevea* latex it is preferable to use the *Hevea* proteins themselves as the reference proteins although, bovine serum albumin has been widely mentioned in literature as the standard protein.

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