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The Proteins of *Hevea brasiliensis* Latex

2. ISOLATION OF THE α -GLOBULIN OF FRESH LATEX SERUM

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In part 1 of this series (Archer & Sekhar, 1955) some properties of the aqueous serum obtained by freezing or centrifuging the fresh latex of *Hevea brasiliensis* were reported and the number of individual protein fractions identifiable by paper electrophoresis was determined. The present investigation is concerned with the isolation and properties of one of the two main constituents, namely component 5, which is shown to be the chief globulin component of fresh latex serum. To avoid confusion between the lettering and numbering systems of different workers, it is proposed that protein 5 be referred to as the α -globulin component of latex serum. It appears to correspond to component V in the nomenclature of Roe & Ewart (1942) and was almost certainly the main constituent of 'protein A' described by Bishop (1927), Kemp & Straitiff (1940) and Bondy & Freundlich (1938).

EXPERIMENTAL

Materials

Freeze-dried serum solids prepared by the Rubber Research Institute of Malaya were used throughout. The aqueous serum phase of fresh unammoniated latex was obtained by prolonged freezing or by centrifuging the latex, the serum being then freeze-dried, in the way already described (Archer & Sekhar, 1955). As before, *F* solids and *C* solids refer to the serum solids obtained from fresh serum prepared by the freezing and centrifuging techniques respectively.

Methods

Elementary analyses. N was determined by the micro-Kjeldahl method, and C, H and ash by Pregl micro-combustion. The S content of the ash and gaseous combustion products was measured turbidimetrically as BaSO₄, after complete dissolution of the ash in dil. HCl. P was determined colorimetrically with ammonium molybdate, after wet oxidation. All samples were dried to constant weight at room temp. *in vacuo*, before analysis.

Electrophoresis. Paper-electrophoresis measurements were carried out as described in Part 1 (Archer & Sekhar, 1955), 0.05 M sodium veronal buffer (pH 8.6) being used. Measurements in the Tiselius apparatus were made at 0° and current 5 mA. Solutions for electrophoresis were prepared in veronal buffer (pH 8.6, ionic strength 0.1), the solutions being dialysed against a large volume of the buffer for 24 hr. at 0° before use.

Fractionation of serum proteins. Two methods were examined:

(a) Following Kemp & Straitiff (1940), solutions of the *F* and *C* serum solids, in distilled water, were partially precipitated with different concentrations of ammonium sulphate at 0°. After 16 hr., the precipitates were centrifuged down and dialysed for 24 hr. against 0.05 M veronal buffer (pH 8.6) at 0°. The resulting solutions were analysed by paper electrophoresis.

(b) Total protein was first precipitated from a solution of *F* solids in water by saturation with ammonium sulphate, and the precipitate dialysed against 0.01 M trisodium citrate at 0° until free from sulphate. The resulting protein solution was clarified centrifugally and its N content determined. Portions of the solution were then diluted with sodium citrate buffers so as to cover the pH range 4.0–5.2

at ionic strengths varying from 0.02 to 0.25, or the pH range 2.1–3.6 at ionic strength 1.0 (due allowance was made for the trisodium citrate in the protein solution after dialysis). After centrifuging down the precipitates, the clear supernatants were analysed for N and their pH values checked by means of a glass electrode. All processing was carried out at 0°. The precipitates were dissolved in 0.1M veronal buffer (pH 8.6) and examined for homogeneity by paper electrophoresis.

Isolation of purified α -globulin. The procedure finally adopted for isolating the α -globulin of the latex-serum solids was based on method (b) above. Approx. 40 g. of *F* solids were dissolved in an equal weight of water, to give a practically clear solution. Saturated aqueous ammonium sulphate (A.R., 600 ml.) was then added and the mixture maintained at 0° for 1 hr. The precipitated protein was centrifuged off and washed by resuspending in fresh saturated ammonium sulphate. The recentrifuged protein was stirred to a cream with a little distilled water, and dialysed in cellophan bags against 0.01M trisodium citrate at 0° for 48 hr., with several changes of the latter solution, to remove most of the contaminating ammonium sulphate. The resulting solution was centrifugally clarified and diluted with 0.01M trisodium citrate to give protein N approx. 1.5 mg./ml. The solution was then mixed with 2 vol. of a mixture of equal volumes of 0.0032M trisodium citrate and 0.054M sodium dihydrogen citrate to give a final pH of 4.5. All solutions were cooled to 0° before use.

The precipitated α -globulin was centrifuged off immediately, stirred to a smooth cream with a little water and redissolved by the gradual addition of 0.1N-NaOH with constant stirring. The pH of the mixture was not allowed to rise above 7.0. Any protein remaining undissolved at this pH was rejected. Provided that the time during which the protein-buffer mixture was kept at pH 4.5 did not exceed 30 min., and the temperature was kept at approx. 0°, the amount of residue insoluble at pH 7.0 was very small. Washing the precipitated globulin with fresh pH 4.5 buffer tended to decrease the solubility of the product.

The solution of α -globulin was reprecipitated three times at pH 4.5 under the same conditions as those described above. The proportions of the two citrate solutions added were adjusted to allow for the amount of caustic soda used in dissolving the protein. The final solution was then freeze-dried or preferably used at once, since the solubility of a dry sample of the protein tended to fall on storage, even at 0°.

The serum solids obtained from fresh latex by centrifuging (*C* solids) usually still contained approx. 0.5% of rubber of very small particle size, which redispersed when the solids were dissolved in water. To prevent contamination of the α -globulin with this residual rubber, the *C* solids were first dialysed in a cellophan bag against 0.01M trisodium citrate solution at 0° for 72 hr. The resulting solution contained the rubber in a slightly flocculated state; most of it could be removed by centrifuging at 20 000 g. The last traces of rubber were removed by the addition of ammonium sulphate until the concentration was between 20 and 30% saturated (a small amount of protein was also precipitated). The protein solution was then clarified by centrifuging and the α -globulin isolated as described above, starting with the precipitation by saturated ammonium sulphate.

Results

Fractionation with ammonium sulphate. Fig. 1 shows the paper-electrophoresis diagrams for fractions i–iv of *F* serum solids obtained by successive precipitation with 25, 50, 70 and 100% saturated ammonium sulphate respectively. Fraction i appears to consist mainly of proteins 5 (α -globulin) and 3, in the nomenclature of Archer & Sekhar (1955), but the amount of this fraction is small. Fractions ii and iii contain most of the α -globulin, but are very heterogeneous. Fraction iv consists mainly of two components, one of which is protein 4, and contains little or no α -globulin. Similar results were obtained by fractionation of the *C* solids.

Fig. 1. Paper electrophoresis diagrams of protein fractions from *F* solids by precipitation with ammonium sulphate. Electrophoresis was carried out for 1000 min. in 0.05M sodium veronal buffer (pH 8.6); potential gradient 4.5v/cm. (i), 0–25%; (ii), 25–50%; (iii), 50–70%; (iv), 70–100% saturated ammonium sulphate. The arabic numerals refer to the components of Archer & Sekhar (1955).

Solubility of serum proteins in citrate buffer. The over-all solubility of *F* serum proteins in citrate buffer at 0°, as a function of pH and ionic strength (*I*), is shown in Fig. 2. The pH of minimum solubility is well-defined at *I* = 0.02 or 0.04, but less so at higher ionic strengths. Above

Fig. 2. The effect of pH and ionic strength on the percentage of *F* serum proteins precipitated from sodium citrate buffer at 0°.

Fig. 3. Paper-electrophoresis diagrams of α -globulin isolated from (a) *F* solids and (b) *C* solids. Electrophoresis was carried out for 1000 min. in 0.05M sodium veronal buffer (pH 8.6); potential gradient 4.5V/cm.

approximately pH 4.5, the solubility of the total protein increases with increasing ionic strength, while at lower pH values the reverse is true. The pH of minimum solubility falls from 4.7 at $I=0.02$, or 4.5 at $I=0.04$, to about 3.4 at $I=1.0$. Paper-electrophoretic measurements indicated that an increase in ionic strength gave precipitates of increasing heterogeneity. These results apply to solutions in which the initial protein concentration was low (approx. 0.2%). Further experiments at pH 4.5 showed that a gradual increase in the heterogeneity of the precipitates occurred if the initial protein-N content of the solutions exceeded 0.5 mg./ml. Use was made of these results in choosing conditions for the isolation of the α -globulin, as described in a previous section.

Fig. 4. Schlieren diagrams of α -globulin from (a) *F* solids and (b) *C* solids. Tiselius electrophoresis was carried out at 0° and 5 ma, in sodium veronal buffer (pH 8.6, I 0.1).

α -Globulin of latex serum. Typical paper-electrophoresis diagrams for the freshly prepared α -globulin of *F* and *C* serum solids are given in Fig. 3. Pronounced 'tailing' of the α -globulin spot occurs, when veronal buffer at pH 8.6 is used, owing to the ease with which the protein is adsorbed on filter paper. The globulin of the *F* solids is indistinguishable from that of *C* solids, and the adsorption prevents

ready detection of heterogeneity due to slower-moving components. However, using the Tiselius apparatus, the schlieren diagrams (Fig. 4) showed the presence of two minor components in both preparations, amounting to approx. 10% of the total. The faster-moving impurity is probably protein 6, detected previously by Archer & Sekhar (1955).

Elementary analysis. Analytical data for α -globulin (from *F* solids), after exhaustive dialysis against distilled water, are given in Table 1. Also included in the Table are analyses by other workers of the 'protein A', the main component of which is believed to be α -globulin.

Table 1. *Elementary analyses of α -globulin and 'protein A'*

Element	α -Globulin (%)	'Protein A'	
		Bishop (1927) (%)	Kemp & Straitiff (1940) (%)
N	15.7 $\pm$ 0.1	14.0-15.1	12.7
C	52.1	—	50.7
H	6.7	—	7.3
S	0.06	—	1.32
P	<0.01	—	0.01
Ash	0.4	0.2-0.5	2.26

Qualitative analysis of the ash showed that the chief metal present was Na (probably left from the sodium citrate buffer used in isolating the protein) with traces of Mg, K and Ca.

Colour and precipitation reactions. Positive reactions to the following tests were obtained: biuret, ninhydrin, xanthoproteic, Millon, Sakaguchi and Adamkiewicz. Reactions to the Molisch and Benedict tests were negative, indicating the absence of carbohydrate. These results are in agreement with the findings of Bishop (1927), Bondy & Freundlich (1938) and Kemp & Straitiff (1940), except that the last authors reported a negative Millon test.

Solubility and isoelectric range of α -globulin. When freshly prepared, α -globulin is readily soluble in neutral or alkaline salt solutions and in acid solutions of pH less than about 3. It is insoluble in distilled water. Determinations of solubility were made over the pH range 4.1-5.9, in sodium acetate buffers of ionic strength 0.04, with a freshly prepared solution of the protein which had been exhaustively dialysed against 0.01M sodium acetate solution. All measurements were carried out at 0°. Between pH 4.3 and 5.4, the solubility of the protein was extremely small (approx. 0.03 g./l.); very similar results were obtained at ionic strength 0.1, except that the solubility was slightly greater. On each side of these pH limits, the solubility increased very rapidly. From these solubility characteristics, the protein can be classified as a globulin with an isoelectric point in the region of 4.8.

The effect of temperature on the solubility of the protein was examined over the pH range 5.5-8.7, the ionic strength being varied by means of sodium acetate. At $I=0.07$, the coagulation produced by heating at 70° for 2 hr. was almost

complete (>94%) at pH values between 5.5 and 7.0. Under the same conditions at pH 8.7, 80% of the protein was coagulated. As the ionic strength was decreased, the percentage of protein coagulating decreased, becoming negligibly small (at pH 7) at ionic strengths less than 0.01.

DISCUSSION

Kemp & Straitiff (1940) isolated 'protein A' by half-saturation of fresh latex serum with ammonium sulphate. From Fig. 1, it is clear that this procedure yields a very heterogeneous fraction, containing all but one of the seven protein components detected by Archer & Sekhar (1955) in fresh serum solids. The electrophoretic diagrams for fractions precipitated at other concentrations of ammonium sulphate show that the method is not suitable for purifying the α -globulin component. The difference in composition between α -globulin and Kemp & Straitiff's fraction (Table 1) is particularly marked in respect of nitrogen, sulphur and ash contents, although the higher values for sulphur obtained by Kemp & Straitiff may be due in part to their preparation not being completely freed from ammonium sulphate.

The 'protein A' isolated by Bishop (1927) from acidified latex serum was reported to contain phosphorus, whereas α -globulin has a negligible phosphorus content after thorough dialysis of the protein. Bishop's data for the ash content of his 'protein A' is in good agreement with that of α -globulin. However, it is probable that the material obtained by Bishop was at least partly denatured, since its solubility was reported to be small except at very high pH values. When freshly prepared, α -globulin is readily soluble in salt solutions at pH 7, and in latex serum reconstituted from *F* or *C* serum solids.

The value of 15.7% for the nitrogen content of α -globulin, the negligible phosphorus content and the negative reaction for carbohydrate indicate that it is not a conjugated protein.

SUMMARY

1. One of the two major protein components of fresh *Hevea*-latex serum has been isolated by an isoelectric precipitation method and the optimum conditions for the precipitation have been determined.

2. Attempts to isolate this component by fractional precipitation of the total serum proteins with ammonium sulphate gave markedly heterogeneous products.

3. A preliminary examination has been made of the purified protein. Analytical and solubility data indicate that it is a globulin lacking non-protein prosthetic groups.

This work forms part of a programme of research undertaken by the Board of the British Rubber Producers' Research Association. Our thanks are due to the Rubber Research Institute of Malaya for the supplies of latex serum solids used in the investigation, to Dr A. D. Patrick for carrying out the electrophoretic analyses shown in Fig. 4, and to Professor M. Stacey for permission to use the Tiselius apparatus at Birmingham University.

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Studies in Detoxication

66. THE METABOLISM OF HALOGENOBENZENES. 1:2:3-, 1:2:4- AND 1:3:5-TRICHLOROBENZENES*

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The trichlorobenzenes are decomposition products of the insecticide benzene hexachloride (gam-mexane). On treatment with alkali, the α -, β -, γ - and δ -isomers of benzene hexachloride are dehydrochlorinated to yield a mixture of the three isomeric trichlorobenzenes in which the 1:2:4-isomer predominates and amounts to about 80 % of the total (cf. Hughes, Ingold & Pasternak, 1953). Benzene hexachloride also decomposes under field conditions, and it has been shown that the deleterious effects of crude benzene hexachloride on wheat seedlings and coniferous seedlings may be due to trichlorobenzenes, the 1:2:4-isomer being the most active towards wheat and the 1:3:5-isomer towards coniferous seedlings (Hocking, 1950; Simkover & Shenefelt, 1952). Benzene hexachloride is also decomposed in animals and insects, but nothing is known about the nature of the metabolites. However, it is known that the different isomers of benzene hexachloride are metabolized at different rates in mice and in flies (Asperen, 1954; Oppenoorth, 1954, 1955). When the common isomers of benzene hexachloride are fed to rats over a long period, the foreign material which accumulates in the tissues, particularly the fat, does not contain 1:2:4-trichlorobenzene, but consists of unchanged benzene hexachloride (Davidow & Frawley, 1951).

The present work was undertaken with the view in mind that benzene hexachloride may be converted into trichlorobenzenes *in vivo* and that these may be further metabolized to phenolic substances. A knowledge of the metabolism of the trichlorobenzenes could thus be useful in elucidating that of benzene hexachloride. The trichlorobenzenes themselves have been used as pesticides and they

are also employed in industry for various purposes such as the manufacture of electrical insulating material. From a toxicological point of view, the trichlorobenzenes are less toxic to small laboratory animals than chlorobenzene and *o*-dichlorobenzene (Cameron *et al.* 1937), and this may be correlated with the extent of mercapturic acid formation, which diminishes as the number of chlorine atoms in these compounds increases.

The metabolism of the isomeric dichlorobenzenes in rabbits has already been described (Azouz, Parke & Williams, 1955; Parke & Williams, 1955).

EXPERIMENTAL

Materials and reference compounds

1:2:3-Trichlorobenzene, m.p. 53°, was purchased (L. Light and Co., Colnbrook, Bucks.); 1:2:4-trichlorobenzene, m.p. 17°, and 1:3:5-trichlorobenzene, m.p. 61°, were prepared according to Holleman (1918).

The following compounds were prepared by standard methods (m.p.'s are corrected; references to the known compounds are given by Huntress (1948)).

Compounds related to 1:2:3-trichlorobenzene. 2:3:4-Trichlorophenol, m.p. 81°, and benzoate, m.p. 142°; and 3:4:5-trichlorophenol, m.p. 98°, and benzoate, m.p. 119°, were prepared.

3:4:5-Trichlorocatechol was synthesized as follows. 2:3:4-Trichlorophenol was converted into 3:4:5-trichlorosalicylaldehyde as described by Duff (1941) for the preparation of *o*-hydroxyaldehydes. The aldehyde (yield 20%), m.p. 87°, formed yellow needles from aqueous ethanol. (Found: C, 37.4; H, 1.6; Cl, 47.0. $C_7H_3O_2Cl_3$ requires C, 37.3; H, 1.3; Cl, 47.2%). The semicarbazone, m.p. 236°, formed pale-yellow needles from aqueous ethanol. (Found: C, 34.1; H, 2.4; Cl, 37.7. $C_7H_4O_2N_2Cl_3$ requires C, 34.0; H, 2.1; Cl, 37.7%). The aldehyde in *n*-NaOH was treated with H_2O_2 , as described by Dakin (1909) for the oxidation of *o*-hydroxyaldehydes, to yield 3:4:5-trichlorocatechol (yield

* Part 65: Smith & Williams (1955).