VOLUME 16 PART 4

Detection and Estimation of Growth Substances in Hevea Latex

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Methods are described for the extraction, chromatography and bio-assay of plant growth substances in Hevea latex. There is evidence for the occurrence of at least two, and probably three active compounds in the extracts, but the commonly-occurring auxin, 3-indolylacetic acid (IAA), does not appear to be present in detectable amounts.

CHAPMAN (1951) has described a technique for the extraction of natural hormones from serum of *Hevea* latex, the growth substance activity of the extracts being determined by the classic *Avena* curvature method. He noted that the curvature obtained was not proportional to extract concentration and that the maximum response varied with the source of latex, never being as high as that induced by optimal concentrations of 3-indolylacetic acid (IAA). Chapman was thus led to believe that he was dealing with a mixture of active compounds. Nevertheless, he was able to demonstrate considerable variations in hormone content and to suggest that a relationship existed between latex yield and hormone level.

Since Chapman's work, the use of synthetic growth substances such as 2,4-dichlorophenoxyacetic acid (2,4–D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5–T) to stimulate latex flow, pioneered by CHAPMAN himself and by BAPTIST AND DE JONGE (1955), has become an accepted part of Malayan estate practice. The success of these yield stimulant mixtures has led to renewed interest in the possible presence, within the tree, of naturally-occurring substances which might themselves influence latex yields. The present paper describes the application of paper chromatographic methods in a re-examination of the problem.

MATERIALS AND METHODS

Reagents

All reagents were of Analytical Reagent grade. Water was glass-distilled.

Latex Collection

Latex samples employed in the preliminary studies reported here were taken from a compact group of twenty-eight-year-old clonal seedling trees tapped alternate daily on a half-spiral cut in good renewed bark. The group contained trees of a wide range of yield capacity with a mean yield of approximately 25 grams dry rubber per tree per tapping. Latex was collected from all trees in the group, mixed thoroughly and sieved to remove bark fragments. Aliquot samples were then taken for extraction. The extraction process was started within two and a half hours of tapping.

Extraction of Latex

An essential preliminary to any extraction procedure is the removal of the rubber hydrocarbon. Chapman achieved this by coagulation with phosphoric acid at 40°C, followed by extraction of the expressed serum. During the course of the present investigation comparisons were made between coagulation and extraction in cold ethanol and various acidic coagulation procedures and it was invariably

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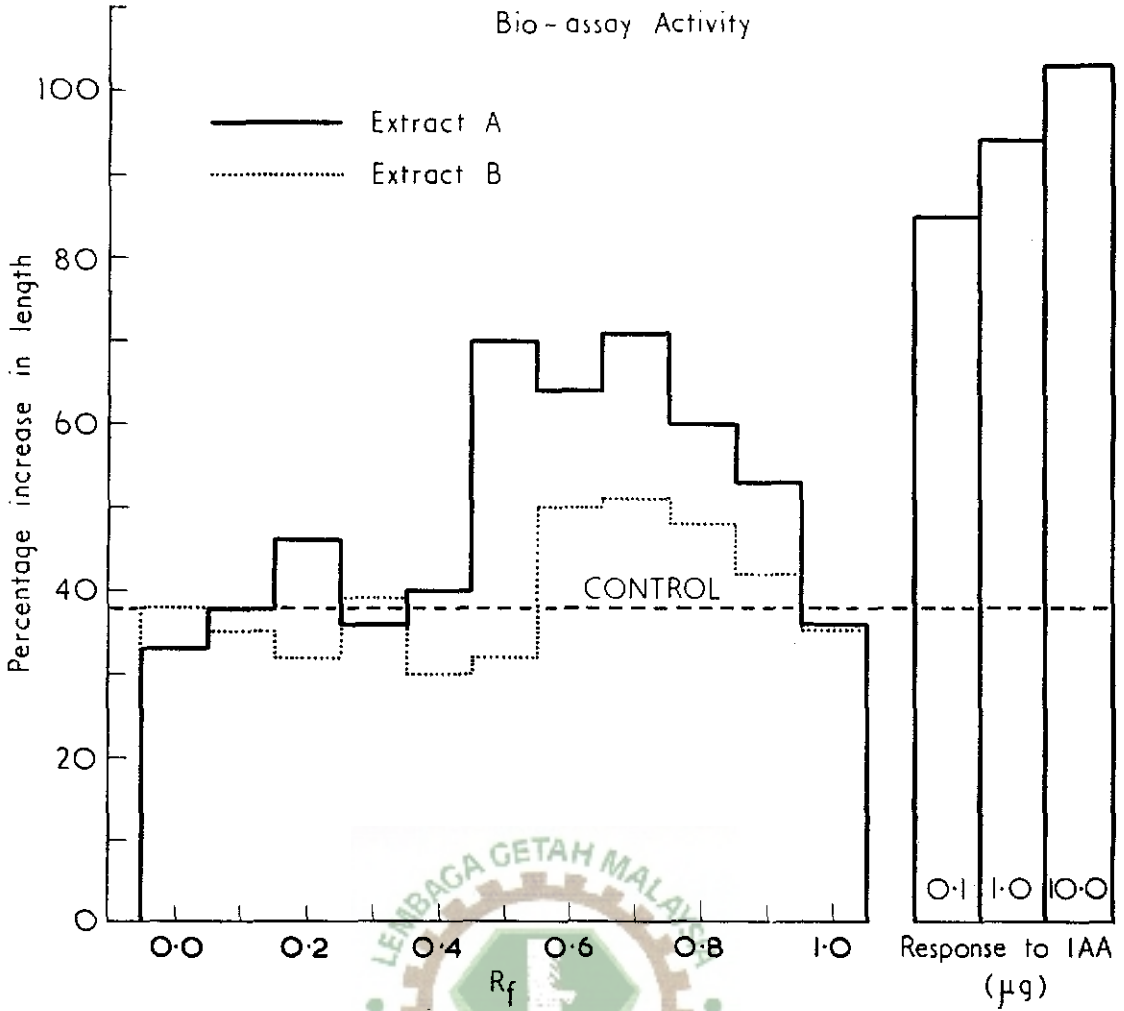

Figure 1. Comparison of the activity of chromatograms of two ether extracts of Hevea latex. Extract A was made by the alcoholic coagulation process described in the text, while Extract B was prepared by acidic coagulation followed by ether extraction of the expressed serum.

found that acidic coagulation resulted in an extract of lower bio-assay activity. Thus Figure 1 illustrates a comparison of extracts prepared by alcoholic coagulation, followed by ether extraction at a later stage (as described below) and by coagulation with phosphoric acid at 40°C and extraction of the serum with ether. The higher activity of the first extract is evident. It was noted that there was little or no sign of enzymic darkening in the case of extracts prepared by the use of ethanol, whereas this was marked in those made following coagulation with acid.

Latex samples of 250 ml were normally employed, although larger or smaller samples could readily be handled with a suitable adjustment of the quantities of reagents.

Standardised procedure. The latex sample was slowly mixed with an equal volume of cold ethanol with constant stirring, the hydro-

carbon coagulating almost at once. The sample was stored at −15°C for approximately 24 hours. (Longer or shorter periods of extraction at this low temperature had little apparent effect on the activity of the extract). The extract was squeezed through muslin or similar cloth, filtered and the alcohol evaporated under reduced pressure. It was found that further extraction of the rubber coagulum with fresh ethanol yielded only a negligible amount of additional activity and this procedure was therefore omitted. The aqueous residue after evaporation of the alcohol was made up to 150 ml with distilled water, adjusted to pH 2.0 with phosphoric acid, and extracted four times with peroxide-free ether. The ether extract was washed once to remove acid, dried over anhydrous sodium sulphate, and evaporated to dryness under a stream of nitrogen.

Paper Chromatography

Extracts were streaked along the starting line of a sheet of Whatman No. 1 paper (18 × 20 inches). A concentration equivalent to 20 ml latex per cm was found convenient. Two extracts could be compared on a single sheet, together with standard spots of 10 μg each of 3-indolylacetic acid (IAA) and 3-indolylacetonitrile (IAN). The solvent employed was *n*-butanol: ammonia (0.880) : water (100: 3 : 18 by volume). Operations were carried out in an air-conditioned laboratory with extreme temperature fluctuations of 22 – 27°C. The variation in temperature on any one occasion never exceeded 2°C. After equilibration for eight hours, the chromatograms were developed overnight by the descending method until the solvent front had travelled some 25 cm. The position of the solvent front was marked and the papers dried in a stream of air.

Preliminary Examination and Subdivision of Chromatograms

The dry chromatograms were examined under ultra-violet light and the positions of fluorescent areas lightly pencilled in and their descriptions noted. A strip 2.5 cm in width was taken from the centre of the chromatogram for bio-assay. This strip was subdivided into eleven equal portions of mean R_f 0.0 (starting line), 0.1, 0.2, etc., up to R_f 1.0 (the solvent front). In some of the earlier experiments the chromatogram strips were subdivided according to the u.v. fluorescence pattern rather than by R_f, but this proved unsatisfactory because there was no strict correlation between activity and fluorescence and because comparison between chromatograms was difficult owing to uneven division. Each paper square was placed in an appropriately-labelled bio-assay dish.

Parallel strips from each chromatogram were taken for test with chromogenic reagents.

Bio-Assay

Ideally, before any conclusions are reached concerning the physiological role of a compound in a particular plant, it should be tested on the plant itself under conditions as similar as possible to those obtaining in nature. Thus in the present investigation, if we are interested in the presence of natural compounds in *Hevea* capable of influencing latex flow after tapping, we should demonstrate their effect on that process. Unfortunately the amounts of active material separated on our chromatograms would be quite inadequate to produce any effect when applied to the rubber tree as a yield stimulant (as much as 0.1 g of active material is required for each tree in normal yield stimulation experiments). Even if such a procedure were practicable, it would be extremely cumbersome and time-consuming. Nor has any laboratory test method been devised which employs *Hevea* tissues and which gives a reliable estimate of the effect of a given compound on latex flow.

In these circumstances, it was decided to employ one of the conventional growth substance assays, the *Avena* coleoptile straight-growth test. It is known from a study of a wide range of synthetic compounds (BLACKMAN *et al.*, 1960), that activity in this test is reasonably well correlated with yield-stimulating ability. It is also well-suited to routine

laboratory use. The straight-growth test was preferred to the *Avena* curvature test employed by Chapman, partly because of the relative simplicity of the techniques employed and partly because a number of synthetic compounds which are highly active yield stimulants, such as 2,4–D, are inactive in the curvature test.

The procedure adopted was as follows: Oat seeds (variety 'Victory' from Svalof) were husked and soaked in water for two hours at room temperature. All subsequent operations were carried out in a dark room maintained at 25 ± 1°C. The seeds were sown, embryos uppermost, on several layers of water-saturated paper tissue contained in half glass bricks. The upper half of each glass brick was then placed in position, maintaining a suitable high humidity. For the first 48 hours the seeds were submitted to low intensity red light to suppress mesocotyl growth. Thereafter they were grown in darkness and handling operations were conducted under dim illumination from tungsten lamps filtered through Kodak OB yellow-green filters.

When the coleoptiles had reached some 2.5 cm in length (the time taken for this stage to be reached varied widely with seeds from different harvests and with different storage conditions), those of uniform length were selected and a 5 mm section cut 3 mm from the tip of each coleoptile with a suitable cutting device. The primary leaves were allowed to remain within the sections which were then floated for three hours on a dilute solution of manganese sulphate (1 mg $MnSO_4.H_2O$ per litre).

Ten sections were placed in a small covered culture dish containing one of the paper segments from the chromatogram and 1 ml of 2% sucrose solution adjusted to pH 5.0 with potassium phosphate/citrate buffer (10^{-2} *M* K_2HPO_4). Initially, 'blank' chromatograms were developed and bio-assayed for control purposes. Since the results so obtained did not differ significantly from those using only a single blank square of filter paper, the latter procedure was adopted for the sake of simplicity.

In addition to the control, standards of 0.1, 1.0 and 10.0 μg IAA were spotted on filter paper squares for bio-assay with each batch of chromatograms. It was not usually practicable to perform duplicate assays on the chromatograms, but wherever possible the controls and standards were replicated to give a measure of variation between dishes.

It was found that straighter growth was obtained by gentle agitation of the dishes on an oscillating table. After about twenty hours, the sections were removed from each dish in turn, blotted lightly, placed on a glass slide in a photographic enlarger and the length of the images measured at × 4 magnification using a transparent plastic ruler. The mean lengths were expressed as a percentage of the original section length and plotted as a histogram against R_f on the chromatogram. The control sections normally increased in length by some 40%. Significant increases over this control growth were attributed to the presence of growth-promoting substances.

Spray Reagents

The following chromogenic reagents were employed:

Modified Ehrlich's reagent. A 1% solution of *p*-dimethyl-amino-benzaldehyde in ethanol, mixed with an equal volume of concentrated hydrochloric acid immediately before use.

Ferric/perchloric reagent. Five per cent perchloric acid and 0.05*M* ferric chloride solution mixed in the proportions 50 : 1 by volume just prior to use.

Nitrite/nitric. Equal volumes of concentrated nitric acid and 0.5% aqueous sodium nitrite mixed immediately before spraying.

RESULTS

A typical histogram obtained from a chromatogram of the latex extract is shown in Figure 2. It will be seen that the principal active area lies between R_f 0.6 and R_f 0.9

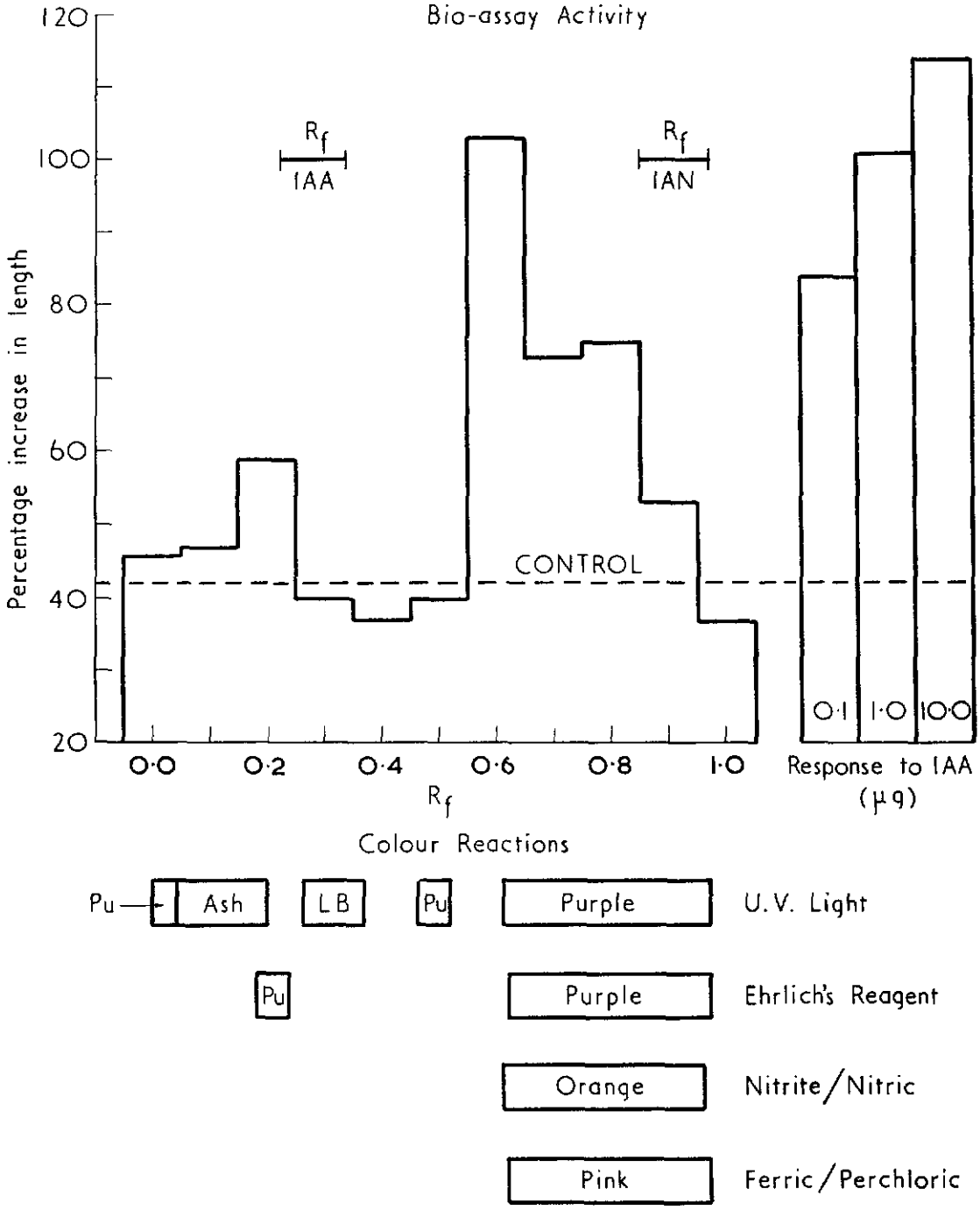

Figure 2. Histogram of bio-assay activity along the length of a chromatogram of a latex extract, compared with controls in sucrose-buffer alone and with the addition of known amounts of IAA. Colour reactions obtained on parallel strips of the chromatogram are also shown, drawn to the same R_f scale.

and that this is accompanied by strong colour reactions with indole reagents. These colour reactions differ from those of IAN (Ehrlich's ash, ferric/perchloric faint buff and nitrite/nitric dark blue) and other synthetic indole compounds of similar R_f (SEN AND LEOPOLD, 1954). In certain chromatograms there is a suggestion of a partial separation of the active zone into two, with peaks at R_f 0.6 and 0.8. It has also been noted that there is little correlation between the intensity of the chromogenic reactions in different chromatograms and the degree of bio-assay activity recorded. It is thus thought likely that we are dealing with at least two compounds in this region of the chromatograms. It is hoped that further investigations, employing other solvent systems, will provide more evidence on this point.

The slight activity found at about R_f 0.2 and associated with a purple Ehrlich's reaction, was thought at first to be due to a small amount of IAA (whose R_f in the standard spot is only slightly higher). However the colour reactions with Ehrlich's reagent are not identical, the unknown compound giving an immediate purple colouration as opposed to the blue colour formed by IAA on warming or standing. No colour reactions were observed with the other sprays until larger quantities were chromatographed, when a faint ash colour was obtained with ferric/perchloric and a yellowish colouration with the nitrite/nitric reagent. IAA (1 μg or less) gave a pink and a red colouration, respectively, with these two sprays under identical conditions.

In another experiment, synthetic IAA was added to latex before extraction (125 μg in 250 ml). The IAA was readily detected in the resulting chromatograms, giving typical colour reactions. A partial separation (as judged by their differing colour reactions) was achieved between the IAA and the naturally-occurring compound, the latter having a somewhat lower R_f.

It was therefore concluded that the unknown compound was not IAA and that the latter substance was not present in detectable amounts.

Some colour reactions were often noted in the initial streak (R_f 0.0) and these were occasionally accompanied by slight bio-assay activity.

DISCUSSION

The techniques described provide a comparatively simple method for the comparison of the growth substance content of latices from different sources. Work employing these methods to investigate growth substance levels in trees of varying yield capacity and following yield stimulation with synthetic compounds is in progress and will be reported elsewhere.

Like most methods employed for auxin estimation (BENTLEY, 1958), the work reported here was confined to ether-soluble substances. It is at present being supplemented by an investigation of ether-insoluble material, the results of which will also be described in a further publication.

The presence of IAA in *Hevea* leaves has been reported by BOLLE-JONES (1954) and it was naturally anticipated that this apparently ubiquitous plant auxin would also be found in latex. However, no trace of it has been detected in the latex samples examined to date, despite the use of a variety of extraction procedures. When IAA was artificially added to latex before extraction it was readily recovered and identified, thus confirming that the methods employed were adequate for its detection.

A few experiments, of a preliminary nature, also failed to detect IAA in extracts of *Hevea* leaves at various stages of development. While the possibility of the occurrence of free IAA in *Hevea* tissues cannot be ruled out, it does appear possible that IAA was liberated from other indole compounds during the extraction procedure employed by Bolle-Jones.

As will be seen from the review by Bentley, cited earlier, the absence of detectable amounts of IAA has already been reported for a number of plant tissues. It appears possible that IAA may not be the auxin

operative in certain tissues, at least in some stages of their development.

Similarly, there have been reports of the detection of unidentified growth-promoting substances, some giving indole-type reactions, from a wide range of plant tissues. When further knowledge has been accumulated, it would be of interest to observe whether the *Hevea* auxins can be equated with any of those isolated from other plants.

In conclusion, it should again be emphasised that the activity of these naturally-occurring compounds in the *Avena* coleoptile straight-growth test constitutes no proof of their activity on latex flow, despite the general correlation between the activity of synthetic compounds in the two systems. Reasonable certainty on this point could only be achieved by the isolation of very much larger amounts of material, permitting tests on the tree itself.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the encouragement and interest of Mr C. W. Brookson, Head of Botanical Division. Mr P. Balasubramaniam provided valuable technical assistance.

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