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The Proteins of *Hevea brasiliensis* Latex

4. ISOLATION AND CHARACTERIZATION OF CRYSTALLINE HEVEIN*

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Fresh rubber latex obtained from *Hevea brasiliensis* consists of a suspension of several types of particles in an aqueous serum, and can be separated into fractions by high-speed centrifuging (Cook & Sekhar, 1953). The top layer in the centrifuge tube consists mainly of rubber particles, below which is a layer of almost clear serum. At the bottom of the tube is a layer referred to in this paper as the 'bottom fraction', which contains sedimentable bodies including the lutoid particles described by Homans & van Gils (1948).

In preliminary work an aqueous extract of the bottom fraction was found to contain several electrophoretically distinct protein components. This observation has been confirmed and elaborated by Moir & Tata (1960). This paper describes the isolation and characterization of a major component which is anionic at pH 8·6 and for which the name hevein is proposed.

EXPERIMENTAL

Materials. Freeze-dried samples of the bottom fraction of freshly tapped latex were used throughout. Collection of the latex and centrifugal separation and freeze-drying of the bottom fraction were carried out by the staff of the Rubber Research Institute of Malaya, as described by McMullen (1959). All freeze-dried fractions were sealed under high vacuum in Malaya, and on arrival at our Laboratory were stored at $-15°$ until required.

Elementary analyses. Total N was determined by the micro-Kjeldahl method and C, H and ash by Pregl microcombustion. The S content of the ash and gaseous combustion products was measured turbidimetrically as $BaSO_4$ after complete dissolution of the ash in dilute HCl. Where the amount of material available for analysis was limited, and the ash content was known to be low (as in purified hevein), C, H and S were determined on the same sample with a microcombustion tube containing a roll of silver and PbO_2 only. P was determined colorimetrically with ammonium molybdate after wet oxidation.

Soluble proteins were extracted from the freeze-dried solids with 0·05 M-phosphate buffer, pH 6·8, or with 0·05 M-$Na_2B_4O_7$, pH 9·3. The protein-N content of the extract was then determined by tannic acid precipitation and Kjeldahl analysis (Archer & Sekhar, 1955). Purified protein samples were dried to constant weight *in vacuo* at 80° before analysis.

Paper electrophoresis. For determining the number of electrophoretically distinct proteins in a solution, the method of Kunkel & Tiselius (1951) as modified by Archer & Sekhar (1955) was used. The isoelectric point of hevein was also determined with a paper-strip-electrophoresis technique. This method was chosen because sufficient purified material was not available for the classical Tiselius method in free solution. The buffers used for determining the isoelectric point contained 0·02 M-sodium acetate, together with sufficient acetic acid to give the required values over the range pH 4·38–5·39. All pH values were determined at 20° with a Cambridge pH meter and glass electrode. Current was applied to the standard paper-electrophoresis apparatus by means of platinum electrodes immersed in beakers of electrolyte connected via agar bridges to the buffer compartments of the apparatus. This modification was necessary to prevent pH changes in the buffer solution reaching the paper strips during the long periods of electrophoresis (about 48 hr.), needed because of the low mobility of the protein near the isoelectric point. The voltage across the strip was measured with platinized electrodes immersed in the buffer close to the strip, and connected to a recording voltmeter, thereby enabling the average voltage during an experiment to be determined. Approximately 700 v was applied across the current electrodes, resulting in a potential gradient in the paper itself of 8·5 v/cm. The electrophoretic movement of the protein in a given time was taken as the difference between the distances moved by the protein and a dextran spot which was applied to a second strip placed alongside that containing the protein. All determinations were carried out in duplicate. The pH of zero mobility was obtained by graphical interpolation of the results obtained at five values in the range pH 4·38–5·38.

Isolation of hevein. Approx. 40 g. of freeze-dried bottom fraction was mixed with 360 ml. of O_2-free water, 0·2 g. of sodium dithionite (to deactivate the polyphenol-oxidase enzymes, which rapidly turn the solutions black), 0·05 ml. of octan-1-ol (to reduce frothing) and a few small pieces of solid CO_2 in a high-speed blender (MSE Ato-Mix) at 0° for 1 min. The mixture was then centrifuged below 5° and the residue re-extracted twice more with 80 ml. of cold water as described above. A.R. $(NH_4)_2SO_4$ was then added to the combined extracts in the proportions of 43 g./100 ml. of liquid, with constant stirring. The pH was adjusted to 6·0 and the mixture stored at 0° for 16 hr. The precipitate was rejected after centrifuging and washing it with 65% saturated $(NH_4)_2SO_4$ solution, and the hevein was precipitated from the combined supernatants by saturating with $(NH_4)_2SO_4$. This precipitate was washed with saturated $(NH_4)_2SO_4$ solution and mixed with water until dissolution was almost complete. An equal volume of 65% saturated

* Part 3: Moir & Tata (1960).

$(NH_4)_2SO_4$ was then added and, after standing for 2 hr., any insoluble material was centrifuged off and rejected. The supernatant was again saturated with $(NH_4)_2SO_4$, the precipitated hevein redissolved in 65% saturated $(NH_4)_2SO_4$ and reprecipitated as before. The final precipitate was then dissolved in water and desalted. This could not be achieved satisfactorily by our usual dialysis technique because of leakage of protein through the cellulose membranes, so electrodialysis was employed with membranes whose permeability to hevein was negligible.

Electrodialysis. An apparatus similar to that of Wood (1956) was constructed from Perspex. The 5 cm. diameter electrodes were of carbon and the anode and cathode membranes were Permaplex ion-exchange membranes (The Permutit Co. Ltd., London) A. 10 and C. 10 respectively. Soft water previously cooled to 0° was circulated past the electrodes at a combined rate of about 18 l./hr. The initial protein concentration was restricted to that equivalent to $E^{280\,m\mu}_{1\,mm.}$ 0·3 to reduce membrane blockage, which occurred at higher concentrations. The d.c. voltage applied to the cell from a full-wave rectifier unit (unsmoothed) was initially 60 v and rose to 150 v as the salt was removed. The initial current was 300 mA, which decreased to 7 mA at the end of the electrodialysis.

Crystallization. After electrodialysis, the protein solution was freeze-dried, and the resulting solid dissolved in the minimum volume of water at 40°. The solution was then centrifuged and allowed to crystallize at 0° overnight. The hevein was centrifuged off and recrystallized twice more. The crystals were finally dried *in vacuo* over P_2O_5.

Paper chromatography. The amino acids present in hevein hydrolysates were identified by two-dimensional paper chromatography on Whatman no. 1 paper with phenol–$NaHCO_3$, followed by lutidine–propan-2-ol–diethylamine–water as solvents (see below).

For quantitative determination of the amino acids, a simple one-dimensional descending technique with a variety of solvents was used, similar to that of Roland & Gross (1954) as modified by Drake, Giffee, Johnson & Koenig (1957). Approx. 10 mg. of crystalline hevein (dried to constant weight at 70° *in vacuo*) was accurately weighed and hydrolysed in a sealed tube *in vacuo* with 1 ml. of 90% formic acid and 1 ml. of 17% HCl for 24 hr. at 100°. The hydrolysate was then transferred to a small distillation apparatus and evaporated to dryness under reduced pressure in a stream of N_2. The residue was dissolved in a few drops of water and transferred to a small round-bottomed flask of calibrated volume (0·591 ml.), sealed to the end of a B. 14 standard ground-glass socket. This enabled a volume of up to 2 ml. of water to be used for washing the distillation flask, excess of water in the calibrated flask being removed in a stream of N_2. Finally 0·1 ml. of propan-2-ol was added and the solution diluted with water to the standard volume. This microtechnique was necessitated by the small amounts of hevein available. A micropipette delivering 0·005 ml. was used to put the spots of hydrolysate on the paper and the same pipette was used for the spots of standard amino acids prepared in 10% propan-2-ol solution by direct weighing. The standard amino acid solutions were 20 mM except for tyrosine and glutamic acid, which were 10 mM. Dilutions were prepared at 16 mM, 10 mM, 6 mM and 4 mM. Four sheets of Whatman no. 1 paper, 36 cm. × 54 cm., carried a total of nine spots of hydrolysate and nine spots of each of the standards. After solvent development the sheets were air-dried for 24 hr. and then immersed in 0·25% of ninhydrin in acetone containing 1% of acetic acid. For the determination of proline 0·2% of isatin in acetone was used instead of ninhydrin. The sheets were left in a dark box at 50% relative humidity at room temperature overnight, and the maximum densities of the spots were determined with an EEL scanner converted for use with a 3 mm. diameter aperture instead of the standard slit. Amino acid concentrations were determined (probable accuracy ±5–10%) from curves of maximum scanner-galvanometer readings against standard amino acid concentrations. The solvents used for development were:

A. Phenol–$NaHCO_3$ (Drake *et al.* 1957) in an atmosphere of coal gas, with solutions of NH_3 (3%) and KCN (10%) in beakers in the tanks. Aspartic acid, glutamic acid, serine, glycine, threonine, alanine, tyrosine, histidine and arginine were determined by this method.

B. Butan-1-ol–acetic acid–water (25:6:25, by vol.). The upper phase was used for development and the lower phase for equilibration in the tank. Proline and lysine were estimated with this system.

C. *iso*Amyl alcohol–pyridine–water–diethylamine (100:100:70:4, by vol.) with beakers containing 1% KCN soln. and an aqueous solution of phenol in the tank. This method was used for leucine and to prove the absence of isoleucine.

D. Butan-2-ol–3% NH_3(aq.) soln. (3:1, v/v) was used for determination of valine.

Determination of thiol groups. The amperometric-titration method originally developed by Kolthoff & Harris (1946), as modified by Benesch & Benesch (1948) and Weiseman, Schoenbach & Armistead (1950), was used.

Spectrographic analysis. The spectrum of hevein was studied in neutral, acid and alkaline media in the range from 215 to 350 mμ with a Hilger Uvispek spectrophotometer. The tyrosine and tryptophan contents of the unhydrolysed protein were calculated from the spectral curves in acid and alkaline media as described by Beaven & Holiday (1952), Goodwin & Morton (1946) and Bencze & Schmid (1957).

Ultracentrifuge analysis. Sedimentation measurements were carried out at 21° on a 0·7% solution of hevein in 0·2M-NaCl at pH 6·0 in a Spinco model E ultracentrifuge, with an artificial boundary cell, at 59 780 rev./min.

Osmometry. The apparatus used was similar to that of Adair (Alexander & Johnson, 1949). To prevent diffusion of hevein through the nitrocellulose membranes the latter were treated with an 18% glycerol solution in water (Adair, 1955) to reduce their specific permeability. The most suitable value for the latter was found to be $1–4 \times 10^6$ c.g.s. units. Equilibrium osmotic pressures were determined at 0° in 0·2M-NaCl at pH 5·0 over a range of concentrations with recrystallized hevein. The concentration of the solution inside the membrane was determined by u.v. absorption after the osmotic pressure had been measured.

RESULTS

The paper-electrophoresis pattern (Fig. 1) and the sedimentation diagram (Fig. 2) of crystalline hevein give no indication that more than one protein component is present. The elementary

analysis of hevein (Found: C, 46·0; H, 6·0; N, 15·7; S, 5·0; $P < 0·006$; ash, 0·2%) is notable for the high sulphur content.

Reactions of the protein to the Molisch, Benedict and Fehling tests were all negative, indicating the absence of carbohydrate.

Amino acid analysis. The 15 amino acids detected in hevein hydrolysate by paper chromatography are listed in Table 1. No evidence was found for the presence of isoleucine, norleucine, norvaline, asparagine, ornithine, phenylalanine, cysteine, methionine, dihydroxyphenylalanine or hydroxyproline, and they are assumed to be absent.

The thiol-titration method was shown to give consistent results with '*tert.*-dodecylmercaptan' on samples containing as little as 50 μg. of S. With bovine-serum albumin the method gave 0·65 mole of SH/mole of albumin (uncorrected for water content of the protein) as compared with a value of approx. 0·67 obtained by Hughes (1947). When 6 mg. of hevein, containing 330 μg. of total S, was titrated a zero end-point was obtained. A similar titration with standard mercuric chloride solution instead of silver nitrate gave the same result. This confirms the absence of cysteine, and since methionine is also absent the whole of the S in hevein has been attributed to cystine.

All the figures in Table 1 were obtained from the paper-chromatographic maximum-density results with the exception of cystine, tryptophan and tyrosine. The last two were calculated from the spectral curves (see below).

Ultraviolet-absorption spectra. Fig. 3 shows the spectra of hevein in 0·05 N-sodium hydroxide and in 0·05 N-hydrochloric acid. The curve in pure water is indistinguishable from that in the acid medium. The absence of an inflexion at 257·5 mμ in aqueous or acid solutions corroborates the chromatographic evidence that phenylanine is absent ($E^{1\%}_{1\,cm.}$ 25·6 in aqueous solution for the main peak at 280 mμ). The percentages of tryptophan in the hevein molecule, calculated from the spectra by the three methods referred to in the Experimental section, were 9·6, 9·0 and 8·6 respectively; the corresponding values for tyrosine were 4·0, 4·3 and 4·0.

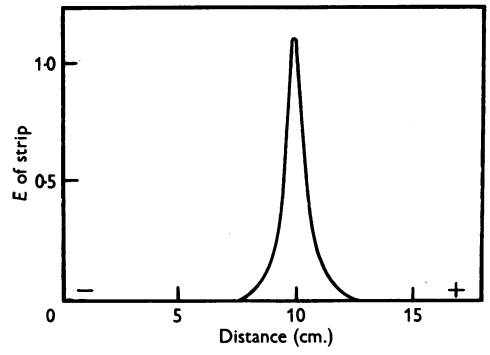

Fig. 1. Paper electrophoresis of hevein. Electrophoresis was carried out for 16 hr. at pH 8·6 in 0·05 M-veronal buffer.

Table 1. *Amino acid analysis of hevein*

	Percentage (w/w) of residues	Relative proportions of amino acid residues
Alanine	1·1	1·6
Arginine	5·7	3·7
Aspartic acid	11·6	10·1
Cystine	19·2	8·0
Glutamic acid	9·9	7·8
Glycine	4·4	7·8
Histidine	3·8	2·8
Leucine	4·7	4·2
Lysine	5·4	4·2
Proline	4·1	4·2
Serine	8·7	10·0
Threonine	1·8	1·8
Tryptophan	9·1	4·6
Tyrosine	4·1	3·8
Valine	1·3	1·3
	94·9	

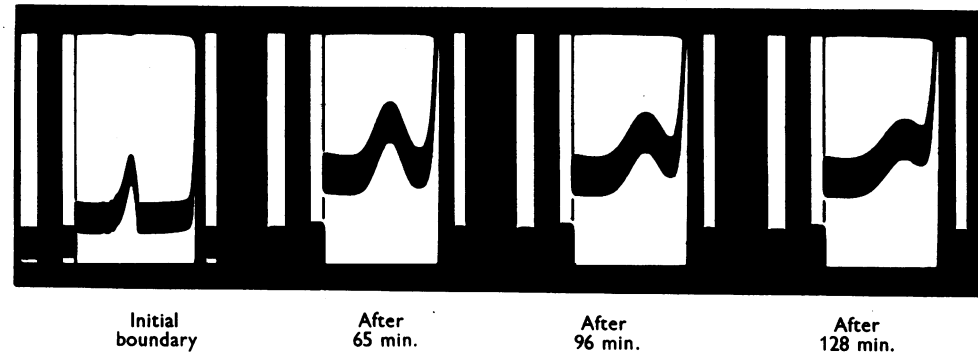

Fig. 2. Ultracentrifugal analysis of hevein at 59 780 rev./min. with a 0·7% solution of the protein in 0·2 N-NaCl at pH 6·0. S 1·19 × 10^{-13}.

Osmotic pressure. Equilibrium was reached in the osmometer in about 12 days, and by extrapolating a plot of π/c against c to zero concentration, a value of the apparent molecular weight in 0·2 M-sodium chloride at pH 5·0 of 10 500 was obtained.

Isoelectric point. The pH at which hevein had the same mobility as dextran in 0·02 M-sodium acetate buffer, in paper-electrophoresis experiments, was 4·7.

Solubility. The solubility of pure hevein in water at 0° is less than 1 %, but the solubility increases rapidly in the presence of neutral salts. The protein crystallizes readily from salt-free cold water in the form of hexagonal needles (Fig. 4), which show birefringence. It is appreciably soluble in ammonium sulphate solutions which are below saturation and its solutions are not coagulated by heat, although its solubility in strong ammonium sulphate solutions is lowered by this treatment.

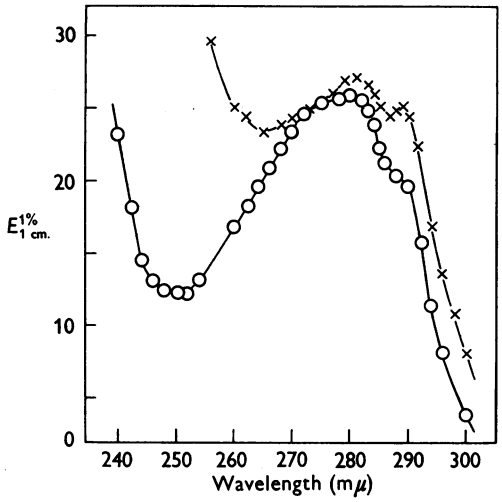

Fig. 3. Ultraviolet-absorption spectra of hevein: ○, in 0·05 N-HCl; ×, in 0·05 N-NaOH.

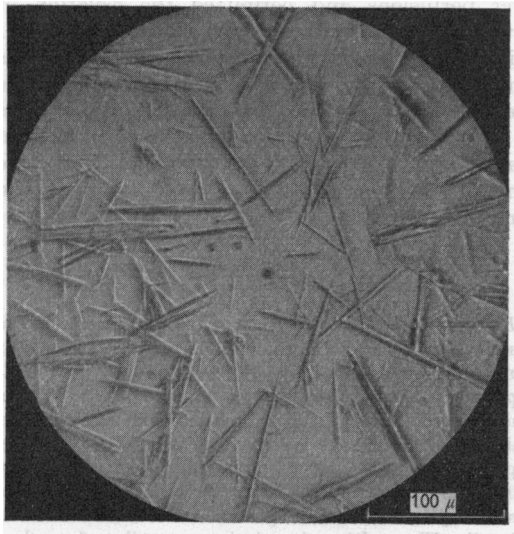

Fig. 4. Photomicrograph of hevein crystals.

DISCUSSION

Assuming that the nearest integers to the numbers in the third column of Table 1 represent the numbers of amino acid residues in the hevein molecule, a minimum molecular weight of 9512 is obtained, which may be compared with the values of 10 500 calculated from the osmotic data.

In respect of its high sulphur content and low molecular weight, hevein resembles the trypsin-inhibiting protein from lima beans reported by Fraenkel-Conrat, Bean, Ducay & Olcott (1952).

The electrophoretic and solubility properties of hevein suggest that this protein may be identical with a protein component (no. 4) identified by Archer & Sekhar (1955) in the aqueous serum of *Hevea brasiliensis* latex, and work is in progress to elucidate this point. It also appears that hevein is largely responsible for the band designated component (viii) by Moir & Tata (1960).

SUMMARY

1. A crystalline protein has been isolated from fresh rubber latex (*Hevea brasiliensis*), for which the name hevein is proposed.

2. This protein contains 5 % of sulphur, has a molecular weight of approximately 10 000, a sedimentation constant of 1·19 S at a concentration of 0·7 %, and $E^{1\%}_{1\,cm.}$ at 280 mμ 25·6.

3. Hevein contains 15 amino acids including cystine but no cysteine or methionine.

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Methods for Measuring Rates of Synthesis of Albumin by the Isolated Perfused Rat Liver

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Although the ability of the isolated perfused rat liver to synthesize plasma proteins was clearly demonstrated in 1951 by Miller, Bly, Watson & Bale, few such experiments have subsequently been reported. The potential use of this system for studies on protein synthesis was, however, recognized by Jensen & Tarver (1956). These authors added ^{14}C-labelled histidine and lysine to the blood used for perfusing a liver, and then measured the specific activity of the free amino acids in the plasma at regular intervals. With these data, and by measuring the corresponding amounts of the [^{14}C]amino acid which had become protein-bound, they were able to calculate a rate of protein synthesis.

However, their measurements of the specific activities of the free amino acids in the plasma, which were obtained by means of the bacterial decarboxylase method, may have been subject to considerable experimental error. For this reason it seemed worth while to repeat some of their measurements with the aid of a more accurate estimation for the specific activity of the free amino acids in the plasma.

The results thus obtained could be compared with the rate of albumin synthesis *in vivo* found by Campbell, Cuthbertson, Matthews & McFarlane (1956). Since it seemed possible that the perfused liver may on average synthesize albumin either faster or slower than it does *in vivo*, it seemed desirable also to measure albumin synthesis directly in the perfusion system. To achieve this, isolation of the newly formed albumin was necessary. Newly formed rat albumin cannot be separated from the rat albumin initially present in the system when the perfusion is conducted with rat blood. Thus the expedient of perfusion with a heterologous blood became necessary. Human blood from which most of the white cells and platelets had been removed proved satisfactory for liver perfusions and permitted the isolation of newly formed rat albumin by means of an appropriate antiserum. Although such a system suffers from certain disadvantages mentioned below, its use has permitted direct comparison between rates of synthesis of albumin calculated from changes in plasma-amino acid specific activity and from the amount of albumin obtained by isolation in the same experiment.

METHODS

Perfusion technique. Perfusions were carried out as described by Cohen & Gordon (1958), except for the use of human blood in certain cases. Carrier lysine was used in all experiments except rat-liver perfusion (RLP) 87 (for amounts see Table 1). When human blood was used the white cell and platelet layer, which separated from the heparinized blood during preliminary centrifuging at 1800 *g* for 20 min. at 20°, was removed as completely as possible by suction and the plasma was re-mixed with the red cells. Warm Ringer's solution was allowed to flow through the liver for 1–2 min. before its removal from the