The Proteins of Hevea brasiliensis Latex.
Part 5*. Starch Gel Electrophoresis of C Serum Proteins

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'C serum' has been prepared from latex by high speed centrifugation; after freeze drying and redissolving at higher concentration it has been examined by a simple technique of starch gel electrophoresis. The patterns obtained are complex and highly dependent on the concentration of starch in the gel. Even with the optimum concentration of starch some features of the pattern are difficult to reproduce. Nevertheless, twenty-one (possibly twenty-two) components staining with naphthalene black have been found in C serum. No definitely significant differences have yet been detected in the patterns with C sera from collections of latex from the same trees made at different times.

In the first paper of this series ARCHER AND SEKHAR (1955) reported an investigation of the proteins of 'C serum', the aqueous phase separated from fresh Hevea latex by high speed centrifugation. They showed that seven protein components could be distinguished in this phase by paper electrophoresis.

After the advent of zone electrophoresis in starch gel (SMITHIES, 1955), it became of interest to apply the superior resolving power of this technique to C serum proteins. Elsewhere we have referred briefly to work along these lines (MOIR AND TATA, 1960; RUBBER RESEARCH INSTITUTE OF MALAYA, 1960a, 1960b; KARUNAKARAN et al., 1961). These references are all to experiments using gels prepared from an ordinary commercial soluble starch of analytical reagent grade (from Messrs British Drug Houses). Such experiments showed that C serum proteins could be resolved into many more than the seven components detected by paper electrophoresis: a maximum of twenty-two bands was observed, but these electrophoretic patterns later proved impossible to reproduce, perhaps because of batch variation in the starch and/or variation in its water content (see below). More consistent results have since been obtained using starch prepared for electrophoresis by the Connaught Medical Laboratories; the present paper describes these.

EXPERIMENTAL METHOD
Preparation of C Serum for Electrophoresis

The source of latex for these experiments was a stand of mature mixed clonal seedlings, in Field 20A of the R.R.I.M. Experiment Station, which has been used in earlier work reported in this series. In the present work eighteen of the trees were used and these were mostly taken from the group of twenty mentioned in earlier publications (MOIR AND TATA, 1960).

The trees were tapped alternate-daily on a half spiral, and the latex was collected into aluminium cups for 30-60 minutes. The pooled latex was sieved to remove bark particles and centrifuged, without full refrigeration, as described earlier (MOIR AND

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COMMUNICATION 362
Tata 1960, pp. 155, 156). After centrifugation, the lusteroid tubes were pierced near the top and bottom of the serum phase and this was drained out through the lower hole. The upper portion of the serum in each tube is densely turbid and contains visible particulate material, including the 'yellow orange layer' of Cook and Sekhar (1953). This portion of the serum was not collected.

C serum from several tubes was pooled, filtered and freeze-dried. The resulting hygroscopic powder was stored, usually under vacuum, at —15° to —20°C until used.

Preparation of Starch Gel

Except when otherwise noted, all the results discussed below were obtained with gels made from a single batch (Lot 141) of *Starch-Hydrolysed* from the Connaught Medical Research Laboratories, Toronto, Canada. The material is markedly hygroscopic; it was dried in vacuo over calcium chloride, in lots of 50 g, for at least a fortnight before use (see further below).

Some gels were prepared with 12.4 g of starch per 100 ml of buffer, the concentration recommended by the manufacturers in using Lot 141 for the electrophoresis of human blood serum. Gels were also made with 1 and 1.5 times this concentration of starch, i.e. with 15.5 g and 18.6 g respectively, per 100 ml of buffer. The electrophoretic patterns obtained with C serum were strikingly different with the three different concentrations of starch; in most respects the results seemed best with the medium concentration (15.5 g per 100 ml of buffer).

The buffer used was 0.03M in H$_3$BO$_3$ and 0.012M in NaOH (cf. Smithies, 1955) and had pH approximately 8.9. The required amount of desiccated starch was mixed with 150 ml of this buffer and heated on a boiling water bath, with shaking, until a thick paste was obtained. This was stirred mechanically for 30 min. with the containing flask immersed in the boiling water bath. A negative pressure was applied at the mouth of the flask for a few seconds before pouring the hot gel into a lightly greased tray.

The tray consisted of a 'Perspex' frame resting on a lightly greased glass base-plate (approx. 0.2 cm thick) and enclosing a space 23.4 × 8.1 × 0.6 cm. It was overfilled with the gel and after a few seconds excess gel, containing froth, was skimmed off. The surface was then completely covered with a glass plate (also greased) and the gel was allowed to set overnight.

Introduction of Protein Samples

For electrophoresis 0.5 g of freeze-dried C serum was dissolved in 0.5 ml of chilled water giving a viscous, turbid solution which was used without further treatment. 0.02 ml portions were pipetted on to slivers of Whatman No. 1 filter paper (1.8 × 0.6 cm) and these were inserted in a vertical slit cut across the centre of the gel. Two or three samples could be conveniently accommodated in gels of the size described. After they were inserted, the slit was closed by pressing the two halves of the gel together from the ends and the gel was partly covered with a layer of 'Polythene' film and a glass plate. The covered portion of the gel was 20 cm long, leaving 1.7 cm exposed at each end for contact with the filter-paper wicks.

When 0.5 g of freeze-dried C serum is dissolved in 0.5 ml of water the volume of solution obtained is about 0.83 ml. The solids content of the solution is therefore approx. 60% w/v which is about ten times that of the C serum before freeze-drying (6-7% w/v). Archer and Sekhar (1955) found 17.8% w/w as an average value for the protein content of freeze-dried C serum. Hence it may be calculated that the 0.02 ml of concentrated solution used to prepare our electrophoretic patterns contained about 2 mg of protein.
Electrophoresis

The apparatus used is shown in Figure 1. It is essentially only a modification of that of Smithies (1955). Apart from the addition of lids the buffer tanks were identical with those used in an earlier paper electrophoresis apparatus (Moir and Tata, 1960). The two compartments in each tank are separated only by a single central 'baffle' reaching to within 0.6 cm of the bottom, but the capacity of the tanks is large (approximately 1 litre each). The electrodes were carbon rods.

The buffer used in the tanks was 0.3M in H$_3$BO$_3$ and 0.06M in NaOH and had a pH of approximately 8.3. It was brought to the same level in each tank with a siphon. Electrical contact between the gel and the buffer in the tanks was made with strips of Whatman No. 3 MM filter paper soaked in this buffer. A single strip the same width as the gel was used at each end. The hanging portions of the strips were sheathed in 'Polythene' film which also covered the top surface of the strip over the area of its contact with the gel.

The electrodes were connected to a voltage-stabilising power pack; this was set so that the potential difference across the 20 cm portion of the gel covered by the top glass plate was 100 volts (5V/cm). This potential difference was measured through platinum wire probes which were kept embedded in the gel at the ends of the top glass plate, in the narrow (approx. 2 mm) gap between this and the paper wicks.

The current was passed for six hours. The resistance of the whole system varied during this period: manual adjustments were

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Figure 1. Apparatus for starch gel electrophoresis used in this study. For the dimensions of the gel itself, see text.
therefore made periodically to the setting of the power pack to maintain the potential difference across the 20 cm section of the gel close to 100 volts. The potential difference rarely drifted out of the range 95—105V between adjustments. The runs were carried out in an air-conditioned room where the maximum range of temperature was 19°—25.5°C. (The usual range was less, and within these limits).

After electrophoresis the top glass plate and the 'Perspex' frame were removed and the two halves of the gel transferred to a cutting block of the type described by Smithies (1955). Each was then cut horizontally into two slices of equal thickness and these were stained with a saturated solution of Naphthalene Black 10B (G.T. Gurr Ltd) in ethanol/water/acetic acid (50/50/10 v/v). After two minutes in the staining bath the four slices of gel were washed four times with ethanol/water/acetic acid of the same composition. The first two washings were done for five minutes each, the third overnight and the fourth for two hours. The gel washing tray was rocked either manually or mechanically during each washing except the third.

After washing, the gels were blotted dry and photographed under illumination from two 100W, frosted, incandescent lamps, using 35 mm Kodak Micro-File film. A centimetre scale was included in each photograph beside the gel. A red filter was used in the later stages of the work reported here, but its effect on the final prints was slight.

The film was developed with Kodak developer D158 and printed on Kodak Bromide WSG3S paper.

The range of intensity of the bands in each electropherogram was very wide (from very dark to almost invisible) and it was difficult to reproduce this satisfactorily on a single photographic print. In general more detail was visible on the electropherograms and photographic negatives than in even the best prints; however some bands were clearer on the prints than in the original subject.

Most of the faint bands occurred in two regions of the electropherograms which were separated from the main pattern of darker bands (see below). After printing the main pattern from the negative it was therefore possible to mask it and continue printing the regions containing the fainter bands. By this means these fainter bands could sometimes be made visible on the final prints, but the procedure of course distorts their relative intensities. The photographs composing Figure 3 were 'shaded' in this manner; those of Figure 2 were not.

RESULTS AND DISCUSSION

Effect of Starch Concentration on Electropherograms

Figure 2 shows the results obtained by electrophoresis of the same sample of freeze-dried C serum in gels made with three different concentrations of starch: the concentration recommended for electrophoresis of blood serum (12.4 g/100 ml of buffer, Figure 2(a)); 1.5 times this concentration of starch (15.5 g/100 ml of buffer, Figure 2(b)); 1.5 times this concentration of starch (18.6 g/100 ml of buffer, Figure 2(c)). The other conditions of electrophoresis (buffer, time, voltage, etc.) were essentially the same in each case, and as described above.

It is of course well known that the concentration of starch present in the gel influences the nature of the electrophoretic patterns finally obtained with a given protein mixture. (See also Raymond et al., 1962) for reference to a similar effect in polyacrylamide gels). However, the differences between the patterns in Figure 2 are rather more pronounced than those illustrated by Smithies (1955) in his Figure 7, with electropherograms of blood serum on 12.5% and 15% starch gels. (They are also more pronounced than those recently illustrated by Smithies (1962) with electropherograms...
Figure 2. Effect of starch concentration on electrophoretic patterns. The three electropherograms shown were all prepared with the same sample (No. 4) of C.e.r.u.m, applied in triplicate to each gel. The starch concentrations in the gels were:

(a) 12.4 g/100 ml of buffer
(b) 15.5 g/100 ml of buffer
(c) 18.6 g/100 ml of buffer

(The photographs were not 'shaded' and the fainter bands are not visible in the reproduction).
of purified proteins at various concentrations of starch in the presence of urea and mercaptoethanol). It is difficult to say with confidence that any particular band in Figure 2(a) is identical with one in Figure 2(b). A similar uncertainty arises in comparing Figure 2(b) with Figure 2(c), although in this case a few bands do appear to correspond.

The theoretical reasons for the profound effect of starch concentration upon the electrophoretic patterns obtained from C serum proteins are of interest (cf. SMITHIES, 1962), but we are here concerned more with the practical consequences. These are that for a series of observations a suitable concentration of starch must be selected as standard, and this concentration must be accurately maintained.

In a series of runs with the 'recommended' concentration of starch, as in Figure 2(a), the reproducibility of the patterns and the resolution of bands both seemed inferior to results obtained with '1½ times' concentration as in Figure 2(b). With '1½ times' concentration, as in Figure 2(c), a series of runs gave fair reproducibility, but the pattern was rather too compressed and in some regions showed inferior resolution.

For further study the '1½ times' concentration (15.5g starch per 100 ml of buffer) was therefore selected as standard. A detailed discussion of results with this concentration of starch is given below and illustrated further by Figure 3.

With regard to accurate maintenance of starch concentration in the routine preparation of gels, the very marked hygroscopy of the starch has to be considered. As received by us, Connaught starch was partly hydrated: samples dried for two weeks in vacuo over calcium chloride, to approximately constant weight, lost 2.5 to 2.9% of their original weight. Samples exposed to the (very humid) air of our laboratory increased in weight, over the same period, by about 15%. A very marked variation in the electropherograms is produced by varying the starch concentration (Figure 2); hence it was important to avoid accidental variation in starch concentration, arising from uncertain hydration of the solid starch, if reproducible results were to be obtained. We therefore dried the starch in vacuo over calcium chloride before use, as noted earlier.

**Detailed Results with Standard Starch Concentration**

The results reported here concern freeze-dried C serum prepared from nine different lots of latex collected at irregular intervals over a period of thirteen months. Each sample of C serum was examined on four or more electrophoretic runs using gels with '1½ times concentration' of starch.

Samples Nos. 1 to 8 were first examined individually on separate gels to which the sample was applied in triplicate. (Figure 2(b) shows one of these electropherograms, on sample No. 4). When the electropherograms were compared it appeared obvious that there was a recurring pattern of bands, which were identical as between different preparations of C serum from the same group of trees. Measurements of mobility did not seem a reliable means of characterising particular bands however, since the absolute mobilities varied somewhat between different runs. A number of runs were therefore performed in which each gel carried two different samples of C serum side by side. The resulting electropherograms left no doubt of the correspondence of bands between individual patterns. (The total number of electrophoretic runs under consideration, including both the 'triplicate' and the 'comparison' type was forty-six).

Electropherograms of the 'comparison' type are illustrated in Figure 3. In the diagram, Figure 3(d), the mobilities have been made to correspond with those in the electropherogram shown immediately above in Figure 3(c). (As noted, there is some vari-
tion in absolute mobilities between runs). In other respects, Figure 3(d) represents the general pattern which emerges from consideration of all electropherograms on the nine samples of C serum; it includes all the bands—twenty-two in number—detected in the course of this work. All the bands were not detected on all the electropherograms: their reproducibility is discussed below. However, the original electropherogram and photograph corresponding with Figure 3(c) were exceptionally clear: all the bands of the generalised pattern appeared to be present in the upper row of this electropherogram (arising from sample No. 2); in the lower row arising from sample No. 1, band 7 was the only one not observed. The faintest bands have unfortunately been lost in the printed reproduction.

Bands 6, 8, 9, 10, 11, 12 and 19 appeared on every electropherogram from each of the nine samples of C serum. Bands 15, 16, 17 and 18 were quite intense but varied in their resolution from each other in different runs (compare Figures 3(a) and 3(c)). In some runs these bands were severely blurred together; in these cases, although they did not appear absent, it was very difficult to distinguish them as individuals. This variation was not characteristic of particular samples of C serum, since it occurred as between duplicate runs on the same sample; it must therefore have been due to some variable in the electrophoretic procedure which we did not succeed in controlling. Nevertheless bands 15, 16, 17 and 18 were detected as individuals on one or more electropherograms from each of the nine samples.

The region containing bands 13 and 14 presented a somewhat similar difficulty. Band 13 is indicated in the diagram, Figure 3(d) as a moderately intense, moderately sharp band about equidistant between 12 and 15. A band corresponding with this definition was seen on the majority of the electropherograms including at least one from each of the nine samples. (Figures 3(a), (b), (c) are examples). In about ten of the forty-six electropherograms this region of the pattern was blurred; 13 appeared to be present but not properly resolved from 14. Band 14 itself was observed on one or more electropherograms from each sample of C serum. In addition to the variation in resolution from 13, this band was erratic in apparent intensity: it commonly appeared faint and on many electropherograms it was not definitely visible; in a few cases it was almost as intense as band 13. On several of these points see Figure 2(b) and compare with Figures 3(a), (b), (c). It is possible that the appearance of the 13, 14 region is particularly dependent upon the washing procedure: the background at this point is rather strong. The variations observed did not seem to be characteristic of individual samples of C serum.

On the cathode side of the electropherograms, five faint bands (1, 2, 3, 4, 5) were detected; a group of similarly faint bands (20, 21, 22) was found near the anode end of the patterns. Most of these bands were near the limit of detectability and hence difficult to reproduce, i.e. they did not all appear on all electropherograms. They were defined by reference to the clearest electropherograms, e.g. the one reproduced as Figure 3(c); their approximate positions on the original photograph in this case are shown in Figure 3(d).

A band corresponding with 21 was detected on at least three electropherograms from each of the nine samples of C serum; bands 2, 3, 4 and 5 were detected, together, on at least two electropherograms from each sample. (Bands 2, 3, 4, 5 and 21 were all visible in both rows of each of the original photographs of Figures 3(a), (b) and (c)). Band 4 was slightly erratic in relative migration, being sometimes closer to 3 than to 5 and sometimes closer to 5 than to 3. On some
Figure 3. (a), (b), (c): Electropherograms comparing different samples of C serum; starch concentration: 15.5 g/100 ml of buffer. (The photographs were 'shaded' as described in the text to emphasise the fainter bands).
(d): Diagram representing generalised electrophoretic pattern from C serum at this starch concentration.
electropherograms it failed to resolve from 5. On one or more electropherograms from each sample a band with a higher cathodic mobility than band 2 was seen. It was very faint and rather broad, with ill defined boundaries; in a few cases it appeared possibly double. Since there is clearly at least one component in this region it is included, as band 1, in the generalised pattern (Figure 3(d)). (It was visible in both rows on the original photographs of Figures 3(a) and 3(c)).

Bands 20 and 22 were extremely faint with ill defined boundaries but were seen on at least one electropherogram from each sample (including both rows of those reproduced as Figures 3(b) and 3(c)).

Band 7, in the interior of the pattern, was also faint and barely resolved from band 8, but was seen on some electropherograms from each sample. (It was visible in the original photographs of Figure 3(a) - top row; Figure 3(b) - both rows; Figure 3(c) - top row).

To summarise, the main part of the pattern, comprising bands 6, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18 and 19, is fairly readily reproducible except that the region including band 13 is sometimes obscure and the resolution of 15, 16, 17, 18 from each other is unsatisfactory in some runs. To ensure detection of the remaining, fainter bands it seems essential to prepare several electropherograms from any given sample.

Our earlier electropherograms (RUBBER RESEARCH INSTITUTE OF MALAYA, 1960b) of C serum, using gels prepared with B.D.H. soluble starch, showed a maximum of twenty-two components. The apparent close agreement between this and the present results is partly fortuitous - the details of the patterns on B.D.H. starch were different on numerous points from those illustrated in Figure 2(b) and Figure 3; since they cannot now be reproduced they are of little further interest.

In the experiments reported here, the solution of C serum applied to the gels contained colloidal material since it was not convenient to clarify the small volume prepared, e.g. by high speed centrifugation. Band 6 in the patterns may be due to this material. A similar effect is described by ELTON AND EWART (1960) in the starch gel electrophoresis of wheat proteins. This band may, however, also contain soluble protein material of high molecular weight which cannot penetrate far into the gel (cf. the β-lipoprotein of blood serum: SMITHIES, 1959b). The point can be decided only by further work.

It seems safe to assume that the other twenty-one bands staining with naphthalene black each represent at least one distinct protein in C serum, and it is interesting to compare this conclusion with those of ARCHER AND SEKHAR (1955) using paper electrophoresis.

The trees used in our experiments were mostly the same as theirs and our procedure of latex collection, centrifugation, freeze-drying etc., was essentially identical. Their subsequent procedures differ widely from ours not only in respect of the supporting medium used for electrophoresis but also in other ways: in particular it is impossible to compare the amounts of protein submitted to electrophoresis in the two cases, since it was not determined in either (although an estimate of the amount of protein used in our procedure has been given above); the stains used were different and the sensitivities are unknown. It may be that some of the faint bands in our patterns are observed because of a higher overall sensitivity and that they have no counterparts in Archer and Sekhar's paper electropherograms. However, even if only the section of our pattern containing the stronger bands (8 to 19) is compared with the paper electropherograms of C serum proteins, it seems obvious that some or all of the seven components in the latter must be inhomogeneous and that they
are further resolved in starch gel. Similar results have generally followed when the starch gel and paper electrophoresis of a given biological extract have been compared. The relationship between the components observed by the two procedures in the case of C serum may be determinable by two-dimensional electrophoresis as described by POULIK AND SMITHIES (1958) for blood serum; we have not yet attempted this; it should be noted that the paper electrophoresis of C serum proteins is considerably more difficult than that of blood serum, because of the pronounced 'tailing' of one of the components (known as α-globulin: ARCHER AND COCKBAIN, 1955).

FRANGLEN AND GOSELIN (1958) discussed the possibility that starch gel electrophoresis might overestimate the number of really distinct substances in a mixture. But in practice it seems to be generally true that starch gel electrophoresis in one dimension is more likely to underestimate the number of individual proteins in a biological extract. For example, some of the components seen on a one-dimensional starch gel electropherogram of blood serum are inhomogeneous, as is shown by two-dimensional electrophoresis (POULIK AND SMITHIES, 1958 and SMITHIES, 1959b). Some components of diphtheria toxin which form single bands on a one-dimensional starch gel electropherogram are found to be complex by immuno-electrophoresis in starch gel (POULIK AND POULIK, 1958).

It is therefore very likely that the application of more refined techniques to C serum would reveal more than the twenty-one or twenty-two components here reported. Among the numerous improvements on simple starch gel electrophoresis which suggest themselves for future work with latex proteins are the ones just mentioned (two-dimensional and immuno-electrophoresis), but also the use of vertical starch gels (SMITHIES, 1959a), discontinuous buffer systems (POULIK, 1957) and polyacrylamide gels (RAYMOND et al., 1962; CHANG et al., 1962).

In retrospect the procedure we have used might be improved on some points. Apart from closer control of voltage, temperature and the washing procedure, the starch would probably be better dried before weighing with a more reliable desiccant than calcium chloride. Longer gels and longer times of run than we have used might improve resolution, although there is always the possibility in long runs of a minor component diffusing to an undetectable concentration. Finally, the elimination of the filter paper insert used to apply the sample in the present work would be desirable, especially since the α-globulin of C serum is reported to be adsorbed on filter paper (ARCHER AND COCKBAIN, 1955; cf. SMITHIES, 1959b, p. 70).

As noted earlier, all the electropherograms so far discussed were prepared in gels made with Lot 141 of Connaught starch. It is of interest to know whether the pattern can be reproduced on other lots of this starch. Our investigations of this question are incomplete, but some electropherograms of C serum have been prepared with Lots 144 and 148 of Connaught starch. The starch was desiccated before weighing and the gels were made with 1½ times the concentration of starch recommended by the manufacturers. The main portion of the patterns obtained was essentially identical with that already described, i.e. bands recognisable as 6, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18 and 19 were present in every run. Bands 7 and 14 were also detected in all samples of C serum examined. Bands 20, 21 and 22 were detected in several samples but not in all. However we believe that this reflects the difficulty of detecting these faint bands rather than any genuine difference between samples of C serum.

It appears then, that at least the anodic portion of the electrophoretic pattern is reasonably reproducible as between different batches of starch. Results with the cathodic
portion of the pattern have so far been less satisfactory, with both Lot 144 and 148; further work will be necessary on this point.

The results as a whole suggest that there are no gross qualitative differences between the proteins of C serum in latex collected at different times during the year from the same set of trees. It was earlier thought that seasonal variation in latex proteins was likely (Moir and Tata, 1960; Karunakaran et al., 1961) and might possibly present difficulties in the comparison by starch gel electrophoresis of proteins from different clones of Hevea. Since this no longer seems so, one of us (S.J.T.) has initiated work on clonal samples of C serum, using the electrophoretic technique described above.

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