Nucleotides of *Hevea brasiliensis* Latex

A RIBONUCLEOPROTEIN COMPONENT

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Natural rubber latex is obtained from the tree *Hevea brasiliensis* by making an incision in the bark extending in depth to within about 1 mm. of the cambial tissue (see Riches & Gooding, 1952). The latex which flows from the cut is believed to consist mainly of the content of the laticiferous-vessel system (Ruinen & de Haan-Homans, 1950). The collected latex is a suspension of several particulate phases in an aqueous serum and can be separated into fractions by high-speed centrifuging (Cook & Sekhar, 1953). The rubber particles form the uppermost layer; the 'bottom fraction' consists of sedimented material, while the intermediate layer, termed 'C-serum', represents the aqueous phase. Bottom fraction includes the 'lutoid particles' described by Homans & van Gils (1948); it is highly hydrated and contains a variety of non-rubber metabolites. The present work describes the isolation and characterization of a nucleic acid which is present as a ribonucleoprotein in C-serum and bottom fraction.

EXPERIMENTAL

The collection, centrifugal separation and freeze-drying of all the latex fractions used in the present work were carried out by the staff of the Rubber Research Institute of Malaya. The source of supply was a group of twenty mature mixed clonal seedlings growing at the Experiment Station of the Institute. These trees were tapped on a half-spiral, alternate daily system (cf. Riches & Gooding, 1952) and the latex was collected in aluminium cups at ambient temperature for 30–60 min. The latex was sieved on reception at the laboratory, to remove any particles of bark, and twenty ultracentrifuge tubes were filled with it. Ten of these were centrifuged as soon as possible in Rotor no. 21 of a Spinco Model L ultracentrifuge. A speed of 20 000 rev./min. ($g_{max.}$ 53 620) was maintained for 40 min.; acceleration and deceleration time together totalled a further 40 min. The refrigeration system of the centrifuge was operated during the run but the latex and rotor were not pre-chilled. The second batch of ten tubes was kept in the refrigerator and centrifuged in a similar manner after the first batch. In this second run the temperature of both latex and rotor was somewhat lower than in the first.

C-serum was collected from the tubes by piercing and draining. It was pooled, filtered and freeze-dried. The bottom fraction was removed after cutting up each tube and lightly washing the surface of the layer with water. Pooled bottom fractions were slurried with water and the slurry was freeze-dried. The freeze-dried materials were sealed under high vacuum and despatched to this laboratory, where they were stored at −25° until required.

METHODS

Elementary analysis. Carbon, hydrogen and ash determinations were made by a Pregl-type microcombustion and total nitrogen was determined by the micro-Kjeldahl method with a mercury catalyst. Phosphorus was measured colorimetrically with ammonium molybdate after wet digestion with sulphuric acid and nitric acid and with Metol (*p*-methylaminophenol sulphate) as reducing agent. The sulphur content was obtained by turbidimetric estimation as barium sulphate, after wet digestion in nitric acid–perchloric acid mixture. The use of this method on a micro scale was examined by Dr W. T. Chambers and a reproducibility of $\pm 5\,\mu g.$ over the range 10–150 $\mu g.$ was obtained. Standards used for calibration were mixtures of pure adenylic acid and insulin, of suitable phosphorus/sulphur ratio.

Hydrolysis. Nucleic acid hydrolyses were carried out in N-HCl at 100° for 1 hr. and in 0·3 N-NaOH at 37° for 16 hr. Samples of approximately 5 mg. of solid were sealed in Pyrex tubes of 0·75 ml. capacity and hydrolysed in 0·2 ml. of acid or in 0·5 ml. of alkali.

To hydrolyse the protein component a sample of about 2 mg. of nucleoprotein was sealed under N_2 in a 0·7 ml. thick-walled tube with 0·4 ml. of a 50:50 (v/v) mixture of 57% HI and 95% formic acid. This was heated at 90–100° for 22 hr.

Chromatography. The nucleic acid constituents were chromatographed according to Wyatt (1951) and the amino acid hydrolysates with both butan-2-one–propionic acid–water (15:5:6, by vol.) (see Clayton & Strong, 1954) and phenol–0·1 N-$NaHCO_3$–water (72:19:9, by vol.) in two-dimensional chromatograms with 2:6-lutidine–propan-2-ol–water–diphenylamine (50:20:25:0·66, by vol.) as the second solvent.

An atmosphere of NH_3 and coal gas, in the presence of KCN solution, was maintained in the tank throughout the phenol runs (see Block, Le Strange & Zweig, 1952).

In all cases hydrolysate solutions were applied and measured with an Agla micrometer syringe (Burroughs Wellcome and Co., London).

Ionophoresis. No. 1 filter paper was used in an apparatus similar to that described by Markham (1955). Sodium citrate or sodium formate buffers (0·3 M) at pH 3·3 were employed.

Detection and estimation of hydrolysis products. Chromatograms or electrophoresis diagrams of the hydrolysis

products were photographed according to Markham & Smith (1949). The resulting prints enabled the components absorbing in the 260 mμ region to be located, and these were then eluted into 0·1 N-HCl and quantitatively estimated in a Hilger spectrophotometer, by using the extinction data of Markham & Smith (1951), which was correct for a 5% loss of pyrimidine components. Alternatively, the location and semiquantitative estimation of the purine and pyrimidine components could be made directly and automatically by passage of the filter-paper strips through a short-wave ultraviolet scanning photometer (A. I. McMullen, unpublished work). The nucleic acid content of the original sample was calculated from the total weight of the individual components.

Isolation of ribonucleoprotein. To remove low-molecular-weight nucleotides, sugars etc., the latex freeze-dried C-serum was homogenized in an Ato-mix apparatus (M.S.E. Ltd., London) with HCl–sodium dihydrogen citrate buffer at 0° and pH 3·0 (approximately 20 g./100 ml.). After 3 min. the mixture was centrifuged at 6000 *g* for 15 min. at the same temperature and the insoluble residue extracted twice more with the cold buffer. No nucleic acid was removed at this stage. The residue was then dropped into either (*a*) 0·5 M- or 2 M-NaCl at 5° or (*b*) 2 M-NaCl solution at 95–100°, stirred and maintained at this temperature and kept at pH 6–8 by the addition of NaOH, for 30 min. The insoluble material was separated by centrifuging at 13 000 *g* for 20 min. and re-extracted thrice more with the NaCl solution. The combined extracts were filtered to remove a small quantity of aggregated rubber and brought to 66% (v/v) ethanol content. The ribonucleoprotein (RNP), together with some free protein, was precipitated on standing for a short time at 5° and was centrifuged down. This impure product was redissolved and precipitated with ethanol as before. Repeated precipitation with 0·1 N-HCl at pH 3·5 gave a material containing nucleic acid associated with protein. Attempts to remove this protein by denaturation with octanol, chloroform and water (Sevag, Lackman & Smolens, 1938) were unsuccessful, as much of the ribonucleic acid (RNA) was carried down with the denatured protein.

Removal of further protein was achieved by extracting the preparation with a 50:50 (w/w) mixture of A.R. phenol and water at pH 7·0, after the manner of Kirby (1956). By this means a product containing 62% of nucleic acid could be prepared. However, by repeated precipitation of the impure RNP with cetyltrimethylammonium bromide (CTAB), products were finally obtained containing in method (*a*) 60–65%, and in method (*b*) 67–72%, of nucleic acid (as determined by the hydrolytic procedure described above). These latter products were homogeneous in the ultracentrifuge.

The use of CTAB as precipitant was adopted after trials with several cationic compounds. For example, an aqueous solution of 2-methyl-5-vinylpyridine polymer formed such a stable complex with the RNP that it was impossible to separate the two by precipitation methods. This complex was insoluble above pH 5·7 and the nucleic acid could not be precipitated below this pH. The use of CTAB had the further advantage of not absorbing appreciably in the short-wave ultraviolet region.

In carrying out the purification procedure with CTAB about 10 mg. of the impure nucleoprotein was dissolved in 20 ml. of water, dilute NaOH being used to maintain the solution at or near pH 7·0. After stirring for 1 hr. at 5° the insoluble residue was rejected. A 2% CTAB solution (3–5 ml.) was then added with stirring and the colloidal precipitate first formed was converted into a floc by excess of CTAB. When this occurred the complex was centrifuged off, resuspended in ice-cold water and immediately recentrifuged for 10–15 sec. at 2000 *g*. (Some nucleic acid may be lost as a colloidal dispersion if washing is prolonged.) The complex was next dissociated by dissolving in M-NaCl at approximately pH 7·0 and the clear solution, after filtration, adjusted to pH 3·0 with HCl. This caused precipitation of the ribonucleoprotein on standing for a few minutes at 5°. The process was repeated at least once, and until no material insoluble in the salt solution remained. Inorganic salts and residual CTAB were finally removed by precipitation with HCl solution at pH 3·5, after which the RNP was washed with 66% aqueous ethanol and then with absolute ethanol, and dried *in vacuo*.

Sedimentation. The ultracentrifuge runs were carried out on a Spinco machine by Mr B. Boon of the Department of Biochemistry, Cambridge. The runs were performed at a maximum *g* of 270 000 and an average temperature of 20–21°. The solvent was 0·2 M-NaCl in all cases, and with the nucleoprotein of the serum solids measurements were made at both pH 7·0 and pH 4·0.

Osmotic molecular weight. This was determined at 0° and at pH 4·0 in the presence of 0·2 M-NaCl, with the improved toluene osmometer of Adair (Alexander & Johnson, 1949). Membranes suited to the molecular weight were prepared from collodion solution, the volume of the 'thimble' being approximately 0·7 ml. To reduce loss of solute from this small volume by diffusion into the main osmometer buffer, a glass ball was inserted in the capillary tube just above the membrane. Suitable temperature control at 0° was obtained by placing the osmometer in a vacuum flask containing ice, ensuring that the outer buffer level was appreciably below the level of the ice, and installing the whole in a laboratory refrigerator.

RESULTS

Ultraviolet absorption and fluorescent emission photographs of descending chromatogram patterns of the purified ribonucleoprotein, after acid hydrolysis, showed the presence of four components which were identified from their R_F values as guanine, adenine, cytidylic acid and uridylic acid. Confirmation of the presence of these components was obtained by paper electrophoresis. Corresponding data obtained after alkaline hydrolysis of the nucleoprotein showed, as expected, the presence of the four mononucleotides—guanylic acid, adenylic acid, cytidylic acid and uridylic acid. Further confirmation of the identity of the hydrolysis products was obtained by elution of the spots from the chromatograms and electrophoresis patterns, followed by determination of the spectra over the range 220–300 mμ at pH 1·0 and pH 7·0 or 11·0. Alterations in $\lambda_{max.}$ and in $E_{max.}/E_{min.}$ similar to those expected (Beaven, Holiday & Johnson, 1955) were obtained. In some of the hydrolysates the purine components were also precipitated as silver

Table 1. *Base composition of* Hevea *ribonucleic acid*

Samples 2, 4 and 5 were finally purified by the CTAB procedure and sample 3 by the two-phase phenol treatment.

Sample	Temp. of extraction	Nucleic acid content (%)	Base molar ratio (adenine = 1·0)			Purine / Pyrimidine	6-Oxo / 6-Amino
			Guanine	Cytosine	Uracil		
1	5°	43·9	1·43	1·13	0·95	1·17	1·12
2	5°	59·6	1·50	1·32	1·00	1·08	1·08
3	100°	62·1	1·40	1·21	1·00	1·09	1·09
4	100°	72·1	1·41	1·36	0·80	1·12	0·93
5	100°	71·1	1·51	1·38	0·91	1·10	1·02

salts at pH 1·0, redissolved in boiling HCl solution and determined spectroscopically as above.

Table 1 gives the purine and pyrimidine base composition of typical preparations obtained by different purification procedures. Sample 1 was purified by precipitation with aqueous ethanol and with the addition of 0·1 N-HCl to pH 3·5. Samples 2, 4 and 5 were also subjected to precipitation with CTAB, and sample 3 to phenol treatment similar to that of Kirby (1956), without purification by CTAB. The starting material for samples 1–4 was freeze-dried C-serum, and for sample 5 was bottom-fraction solids. The base composition of the products obtained by hot extraction of the starting materials is very similar to that of the products obtained by extraction at 5°, although the percentage of nucleic acid in the latter samples is lower. A further difference, not shown in Table 1, is that not all the nucleic acid was extracted from the original materials by sodium chloride solution at 5°, even when the concentration of sodium chloride was increased to approximately 2 M.

Hydrolysis of the above samples by the mixture of concentrated HI and formic acid, followed by two-dimensional paper chromatographic analysis, enabled the following amino acids to be identified from their R_F values: aspartic acid, glutamic acid, glycine, serine, cystine, threonine, alanine, lysine, histidine, tyrosine, tryptophan, leucine/isoleucine and phenylalanine. The presence of the aromatic amino acids was confirmed by their ultraviolet absorption. The sulphur content of the preparations containing about 70 % of nucleic acid was 0·3 % and the N/P ratio approximately 5·0.

That the polynucleotide is not deoxyribonucleic acid is apparent from its degradation by alkali, and this is confirmed by a negative diphenylamine reaction. The presence of a pentose sugar in the purified nucleoprotein was confirmed by a positive orcinol test.

The average polynucleotide content of the freeze-dried C-serum was approximately 0·1 %, representing 0·007 % of the original normal latex centrifuged serum.

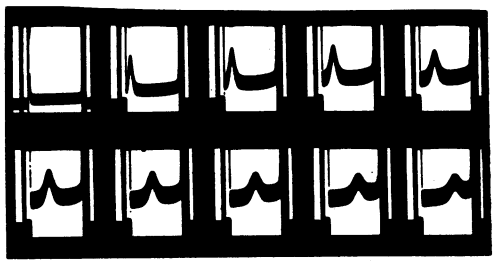

Fig. 1. Sedimentation pattern of *Hevea* ribonucleoprotein in 0·2 M-NaCl at 20° and pH 4·0. (Exposures at 14 min. intervals.) $g_{max.}$, 270 000; rev./min., 59 780.

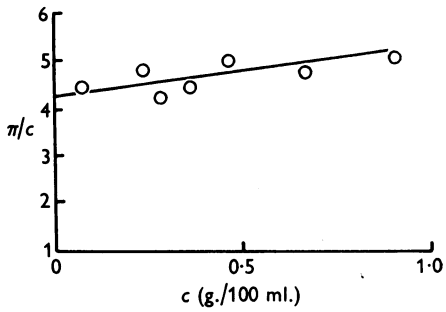

Fig. 2. Osmotic pressure of *Hevea* ribonucleoprotein in 0·2 M-NaCl at 0° and pH 4·0 (mol.wt. 39 400). Ordinate: the 'reduced pressure'.

Incubation of the pure nucleoprotein with 8 M-urea at 37° for several days caused no change in its composition (see Elson, 1959).

Centrifuging of the C-serum solution for 8 hr. at 0° and 25 000 *g* gave a sediment containing about 2 % of the total polynucleotide content.

Molecular weight. Sedimentation data for the purified nucleoprotein (Fig. 1) showed the presence of a single component. Sedimentation coefficients (S) for different preparations varied only from 3·55 to 3·82 s over the pH range 4·0–7·0. Determinations made on a single purified product from freeze-dried bottom fraction gave a similar value of S (3·97).

No diffusion or molar-density data are yet available for this nucleoprotein, but the average value of the sedimentation coefficients at pH 7·0 can be used to obtain a rough estimate of the molecular weight by employing a diffusion coefficient of $D_{20} = 6{\cdot}5 \times 10^{-7}$ and a specific molar volume of $V_\rho = 0{\cdot}58$. These values were obtained for yeast nucleic acid by Watanabe & Iso (1950) and give a molecular weight of 34 700 in the present instance. This result is almost certainly appreciably lower than the correct value, since the *Hevea* nucleoprotein, containing 30 % of protein, must have a smaller diffusion coefficient and a larger specific volume. Osmotic determinations of mol.wt. (Fig. 2) gave a value of 39 400.

DISCUSSION

Although the analytical results given above provide strong evidence that at least a part of the nucleic acid in *Hevea brasiliensis* latex is present as a soluble ribonucleoprotein, it is difficult to assess the extent to which the different isolation and purification procedures degrade or otherwise affect the native nucleoprotein.

Table 1 shows that the base composition of the purified products is not greatly altered by the method of isolation. The guanine/adenine ratio is considerably higher than in yeast RNA (Magasanik, 1955) or some plant-leaf RNA (Thomas & Sherratt, 1956; Lengyel & Ochoa, 1958) and plant-virus RNA (Dorner & Knight, 1953). It is similar, however, to the ratio found by Cooper & Loring (1957) in plant chloroplasts, to that found by Eggman, Singer & Wildman (1953) in tobacco-leaf cytoplasm, and to that in the cytoplasm of some rat cells (Magasanik, 1955). The cytosine/uracil ratios of Table 1 are greater than unity, in contrast with a ratio of less than unity for yeast RNA.

Since the nucleic acid content of the *Hevea* RNA preparation of molecular weight 39 400 was 67 %, it follows that the polynucleotide of the nucleoprotein has a maximum molecular weight of approximately 26 400, representing about 82 nucleotide units. Similarly, the molecular weight of the amino acid component (if protein) cannot exceed 13 000.

Clearly, also, the protein component is not a ribonuclease similar to that described by Elson (1959), since no hydrolysis of polynucleotide occurs on incubation with urea.

An interesting feature of the present results is the relatively high percentage of nucleic acid in the samples purified by the CTAB procedure. Even so, these samples contained 30 % by weight of non-nucleotide component, which is evidently very firmly bound to the polynucleotide to give a product showing only one peak in the ultracentrifuge. It has not yet been possible to determine quantitatively the amino acid content of the hydrolysed products, but, assuming that all the non-nucleotide portion of the purified samples is composed of amino acid residues with an average molecular weight of 118, the ratio of mononucleotide to amino acid residues in these samples can be calculated readily from the nucleotide base compositions in Table 1. This ratio is approximately 1·0 for the preparations with the highest nucleic acid content, suggesting the possibility that removal of protein by methods which do not affect the polynucleotide composition results in a 'core' of adhering amino acid residues where each residue is associated with one nucleotide. The mechanism of this adherence has not been investigated in this work, but a theory such as that postulated by Dounce (1952) for amino acid and polynucleotide interaction, or the possibility of polypeptide–polynucleotide combination by a similar mechanism where only the basic residues react (probably enhanced in this case by van der Waals interaction), could be put forward. Carboxyl groups in the former instance or surplus phosphate and carboxyl groups in the latter instance would still be capable of reacting with CTAB to give the insoluble complex described above.

SUMMARY

1. The isolation and characterization of a purified ribonucleoprotein component of *Hevea* rubber latex is described.

2. A method of final purification employing a cationic detergent has been developed, which gives a product of approximately 70 % nucleic acid content.

3. The composition of the polynucleotide portion of the purified product is similar to that of several other plant nucleic acids so far reported in cytoplasm.

4. The molecular weight of the product is approximately 40 000; the protein part of the molecule has a maximum molecular weight of 13 000.

5. The ratio of the number of amino acid residues to the number of nucleotide residues in the purified nucleoprotein is discussed.

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A Study of Protein-Binding in the Metabolism of Vitamin B_{12}

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The mechanism by which intrinsic factor from normal human gastric juice promotes the absorption of cyanocobalamin (vitamin B_{12}) in patients with pernicious anaemia is still obscure. In particular there is no agreement about the part played by that substance in the juice which combines with cyanocobalamin and makes it non-diffusible. Substances that combine with cyanocobalamin have been reported in other body fluids and tissues. Rosenthal & Sarett (1952) and Wolff, Karlin & Royer (1952) have shown that human serum contains its vitamin B_{12} in bound form and that the serum will further bind limited amounts of added cyanocobalamin. With an ultrafiltration technique we showed that milk of several different species contains protein-bound vitamin B_{12} and we measured the quantity of additional vitamin that the milks would 'bind' (Gregory & Holdsworth, 1955*a*).

Confusion has arisen because different authors use the term 'free vitamin B_{12}' to mean quite different entities (cf. Heathcote & Mooney, 1958). It seems to us that it can be resolved only if authors would restrict the term to vitamin B_{12} itself, i.e. either cyano- or hydroxo-cobalamin, and to use the term 'available vitamin B_{12}' when implying that the test material can provide a substance that will support the growth of an assay organism.

Even then it is necessary to define carefully the conditions of test since we have shown that the available vitamin B_{12} measured depends on the concentration of the test substance and on the organism and method of assay (Gregory & Holdsworth, 1957). Unfortunately there is no completely satisfactory general method for the measurement of 'free vitamin B_{12}'. Gregory & Holdsworth (1957) concluded that the ultrafiltration method was the most reliable technique for measuring the binding capacity of a substance for additional cyanocobalamin. But it must be remembered that this technique will differentiate only those bound forms too large to pass through the membrane. Certain peptide conjugates of the vitamin will pass through Visking tubing although they are obviously bound forms of vitamin B_{12}. Butan-1-ol extracts free vitamin B_{12} from test material saturated with ammonium sulphate (Ellis, Petrow & Snook, 1949) and also certain small peptide conjugates.

In the experiments reported in this paper we have used the ultrafiltration method to determine whether tissues contain any ultrafiltrable vitamin

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