apparatus. However, an immunological method showed the presence of three additional minor components.

2. An antiserum, prepared by injection of chick serum albumin into rabbits, was used to demonstrate the net synthesis of serum albumin by chick-liver slices. The results of Peters & Anfinsen (1950) were thus confirmed.

3. The incorporation of radioactivity into serum albumin and a soluble protein fraction was studied after incubation of chick-liver slices with [^{14}C]glucose and [^{14}C]alanine respectively. The specific radioactivity of the alanine in each protein fraction was determined.

4. The incorporation of radioactivity into a soluble protein fraction of rat hepatoma and rat liver was studied after incubation of the tissue slices with [^{14}C]glucose. The specific radioactivity of the glucose during the incubation period was followed. It was thus shown that under these conditions there is a better utilization of glucose by hepatoma tissue for the synthesis of protein than there is by liver tissue.

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The Proteins of *Hevea brasiliensis* Latex

1. PROTEIN CONSTITUENTS OF FRESH LATEX SERUM

By B. L. ARCHER

British Rubber Producers' Research Association, 48, Tewin Road, Welwyn Garden City, Herts.

AND B. C. SEKHAR

*The Rubber Research Institute of Malaya, 3rd Mile Ampang Road,
(P.O. Box 150), Kuala Lumpur, Malaya*

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Analytical studies (Altman, 1946) have shown that fresh unammoniated *Hevea* latex contains about 1% of protein, part of which is adsorbed at the surface of the rubber particles while the remainder is distributed between the aqueous serum phase of the latex and the luteoid bodies first described by Homans & van Gils (1948). Although it is generally

recognized that the presence of protein in fresh latex is an important factor controlling its colloidal stability, further information on the concentration and properties of the individual protein components, and their distribution between the above three phases, is necessary for a better understanding of the colloidal behaviour of latex.

The present paper describes investigations on the proteins of fresh latex serum. Before 1942 the presence of three protein fractions in fresh serum had been demonstrated (Bishop, 1927; Kemp & Straitiff, 1940). Similar fractions were also obtained from ammonia-preserved latex, though the presence of ammonia modified the properties of the proteins in some respects (Kemp & Straitiff, 1940; Bondy & Freundlich, 1938). Later, a more detailed electrophoretic analysis of the proteins in un-ammoniated serum was carried out by Roe & Ewart (1942), who demonstrated the presence of seven electrophoretically distinct protein constituents.

The present paper confirms and extends many of Roe & Ewart's results. It is shown that the properties and composition of latex serum prepared by prolonged freezing of fresh latex differ in certain important respects from the serum obtained by centrifuging the latex.

EXPERIMENTAL

Materials

Freeze-dried serum. All experiments were carried out with latex from the same eighteen mixed clonal seedlings growing at the Rubber Research Institute of Malaya Experiment Station, and tapped regularly on a half-spiral, alternate-day tapping system. Latex collected in glass vessels surrounded by a mixture of ice and salt did not separate into three distinct fractions on high-speed centrifuging (see below) and did not yield a clear serum after coagulation by deep freezing. The latex was therefore collected at normal temperature in clean aluminium tapping cups. The collection period was limited to 30 min. and the first runnings from the tree were not included in the samples.

Serum was obtained from the latex in two ways. The first consisted in freezing the latex at -25° for a period of 15 days or more (Sekhar & McMullen, 1951). The frozen mass was broken up into small pieces to expose a large surface to the air; after thawing, the rubber separated completely, leaving a clear serum which could be decanted and freeze-dried. The serum so obtained is described here as *F* serum. The method of preparation is essentially the same as that used by Bishop (1927) and by Roe & Ewart (1942). If appreciably less than 15 days' freezing at -25° was allowed, or the frozen latex was not broken up, coagulation was often incomplete and a milky serum resulted.

Alternatively, the freshly tapped latex was treated as rapidly as possible, by centrifuging at 59 000 *g* in an ultracentrifuge at 5° . After 45 min. at speed, the serum had separated as an almost clear 'middle' layer, with a 'heavy fraction' at the bottom and a rubber layer at the top of the centrifuge tube (Cook & Sekhar, 1953). The serum could be run off by making two small punctures in the celluloid centrifuge tube, one near the bottom of the serum layer, the other near the top. This material, described in the present paper as *C* serum, was stored at -25° until a second collection and centrifuging had been made two days later. The sera from the two collections were bulked and freeze-dried together.

When dry, the serum solids were transferred to smaller glass tubes, the moisture absorbed during transfer was removed under vacuum and the tubes sealed off. Samples were stored in this condition, preferably at 0° , until ready for use.

Methods

Elementary analyses. C, H and ash determinations were made by Pregl micro-combustion, and total N by the micro-Kjeldahl method. P was determined colorimetrically with ammonium molybdate, after wet oxidation. The S content of the ash and gaseous products after micro-combustion was determined turbidimetrically, as BaSO_4 after complete dissolution of the ash in dil. HCl. All samples were dried to constant weight *in vacuo* at room temp. before analysis.

Protein nitrogen. Three methods were examined for the determination of protein N, namely, precipitation with saturated ammonium sulphate, with 20% (w/v) trichloroacetic acid or with 0.5% tannic acid:

(a) A 1% solution of the serum solids was saturated with ammonium sulphate and set aside for 24 hr. at 0° . The precipitate was centrifuged off and freed from sulphate by dialysis in cellophane at 0° . The resulting suspension was then freeze-dried, brought to constant weight at 105° and analysed for N.

(b) A 40% (w/v) solution of trichloroacetic acid was added to an equal volume of the serum-solids solution (1%, w/v) and the precipitate centrifuged down. In this case, protein N could be determined conveniently by the difference in N content of the clear supernatant and the original solution. Alternatively, the precipitate was washed by resuspending in dilute trichloroacetic acid and recentrifuging; the product was then dried as in (a) and analysed.

(c) The same procedure was followed as in (b), but a 1% solution of tannic acid was used instead of trichloroacetic acid. Protein N was normally determined by difference. Corrections were made, when necessary, for the N content of the tannic acid (or trichloroacetic acid in (b)).

The *C* solids were usually contaminated by small amounts of rubber, present in the original latex as very small particles which were not separated completely at the highest centrifuge speeds attained. On analysis of the precipitated protein directly for N, it was therefore necessary to extract the precipitate with benzene before drying it at 105° .

Paper electrophoresis. For determining the number of electrophoretically distinct proteins in the serum solids, paper electrophoresis on Whatman no. 1 paper was used. The apparatus was a modification of that described by Kunkel & Tiselius (1951), with 0.05 M sodium veronal buffer (pH 8.6) as the solvent medium. To improve resolution of the proteins, the paper was separated from the supporting plates by narrow strips of polythene 0.06 in. thick, parallel to the edges of the paper. After electrophoresis had proceeded under a potential gradient of 4.5 v/cm. for 1000 min., the paper was dyed with 1% bromophenol blue in ethanol, as recommended by Kunkel & Tiselius. Electroendosmotic flow was corrected for by the use of dextran.

After drying, the paper was cut into strips and the amount of dye on each strip determined absorptiometrically after elution with 0.01 M-NaOH. Later, an EEL photoelectric scanner (Evans Electroselenium Ltd., Harlow, Essex), which gave much more rapid results than the dye-elution technique, was used.

The quantitative determination of protein concentration along the paper has not been attempted, in view of the arbitrary assumptions involved in such determinations (Crook, Harris, Hassan & Warren, 1954; Franglen & Martin, 1954).

RESULTS

Analyses of latex-serum solids. An extensive analysis of thirty-three samples, collected over a period of 18 months, is summarized in Table 1.

At concentrations of 5 and 0.5% (w/v) the *F* solutions had pH values of 6.4 ± 0.1 and 6.6 ± 0.1 respectively immediately after dissolution. No change in pH occurred on allowing these solutions to stand in air at 20° for several hours. The pH values of the *C* samples immediately after dissolving were considerably higher and varied between pH 7.3 and 8.1, at 5% concentration. On standing in air at 20°, the pH of these solutions decreased

Table 1. *Elementary analysis of dry serum solids*

	% C H N S P Ash						Protein N*	Non- protein N*	Protein content (see text)*
Average for 27 <i>F</i> samples	37.6	6.19	4.01	0.23	1.04	13.5	1.93	2.08	12.8
Standard deviation	0.66	0.10	0.37	0.07	0.25	1.10	—	—	—
Average for 6 <i>C</i> samples	39.7	6.43	4.52	0.16	0.74	11.4	2.7	1.82	17.8
Standard deviation	0.38	0.15	0.20	0.02	0.14	1.20	—	—	—

* Determined on samples of average total N content.

With the *F* solids, the three methods of determining protein N, referred to above, gave concordant results. Completeness of precipitation was shown by dialysing the filtrates against water at 0° for 72 hr., after which a negative biuret test was obtained. The N content of the dried precipitate was $15.2\% \pm 0.2\%$. For converting protein N into % protein, a conversion factor of 6.58 is therefore recommended for the total proteins.

With solutions of the *C* solids, precipitation of protein by saturated ammonium sulphate was incomplete, the filtrate yielding a further precipitate on heating to 100°. Precipitation with 20% trichloroacetic or 0.5% tannic acid yielded filtrates which gave no further precipitate on heating, and methods (b) and (c) were therefore used for the protein N determinations. The N contents of the dried products were in good agreement with those found for the precipitated proteins from *F* solids. The same conversion factor of 6.58 can therefore be used to convert protein N into percentage protein. However, the yield of protein from the *C* solids was relatively high (17.8%), corresponding to a protein N content of 2.7%. Determination of the protein N by difference, from analysis of the supernatant, gave the same result (Table 1).

pH changes in reconstituted serum. The *F* solids dissolved readily in distilled water to give clear, pale-yellow solutions. *C* solids also dissolved readily, giving almost colourless solutions which were slightly hazy. In both cases the solutions, when freshly prepared, possessed a slight but pleasant odour.

Fig. 1. pH changes in 5% (w/v) solutions of *Hevea* latex-serum solids in distilled water: (a) *C* serum heated to 90° and cooled; (b) *C* serum in air (O) or N₂ (x); (c) *F* serum.

rapidly (Fig. 1), all reaching pH 6.6 ± 0.15 after 3 hr. (curve b). An identical rate of fall of pH occurred when the solutions were kept under pure nitrogen. When a freshly prepared solution of the *C* solids was heated at 90° for 5 min. (curve a) no subsequent fall in pH occurred after cooling (the heat treatment itself usually produced a small increase in pH of approx. 0.3 unit). The fall of pH in solutions of the *C* solids is probably due in part to production of hydrogen sulphide, which could be detected by lead acetate paper after the solutions had been set aside for half an hour or more in a closed vessel.

Protein components of reconstituted serum. The number of individual proteins (or proteoses) in the *F* solids, detectable by electrophoresis, was determined by first precipitating the total protein with saturated ammonium sulphate. Excess of sulphate was removed by dialysis at 0° against 0.05M veronal buffer (pH 8.6). A solution of the total protein in the buffer was then subjected to electrophoresis on paper. A typical electrophoretic diagram is shown in Fig. 2, in which the individual

Protein no. 7 has not been detected in all samples tested.

Effect of ammonia on the proteins of F serum. Roe & Ewart (1942) found that when the serum from frozen latex was treated with ammonia, the number of protein components detectable by electrophoresis decreased until, after several months, only two components of the original seven could be detected. Although it is not entirely

Fig. 2. Paper electrophoresis of *Hevea* latex-serum proteins. Electrophoresis for 1000 min. in 0.05M sodium veronal buffer (pH 8.6) at 4.5 v/cm. The ordinates represent the optical density of the strip in arbitrary units.

protein fractions have been numbered 1-7 in order of increasing anionic mobility. It is clear that fraction 1 is positively charged at pH 8.6. Fig. 2 shows many resemblances to the schlieren diagrams at pH 8.43 obtained by Roe & Ewart (1942) for the frozen latex serum of Florida and Sumatra latex. The lettering of the individual protein fractions given by these authors appears to be related to ours in the following way (our protein no. 2 was not detected by Roe & Ewart; protein VII described by them has not been detected by us):

Roe & Ewart	...	I	II	III	IV	V	VI	VII	—
Present work	...	4	6	1	3	5	7	—	2

Since precipitation by trichloroacetic acid or tannic acid results in the irreversible coagulation of some of the protein components, and precipitation by saturated ammonium sulphate is incomplete with the *C* solids, solutions of the latter were prepared for electrophoresis by dialysing, at 0°, a concentrated solution of the solids against 0.05M veronal buffer (pH 8.6) for 24 hr. A typical electrophoretic diagram is included in Fig. 2.

Fig. 3. Effect of ammonia (1.2%, w/v) on the electrophoresis diagram of *F* serum. Initial position of the proteins is indicated by the vertical line. The times refer to the period of contact with ammonia before electrophoresis. Electrophoresis carried out in 0.05M sodium veronal buffer (pH 8.6) for 1000 min. at 4.5 v/cm.

clear which these two components were, the one present in the larger concentration appeared to be either protein 4 or 5, in our nomenclature. It was noticed that the ammonia also produced a gradual increase in the anionic mobility of this constituent.

In the present work, samples of the same sample of *F* solids were stored in 1.2% (w/v) aqueous NH_3 at 18° for different times. The solutions were then neutralized with dilute H_2SO_4 and the total protein precipitated by saturation with ammonium sulphate. After centrifuging, the precipitates were mixed to pastes with water and dialysed against 0.05M veronal buffer, and the resulting solutions analysed by paper electrophoresis. The results (Fig. 3) show:

(a) The concentration of one of the main protein components (no. 4) of the original solution decreases rapidly and is barely discernible after 15 days' ammoniation. The concentration of the other main component (no. 5) appears to be largely unaffected by the ammonia.

(b) Components 2 and 3 are no longer detectable after 15 days' ammoniation.

(c) The concentrations of components 1 and 6 diminish slowly.

(d) The anionic mobilities of components 5 and 6 increase, and the cationic mobility of component 1 decreases slightly, as the time of contact with ammonia increases. The mobility of component 4 does not change appreciably with time.

DISCUSSION

It is apparent from Table 1 that the percentage of oxygen in fresh latex-serum solids must be high (about 40%), as would be expected from the fact that the polyhydric alcohol quebrachitol is known to be a major constituent of the serum (Tunnicliffe, 1954). At least part of the difference between the carbon and hydrogen contents of the *F* and *C* material was due to the *C* material still containing small amounts of rubber hydrocarbon. The ratio of protein nitrogen to total nitrogen is approximately 50% in the *F* and 60% in the *C* solids. The difference in the actual protein contents indicates that nearly 30% of the serum protein in fresh latex is lost by coagulation during long freezing of the latex. The possibility of some hydrolysis of protein during this process cannot be ruled out, in view of the slightly higher non-protein nitrogen content of the *F* solids. However, the fact that the phosphorus content is also appreciably higher in the *F* material suggests that it is phospholipid, initially associated with the rubber particles, which is partially hydrolysed during the freezing process with passage of phosphorus and base nitrogen into the serum phase. The ready hydrolysis of latex

phospholipids has been demonstrated by previous workers (Tunnicliffe, 1954; Smith, 1953).

The decrease in the pH value of solutions of the *C* solids in distilled water is attributed to the presence of an acid-producing enzyme or bacteria. With the *F* material, this enzymic or bacterial action has already proceeded sufficiently far, apparently, to give a constant pH of approximately 6.5. Roe & Ewart (1942) also observed that the pH of unammoniated serum prepared from frozen latex was 6.4 and that the serum could be kept at -20° for several months without change.

The electrophoretic diagrams for the *F* and *C* solids (Fig. 2) are very similar, indicating that the difference between the solids is mainly one of protein concentration. However, a difference in the nature of one or more proteins is indicated by the incomplete precipitation of protein from the *C* solids by saturation with ammonium sulphate and the appearance of a further precipitate on heating to 100°. From Fig. 2, proteins nos. 4 and 5 appear to be the main components in both *F* and *C* solids.

Our results showing the effect of ammonia on the proteins of *F* serum are consistent with those of Roe & Ewart (1942), if allowance is made for the different storage periods. The two proteins observed by these authors after prolonged ammoniation were presumably components 1 and 5. The predominating importance, as regards concentration, of protein no. 5 in the ammoniated serum is apparent.

SUMMARY

1. The aqueous serum phase of unpreserved rubber latex (*Hevea brasiliensis*) has been separated in two ways: (a) by high-speed centrifuging, and (b) by prolonged freezing at -25°. The protein contents of the serum solids are approximately 18 and 13% respectively; the difference is attributed to coagulation of protein during prolonged freezing of the latex.

2. There is evidence that organic phosphorus compounds (probably phospholipids) are partially hydrolysed during the freezing process, and that enzymic or bacterial reactions occur, leading to a fall in pH of the serum.

3. In both types of serum, seven electrophoretically distinct protein components have been detected, one of which is positively charged at pH 8.6.

4. After treatment with dilute ammonia solution for 15 days, only two of the original protein components are readily resolvable electrophoretically. One of these (no. 5) is a major component of the unammoniated serum.

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Malaya and the British Rubber Producers' Research Association. The authors wish to thank Dr E. G. Cockbain for his interest in this work and for his help in the preparation of this paper. Our thanks are also due to Mr Ho Kam Leng for assistance in some of the experimental work, to Mr G. W. Drake and Dr A. I. McMullen for many useful discussions, and to Dr W. T. Chambers and Mr R. J. Thompson for most of the elementary analyses.

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The Proteins of *Hevea brasiliensis* Latex

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

BY B. L. ARCHER AND E. G. COCKBAIN

British Rubber Producers' Research Association, 48 Tewin Road, Welwyn Garden City, Herts.

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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