The Proteins of *Hevea brasiliensis* Latex. Part 3*

The Soluble Proteins of ‘Bottom Fraction’

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*Paper electrophoresis has been used to examine aqueous extracts of the sedimentable material (bottom fraction) from fresh latex. A total of eight electrophoretically distinct protein components were observed in the extracts. Six of these were of general occurrence in bottom fraction from various different latices; the other two were detected sporadically.*

The introduction by COOK AND SEKHAR (1953) of ultracentrifugation as a means of separating latex into its constituent phases has led to a number of investigations of the chemistry and enzymology of these phases. (ARCHER AND SEKHAR, 1955; ARCHER AND COCKBAIN, 1955; ARCHER, 1960; GASCOIGNE et al., 1958; HSIA, 1958; McMULLEN, 1959). In the first part of the present series, Archer and Sekhar described a study of the proteins of ‘C serum’, the non-sedimentable aqueous phase of latex. This was found to contain a mixture of proteins which could be resolved into seven components by paper electrophoresis. We are now reporting an investigation by paper electrophoresis of the proteins of ‘bottom fraction’, the particulate material sedimented from latex by ultracentrifugation.

The published data on C serum (Archer and Sekhar) were obtained with freeze-dried samples prepared in this laboratory from the latex of mature seedling trees growing in Field 20A of the R.R.I. Experiment Station. Using freeze-dried bottom fraction from the same source, ARCHER (1960; and private communication) found that a mixture of proteins could be extracted from it with water or buffer solutions. Paper electrophoresis of such extracts showed two major components and suggested the presence of several minor ones as well.

We have used a technique of paper electrophoresis somewhat different from Archer’s, have confirmed the presence of two major soluble protein components in freeze-dried bottom fraction and have obtained further information on the minor components. Our results include data on bottom fraction from various clonal latices as well as on material from seedling latex.

**EXPERIMENTAL METHOD**

**Collection of Latex**

Seedling latex was obtained from twenty mature mixed clonal seedlings in Field 20A of the R.R.I. Experiment Station. With a few exceptions, these were the same trees which had supplied latex for the earlier studies on proteins by Archer and his collaborators. They were tapped alternate daily on a half-spiral cut and the latex was collected into clean aluminium cups for 30-60 minutes after tapping. It was then pooled and brought to the laboratory.

Clonal latex was obtained from groups of mature buddings, each of 18 to 20 trees.

*The Natural Rubber Producers’ Research Association and the Rubber Research Institute of Malaya have cooperated in research on latex proteins for some years. Other papers in the present series have appeared in the Biochemical Journal, London, as follows:*

Part 1: ARCHER AND SEKHAR (1955)
Part 2: ARCHER AND COCKBAIN (1955)
Part 4: ARCHER (1960)

(see the list of references at the end of the present paper for further details).
representing the clones Glenshiel 1 (Gl 1),
RRIM 501, Tjirandji 1 (Tjir 1) and Pilmoor
A44 (Pil A44). These trees were also tapped
alternate daily whether or not samples were
required for the work described here, i.e.
the trees were in commercial tapping and not
reserved for these experiments. In some cases
tapping was not on a half spiral: details are
given below. The latex was collected into
clean aluminium cups for approximately one
hour after tapping, then pooled and brought
to the laboratory.

Isolation and Extraction of 'Bottom Fraction'

Latex samples were centrifuged in lusteroid
tubes in Rotor No. 21 of a Spinco Model L
ultracentrifuge. The rotor was accelerated as
quickly as possible (about 20 minutes) to
20,000 r.p.m. \(g(\text{max}) = 53,620\) and this
speed was maintained for 40 minutes; decelera-
tion to rest occupied a further 20 minutes.
Neither the latex nor the rotor was usually
pre-chilled, but the refrigeration system of the
centrifuge was operated during the run and
slight cooling of the rotor and contents took
place. (This procedure is substantially the
same as was used in all the previous investi-
gations cited at the beginning of this paper.
It differs in detail from the 'fully refrigerated'
centrifugation of latex mentioned by Môr
(1959), which is a more recent development).
Separation of the latex occurred essentially
as described by Cook and Sekhar. After the
tubes had been pierced and the serum drained
out, they were cut up and the surface of the
gelatinous bottom fraction was lightly rinsed
with distilled water. Bottom fractions from
several tubes were then pooled, slurried with
distilled water and the slurry freeze-dried.

Simple aqueous extracts of this freeze-
dried material were used throughout the
work: 0.5 g was extracted for 30 minutes,
with stirring, at 0°C with 1.5 ml of distilled
water. The paste obtained was then centri-
figed at approximately 25°C for 30 minutes
at 2,000 g (max) in an MSE centrifuge
(Measuring and Scientific Equipment Ltd.,
London). Refrigerated centrifugation (0°C)
did not seem to give a good separation of the
aqueous extract from the insoluble residue.
After centrifugation the extract was filtered
before application to the paper strips used
for electrophoresis. A turbidity passing the
filter paper was occasionally observed. The
total (Kjeldahl) nitrogen was determined in a
very few extracts: figures of the order of
10 mg N per ml were obtained.

Electrophoresis

The apparatus employed the principle of
Durrum (1950) and was made locally from
Perspex' sheet to a design freely adapted
from that of Flynn and De Mayo (1951). It
is shown in Figure 1. The electrodes were
mantles of platinum wire on glass frames in
the earliest experiments; they were later re-
placed by carbon rods without observable
effect on the results. The tanks were filled
with barbiturate buffer, pH 8.6, \(\mu = 0.05\)
(3.68 g of barbitone and 20.6 g of sodium
barbitone in 2 litres of solution; 1 litre in
each tank).

Figure 1. Apparatus for paper electrophoresis
used in this study. The whole of the prismatic
cover is removable.
Whatman No. 1 filter paper strips 3.1 cm (1\(\frac{1}{4}\) in.) wide and 50 cm long were used. Bottom fraction extract (0.01 ml) was applied across each of four dry strips at the centre, as a narrow band from edge to edge, by means of a micropipette. The strips were then placed in the apparatus with the centres resting on the horizontal glass rod at the apex, and the ends dipping into the buffer in the tanks. The effective length of paper, from one buffer surface to the other, was approximately 44 cm. Before the strips were mounted, the buffer was brought to the same level in each tank (siphon). The strips were then wetted with buffer on each side to within half an inch of the apex, the cover of the apparatus was placed in position and 90-120 minutes were allowed to elapse before the current was switched on. During this period the buffer fronts reached the apex, sharpening the initial band, but sometimes shifting it slightly. A constant current of 2 mA was then passed for 22 hours. The initial voltage across the electrodes varied but was generally in the range 195-245 V; this dropped to 140-180 V by the end of the run. Facilities for accurate temperature control were not available; the runs were performed in air-conditioned laboratories where the temperature was a few degrees below tropical room temperature.

At the end of the run the paper strips were immersed in 95\% ethanol for 5 min. and then stained for 10 min. in a solution of 1\% bromophenol blue in 95\% ethanol saturated with mercuric chloride. Excess dye was washed off with 5\% acetic acid and the strips were then dried in a horizontal position by a current of warm air and exposed to ammonia vapour before observation.

Some strips were treated with paraffin oil under partial vacuum in the usual way and passed through an EEL photoelectric scanner (Evans Electroselenium Ltd., Harlow, Essex). The curves obtained were of some use as records and for comparison with direct visual observations, but no quantitative significance was attached to them because it was obvious that the intensity of at least one of the bands on the finished electropherograms (component (viii), see below) varied uncontrollably with the time of washing in acetic acid.

**Photography and Reproduction of Figures**

The electropherograms were rarely if ever as clear as those obtainable with (for example) blood serum. Medium or low contrast photographs of the strips proved impossible to reproduce through the medium of a printing block. The blocks for Figures 3(a) to (g) were obtained after re-photographing the original photographs on a high contrast film (Kodak Microfile) and printing for maximum contrast. In some cases the figures exaggerate the clarity of the bands and in others slightly distort their relative intensities; their positions are, however, shown correctly.

**RESULTS AND DISCUSSION**

The procedures just described were developed using material from seedling (Field 20A) latex. The extracts proved fairly difficult to resolve; migration was rather limited even with the long time of run adopted. However, when the main details of the method had been worked out, it appeared that the proteins in the bottom fraction extract from this source could be resolved into two major components, with four fainter bands between them. This pattern of six bands, which is represented diagrammatically in Figure 2, recurred continually in the examination of further samples from the seedlings and from the four clones mentioned above. (See Figures 3 (a) to (g)). We have therefore found it convenient to interpret all results as repetitions of, or variations on this theme. The most important variation was the occasional appearance of two additional minor components; their positions are indicated by the arrows in Figure 2. For simplicity the bands are numbered from (i) to (viii) beginning with the band nearest the cathode end of the strip; with this system of nomenclature the fundamental pattern in our results is (i)-(iii)-(iv)-(v)-(vi)-(viii). All eight bands were never clearly seen together on a single freshly prepared electropherogram. Patterns showing the additional components as well as the fundamental ones were recorded as either (i)-
Figure 2. Diagram of stained paper electropherogram of bottom fraction extract, showing the basic pattern of six bands. The arrows indicate the positions of two additional bands of sporadic occurrence (see text). (ii)-(iii)-(iv)-(v)-(vi)-(viii) or (i)-(iii)-(iv)-(v)-(vi)-(vii)-(viii). However, later re-examination of the strips suggested that some samples did in fact contain all eight components, one of the additional ones being very faint. See further below.

In Figure 2, the centre of the original strip, where the sample was applied, is represented by a dotted line. Around this position the final electropherograms always showed a stained smudge which is also indicated in Figure 2, and is visible in the photographs of Figure 3. It is due to insoluble material deposited at the origin during or shortly after application of the sample, and probably consists at least partially of denatured proteins. It is not, of course, due to a component of zero electrophoretic mobility since such a component would be expected to move away from the origin towards the cathode in consequence of electro-osmosis. We have neglected this band near the starting line, regarding it as ‘false’, but it is not impossible that a real band was present in some cases and concealed by the stained insoluble matter.

Using dextran we confirmed that electro-osmotic displacement was in the expected direction, cf. Archer and Sekhar, but no attempt was made to measure it or to determine the absolute mobilities of individual components. Our interest was, at this stage, mainly in resolving the samples, and a hanging strip apparatus such as ours is not suitable for mobility measurements. Even the apparent mobilities of the bands, i.e. their final distances from the origin, were not at all accurately reproducible: the distances indicated in Figure 2 are only rough approximations.

The major bands (i) and (viii) were observed without difficulty in samples from all sources (a partial exception was found only with some of the samples from Tjir 1, discussed below, in which the region around (i) appeared abnormal and was very difficult to interpret); some of the minor bands were often harder to see because of faintness, poor resolution or a general blurring of parts of the stained paper strip. Several fresh extracts were therefore prepared from almost all samples of freeze-dried bottom fraction, and each extract was also usually examined in quadruplicate (four strips). A number of electropherograms were thus available for all samples of bottom fraction and points of doubt were resolved as far as possible by comparisons between them. In some instances, when tests for polyphenoloxidase or ultraviolet fluorescence were performed, some of the strips were not stained, but even in such cases at least three stained strips were prepared.
The electropherograms were always kept after observation, not discarded. When they were reammoniated after storage they sometimes seemed a little clearer than when quite fresh, and sometimes showed traces of minor components which had not been noticed earlier. This effect may be due in part to a slight overall fading with consequent reduction of background but there is of course a subjective element in the examination of paper electropherograms which cannot be eliminated in respect of the faintest bands. Thus a faint band whose presence is unexpected is likely to pass unnoticed, but to be revealed on careful re-examination of the same electropherogram. An example of this occurs in regard to component (vii) in some Tjir 1 samples (see below).

The aqueous extracts of bottom fraction from the seedling latex and from some of the clonal latices darkened during preparation and on the paper strip during the period of 90-120 minutes after application. This effect is ascribed to polyphenoloxidase activity. HSIA (1958) has shown the presence of the enzyme, accompanied by natural substrates, in bottom fraction; CHONG (1956) has confirmed the presence of natural substrates and obtained evidence that they vary considerably in amount in different samples. The degree of darkening of a particular bottom fraction extract is thought to be related to the concentration of these (unidentified) substrates of the enzyme.

Quinones, including some which occur as intermediates in the enzymic oxidation of phenols, are well known to react with (tan) proteins. Hence a system of polyphenoloxidase plus substrate could, in principle, seriously modify proteins occurring in the same mixture. There is strong evidence that a reaction of this type occurs naturally in the cuticle of arthropods: for reviews of the extensive literature see MASON (1955) and WIGGLESWORTH (1957). COHEN et al. (1956) have described an apparently similar effect in leaf extracts which contain soluble proteins and also display a browning reaction attributed to the presence of polyphenoloxidase and natural substrates. These authors developed an anaerobic extraction technique for leaves, which resulted in suppression of oxidase activity and thereby prevented any secondary attack on the other proteins in the extract. It was shown that such attack, if uncontrolled, discoloured some of the proteins and reduced their solubility.

It seems quite likely that similar reactions occurred in some of our bottom fraction extracts, with consequent damage to the soluble proteins. Such an effect is probably partly responsible for the 'false' band on the starting line of some of our electropherograms; we have, however, made no attempt to suppress it, and it appears that except in the case of one clone (Pil A44, q.v.) destruction of soluble proteins was not extensive enough to prevent their being resolved and observed.

The detailed results on bottom fraction from the various sources mentioned above are given below.

Figure 3. Photographs of stained paper electropherograms of bottom fraction extracts. Each photograph is the same size as the subject.
Figure 3 (b) Glenshiel 1.

Figure 3 (c) RRIM 501

Figure 3 (d) RRIM 501 (another sample showing additional band, (vii); see text).
G. F. J. Moir and S. J. Tata: The Proteins of *Hevea brasiliensis* Latex

Figure 3 (e) Tjirandji 1.

Figure 3 (f) Pilmoor A44.

Figure 3 (g) Pilmoor A44 (another sample; see text).
Field 20A seedlings

As noted earlier, material from this source was used during the development of the experimental method, and the presence of the six components, (i)-(iii)-(iv)-(v)-(vi)-(viii) was detected before the procedures of extraction and electrophoresis described above had been standardised in all details. Six samples which were then examined by the standard procedure or by very slight modifications of it (application of extract to strips pre-wetted with buffer; centrifugation of extract at room temperature which was above 25°) confirmed the occurrence of this pattern, which is shown in Figure 3(a).

Two other samples were used in experiments which included alterations in the standard procedure not adopted in subsequent work (modifications in the design of the cover of the apparatus; longer times of run); these runs were performed at room temperature without air conditioning. Although non-standard, they supplied further confirmation of the occurrence of the six 'fundamental' components.

Component (vii), which was first detected in RRIM 501 material (see below), seemed to be present on some electropherograms of Field 20A material as well, but the band was extremely faint. This component probably occurs in bottom fraction from the seedling trees at a concentration near the level component probably occurs in bottom fraction from this clone was taken from trees, in Field 30, on which the cuts were close to the union. Later a new half spiral panel was opened on the other side of the trees in the usual way. The second sample of latex was collected about six months after the new panel had been started and the remaining five samples over a period of eight months thereafter from the same panel.

Extracts of bottom fraction from this clone showed little or no tendency to darken on the paper strips.

When the first sample was examined only the six common components (i)-(iii)-(iv)-(v)-(vi)-(viii) were detected (see Figure 3(c)). These were present in all subsequent samples, but with the second sample the additional component (vii) was encountered for the first time and it recurred in samples three, four and five (see Figure 3(d) (from sample three)). This extra band was faint and in the case of sample four was definitely visible only after long storage of the electropherograms. With samples six and seven the band was extremely faint, though probably present.

Component (ii) did not appear clearly on any of the electropherograms of RRIM 501 material, but a few of the strips, including some on which band (vii) was visible, showed faint and doubtful indications of its presence.

Tjirandji 1 (Eight samples)

The first collection of latex from this clone was from a stand of trees, in Field 5, tapped on a half spiral, the cuts being already well down on the panel, roughly six inches from the union at the lower ends of the cuts. The aqueous extract of bottom fraction in this case was turbid even after filtration through Whatman No. 1 paper. It did not darken on the paper strips. Seven components were detected after electrophoresis, namely (i)-(ii)-(iii)-(iv)-(v)-(vi)-(viii), this being the first observation of the extra component, (ii) (see Figure 3(e)). Though not widely separated from component (i), the new band was quite unmistakable on almost all electropherograms from this sample (twenty-eight strips were prepared in this case).

Latex for samples two and three was taken from the old panels; bottom fraction from these again gave extracts which showed no darkening; component (ii) appeared to be present, in addition to the six common ones, but it was considerably more difficult to detect than before, and was seen on only a few of the strips; in the case of sample three, component (vi) was very faint and the region around it was blurred.

At this stage, five months from the beginning of experiments on Tjir 1, new panels were opened on the opposite sides of the trees. V cuts were used instead of half spirals, the bottom of the V being initially at about 85 inches from the union. The fourth sample of Tjir 1 latex was taken from these high cuts ten weeks after they were first opened.

In this case, the bottom fraction extract darkened distinctly on the paper strips, in contrast to the behaviour of earlier samples. Component (ii) was again clearly visible, as were components (i), (iii), (iv) and (viii). Components (v) and (vi)
were also present but faint and several of the strips were particularly difficult to interpret in the region around (vi), which was again blurred.

The remaining samples were all taken from the V cuts as these progressed down the tree. The tendency of the bottom fraction extract to darken appeared to lessen during this period; by the time a year had elapsed from the opening of the cuts, the samples obtained showed only a slight browning reaction.

This demonstration that polyphenoloxidase darkening occurs in samples from high cuts when it is less intense or absent with lower cuts on the same trees is novel only in respect of the method used. The tendency of whole latex from high cuts to darken has been known for over forty years (De Vries, 1920). See also next section (Pil A44).

In sample five, components (iv), (v), (vi) and (viii) were all present, (v) being faint. The remainder of the patterns on the cathode side, i.e. the region expected to contain (i), (ii) and (iii), showed various abnormalities and was impossible to interpret with certainty, but all three components were probably present. Samples six and eight gave essentially the same result.

Sample seven showed the six standard components (i)-(iii)-(iv)-(v)-(vi)-(viii) with no abnormalities except that band (iii) was faint. There was practically no evidence of (ii).

Component (vi) was not noticed on fresh electropherograms from Tjir 1 material, but when the strips were re-examined after long storage a considerable number of them (several strips from each of samples one, four, five, six and seven) were found to show this band very faintly. Since samples one and four also showed component (ii) clearly, it follows that these samples probably contained both 'additional' components as well as the 'fundamental' ones.

The tapping history of the Tjir 1 trees during collection of latex samples for these experiments appears rather unfortunate in retrospect, although it was in any case unavoidable at the time. More consistent results, in regard to component (ii), might perhaps have been obtained from a series of samples of latex collected from the middle region of half spirals panels.

Samples of latex from cuts very near the union of stock and scion (e.g. samples two and three in the present series) are objectionable on the ground that any truly clonal characteristics may disappear under the influence of the stock. Samples from high V cuts may have other peculiarities than the obvious browning reaction and may therefore not be strictly comparable with those from half spiral panels.

Pilmoor A44 (seven samples)

This clone was selected for study as a matter of curiosity because it has the reputation for producing a latex abnormally liable to spontaneous darkening. The tapping history was similar to that just given for Tjir 1, the Pil A44 trees being also in Field 5.

The first collection of latex was made from the lower part of a half-spiral panel. The bottom fraction extract showed particularly strong darkening during preparation and after application to the paper strips. Nevertheless the six common components were detectable on the final electropherograms. See Figure 3(i). When these were kept for some time faint indications of the presence of component (vii) were seen (barely visible in this photograph).

The trees were now tapped on new V cuts at about 85 inches above the union. The second sample of Pil A44 latex was taken from these cuts three weeks after opening. Bottom fraction extract prepared from it darkened even more strongly than in the case of the first sample. The electropherograms showed a strong band on the starting line and the overall appearance was obscure. The major components (i) and (viii) were clearly visible, as was component (vi), but in place of the three components (iii)-(iv)-(v) only two faint bands were seen, and their identity was uncertain. There was a slight suggestion of the presence of component (vii).

Five further samples from the continuation of the same panel gave similar results: the darkening reaction was very strong and the electropherograms were all somewhat obscure, though components (i), (vi) and (viii) were obviously present. The region where components (iii)-(iv)-(v) were expected was blurred and faintly stained; with three samples two bands appeared to be present here; with the remaining two samples the region appeared to contain three bands blurred together; in all cases the identity of these bands was uncertain. The extra anionic component (vii) was detectable, though also rather blurred in all of the last five samples. Figure 3(g), from sample seven, is representative.

In the Pil A44 samples only very faint and uncertain indications of component (ii) were seen.

As suggested earlier in this discussion the obscurity of the electropherograms from Pil A44 material is probably connected with the intense browning reaction which occurs in this case.

It will be obvious from the striking resemblance between the electropherograms from numerous samples why we have chosen to interpret our results as explained at the beginning of this discussion and in Figure 2. This resemblance cannot be meaningless; the interpretation adopted provides a convenient nomenclature for bottom fraction proteins, and the paper electrophoretic procedure has immediate application in experiments on their fractionation and purification.

It is recognised that in the absence of measurements of mobilities definitions of the 'components' are quite loose; conse-
sequently it is not certain that (for example) the protein of band (iii) in a Gl 1 sample is fully identical with the one similarly designated in Tjir 1, and it is not possible as yet to establish a relationship between the eight components from bottom fraction and the seven reported in latex serum by Archer and Sekhar. Also, the components (i) to (viii) may not be homomolecular proteins but complexes of molecules of similar mobility which could be separated by more potent techniques. Similar considerations are now well known to apply to the proteins of blood serum.

An attack on these problems requires the characterisation of the components, and hence their separation on a preparative scale. Archer and Cockbain (1955) and Archer (1960) have published work of this kind carried out by classical methods of protein fractionation. In this laboratory we are studying ion exchange chromatography with cellulose derivatives (Peterson and Sober, 1956; Sober et al., 1956) as a means of preparation of individual proteins from bottom fraction.

However, some incidental observations were also made during the paper electrophoretic work which bear upon the characterisation of some of the components and are therefore of interest here.

Stained electropherograms viewed under ultraviolet light showed a fluorescent band in the position of component (i). This was also evident with strips dried at about 100°C immediately after removal from the apparatus, washed with aqueous ethanol to remove buffer salts, redried and compared with strips from the same set stained in the ordinary way. Such fixed but unstained strips showed a fluorescent band in the position of component (i) with starting material from Field 20A seedlings, and each of the four clones. This suggests that component (i) is itself fluorescent.

Component (viii) was usually somewhat diffuse and tended to wash off the strips after staining, if the washing in 5% acetic acid was prolonged. This effect seemed to occur with samples from all sources, and was very much less marked with the other components, (i) to (vii).

When strips which had not been heated or fixed in alcohol were treated with dilute catechol solution immediately after electrophoresis, a rapid browning was produced in the region occupied by components (iii), (iv), (v), suggesting that the enzyme polyphenoloxidase is associated with all three bands. This effect was observed with Field 20A seedling material; samples from other sources were not fully tested but some evidence was obtained that the same association occurs in Gl 1.

Differences in the protein complement of individual organisms within a single animal species have been demonstrated by Smithies (1955) and Ashton (1957) using starch gel electrophoresis. It seems quite likely that analogous differences would occur in the proteins of different clones of a single plant species, for example in the latex proteins of different clones of Hevea brasiliensis. A report has already appeared in the literature (Balai Penjeldikan dan Pemakaian Karet, 1958) that 'difference in the protein patterns of the clonal latices Tjir 16 and PB 186 could be shown' (by paper electrophoresis), but details of this work do not appear to have been published.

The results of the present investigation give some slight evidence in favour of this idea: component (ii) was detected clearly only in material from Tjir 1. However, the technique we have used is not competent to establish intraspecific differences definitely. Furthermore, in our experiments the effects of variations in methods of tapping, in the positions of the cuts on the trees and in the season at which samples were collected are difficult to disentangle from any truly clonal peculiarities of bottom fraction proteins which may occur. Some of these complications, notably the effect of season, would be impossible to eliminate with any plant material. Nevertheless it is of interest to apply a more refined electrophoretic technique to latex proteins for several reasons, and starch gel electrophoresis is now
under study in our laboratories. The results will be reported subsequently, but it may be noted that it is already possible to distinguish a larger number of proteins in both serum and bottom fraction than have been detected by paper electrophoresis.

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