"Growth and Morphogenesis in Tissue Cultures of *Hevea brasiliensis*"

by

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This thesis is my own work unless otherwise acknowledged and has at no time been submitted for another degree

Martin Wilson

I certify that this statement is correct

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CONTENTS

GENERAL INTRODUCTION

Almost all the natural rubber of commerce is produced by the rubber tree, Hevea brasiliensis. The work described in this thesis is designed to provide the foundations for the application of the whole range of plant tissue culture techniques to the specific problems of quantitative, and perhaps qualitative, improvement in the production of rubber from Hevea brasiliensis.

Haberlandt (1902) was the first to consider the possibility of growth of higher plants from their constituent, living, somatic cells, and postulated that cultures of these cells would be capable of giving rise to in vitro embryos. Together with these prescient speculations, Haberlandt studied those cells of mature plants which retain the capacity for division, this becoming apparent during the phenomenon of "wound-healing." He went on to consider the presence of a "wound-hormone" (Haberlandt, 1921) which could chemically stimulate cell division, and the possible implication of this "wound-hormone" in the early stages of parthenocarpy (Haberlandt, 1921a) and adventive embryogeny (Haberlandt, 1921b, 1922) from these dividing cells. The philosophy that a complete knowledge of the nutritional requirements of cells and their responses to stimuli, permits the selection of conditions which will cause them to recapitulate in vitro their behaviour in vivo, stems from Haberlandt's original studies.

Gautheret (1939), using segments of the roots of Daucus carota established the first callus cultures of potentially

unlimited and undifferentiated growth. The formation of callus is initially a response to wounding, with cells in the vicinity of the wound becoming active meristematically, proliferating, and giving rise to a mass of undifferentiated tissue. Plant organs which do not form callus after wounding can be induced to do so by chemical stimulation, using such compounds as natural and synthetic auxins and cytokinins. After excision of the parent plant material the growth of callus tissue may be prolonged indefinitely on a suitable culture medium. The parent explant may stamp its own distinct, physiological features on the derived tissue as, for example, callus cultures derived from either adult or juvenile phases of Hedera helix (Stoutemeyer and Britt, 1965), while the gross morphlogical appearance of callus tissue initiated from various regions of a plant may be uniform (Barker, 1969).

Medium selection is the major problem in the establishment of a new tissue in culture. As well as satisfying the criteria of establishment it is also desirable, from an experimental viewpoint, that the rate of growth be above a certain lower limit. Since the pioneer work of Gautheret (1939) and others, the difficulties of medium selection have been eased by the development of basal media which have proved satisfactory, with only minor modifications, for a variety of plant tissues. Useful have been those developed by Heller (1953), White (1963), Gautheret (1959) and particularly Murashige and Skoog (1962). Some studies aimed at the production of a medium with maximum versatility have been carried out by Schenk and Hildebrandt (1972), and when

more work of this kind has been undertaken the problems of introducing new tissues into culture will be greatly diminished.

However, the requirements for growth of different plant species are inevitably various and so it is not to be expected that any one basal nutrient medium could support growth of all cultured plant tissues at a satisfactory level for experimental purposes. More realistically, the ideal basal medium would be one which supports some growth of the majority of plant species and tissues. The rate of growth could then be improved by modifying the balance and levels of plant growth regulators in the medium, together with altering the temperature and light regime.

Many plant tissue culture media incorporate natural products such as coconut milk, yeast extract and casein hydrolysate which are incompletely characterised. This situation is unsatisfactory in view of the fact that when the constituents of a medium are exactly defined it is possible to monitor changes that occur in media as a consequence of tissue growth and thus to identify precisely the factors which limit the modes and extent of this growth. The effect of coconut milk was first noted by Caplin and Steward (1948) when an eighty-fold increase in the fresh weight of callus proliferating from small explants of the secondary phloem of carrot root was obtained. Subsequently much work has been carried out by Steward and others (Shantz and Steward 1952, 1955) in attempts to characterise the constituents of coconut milk using carrot callus as a bioassay system for synthetic

mixtures, but to date none has exactly reproduced the extent of the stimulatory capacity of coconut milk. Coconut milk has a wide range of effects on growth and differentiation (Kent and Steward 1965) and so complete identification of the substances contained in this natural product will be of value to aid an understanding of the relationship between media constituents and the tissues cultured on them. Some of the important classes of constituent compounds of coconut milk, for example, cytokinins, sugar alcohols and reduced nitrogen, have been identified (Steward, Mapes and Ammirato, 1969). The addition to the culture medium of synthetic mixtures based on these constituents obviates the necessity for the presence of coconut milk in the medium to obtain rapid growth of cultured tissues and the induction of high levels of embryogenesis in carrot, without wholly recapitulating the extent of these responses.

Data relating to the rate of uptake of nutrients from semi-solid media into callus cultures are rare for the following reasons. Such a study is technically arduous and the value of the data obtained to further work would be severely limited, since in all culture systems where the nutrients are supplied in batches control of nutrient levels can only be exercised through the initial concentrations administered. Nutrient studies of this nature are best made using a continuous culture system, such as that developed by Wilson, King and Street (1971) for cell suspensions of Acer pseudoplatanus. Here, it is possible to change the levels of

individual nutrients during growth and to monitor the effects of these changes on the cells in culture.

Steward et al (1969) observe that the need for a simultaneous supply of all the essential nutrients in the same proportions throughout growth is commonly presumed, but that there is no evidence that this situation obtains. Some data suggest the opposite, for example in cell suspensions of Acer pseudoplatanus the calcium/phosphorous ion ratio rises as growth of the cells proceeds (King, 1973). Considerations of these more subtle cell to media relationships may have to be taken into account in order to rationalise the lower growth rates achieved by tissue cultures in comparison with the meristematic regions of the plant from which they originate. For example, the duration of the mitotic cycle in cultured cells of Happlopappus gracilis is 22 hours (Eriksson, 1967) but only 11 hours in root tip meristems (Sparvoli, Gay and Kaufmann, 1966). Preliminary observations suggest a similar situation in cultured cells and root tip meristems of Daucus carota (Bayliss, unpublished).

Cytological instability is a feature of plant tissue cultures (Sunderland 1973), and although polyploid cells may originate from the parental explant tissues (Torrey 1965), the range of chromosome number including aneuploids in established cultures suggests that instability can arise as a consequence of culture conditions (Kao, Miller, Gamborg and Harvey 1970). Torrey (1965) found that by varying the concentrations of auxin and cytokinin it was possible to stimulate preferentially diploid, or both diploid and tetraploid cells

to divide and thus affect the final numbers of diploid and tetraploid cells in cultures of Pisum sativum. The cytological stability of tissues in culture may also be affected by environmental changes. Demoise and Partanen (1969) subcultured callus of Paeonia suffructicosa both in liquid and on semi-solid medium with identical constituents and found that on semi-solid medium there were lower mitotic indices and initially lower percentages of polyploid cells, indicating that the physical state of the medium exerts an effect on the rate of production of polyploids.

Gupta and Carlson (1972) claim that it is possible to maintain stable cultures of haploid cells and preferentially to select haploid cells from mixed populations of cells of varying ploidy. This claim is based on an observation that 9 μg/ml of parafluorophenylalanine added to standard tobacco medium (Linsmaier and Skoog, 1965) allows for a 15-fold increase of haploid Nicotiana tabacum callus with only a 1.5-fold increase for diploid callus tissue. Carlson, Smith and Doering (1972) working with Nicotiana glauca and N. langsdorffii and the amphiploid hybrid of these species were able selectively to regenerate callus from protoplasts of the hybrid by using the protoplast culture medium of Nagata and Takebe (1971). Furthermore the amphiploid callus was unique in its ability to grow on the medium of Linsmaier and Skoog (1965) in the absence of exogenous hormones. In this study the culture medium served as an agent of selection and as such played a different role from, for example, the

one adopted in the stimulation of morphogenesis.

An increase in the growth of cultured tissue has frequently been observed in liquid medium (Caplin and Steward, 1949; Venketeswaran, 1962; Demoise and Partanen, 1969). Some of the factors underlying this enhancement of growth may include a more uniform exposure of the cells to nutrients, with continuous agitation facilitating greater concentration gradients both for the diffusion of nutrients into the cells and for the removal of metabolic wastes which could be inhibitory to growth. Cultures of plant cells as suspensions have been obtained by transferring loosely packed cell masses usually described as "friable" callus to agitated liquid medium. The earliest suspensions of cultured plant cells capable of repeated subculture, those of Nicotiana tabacum and Tagetes erecta, were developed by Muir (1953). Following this early work it became clear that success in the establishment of a cell suspension is largely dependent on the friability of the callus inoculum. The factors influencing the friability of a callus were the object of a detailed study by Torrey and Shigemura (1957). Here, friability was promoted when auxin was removed from liquid medium or when the concentration of yeast extract was increased. It was hypothesised that friability developed from enhanced activity of an enzyme solubilising pectic substances in plant cell walls.

Various other medium modifications have been found to promote cell dispersion from comparatively compact callus masses in the initiation of suspension cultures. In contrast

to the findings of Torrey and Shigemura (1957) a high auxin concentration promoted cell separation in the work of Bergmann (1960), and Torrey and Reinert (1961). Also promotive were the effects of an appropriate balance between auxin and kinetin (Earle and Torrey, 1963) and between auxin and coconut milk (Lamport and Northcote, 1960). Street, King and Mansfield (1971) found that addition of a pectinase combined with a cellulase markedly increased the level of dispersion in cell suspension cultures of Acerpseudoplatanus, supporting in some respects the hypothesis advanced by Torrey and Shigemura (1957). Mansfield (1973) has also shown that cell suspensions initiated from inocula comprised largely of single cells had a high growth rate and that aggregation during the stages of active cell division was not a prerequisite for growth. This is in contrast to the hypothesis of Torrey, Reinert and Merket (1962), and Street and Henshaw (1963) that the potentiality for growth resides mainly in the aggregated fraction of plant cell suspension cultures. The higher frequency of mitotic figures in aggregates provided the basis for this hypothesis. The results of Street and Henshaw (1963) suggest that cells in aggregates start active division earlier than those freely suspended in the medium, and thus make a greater contribution to the growth of these cultures. Mansfields results (1973) indicate that in the absence of aggregates and from comparatively high inoculum densities, suspensions of predominantly free cells may achieve similar growth rates to those measured for less highly dispersed cultures. Aggregation in suspension cultures is a major obstacle in the use of these cultures for physiological and genetical

investigations and in view of this the findings of Mansfield (1973) are very encouraging.

Thomas (1970) has discussed the possibility that a switch in the degree of dispersion in cell suspensions from fine to aggregated is associated with a changing physiology of the tissue related to morphogenetic expression. Suspension culture of plant cells provides an attractive system for the study of morphogenesis, but since the first *in vitro* plant tissues were grown on semi-solid medium, most early studies in morphogenesis involved the use of callus cultures. In 1939 White found that callus tissue from the hybrid *Nicotiana glauca* X *N. langsdorffii* did not differentiate when grown on semi-solid medium but formed buds when submerged in a liquid medium. Following on from this observation came the pioneer work of Skoog and others (1944, 1954a, 1954b, 1955, 1956, 1957) which eventually resulted in the recognition of 6-furfurylaminopurine or "kinetin" as representative of a new group of cell division promoters given the general name of kinins or cytokinins. It was found that by altering the ratios of IAA and kinetin in the culture medium, tobacco pith tissue could be induced to form buds, undifferentiated callus or roots. Skoog and Miller (1957) propounded the view that quantitative relationships between auxins and kinins, and perhaps other factors such as certain amino acids, may provide a common mechanism for the regulation of all types of plant growth from cell enlargement to cell division to organ formation. On this hypothesis it would not appear necessary to postulate the presence of unique compounds essential for morphogenesis. However, the general validity

of this hypothesis remains in question since similar variations in the auxin:kinin ratios and the availability of reduced nitrogen fail to induce organogenesis in many plant tissues which display no regenerative capacity in culture.

Root formation has often been observed in tissues cultured *in vitro* (Buvat 1944), but this phenomenon disappears in the course of subsequent subcultures, with auxin apparently incapable of maintaining rhizogenesis (Delarge 1942, Gautheret 1955b). Jacquiot (1955a, 1955b) working with cambium tissue cultures of *Ulmus*, *Betula* and *Tilia* found that these tissues were unable to produce roots from the initial explants and early subcultures, but acquired this capability in later passages. The former data have been interpreted as due to the exhaustion of a root formation factor and the latter as due to the loss of an inhibitor of root formation (Dore, 1965). Generally, however, reports of root formation from tissue cultures have proved variable, thus restricting their potential use for the identification of any specific rhizogenetic factors which may be involved.

The *in vitro* formation of embryo-like structures was first demonstrated using callus cultures of *Daucus carota* (Steward, 1958; Reinert, 1959) but the steps leading to the production of these structures are still the subject of controversy. Steward (1964, 1970), from his work with *Daucus carota* concluded that expression of totipotence occurs only when cells are grown free and in a medium which closely resembles the natural environment of the carrot embryo in the embryo sac. Reinert (1970), on the other hand, considers

that the first steps in the development of embryos from isolated single cells of carrot are not closely similar to those displayed by an *in vivo* egg cell following fertilisation. He was able to observe typical stages of embryo development only after a number of cells had been formed by divisions of the original isolated mother cell, which suggests that these cells undergo a simultaneous reorientation perhaps under a common polarising influence. Abnormality in development is conspicuously frequent when plant embryos are cultured in nutrient media (Raghaven and Torrey 1963). Thus, the essence of the dilemma is whether abnormality is fostered by cultural conditions and obscures normal patterns of development, or alternatively, unorganised cell growth and division have normal pathways of embryogenesis superimposed upon them through polarisation processes promoted by *in vitro* factors. A compromise solution would entail only certain cell masses being capable of responding to polarising influences with these cell masses in turn derived from isolated totipotent mother cells. Wardlaw (1965) points out that *in vivo* many individual embryos, both of dicotyledonous and monocotyledonous plants, may show "anomalous" segmentation patterns especially in the early stages and yet still give rise to fully formed embryos of entirely normal morphology. McWilliam, Smith and Street (1973) have demonstrated the *in vitro* formation of carrot embryos of normal morphology and showed that the early segmentations do not correspond with those described for zygotic embryogeny in this species by Borthwick (1931). Street (1973)

raises the question of whether, in embryogeny, the cell pattern is to be regarded as secondary to integrated polarised growth and development, and as such subject to quite drastic modification by environmental factors which do not impair the origin of a normal embryo.

While this remains in the precinct of speculation, the question of what substances induce the formation of embryo-like structures in cell cultures can be approached through experiment. Steward (1958) considered that coconut milk may have unique properties in this respect basing this proposal on the analogous relationships of cultured carrot cells to coconut milk, and a fertilised egg to its endosperm. However, since carrot cultures have been shown to produce embryo-like structures on any basal medium which supports growth (Halperin and Wetherell 1964), there seems to be no evidence of a unique relationship between coconut milk and cultured carrot cells.

As well as coconut milk (Kent and Steward, 1965), yeast extract (Kato and Takeuchi, 1966) has been shown to induce embryo development from cultures of hypocotyl segments of carrot, but most recent studies have concentrated on the inorganic constituents of culture media. Ammonium ions (Halperin and Wetherell, 1965) were shown to enhance the induction of embryogenesis in wild carrot cultures. Tazawa and Reinert (1969) described this effect more exactly, demonstrating that the occurrence of ammonium in the medium was not a prerequisite for embryo formation <u>in vitro</u> but rather that a certain level of intracellular ammonium is involved. Some media variations which were designed to investigate embryogenesis by controlling the form and

availability of the nitrogen supply were found to stimulate the formation of roots (Reinert 1963, Halperin 1966). Thomas (1970) presented data supporting the view that ammonium ions are required for the *in vitro* formation of embryo-like structures in *Atropa belladonna*. The concentrations of ammonium sulphate in the culture medium appeared to determine whether the morphogenetic ability of the cells was expressed, and if so whether embryogenetically, or in the formation of roots. High salt concentrations (Butenko 1967) and certain cation levels (Reinert, Tazawa and Semenoff, 1967) have also been implicated. Braun and Wood (1961, 1962) had previously suggested that ions may be involved in activation of hormone biosynthetic systems. Reinert (1959) showed that removal of auxin from the culture medium resulted in the formation of embryo-like structures, while Halperin and Wetherell (1964) showed that low auxin levels (10^{-7} g/ml 2,4-D) could induce embryogenesis in wild carrot cell cultures.

As evinced by the literature referred to here, the study of embryogenesis in tissue culture has been most intensively examined in carrot, mainly because it was the first species to display embryogenic capacity *in vitro*. The hypocotyl epidermal cells of plantlets derived from callus cultures of *Ranunculus sceleratus* (Konar and Nataraja, 1965) offer the clearest example of embryo formation from a specific cell type, while recently embryogenesis in cell cultures of *Atropa belladonna* has been traced to origin from single cells in cell aggregates or from cells of the cultured embryo suspensors (Konar, Thomas and Street, 1972).

Tissue cultures of many plant species remain recalcitrant

when exposed to conditions designed to promote morphogenesis. Woody plants, monocotyledonous plants and plants which are both woody and monocotyledonous appear particularly difficult to establish in culture, while the induction of morphogenesis in these plants is rarely reported. The need for more complex in vitro requirements may be responsible, but this was not found to be the case for Asparagus officianalis where embryogenesis was successfully induced by Wilmar and Hellendoorn (1968). Here, a comparison of the differences between the factors inducing embryogenesis from cultures of Asparagus officianalis, (a monocotyledon) and Daucus carota (a dicotyledon) revealed that these differences were largely of a quantitative nature.

The formation of embryos from cell and callus cultures of plant tissues is of great significance in the study of embryology and in the propagation of certain economic plants such as the Oil Palm. The observation of Guha and Maheshwari (1964) that pollen grains of Datura innoxia could be induced to form embryos by culturing them undisturbed within the anther, and that haploid plants resulted (Guha and Maheshwari 1967), heralds a new era in plant breeding and allied mutation research. Callus and cell cultures initiated from such haploid plants offer unique opportunities for genetical research in higher plants. Haploid plants have been produced by anther culture from a number of plant species including Atropa belladonna (Zenkteler, 1971), Nicotiana tabacum (Sunderland and Wicks, 1969), and Oryza sativa (Niizchi and Oono, 1968). Recently, isolated pollen grains of Lycopersicon pimpinellifolium (Debergh and Nitsch, 1973) have been induced to form haploid embryos suggesting that it may soon be

feasible to apply refined mutation and selection techniques to the earliest cell divisions involved in haploid embryogenesis.

There are few data available relating to the morphogenetic abilities of haploid cell and callus cultures, but from a genetical standpoint, at least some of the haploid cells derived from a diploid tissue which undergoes morphogenesis must also show this ability. Thus it should be possible to exploit techniques which have been developed for the induction of morphogenesis in diploid tissues for haploid material, and in doing so, perhaps to gain insight on the diploid status of plant species.

In the production of a haploid plant, a haploid cell (the pollen grain) is preferentially stimulated to divide. Torrey first reported the preferential mitotic stimulation of polyploid cells of cultured pea root segments in 1961. Johri and Nag (in press) have shown that the nutritional requirements for callusing and organogenesis in the diploid embryo and triploid endosperm tissues of Dendrophthoe falcata and Taxillus vestitus were markedly different initially, but that after repeated subculture no differences were detectable. In this work, and that of Torrey (1961) endogenous factors may be implicated, but these are less relevant to the preferential stimulation of haploid callus growth reported in Nicotiana tabacum by Carlson (1972).

It is commonly observed that prolonged maintenance of a tissue culture which initially readily undergoes morphogenesis, brings about an apparent diminution in competence (Syōno, 1965). Sussex and Frei (1968) showed that a 12 year-old carrot callus had retained the ability to produce embryo-

like structures, but this observation is exceptional. In cultured tissues growth rate, nutrient requirements, friability and chromosome constitution are all factors which may change temporally and thus any one of these may be involved in an apparent loss of morphogenetic ability (Murashige and Nakano 1965, 1967. Torrey, 1967). Aneuploid and polyploid cells are frequently found in cultured tissues, and a relationship between their occurrence and a loss of morphognetic ability is suggested by some data (Muir, 1963). However, the low polyploid condition does occur in cultures which readily undergo organogenesis (Blakely, Jennings and Turner, 1968). Torrey (1967), and Murashige and Nakano (1967) consider genomic imbalance due to aneuploidy or high levels of polyploidy to be the basis of the inability of some cultures to form organs. While polyploidy is frequently observed in cultured tissues, Street (1969) points to the analogous situation of enopolyploid cells found in differentiated tissues of higher plants and it may be that some polyploid cells in cultures have arisen due to the retention of _in vivo_ differentiation patterns.

It is clear that if cells in culture are totipotent (Steward, Kent and Mapes, 1966) then any feature of the whole plant may be expressed in appropriate culture conditions and there is every reason to suppose, for example, that the synthetic abilities of differentiated plant tissues can be recapitulated _in vitro_. It is of major technological and biological interest to establish whether chemical differentiation can be induced in culture in the absence of morphogenetic expression. If systems of this nature could be developed then

it is possible to envisage that commercially and pharmaceutically important plant cells could be exploited in the same way as micro oranisms. The synthetic capacities of plants are usually localised in special plant organs. For example, the accumulation of urease in the cotyledons of the jack bean (Canavalia ensiformis), or oil production by the secretory hairs which occur in Mentha and other Labiatae. Alkaloid biosynthesis is not considered to be localised in specific organs but appears to be a characteristic of all organs, although it should be mentioned that not all organs of any one species display such a function. Specialised tissue systems such as laticifers are associated with their own peculiar biochemistry as evinced in the production of opium and rubber.

Progress in inducing plant cell and tissue cultures to simulate whole plant biosynthetic pathways has been slow. There are many examples of cultured tissues showing little or none of the desired metabolite production when compared with that of the whole plant. Cultured cotyledonary tissue of jack bean, for instance, showed no urease activity when compared with intact seed (Krikorian and Steward, 1969). West and Mika (1957), showed that atropine synthesis took place in Atropa belladonna callus tissue which bore roots, but Bhandary, Collin, Thomas and Street (1969) showed that atropine was not synthesised in the absence of root formation. Peppermint tissue cultures (Mentha piperita) and those of Spearmint (M. spicata) are unable to synthesise oils either spontaneously, or when fed appropriate precursors (Krikorian, 1965, Stohs and Staba, 1965). It was concluded that the

metabolic pathways leading to the essential oils are linked to the environment *in situ* and to the development of the oil secreting glands which do not form in cultures (Krikorian and Steward, 1969).

Perreault (1950) and Snyder (1950, 1955) unsuccessfully attempted to use tissue cultures of laticiferous plants to investigate their potentialities as rubber producing systems. Snyder encountered difficulty in establishing cultures of laticiferous plants and Krikorian and Steward (1969) anticipate that laticiferous systems will present special difficulties under culture conditions due to their complexity. Clearly, conditions have yet to be established which will permit the recapitulation of metabolic pathways required for the synthesis of pharmaceutically and commercially important compounds *in vitro*. Indeed it may be that synthesis of comparable amounts of the desired products cannot be achieved outside the framework of the whole plant.

There are three main objectives in the application of plant tissue culture techniques to the commercial exploitation of the rubber plant, *Hevea brasiliensis*. One, to improve the uniformity of propagants by replacing grafting of clonal shoots to seedling roots with clonal plants of embryonal origin; two, to produce mutant plants with increased rubber yields; and three, to study the physiological and biochemical factors affecting laticifer differentiation and latex production.

SECTION 1

MATERIALS AND METHODS

1. General

Glassware for use in medium preparation and aseptic transfers was cleaned by techniques described by Street and Henshaw (1966). "Analar" grade chemicals and double distilled water were used in culture media and the desired pH achieved by addition of 0.1 N.HCl or 0.1 N.NaOH prior to sterilisation. Culture vessels and media were sterilised by autoclaving at 121° (15 psi approximate pressure) for 15 minutes. Solutions of thermolabile constituents were sterilised by passage through an appropriate Millipore filter. General laboratory facilities and procedures for aseptic manipulations are fully described by Street (1973).

2. Sources of Explant Material

Seeds were obtained from the Rubber Research Institute of Malaya. These were the products of random pollinations and thus were genetically variable. The seeds arrived coated with a light dusting of sulphur powder which was removed by washing with water prior to germination. To germinate the seeds they were placed ridged-side-downwards (PLATE 1), half-buried in moist peat or horticultural vermiculite. The seeds were sown in large numbers in trays with subsequent transfer of the germinated seedlings to pots. Viability is retained for about two months in the freshest seed and in practice is very variable, ranging from 10-85% depending upon the age of the seeds when received from the plantations.

A temperature of 25°-27° or above is necessary for greenhouse cultivation and it is preferable that the air is humid. A twelve hour daylength is required for winter cultivation in temperate climates. The young seedlings were cultivated in peat, with applications of liquid fertiliser.

For the sterile germination of seeds the hard testa was removed and the seeds soaked in 1% w/v bromine water, with a trace of detergent to ensure adequate "wetting", for five minutes. This was followed by 5 washes in sterile distilled water. 250 ml Erlenmeyer flasks filled to a depth of approximately 2" with horticultural vermiculite which had been moistened with water, were autoclaved for 40 minutes at an approximate pressure of 15 psi. After allowing the flasks to cool, three sterilised seeds were placed in each flask, with 3-4 day-old seedlings harvested from such flasks being dissected and used as a source of sterile explant material.

Young plants germinated under non-sterile conditions and within the age range of 2-18 months were the most commonly used source of explant material. The stems of these plants were firstly washed down with industrial methylated spirits and then cut into segments of approximately one inch in length. After soaking in 1-2% sodium hypochlorite solution for five minutes, each piece was placed in a sterile plastic Petri dish and bisected longitudinally. The cortex was removed from each half in an attempt to expose the cambial regions. It was found that in older stem pieces (those nearest the base of the plant) the majority of the

cambial region was removed with the coftex, whilst in the younger regions of the stem it proved impossible to cleanly remove the cortex with forceps and scalpel and some cambial activity was retained by the segment core. (PLATES 1 and 2). Bisected petiole segments were also used as explant material.

3. Conditions of Culture

a) Semi-solid medium

All agar medium was solidified with 0.7% w/v Ionagar no. 2. 100 ml or 250 ml Erlenmeyer flasks with 25 ml or 50 ml aliquots of medium respectively, were routinely used. For some callus initiation experiments 28 ml screw-capped vials with 8 ml of medium were preferred.

b) Liquid medium

Routine maintenance of cell suspension cultures involved the inoculation of 5 or 10 ml aliquots of stock culture into 50 ml fresh medium in 250 ml Erlenmeyer flasks. Agitation was achieved using a Gallenkamp orital shaker set at 120 rpm.

For both liquid and semi-solid medium the flasks were autoclaved stoppered with cotton wool bungs and covered with an aluminium dust cap. The bungs were replaced after inoculation with a sheet of sterile aluminium foil which was pressed into close contact with the neck and rim of the flask.

4. Assessment of Growth

a) Callus

Growth was monitored by measuring fresh and dry weight. The fresh weight of the inoculum obtained by weighing the flask, medium and cotton wool bung immediately pre- and post-inoculation. Subsequently the culture was terminated by removal of the callus from the surface of the agar and

Explanation of PLATE 1

The source of parental explants

a Young plant within the age range of 2-18 months.

b Seeds of *Hevea brasiliensis* (r = ridged side upwards).

c Transverse section through the stem of an 18-month old plant, approximately 4 inches below the apical bud.

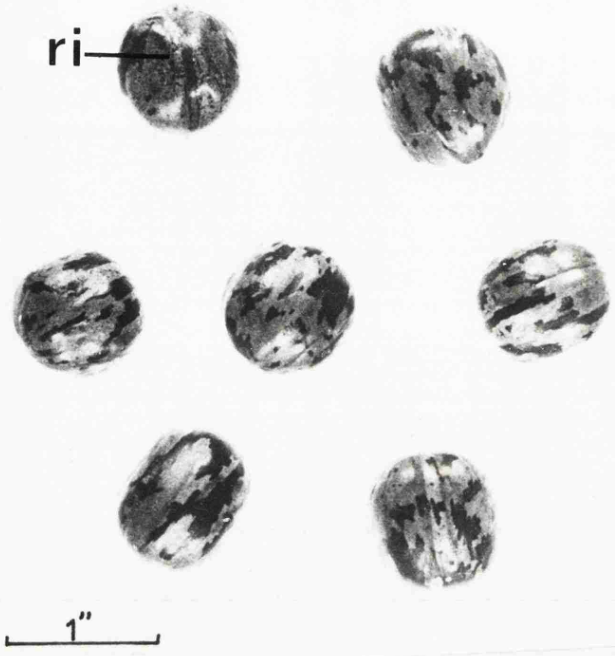
d Transverse section through the basal regions of the stem of an 18 month-old plant.

In c and d the cambial regions (Ca) are indicated by arrows and it is from these regions that callus initiation occurs.

PLATE 1

a

b



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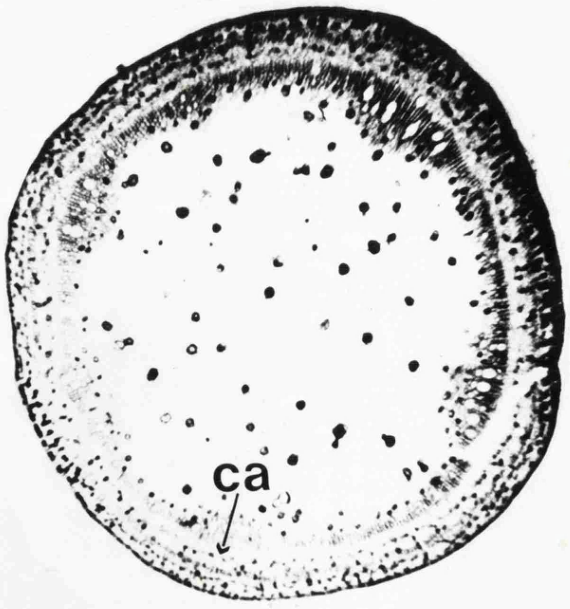

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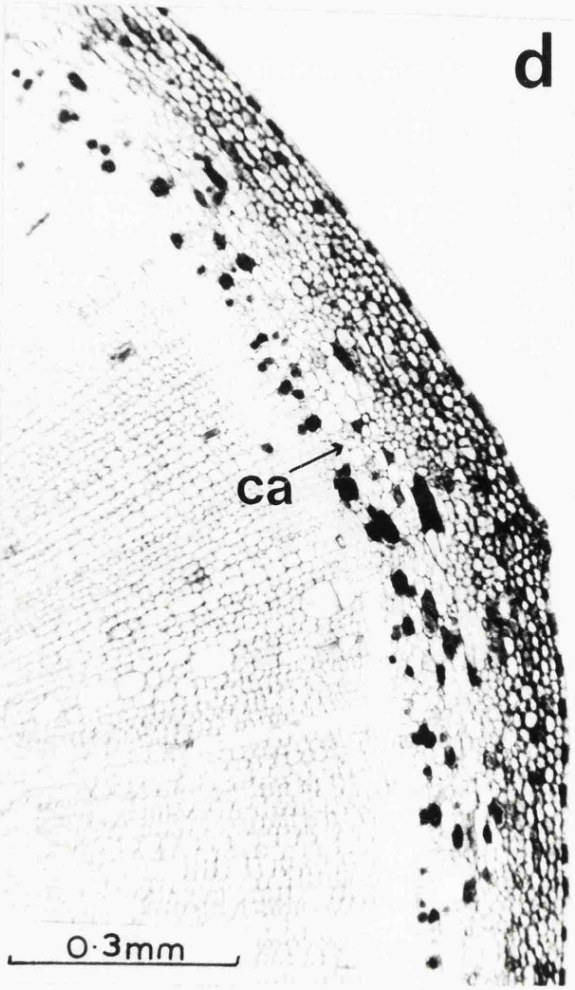


Explanation of PLATE 2

The initiation and first passage of callus cultures

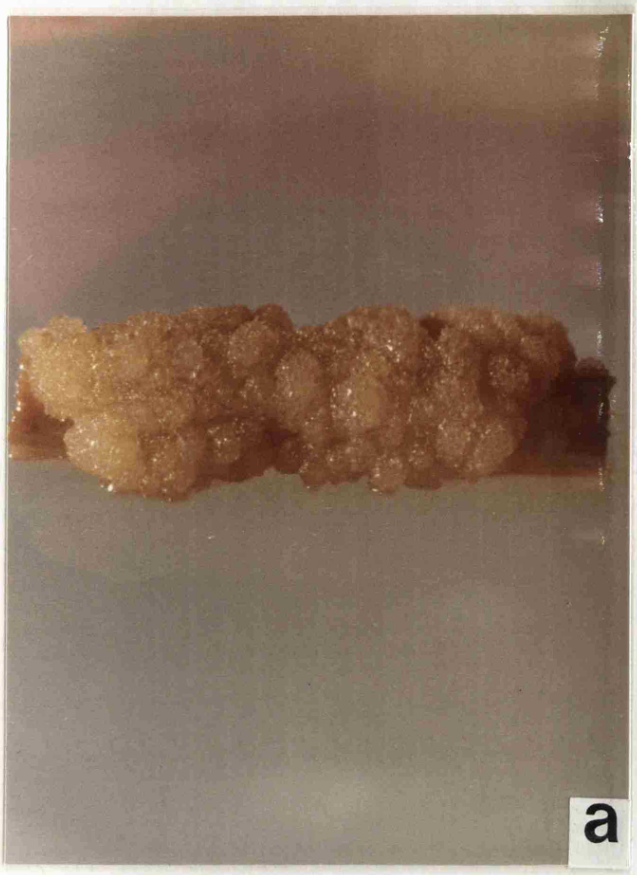
a The initiation of callus from the woody core of stem segments obtained from the apical ends of 2-18 month-old plants.

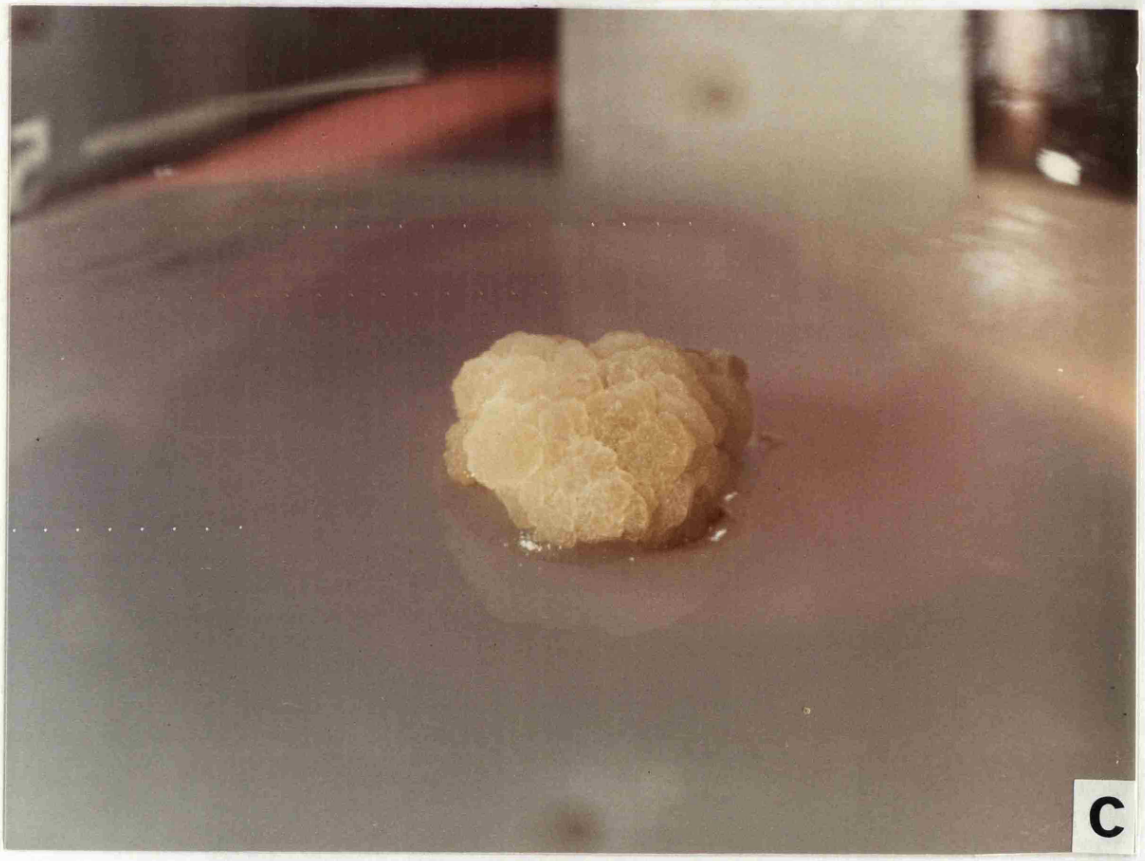
b The initiation of callus from cortical tissue removed by sterile dissection from the circumference of stem segments obtained from the basal regions of 2-18 month-old plants.

c First passage callus tissue. The morphological heterogeneity which is a feature of the callus growth in subsequent passages (PLATE 4) is not apparent in the first passage.

(The one inch scale is a reference for a, b and c)

PLATE 2





1"

weighed on a sheet of preweighed aluminium foil. Dry weights were obtained by placing the aluminium foil and callus in an oven set at 80° for two days, cooling in a desiccator and reweighing to constant weight.

b) Cell suspensions

i) Cell number: the technique used for determining the cell number per ml of the suspension culture was a modification of that described by Henshaw, Jha, Mehta, Shakeshaft and Street (1966). 3 ml of culture was suspended in 6 ml of 10% w/v chromium trioxide solution in a 28 ml screw capped vial. The vial was then immersed for up to 20 minutes at 70° in a water bath, removed and shaken violently using a flask shaker (Baird and Tatlock Ltd.) for up to 15 minutes. The suspensions so prepared were suitably diluted with water and six representative samples from each suspension were transferred to two counting slides using a Pasteur pipette (Henshaw et al 1966). After allowing the cells to settle on the counting slide, sixty random fields were counted (10 fields/sample) at a X100 magnification on a Watson 70 microscope. The cells treated in this way were observed either singly or in small groups of up to four in number. They had contracted, stained cell contents which facilitated individual identification. From the depth of the slide well and the diameter of the field of view, the field volume was calculated to be 0.8 μl. Samples were diluted so that not more than 20 cells were present per field. An estimation of the number of cells per ml of culture was made from the number of cells in one representative sample. The mean and standard error were calculated for the six samples, with the criterion of accept-

ability being satisfied when the standard error was below 10% of the mean value. Cell numbers are expressed as cells per ml x 10^{-6} of culture.

ii) Packed Cell Volume (P.C.V.): a sample of suspension culture (10 ml) was centrifuged in a graduated glass centrifuge tube at 7000 x g for 1 minute. The volume of the cell pellet was expressed as a percentage of the sample volume.

iii) Dry weight: 5 ml of suspension culture was filtered under vacuum through a glass-fibre filter disc, (Whatman GF/C; 25 mm dia., c.a. 25 mg dried weight) washed three times with 5 ml of distilled water and dried in an oven at 80^{o} for 24 hours. The disc was cooled inside a desiccator and reweighed to constant weight.

iv) Estimate of dispersion: Following the work of Mansfield (1973) a quantitative estimate of cell dispersion was made for suspension cultures of Hevea brasiliensis. Samples for the counts of the number of cells in aggregates of 10 or less were made by filtering 2 ml of culture through a 205μ Nybolt gauze, thoroughly washing with 3% sucrose solution and then making up the filtrate plus washings to a known volume with 3% sucrose solution. The filtration removed many of the large aggregates and the counts of cells in aggregates of ten or less were made using the counting technique previously described. These counts were then expressed as a percentage of the total cell number/ml of culture.

5. Histological Techniques

All materials were routinely fixed in 1% chrome acetic acid for 24 hours, except when stained with 1% osmium

tetroxide when this fixation stage was omitted. The fixed tissue was then washed with water for 24 hours, dehydrated in an ethanol series, cleared in xylene and embedded in paraffin wax (M.pt.58°). Sections were cut using a Cambridge Rocking Microtome at a thickness of 10 μ. Sections were stained either with a total lipid stain Nile blue. (Cain 1947) and mounted in a water mounting medium. (G. T. Gurr Ltd., London) or with the general stain Toluidine blue and mounted in "Euparal". The former stain was used since it aids in the identification of latex vessels as does 1% osmium tetroxide (a stain for unsaturated lipids, Cain 1950) which was used both as a fixative and a stain for some sections.

6. Cytological Techniques

a) Chromosome counts

i) Callus: the tissue was pretreated with 0.05% colchicine solution for two hours before fixing in freshly prepared 3:1 absolute alcohol:glacial acetic acid. The chromosomes were stained by the feulgen squash method (Darlington and La Cour, 1962). The optimum hydrolysis time in N.HCl at 60°C was found to be between 10 and 12 minutes. After staining in leuco-basic fuchsin, the tissue was macerated and squashed in 45% acetic acid. When a permanent preparation was made the slide was placed on cardice (Conger and Fairchild, 1953) frozen, and then placed in absolute alcohol, 1:1 absolute alcohol:xylene and finally, xylene, each for five minutes. The preparations were mounted in "Xam" (G. T. Gurr Ltd., London).

ii) Cell suspension: as above except that centrifugation was required to separate the cells from the solutions

before transference.

b) Feulgen cytophotometry. The slides were prepared as above except that no pretreatment was performed and the tissue was washed in SO_2 water for ten minutes to clear it of extra-nuclear leuco-basic fuchsin stain. Maceration and squashing was carried out in 25% acetic acid. The machine used was a Vickers M 85 integrating microdensitometer which incorporates a scanning device that minimises distributional error; extinction is summated as the scanning progresses so that a direct measurement of total absorption is provided in arbitrary units for a single nucleus. The absorptions of 200 randomly chosen interphase nuclei were measured for each tissue at a wavelength of 562 mμ . All measurements are in arbitrary units.

7. Electron Microscopy

For the sections of callus tissue, 1 x 2 mm pieces were cut and immersed in 2% osmium tetroxide in veronal-acetate buffer pH 7.4 for two hours. Dehydration after washing was by means of a graded alcohol series followed by impregnation with Araldite (Glauert and Glauert, 1958). Ultra thin sections ranging from about 300 Å-800 Å in thickness (grey or silver interference colours) were cut from the blocks on a Siroflex ultra-microtome using glass knives and then supported on carbon films mounted on copper specimen grids. The electron microscope used was a Siemens Elmiskop Mk. 1.

8. Amino Acid Analysis of Liquid Medium

a) Sample preparation

Lignin and hemicellulose which are found in small amounts in a "conditioned" medium (H. A. Collin unpublished observa-

tions) would be deposited on the exchange resins used in this analysis with resultant resin malfunction and thus must be removed from the medium to be analysed. 200 ml aliquots of the medium were freed from such colloidal material by the addition of ethanol to 80 per cent (Tulecke and Rutner, 1965) with removal of the resulting precipitate by filtration. Amino acids are soluble in this concentration of ethanol and a high recovery is achieved by ethanol extraction (Boulter 1966). The ethanol was removed by rotary vacuum evaporation at a temperature of 37^{o} and the resulting residue made up to 1 litre in distilled water. This dilution of the original 200 ml medium aliquot up to 1 litre aids adsorption of amino acids on to the column used for desalting. The desalting procedure was as described by Melhuish (1962). The column resin used was Zeocarb 225 ($[H^{+}]$ form-dimensions 20 x 1 cm). After washing with distilled water and checking for neutrality, the column was charged with 200 ml 2N.HCl and washed with 5 litres of de-ionised water. The 1 litre diluted sample was applied to the column and the resultant effluent discarded. Another 500 ml of de-ionised water was then applied to the column to remove any remaining anions. Elution of the column was achieved by the application of 200 ml of $4N.NH_3$, followed by a further 25 ml to ensure that elution was complete. The ammonia was evaporated from the eluate by taking the latter down to dryness three times *in vacuo*. After the third evaporation, the residue was taken up in 21 ml of de-ionised water and equally distributed between 3 x 25 ml rotary evaporator flasks. Thus each flask contained 7 ml of the concentrated eluate, each equivalent to 66.7 ml of the original 200 ml medium aliquot. Following a

final evaporation the residues were each taken up in 1 ml of 0.01 N.HCl.

b) Sample analysis

The 1 ml sample was diluted to 10 ml with double distilled water and 1 ml of this diluted sample loaded on to the acidic cation exchange column which is part of the "Technicon Automatic Amino Acid Analyser." The assembly and operation of this instrument has been described previously (Boulter 1966, Simpkins 1969 and Stuart 1969). Norleucine was incorporated into the samples as an internal standard and calculationsof the amounts of amino acids in the samples were based on values obtained for the norleucine equivalents as described in the Technicon Manual. Four calibration curves were performed for each amino acid before calculations were made of the amounts of these amino acids present in the samples under investigation. The amino acids present in the liquid medium were identified by co-chromatography with standard mixtures. Enhancement of the standard amino acid peaks (relative to norleucine) indicated that the same amino acid was also present in the extract. The amino acid mixture used for the standard runs is given in Table 1.1. and an elution diagram showing the separation of these amino acids by the Auto Analyser is given in Fig. 1.1.

Explanation of Figure 1.1

Separation of the standard amino acid mixture on the Auto analyser

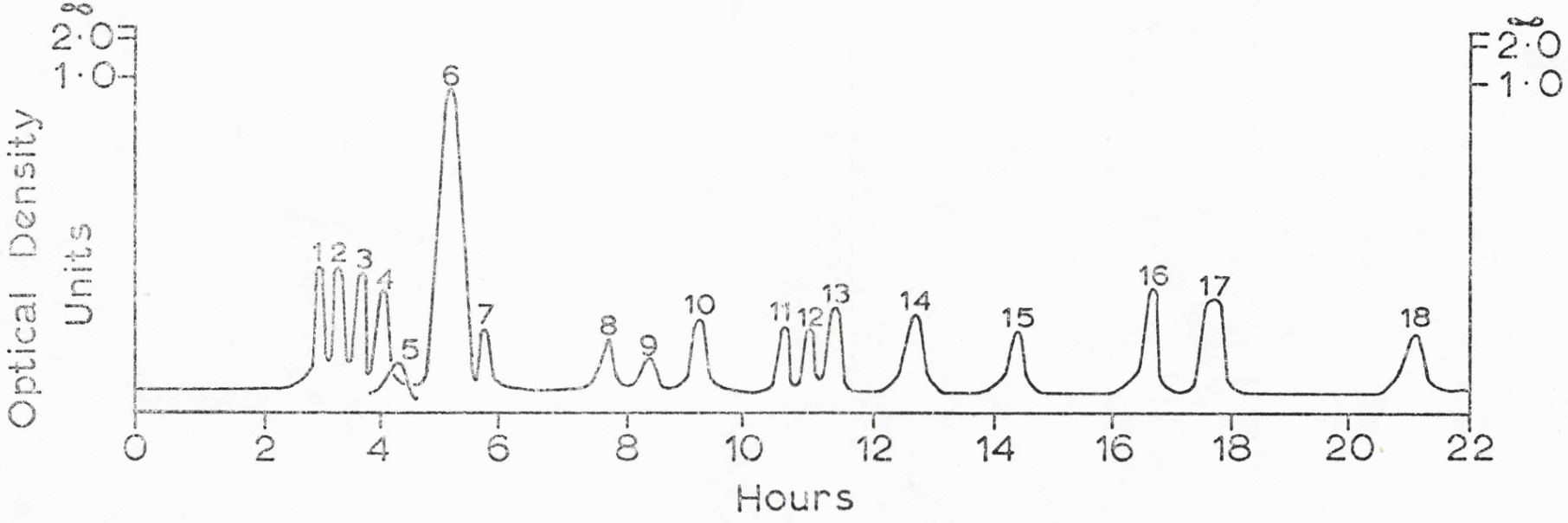
TABLE 1.1

KEY	CONSTITUENT	M.WT.	Mg/l equivalent to 0.1μM/ml
1	L-Aspartic Acid	133.1	13.31
2	L-Threonine	119.1	11.91
3	L-Serine	105.1	10.51
4	L-Glutamic Acid	165.1	16.51
5	L-Proline	115.1	11.51
6	L-Glycine	75.1	7.51
7	L-Alanine	89.1	8.91
8	L-Valine	117.1	11.71
9	L-Cystine	120.2	12.02
10	L-Methionine	149.2	14.92
11	L-Isoleucine	131.2	13.12
12	L-Leucine	131.2	13.12
13	D.L-Norleucine	131.2	13.12
14	L-Phenylalanine	165.2	16.52
15	Ammonium sulphate	132.1	13.21
16	L-Lysine	182.7	18.27
17	L-Histidine	209.6	20.96
18	L-Arginine	210.7	21.07

0.1μMoles of each constitutent was contained in 1 ml of the standard mixture loaded on to the column of the Auto analyser.

Fig 1.1

Elution diagram showing the constituent amino acids present in the standard mixture on the Technicon automatic amino acid analyser



Experimental

S E C T I O N 2

CALLUS CULTURE

INTRODUCTION

Tissue cultures of forest trees have generally proved difficult to maintain *in vitro* when compared with herbaceous species. Jacquiot (1959) isolated cambial tissues from the trunks of 25 forest tree species but was able to establish callus cultures from only 6 of these. Hildebrandt et al. (1956), Pelet et al. (1960) and Mathes, 1964 have successfully grown tissues of various tree species on media supplemented with natural products such as coconut milk, malt extract, yeast extract and casein hydrolysate. Wolter and Skoog (1966) working with cultured tissue of *Fraxinus pennsylvanica* studied the quantitative requirements and interactions of nutrients and growth factors of these cultures on defined medium. Jha (1966) carried out a similar study with *Acer pseudoplatanus* but there are few other reports of the establishment of forest tree callus cultures on synthetic media.

There are two reports of the induction and establishment of callus from tissues of *Hevea brasiliensis*. Bouychou (1953) described the proliferation and growth of *Hevea* callus on the medium of Gautheret (1942). Chua (1966), after failing to reproduce the results of Bouychou (1953), devised an elaborate medium containing a wide range of natural products, amino-acids, vitamins and growth regulators which supported a slow rate of callus growth. Chua (1966) observed that

by increasing the sucrose content in his medium from 2% to 10% an enhancement of callus growth was obtained. At best, plumule discs approximately 1 mm in length, from 3-4 day-old seedlings, produced a callus proliferation of 15 mm in diameter after 3 months. The complexity of the medium of Chua (1966) coupled with the slow growth of callus rendered this system unsatisfactory for the support of stocks of Hevea callus tissue which were to serve as the basis for the exploitation of the whole range of plant tissue culture techniques.

The reports of Bouychou (1953) and Chua (1966) are contradictory with regard to the support of callus growth on the medium of Gautheret (1942). The fact that Chua used the plumule tissues of 3-4 day-old seedlings, while Bouychou worked with stem segments of "young" seedlings may account for the contradiction. This conflict in the previous reports of Hevea callus culture meant that the practical approach for the present study had to be empirical. Initially, therefore, it was necessary to screen a range of media including those of Gautheret (1942) and Chua (1966) in order to assess their suitability as support systems for the callus culture of Hevea brasiliensis.

A. The Initiation and Growth of Callus

The formulae for all media are set out in the Appendix. Unless otherwise stated the explants were stem segments of young plants within the age range of 2-18 months. These segments were between $\frac{1}{2}$" and 1" in length and, after exposing the cambial regions by separating the outer cortical tissue from the woody segment core, were incubated on semi-solid medium at

25° in diffuse light. The first experiment was designed to test the effectiveness of various media for the induction and growth of callus. (Table 2.1.) Whilst a number of the explants showed some callus initiation there was none where this was sufficient to allow transfer of the induced callus after 45 days. One of the woody stem explants on the medium of Linsmaier and Skoog produced roots which appeared to arise from an area of callus growth, rather than directly from the explant itself. This was the most positive growth response that was obtained and thus a range of auxin and kinetin levels were tried (Table 2.2),based on the medium of Linsmaier and Skoog, in an attempt to encourage more active callus growth rather than the initiation and growth of roots. The results obtained from these treatments indicated that 2 mg/l IAA and 0.5 mg/l kinetin were the most effective levels for the initiation and growth of callus. Callus proliferations approximately 5 mm in diameter had formed from the explants after 16 days.

From Table 2.1 it will be seen that 2,4-D had only been applied with the media of White and Chua. Thus the next experiment was to test the effectiveness of 2,4-D as a growth hormone in Linsmaier and Skoog's medium. (Table 2.3.)

TABLE 2.1

The effectiveness of a range of media tested as support systems for the induction of callus growth from stem and petiole explants of Hevea brasiliensis

	MEDIUM			STEM	PETIOLE
1	WHITE (1943)	0.5 mg/l	2,4-D	7/0 *	8/0 *
2		1.0	"	7/2	8/0
3		1.0	NAA	4/3	5/2
4		2.0	"	5/2	6/2
5	MURASHIGE AND SKOOG (1962)	1.0	NAA	11/3	-
6		2.0	"	18/0	10/3
7	CHUA (1966)			54/20	42/6
8	LINSMAIER AND SKOOG (1965)			12/4	4/0
9	WHITE WITH WOOD and BRAUNS SUPPLEMENT (1961)			20/0	-
10	GAUTHERET (1942)			15/10	-

* Number of explants/number showing callus initiation.

The pH of all media was adjusted to 5.6

TABLE 2.2

Combinations of IAA and kinetin tested to determine the optimum levels for callus initiation and growth on the medium of Linsmaier and Skoog from stem explants of Hevea brasiliensis

Treatment	IAA (mg/l)	Kinetin (mg/l)
1.	1.0	0.02
2.	1.0	0.10
3.	1.0	0.50
4.	2.0	0.02
5.	2.0	0.10
6.	2.0	0.50
7.	5.0	0.02
8.	5.0	0.10
9.	5.0	0.50
10.	10.0	0.02
11.	10.0	0.10
12.	10.0	0.50

Ten replicate flasks were used for each treatment and the pH of all treatments was adjusted to 5.6.

TABLE 2.3

Levels of 2,4-D, NAA and kinetin tested for their activity on the initiation and growth of callus from stem explants of Hevea brasiliensis on the medium of Linsmaier and Skoog

Treatment	2,4-D (mg/l)	Kinetin (mg/l)
1.	0.5	0.05
2.	0.5	0.50
3.	2.0	0.05
4.	2.0	0.50
	NAA (mg/l)	
5.	2.0	0.05
6.	2.0	0.50
7.	5.0	0.05
8.	5.0	0.50

Five replicate flasks were used for each treatment and the pH of all treatments was adjusted to 5.6.

The greatest amount of callus initiation and growth occurred with both 2,4-D levels and 0.5 mg/l kinetin. Individual callus growths on these treatments achieved a diameter of approximately 10 mm after 16 days.

The media of Linsmaier and Skoog and Murashige and Skoog are basically similar but differ in that Linsmaier and Skoog's contains 1 gm/l casein hydrolysate and 0.4 mg/l thiamine, while that of Murashige and Skoog contains 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.1 mg/l thiamine and no casein hydrolysate. The absence of casein hydrolysate means that Murashige and Skoog's is a defined medium and thus preferable to that of Linsmaier and Skoog on the basis that cultural requirements can be identified precisely. Thus an experiment was carried out to determine whether the differences between the media of Linsmaier and Skoog and Murashige and Skoog significantly affect the initiation and growth of callus. (Table 2.4.) The greatest amount of callus growth occurred with the three 2,4-D treatments and 0.5 mg/l kinetin and no differences were observed between the media of Linsmaier and Skoog and Murashige and Skoog either of a qualitative or quantitative nature.

The medium of Chua (1966) had already been tested (Table 2.1) and found inadequate as an experimental system for the support of Hevea callus growth. Attempts to improve this medium were made with the following modifications. The sucrose level was reduced from 100 gm/litre to 40 gm/litre, 2,4-D and IAA were alternatively removed with the remaining growth hormone level increased to 2 mg/l, casein hydrolysate

TABLE 2.4

Levels of 2,4-D, NAA, IAA and kinetin compared on the media of Linsmaier and Skoog, and Murashige and Skoog in their effects on the initiation and growth of callus from stem explants of Hevea brasiliensis

TREATMENT	2,4-D(mg/l)	KINETIN(mg/l)	TREATMENT	IAA(mg/l)	KINETIN(mg/l)	TREATMENT	NAA(mg/l)	KINETIN(mg/l)
1	0.1	0.05	7	1.0	0.05	13	1.0	0.05
2	0.1	0.50	8	1.0	0.50	14	1.0	0.50
3	0.5	0.05	9	2.0	0.05	15	2.0	0.05
4	0.5	0.50	10	2.0	0.50	16	2.0	0.50
5	2.0	0.05	11	5.0	0.05	17	5.0	0.05
6	2.0	0.50	12	5.0	0.50	18	5.0	0.50

Five replicate vials were used for each treatment and the pH of all treatments was adjusted to 5.6. Since two basal media were compared a total of 36 treatments was tested.

was increased from 5 mg/l to 1 gm/l, and kinetin was applied at 0.5 mg/l. These modifications made no appreciable difference to callus initiation and growth on Chua's medium.

Although some success had been achieved in callus induction and growth, the rate of callus growth was still very slow and it was not possible to quantify growth under varying conditions due to the shortage of material for inoculation. Further experiments were designed to test the effect of environmental variations with a range of media on callus growth. Firstly stem and petiole explants were inoculated on to filter paper bridges (Heller, 1949) and thus supported above liquid medium in boiling tubes. A range of media was tested but no initiation was observed. This was followed by an experiment devised to test the effect of light/dark and temperature variations using a range of media.

TABLE 2.5

Conditions adopted to test the effects of light/dark and temperature variations with a range of media on callus initiation and growth

MEDIUM	ENVIRONMENTS
Chua	
Murashige and Skoog	
Linsmaier and Skoog	25°Light, 25°Dark
White	30°Light, 30°Dark
White plus Wood and Braun's supplement	

Light intensity was measured at 200 lux. The auxin and kinetin levels used in all media were 2 mg/l 2,4-D, and 0.5 mg/l kinetin. Ten replicate flasks were used for each treatment and the pH of all treatments was adjusted to 5.6.

After 8 weeks callus proliferation was most extensive on the media of Murashige and Skoog and Linsmaier and Skoog at 30° in the dark. Measurement by weight, although desirable,would have involved the risk of contamination and possible wastage of material and was thus not undertaken at this time.

From this last experiment it was possible to excise the callus from the explants and subculture on to fresh medium. Twenty callus growths obtained in this manner and of identical pedigree were used to inoculate twenty flasks each containing the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin. Ten flasks were incubated at 25° in the dark and ten at 30° also in the dark. After four weeks callus growth was much more prolific at 30° than at 25°, and the callus morphology at 30° was visibly different appearing more friable than the hard, compact growth at 25°. In view of this improvement in growth rate it appeared opportune to build up stocks of callus in order to quantify the temperature effect and to assess the apparent suitability of the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin as a support system for the growth of Hevea brasiliensis callus.

B. The Assessment of Callus Growth

The establishment of callus stocks on the medium of Murashige and Skoog with 2.0 mg/l 2,4-D and 0.5 mg/l kinetin at 30° in the dark made the necessary material available for a quantitative assessment of growth under various environmental and media treatments. Although for the preliminary

work 2 mg/l 2,4-D and 0.5 mg/l kinetin appeared to be the optimum concentrations for promoting initiation and growth of callus on the medium of Murashige and Skoog, the response of callus to seven different 2,4-D and kinetin concentrations was tested for two passages to identify more precisely the optimum levels of these substances. Since with more than 2 mg/l 2,4-D callus growth had been very slow and the callus extremely compact, 2,4-D was tested over the range of 0-2 mg/l. Kinetin was tested over the range of 0-2.5 mg/l (Table 2.6). At this time the emphasis was on a rapid increase of callus stocks and thirty days was chosen as the shortest acceptable passage length.

Table 2.6

Concentrations of 2,4-D and kinetin in the medium of Murashige and Skoog for growth of Hevea brasiliensis callus tissue at 30° in the dark

Treatment	2,4-D (mg/l)	Kinetin (mg/l)
1.	0.0	0.0
2.	0.0	0.5
3.	0.5	0.5
4.	1.0	0.5
5.	2.0	0.5
6.	2.0	0.0
7.	2.0	2.5

The pH of all treatments was adjusted to 5.6. Growth was measured by obtaining the fresh and dry weights of five callus growths per treatment at 10 day intervals for two passages, each of 30 days.

The results are expressed graphically (see Figures 2.1 to 2.4). Concentrations of 2,4-D below 2 mg/l were less effective in stimulating Hevea callus growth (treatments 1, 2, 3 and 4, Figures 2.1 and 2.2). Kinetin did not appear to be essential for the maintenance of active callus growth over the first 60 days tested (treatment 6, Figure 2.3) but at a concentration of 2.5 mg/l an inhibition of growth was observed (treatment 7, Figure 2.4). The maximum growth response was obtained with 2.0 mg/l, 2,4-D and 0.5 mg/l kinetin (passage 1, treatment 5, Figure 2.3). Since all the callus tissue used to inoculate the seven treatments had previously been growing in these concentrations of 2,4-D and kinetin, passage 1 of treatment 5 is strictly comparable with the second passage of the other treatments. In the absence of both 2,4-D and kinetin (treatment 1, Figure 2.1) the callus tissues rapidly became moribund and a second passage proved impracticable. In almost all the treatments a retardation of callus growth assessed by the percentage increase in fresh weight was accompanied by a rise in the percentage dry weight. Marked fluctuations in the percentage dry weight occurred when the growth of callus was poor but in those treatments which stimulated the active growth of callus (treatments 5 and 6, Figure 2.3) it was generally lower and remained fairly constant at between 6 and 8%. From their appearance and consistency the callus tissues produced in treatments 5 and 6 (Figure 2.3) were also the most friable. The percentage dry weight is indicative of the amount of water that is held in the callus tissue and one would expect a tissue with a high water content to disperse

Explanation of Figures 2.1 to 2.4

The effect of various treatments with 2.4-D and kinetin on the growth of Hevea brasiliensis callus tissue for two passages on the medium of Murashige and Skoog

Treatment	2,4-D mg/l	kinetin mg/l
1	0.0	0.0
2	0.0	0.5
3	0.5	0.5
4	1.0	0.5
5	2.0	0.5
6	2.0	0.0
7	2.0	2.5

Key:- 0—0 Growth as measured by increase in fresh weight over an incubation period of 30 days.

●—● Dry weight as % fresh weight ($\frac{\text{Dry wt}}{\text{fresh wt}} \times 100$) over an incubtaion period of 30 days.

Each determination plotted represents the mean of five replicate callus growths.

Fig 2.1

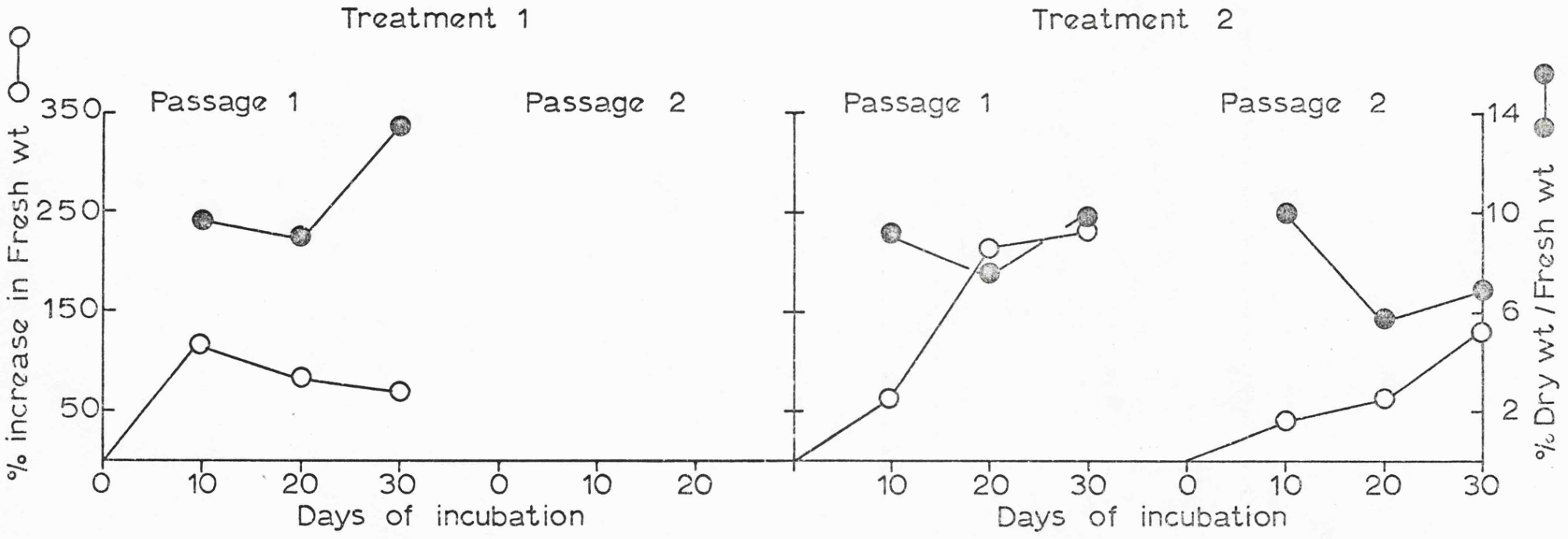

Treatment 1
Treatment 2
Passage 1
Passage 2
Passage 1
Passage 2
% increase in Fresh wt
% Dry wt / Fresh wt
350
250
150
50
14
10
6
2
0
10
20
30
0
10
20
10
20
30
0
10
20
30
Days of incubation
Days of incubation

Fig 2.2

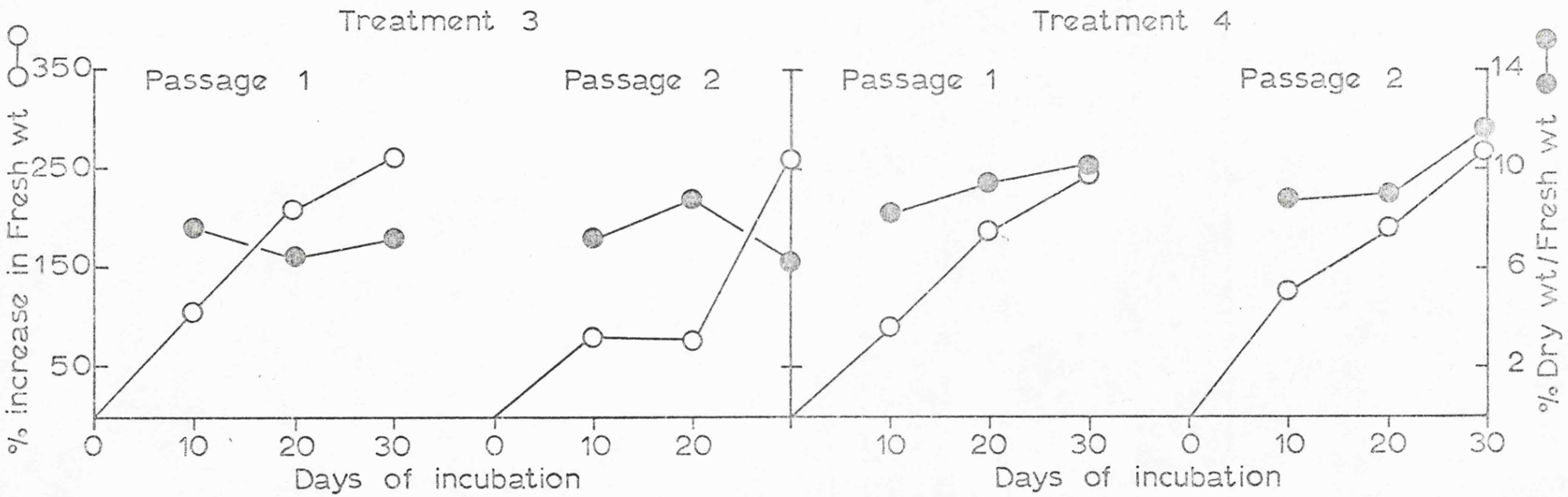

Treatment 3
Passage 1
Passage 2
Treatment 4
Passage 1
Passage 2
% increase in Fresh wt
% Dry wt/Fresh wt
350
250
150
50
14
10
6
2
0
10
20
30
Days of incubation

Fig 2.3

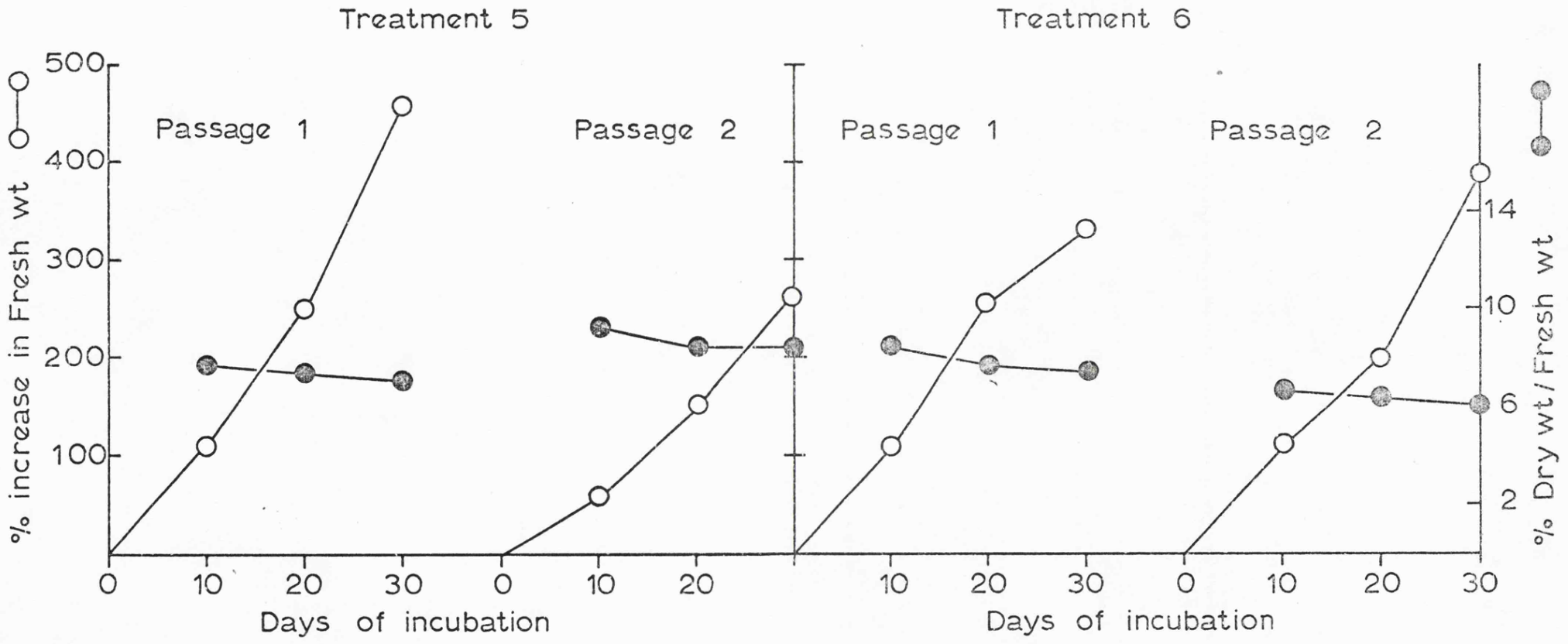
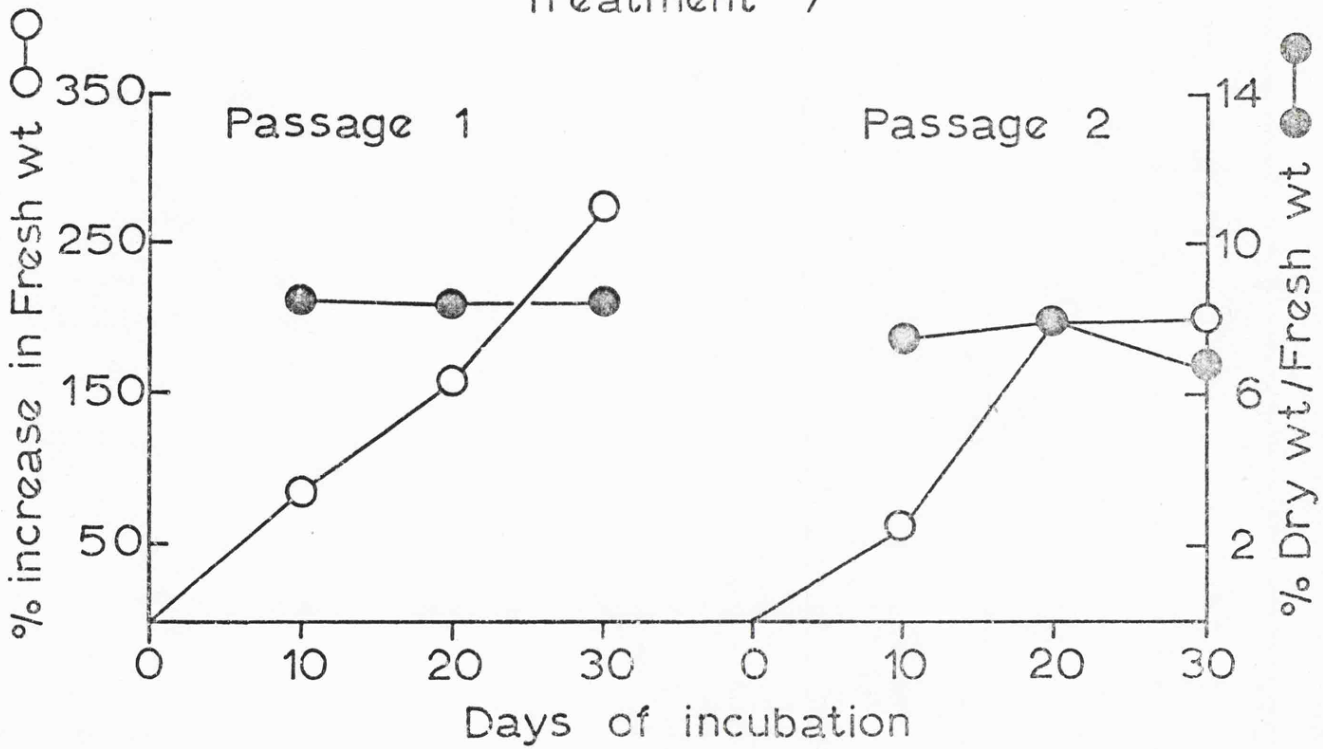

Treatment 5
Treatment 6
Passage 1
Passage 2
Passage 1
Passage 2
% increase in Fresh wt
% Dry wt / Fresh wt
500
400
300
200
100
14
10
6
2
0
10
20
30
0
10
20
10
20
30
0
10
20
30
Days of incubation
Days of incubation

Fig 2.4


Treatment 7
Passage 1
Passage 2
% increase in Fresh wt
% Dry wt/Fresh wt
350
250
150
50
14
10
6
2
0
10
20
30
0
10
20
30
Days of incubation

more readily in liquid medium than a tissue with a low water content. If this proved to be so then the percentage dry weight could be used as a parameter of friability. It can be suggested from these results that friability is related to growth rate since relatively rapidly growing callus tissues show lower percentage dry weights.

These data indicated that the callus of Hevea brasiliensis was most rapidly growing and most friable on the medium of Murashige and Skoog in the presence of 2 mg/l 2,4-D and 0.5 mg/l kinetin.

An enhancement of growth had been observed at 30° as compared with 25° in the dark. In order to quantify this effect callus tissue growing on the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin was exposed to four environments, these being 25° in the light (200 lux) 30° in the light, 25° dark and 30° dark. 40 flasks were inoculated each with approximately 50 mg fresh weight of callus tissue with ten of these flasks incubated in each environment for 30 days. (Table 2.7).

From the results it can be seen that growth of Hevea brasiliensis callus tissue is significantly increased by incubation at 30° when compared with growth taking place at 25°. At 30°, but not at 25°, there appears to be a light/dark and temperature interaction with the result that growth of callus tissues incubated at 30° in the dark is significantly greater than at 30° in the light. Figure 2.5 and the standard deviations of the mean show that there is much greater variation in the percentage increase in fresh weight

TABLE 2.7

A comparison of Hevea brasiliensis callus growth in a range of environments on the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l K.

Environment	Initial Fresh wt.	Final Fresh wt.	% Fresh wt. Increase	S.D. of the Mean	Yield in gm/gm Initial Fresh wt.
1. 25°L	506 mg	1152 mg	131	14.1	1.27
2. 25°D	521	1080	122	15.4	1.07
3. 30°L	547	1656	201	18.2	2.02
4. 30°D	536	2470	366	64.1	3.61

A statistical analysis of the results was made using a modified "t" test (d)

Comparing	d	°F	Probability
1 & 2	0.42	18	> 0.10
1 & 3	2.80	16	> 0.01 < 0.02 *
3 & 4	2.40	11	> 0.02 < 0.05 *
2 & 4	3.70	10	> 0.002 < 0.01 *

* significant

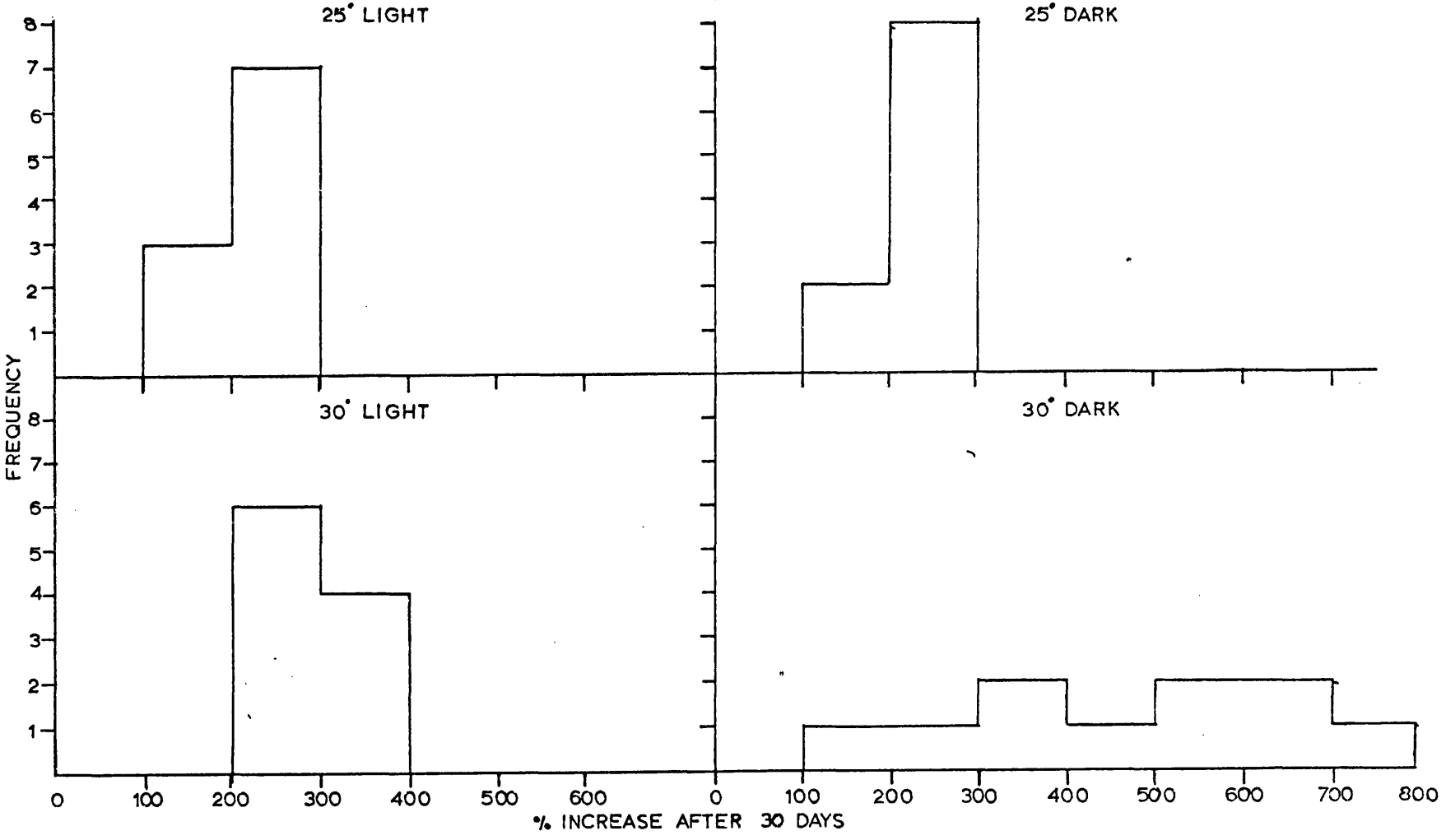
Explanation of Figure 2.5

The effect of light/dark and two temperatures on the callus growth of Hevea brasiliensis

Callus growth as measured by the % increase in fresh weight after an incubation period of 30 days in the four environments. 25° Light (200 lux), 25° Dark, 30° Light (200 lux) and 30° Dark.

Fig 2.5

THE EFFECT OF LIGHT/DARK AND TEMPERATURE VARIATIONS ON ON CALLUS GROWTH



between individual callus growths incubated at 30° in the dark than in the other environments. Thus the most rapidly growing and most friable callus was obtained when the tissues were grown on the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin incubated at 30° in the dark.

It was necessary to test a range of media under those conditions which had proved most satisfactory for callus initiation and growth on the medium of Murashige and Skoog, to ensure that this was the most suitable basal medium. Seven media were tested including that of Murashige and Skoog with all media containing 2 mg/l 2,4-D and 0.5 mg/l kinetin as the auxin and kinin supply. Ten flasks were used for each medium, these being inoculated with a callus fresh weight of approximately 500 mg. At the end of 30 days five of the callus growths were harvested and weighed whilst the remaining five were used as inocula for the second passage. The pH of all media was adjusted to 5.6.

Media which gave yields of below 1 gm/gm of initial fresh weight in the second passage were considered unsuitable as support systems for the experimental culture of Hevea callus tissue. As can be seen from the results (Table 2.8) only on the media of Miller and Murashige and Skoog were yields above 1 gm/gm of initial fresh weight achieved in the second passage, the maximum yields being obtained on the medium of Murashige and Skoog. The media of Miller and Murashige and Skoog are similar, differing only in that glycine is absent from Miller's medium and the constituents of Miller's medium are present in lower concentrations than the equivalent constituents of Murashige and Skoog's medium.

TABLE 2.8

The growth of Hevea brasiliensis callus tissue on a range of basal media at 30° in the dark over two passages

PASSAGE 1

Medium	Initial Fresh wt.	Final Fresh wt.	Yielding gm/gm Initial Fresh wt.
Murashige and Skoog (1962)	2.39 gm	9.76 gm	3.08
White (1943)	5.599	5.90	0.06
Chua (1966)	1.92	3.86	1.01
Modified Chua	2.19	4.59	1.10
Heller (1953)	2.92	7.33	1.51
White with Wood and Braun's Supplement (1961	1.82	4.42	1.43
Miller (1967)	3.24	7.20	1.22

PASSAGE 2

Murashige and Skoog	4.76 gm	14.97 gm	2.14
White	-	-	-
Chua	2.07	3.62	0.75
Modified Chua	3.41	4.91	0.44
Heller	3.61	5.54	0.53
White with Wood and Braun's Supplement	3.63	5.04	0.39
Miller	5.12	14.16	1.77

All measurements tabulated represent the sum of five callus growths except those for the medium of White, where the ten replicate callus growths were moribund after the first passage, and thus made further subculture impracticable.

During this work there was an occasion when difficulty was encountered maintaining healthy stocks; an investigation revealed that an incorrect level of potassium dihydrogen phosphate ($KH_2PO_4.H_2O$) had been used for two passages. Thus an experiment was designed to test whether the level of potassium dihydrogen phosphate was optimal in the standard medium of Murashige and Skoog. The normal quantity of $KH_2PO_4.H_2O$ in Murashige and Skoog's medium is 170 mg/l. The three other levels tested were half-normal, twice and four times normal, i.e. 85 mg/l, 340 mg/l and 680 mg/l. The experiment was carried out over two passages and each treatment was tested with ten flasks, each containing approximately 0.5 gm of callus tissue, per passage. The pH of all media was adjusted to 5.6. (Table 2.9).

The results clearly indicate that the standard concentration of 170 mg/l of $KH_2PO_4.H_2O$ is the optimum of those tested. Callus growth on phosphate levels above this generally were more compact and slower growing.

When the roots, cotyledons, endosperm, hypocotyl and plumular regions of 2-3 day-old sterile seedlings were compared as explant material, the explants from the hypocotyl and plumular regions of the seedling proved to be the most prolific source of callus. Although initiation occurred with explants from other regions of the dissected seedling, the callus formation was insufficient to allow its excision and transfer to a second passage. Viable seed, however, was not always available and so sterile explants obtained from non-sterile young plants as described in the Materials and Methods section were generally used to initiate callus cultures.

TABLE 2.9

The growth of Hevea brasiliensis callus tissue on a range of $KH_2PO_4.H_20$ concentrations on Murashige and Skoog's medium at 30^o in the dark for two passages

PASSAGE 1

Medium	Initial Fresh wt.	Final Fresh wt.	Yielding gm/ gm Initial Fresh wt.
M+S, +85 mg/l $KH_2PO_4H_2O$	4.7 gm	16.8 gm	2.5
" +170 " "	4.4	14.9	2.4
" +340 " "	3.9	12.6	2.2
" +680 " "	5.3	18.7	2.5
PASSAGE 2			
" +85 " "	5.5 gm	11.8 gm	1.1
" +170 " "	6.7	21.3	2.2
" +340 " "	5.6	11.8	1.1
" +680 " "	8.9	15.8	0.8

All measurements tabulated represent the sum of ten callus cultures.

Morphologically the callus tissues of Hevea brasiliensis grown on the medium of Murashige and Skoog appeared very heterogenous (PLATE 4, Section 3) with areas which appeared to be the result of compact callus growth, interspersed with brown necrotic tissue and soft white tissue formations. Sections of these tissues are indicative of their differing physical states (PLATE 3). The soft white tissues appear to be composed of large, loosely packed cells such as those of a friable tissue while the brown necrotic areas are composed of relatively tightly packed, collapsed cells. Attempts which were made to separate these regions by excision and to culture them in mutual exclusion were unsuccessful. The brown necrotic tissue was incapable of growth, while the compact callus growth gave rise to a similarly heterogenous mass of tissues from which it was derived.

The initial objective of establishing callus growth at a suitable rate for experimental purposes on a defined medium had been achieved. The callus tissue has a temperature requirement of 30° or above for satisfactory growth which would appear to be an apposite reflection of the physiology of the rubber plant in vivo. There was great variation in the response of individual callus growths in replicate treatments and there was also evidence of gross morphological and histological variation within each callus growth (PLATE 3). Nevertheless conditions had been established for the long term culture of Hevea callus by repeated subculture every 30 days.

The work in Section Three describes attempts to develop free cell suspension cultures of Hevea brasiliensis from the callus cultures described above.

Explanation of PLATE 3

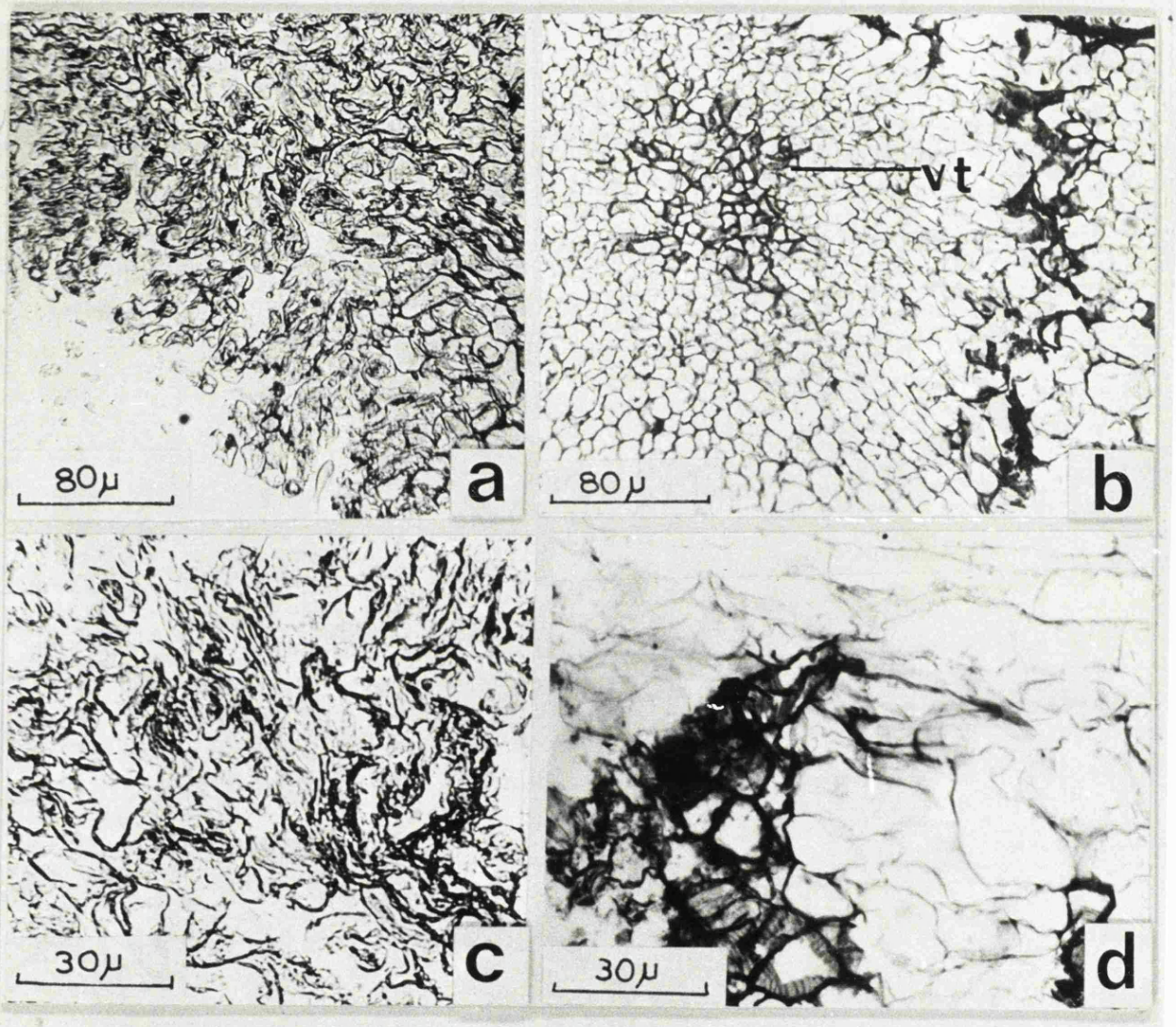
Anatomical variation in callus cultures maintained continuously on semi-solid medium

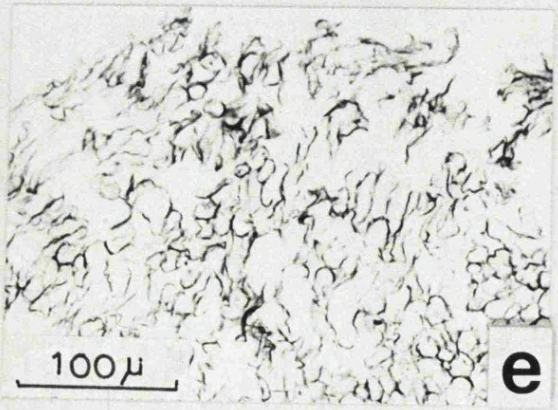
a, c Sections through a region of brown, necrotic tissue. The cells do not appear to have the capacity for further division, and many of the cell walls are ruptured.

b, d Sections through a region of white, compact callus growth. Clumps of vascularised tissue (vt) are commonly encountered.

e Section through a soft white tissue formation composed of large loosely packed cells.

PLATE 3





SECTION 3
CELL SUSPENSION CULTURE

INTRODUCTION

A suspension of plant cells is obtained by placing callus tissues in agitated, liquid medium. The liquid medium constituents are usually identical with those incorporated into the semi-solid medium supporting these callus tissues, e.g. callus and cell suspensions of Acer pseudoplatanus, Nicotiana tabacum and Daucus carota. However, callus stocks of Atropa belladonna are cultured on 0.5 mg/l kinetin while cell suspension stocks are preferably maintained in the presence of 0.1 mg/l kinetin (Thomas, 1970). The subsequent dispersion of the callus tissue when placed in liquid medium is dependent upon its friability but no standard degree of dispersion has been established for a culture to be regarded as a cell suspension. Thus the highly dispersed, enzyme-treated Acer pseudoplatanus cultures developed by Mansfield (1973), and the cultures of Antirrhinum (Melchers and Bergmann 1958) containing large spherical aggregates up to 4 mm in diameter and very few free cells, have both been described as cell suspension cultures.

The advantage of growing plant cells suspended in liquid medium compared with as callus on semi-solid medium is that a greater degree of uniformity is achieved in the responses of these cells to cultural conditions. Clearly, the degree of this uniformity is inversely proportional to the degree of cellular aggregation, with highly aggregated cultures displaying a range of morphologically and physiologically distinct cell types. The purpose of the work presented in this

section was to develop cell suspension cultures of Hevea brasiliensis which would be suitable, ultimately, for the single cell cloning technique.

Experimental

The Initiation and Growth of Cell Suspensions

The most friable callus pieces were selected from stock callus cultures and used in an attempt to initiate a cell suspension. Approximately 0.5 gm fresh weight of callus was placed in 20 ml of liquid medium in a 100 ml Erlenmeyer flask. The medium constituents were identical with those making up the culture medium used for maintaining callus stocks. 30 replicate flasks were incubated at 30° on a Gallenhamp orbital shaker set at 120 r.p.m. After two weeks no appreciable dispersion of the initial callus inoculum into smaller cell aggregates had taken place and any free cells which were present in the medium were found to be plasmolysed. Many subsequent attempts were equally unsuccessful, with lack of friability of the callus inoculum being the most obvious factor involved. Growth of the callus inoculum appeared to continue in liquid medium and occasionally these would fragment to the extent that up to twenty growing callus pieces would result. However, these fragments were such as to preclude transfer of cell material from these cultures using an automatic pipette (A. R. Horwell Ltd., London) fitted with a wide bore canula of 3 mm in diameter.

Increasing the shaking speed from 120 r.p.m. to 130 r.p.m. had no noticeable effect in promoting dispersion and after three weeks the medium in these cultures was removed as completely as possible and replaced with fresh Murashige and Skoog's medium using a 10 ml automatic pipette. After two such "passages" the cultures were returned to semi-solid

medium and the callus masses growing on the semi-solid medium were maintained as callus stocks. Callus recovered from liquid medium in this way was designated as "S" callus, while callus which had not been subjected to growth in agitated liquid medium at any time during its culture was designated as "A" callus.

Using "A" callus as the inocula the treatments tabulated below were tested to assess their effects on the dispersion and growth of Hevea brasiliensis tissue in liquid medium.

TABLE 3.1

The effect of flask size, medium quantity and shaking speed on the dispersion and subsequent growth of Hevea brasiliensis callus tissue in liquid medium

Flask size in ml	Quantity of medium in ml	Shaking speed (rpm)
100	10; 20	70; 120
250	20; 50	70; 120

Five replicates of each of these treatments (8 in all) were incubated at 30°.

From a visual assessment of growth and dispersion in 250 ml flasks with 50 ml of medium, these were chosen for use in subsequent experiments and 120 rpm chosen as superior to 70 r.p.m. as a shaking speed. Nevertheless, combining these three conditions did not give a satisfactory cell suspension when "A" callus tissue was used as the inoculum.

When using comparatively friable tissues as inocula giant, elongated cells were frequently observed in the cultures. These were similar in size and appearance to those noted in cell suspensions of Acer pseudoplatanus by Simpkins, Collin and Street (1970). With Acer cells it was found that when

the sole source of nitrogen supplied to the cells was in the form of nitrate the giant elongated cells declined in frequency, their place being taken by smaller, rounded cells. Growth of the cell suspensions, however, was not significantly affected by the use of nitrate as the sole nitrogen source. In view of these results a similar medium modification was tested with the medium of Murashige and Skoog to assess whether a similar change could be brought about with Hevea brasiliensis cell suspensions (see Appendix for full details of the medium used). Casein hydrolysate (400 mg/l), urea (200 mg/l) and cysteine (10 mg/l) were all added to the medium of Murashige and Skoog to determine whether these substances could improve the growth of the cell suspensions of Hevea brasiliensis, but with all these media modifications no improvement in the numbers of free cells and small cell aggregates produced from the callus inocula was achieved and the cells that were sloughed off retained their giant, elongated form.

Regardless of the media variations, quantities and flask sizes, cell counts indicated that there were never more than 150,000 cells/ml in the medium supernatant and that this cell number remained fairly constant while the callus inocula continued growth. If the callus inocula were removed from such cultures the cell number in the remaining medium showed a very gradual decline. When a particularly friable "A" callus growth developed on semi-solid medium and was used as an inoculum for the initiation of a cell suspension culture, the resulting cell suspensions showed a very slow increase in

number and dry weight over four weeks with considerable variation in growth between individual cultures (Table 3.2).

TABLE 3.2

The initiation of cell suspension cultures using friable "A" callus tissues as inoculum, assessed by measurements of cell number and dry weight per ml in the medium "supernatant"

Culture	Cell No.x10^{-6}/ml		Dry wt. in mg/ml of culture	
	DAY 2	DAY 30	DAY 2	DAY 30
1	0.16	0.23	1.25	2.50
2	0.16	0.23	1.50	1.75
3	0.13	0.29	1.75	2.55
4	0.20	0.22	1.75	1.75
5	0.21	0.27	1.50	1.75

This increase in cell number and dry weight in the medium "supernatant" may be due either to the growth and division of some cells in suspension, or to a slightly increased level of dispersion over the course of 28 days in culture.

Attempts were made to improve upon this slow increase in cell number and dry weight by altering the concentrations of 2,4-D and kinetin in the medium of Murashige and Skoog (Table 3.3)

TABLE 3.3

Levels of 2,4-D and kinetin tested for their effects on the dispersion and growth of Hevea brasiliensis cell suspensions from "A" callus tissue inocula

Treatment	2,4-D (mg/l)	Kinetin (mg/l)
1	0.0	0.0
2	0.0	0.5
3	0.5	0.5
4	2.0	0.0
5	2.0	0.1
6	2.0	0.5
7	5.0	0.5
8	10.0	0.5

Five replicate flasks per treatment and each flask was inoculated with approximately 2 gm fresh weight of "A" callus tissue.

There was no improvement on the levels of dispersion previously observed with 2 mg/l 2,4-D and 0.5 mg/l kinetin and after four weeks the media containing 2 mg/l 2,4-D appeared to be the most suitable of those tested for promoting the dispersion of "A" callus tissues in liquid medium, with the three kinetin levels tested appearing to have no effect.

As mentioned previously, many of the callus inocula from which cell suspensions of Hevea brasiliensis could not be obtained had been returned, usually after two "passages" in liquid medium, to semi-solid medium. After approximately two months large quantities of extremely friable callus tissue were observed proliferating from all of these cultures. A

clear difference was evident between "A" and "S" callus growths (Plate 4) with the latter tissue lighter in colour, softer in texture and generally of a more homogenous nature in appearance. When "S" callus was reintroduced into liquid Murashige and Skoog medium with 2 mg/l 2,4-D and 0.5 mg/l kinetin the tissues readily dispersed, and in some cultures cell counts of 2.5×10^{-6} cells/ml were recorded after 14 days (Plate 4). In view of this favourable response it was decided to test a range of 2,4-D and kinetin concentrations in liquid medium in order to determine those most suitable for the maintenance of stocks of Hevea brasiliensis cell suspensions initiated from "S" callus inocula (see treatments set out in Table 3.3).

After 14 days, 20 ml of the supernatant medium of each treatment together with the suspended free cells and cell aggregates were transferred into 30 ml of fresh medium. These cultures (vol. 50 ml) were considered as the first cell suspension passage with the previous passage being regarded as the initiation passage. Cell counts were recorded for each treatment at Day 0 and Day 14 during this first passage and the results are given in Table 3.4

PLATE 4

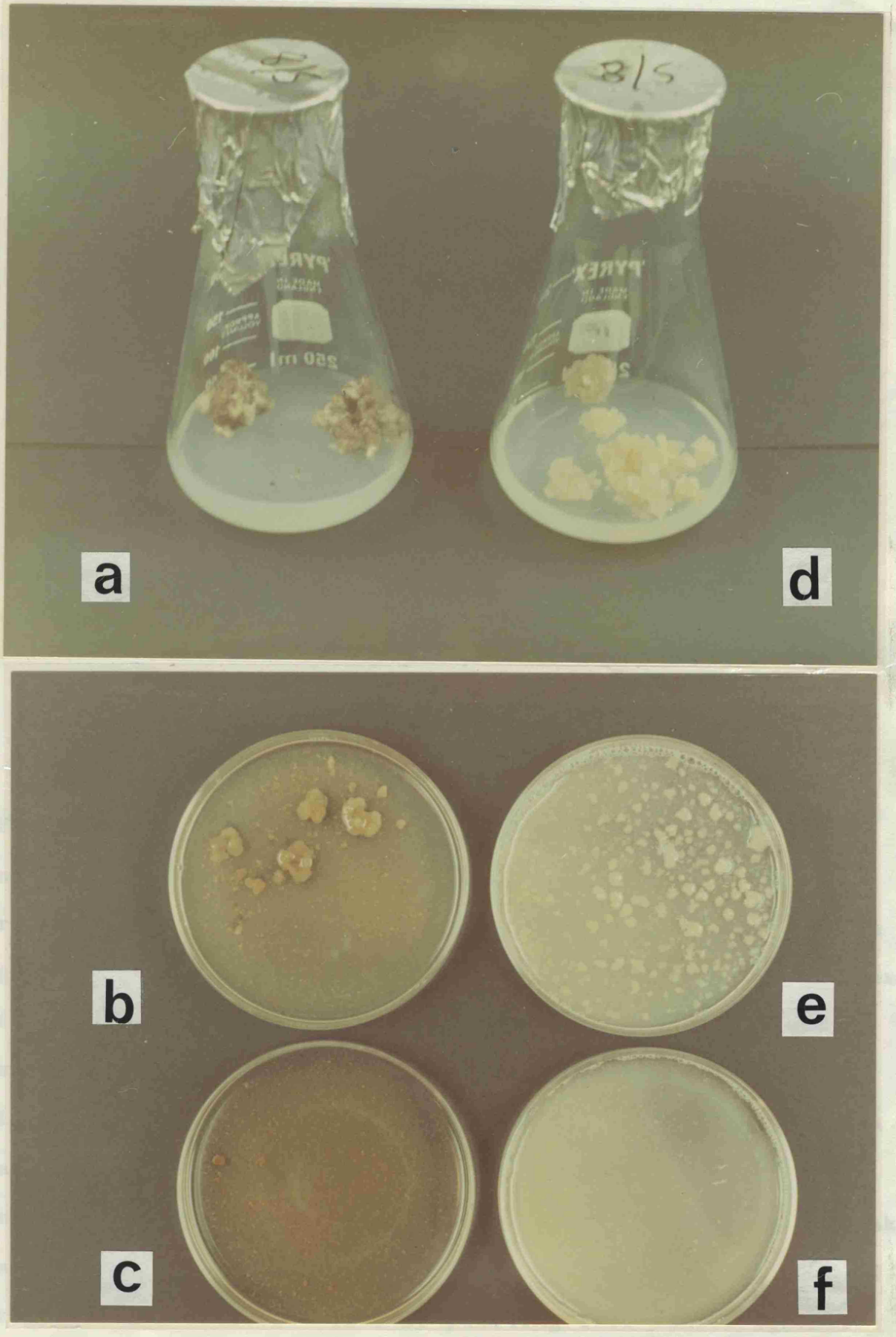


TABLE 3.4

Levels of 2,4-D and kinetin tested for their effects on the growth of cell suspensions of Hevea brasiliensis

Treatment (as in Table 3.3)	Cell No. x 10^{-6}/ml	
	DAY 0	DAY 14
✓ 1	0.21	0.65
2	0.07=	0.78
✓ 3	0.10	0.73
✓ 4	0.10	1.26
5	0.24	1.27
✓ 6	0.26	2.98
✓ 7	0.10	0.80
8	0.03	0.20

Five replicate flasks were used per treatment. The cell counts tabulated are the means of representative samples taken from every flask and pooled for each treatment.

From these cell counts it can be seen that treatments 4, 5 and 6, i.e. 2 mg/l 2,4-D and 0.0, 0.1 and 0.5 mg/l kinetin respectively, supported the highest levels of cell suspension growth as measured by cell number. From each of these fifteen flasks (3 x 5) a representative 10 ml sample of the cell suspension was withdrawn using a 10 ml automatic pipette and inoculated into 50 ml of fresh medium of the same composition as in the first passage. At two or three day intervals sterile representative 3 ml samples were taken from each flask and the samples from flasks of the same treatment were bulked together and counted. Thus by plotting cell number against days of incubation, growth curves were drawn for these second

passage cell suspension cultures in order to determine which of the three kinetin levels was most suitable for maintenance of cell suspension stocks (see Figure 3.1).

The results indicate very little difference in growth between treatments when measured by increase in cell number. However, in these cultures a greater degree of aggregation was evident in the absence of kinetin than with 0.5 mg/l kinetin present. Thus 2 mg/l 2,4-D and 0.5 mg/l kinetin were chosen for the maintenance of stock suspension cultures. As previously noted with callus cultures there appears to be no absolute requirement for kinetin for the in vitro culture of Hevea brasiliensis cells and tissues.

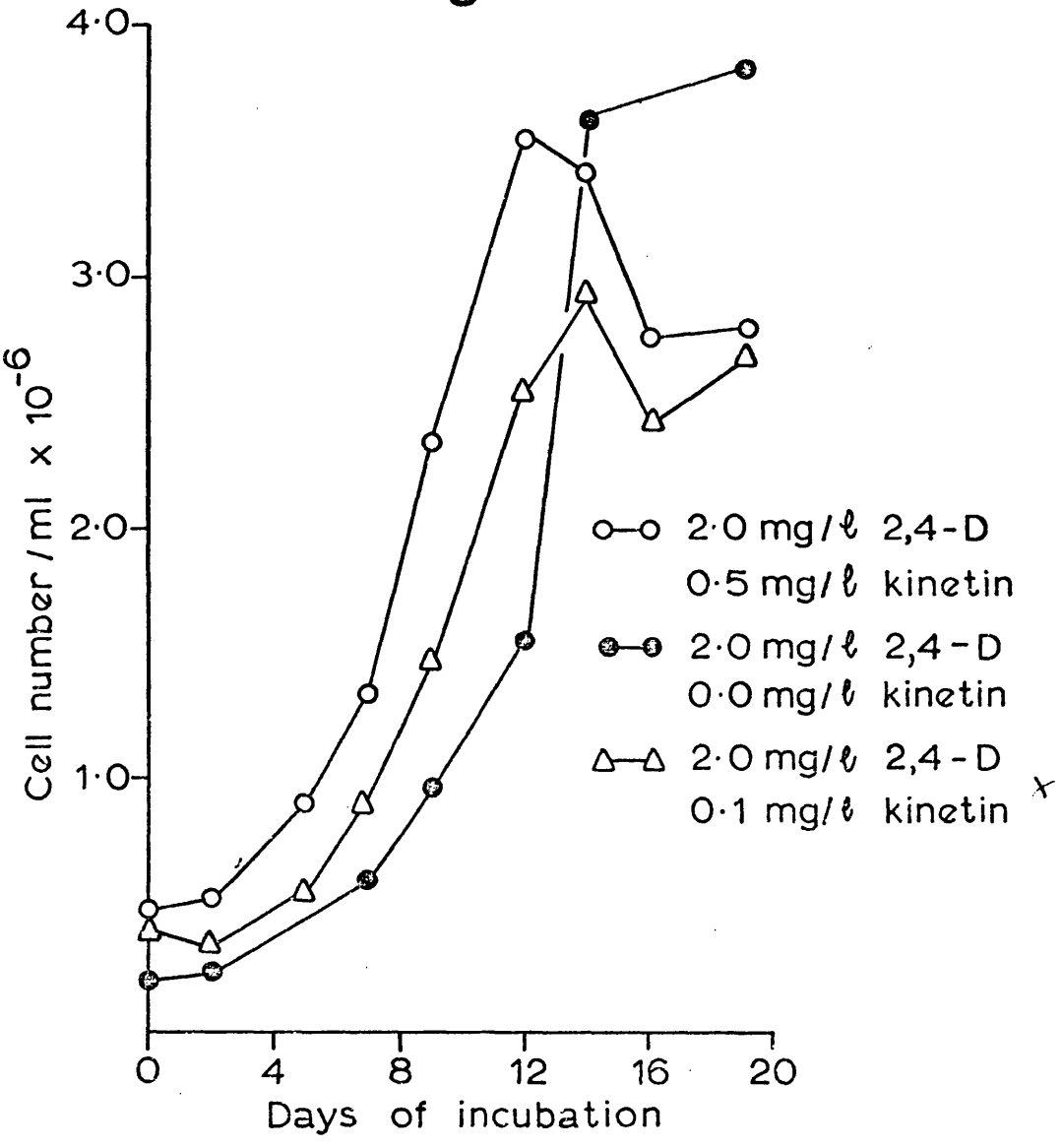
When a 10 ml inoculum of a 4 day-old cell suspension culture was used to initiate futher cultures, the initial cell number was usually between 0.5 and 0.6 x 10^{-6} cells/ml and it was necessary to subculture again after between 12 and 14 days. Initiation of further cultures from such cell suspension stocks which had been left for longer than 14 days was less successful, and proved impossible when these stock cultures had been left for as long as 20 days. The decline in cell number as the cultures proceed can be seen in Fig. 3.1. This decline can be interpreted either as indicative of cell lysis or as due to an increasing proportion of the cells becoming susceptible to destruction by the chromic acid treatment used to achieve cell separation before counting. Davey, Fowler and Street, 1971 have similarly interpreted a comparable phenomenon in the growth of cell suspensions of Atropa belladonna. When a 5 ml inoculum of a 14 day-old

Explanation of Figure 3.1

The effect of kinetin on the growth of Hevea brasiliensis cell suspensions

Growth of Hevea cell suspensions as measured by the increase in cell number. Each cell number plotted represents the mean of the counts for each of five replicate flasks.

Fig 3.1


4·0
3·0
2·0
1·0
Cell number / ml x 10^{-6}
0
4
8
12
16
20
Days of incubation
2·0 mg/ℓ 2,4-D
0·5 mg/ℓ kinetin
2·0 mg/ℓ 2,4-D
0·0 mg/ℓ kinetin
2·0 mg/ℓ 2,4-D
0·1 mg/ℓ kinetin

Hevea cell suspension culture was used, the initial cell number was between 0.25 and 0.35 x 10-6 cells/ml and it was not necessary to subculture again until between 24 and 26 days had elapsed. (See Figure 3.2). Thus, stock suspensions were routinely cultured in the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin on an orbital shaker set at 120 r.p.m. and incubated at 30° in the dark. Cultures were usually maintained as batches of 55 ml in 250 ml flasks and were transferred after 24 days using an inoculum of 5 ml to achieve an initial cell number in the next passage of between 0.25 and 0.35 x 10^{-6} cells/ml.

The major obstacle in the development of these cell suspension cultures had been the lack of friability of the callus inocula. As already mentioned a dramatic change in the friability and appearance of the callus tissue was achieved by reinoculating callus which had been growing in liquid medium for two passages back on to semi-solid medium. An experiment was now carried out to compare "A" and "S" callus growth using the parameters of fresh weight and dry to fresh weight ratio. 30 "A" callus growths and 30 "S" callus growths of approximately equal size were inoculated on to Murashige and Skoog's medium and incubated at 30°. The culture history of the "A" callus inocula covered 16 passages on agar, each of approximately one month in duration, while for the "S" callus inocula 12 passages on agar followed by two passages in liquid medium, followed by a further two passages on agar medium preceded the passage of the experiment. Ten flasks of both the "A" and "S" callus growths were sampled at approximately 10 day intervals for 32 days.

Explanation of Figure 3.2

The effect of inoculum size on the growth of Hevea brasiliensis cell suspensions

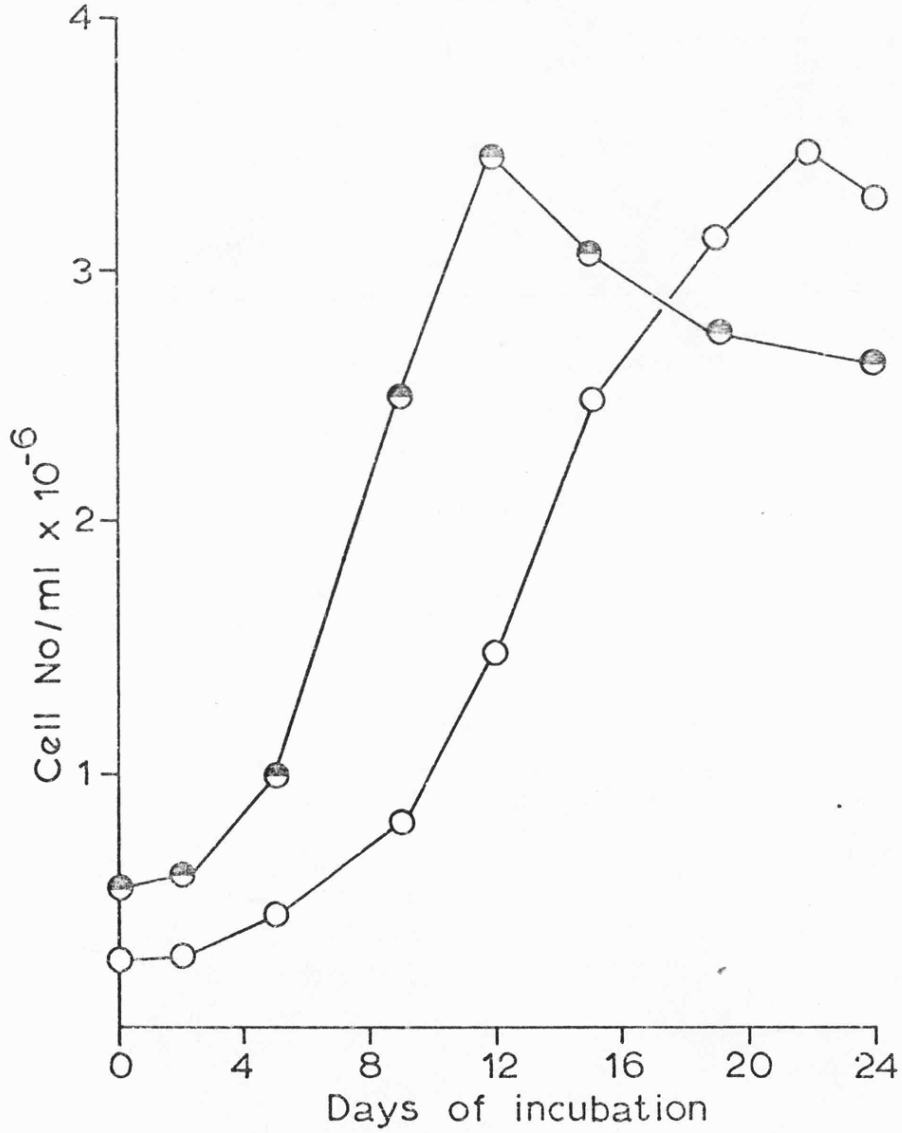
Key:- ●—● Inoculum density of 0.5×10^{-6} cells/ml

O—O Inoculum density of 0.25×10^{-6} cells/ml

Each cell number plotted represents the mean of the counts for each of the five replicate flasks.

Fig 3.2

THE EFFECT OF INOCULUM SIZE ON THE GROWTH CURVE OF CELL SUSPENSIONS OF *HEVEA BRASILIENSIS* AS MEASURED BY CELL NUMBER.



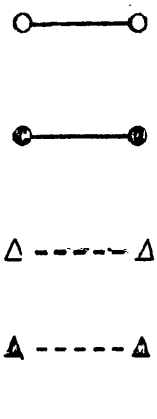
As mentioned above callus inocula of approximately equal size were used, but it was clear that "S" callus growth had a lower weight to volume ratio. Thus the mean ($\bar{x}$) initial fresh weight of the "A" callus inocula was 236.5 mg, while that of the "S" callus inocula was 72.0 mg. This difference could have affected the patterns of growth in two ways. Firstly, the inocula of the "A" callus may be so large as to allow only limited division and growth before medium exhaustion intervenes. Secondly, the "shock" of transfer to fresh medium may be minimised by inocula above a certain critical lower weight limit due to the ability of these heavier inocula to "condition" their immediate environment. However it is doubtful if these factors obscured basic differences in the growth of "A" and "S" callus tissues (Figure 3.3).

From the results it can be seen that the major differences between "S" and "A" callus are that the former tissues grow more rapidly and have a lower percentage dry to fresh weight ratio. As suggested in Section 1 and reaffirmed by these results, a friable callus appears to be one which is comparatively rapidly growing and has a low percentage dry weight. Also clearly indicated here is a low weight to volume ratio since while the volume of "A" and "S" callus tissues taken for inoculation were approximately equal, the weight of the former was 3 times that of the latter (presumably due to extensive "air spaces" in the friable callus). Thus these parameters may be used to predict the facility with which callus tissue will disperse in liquid medium and give rise to actively growing cell suspensions.

Explanation of Figure 3.3

A comparison of the growth of "A" and "S" callus tissues as measured by the % increase in fresh weight and values of dry weight as percentage fresh weight

Key:-



O——O % increase in fresh weight of "A" callus growth

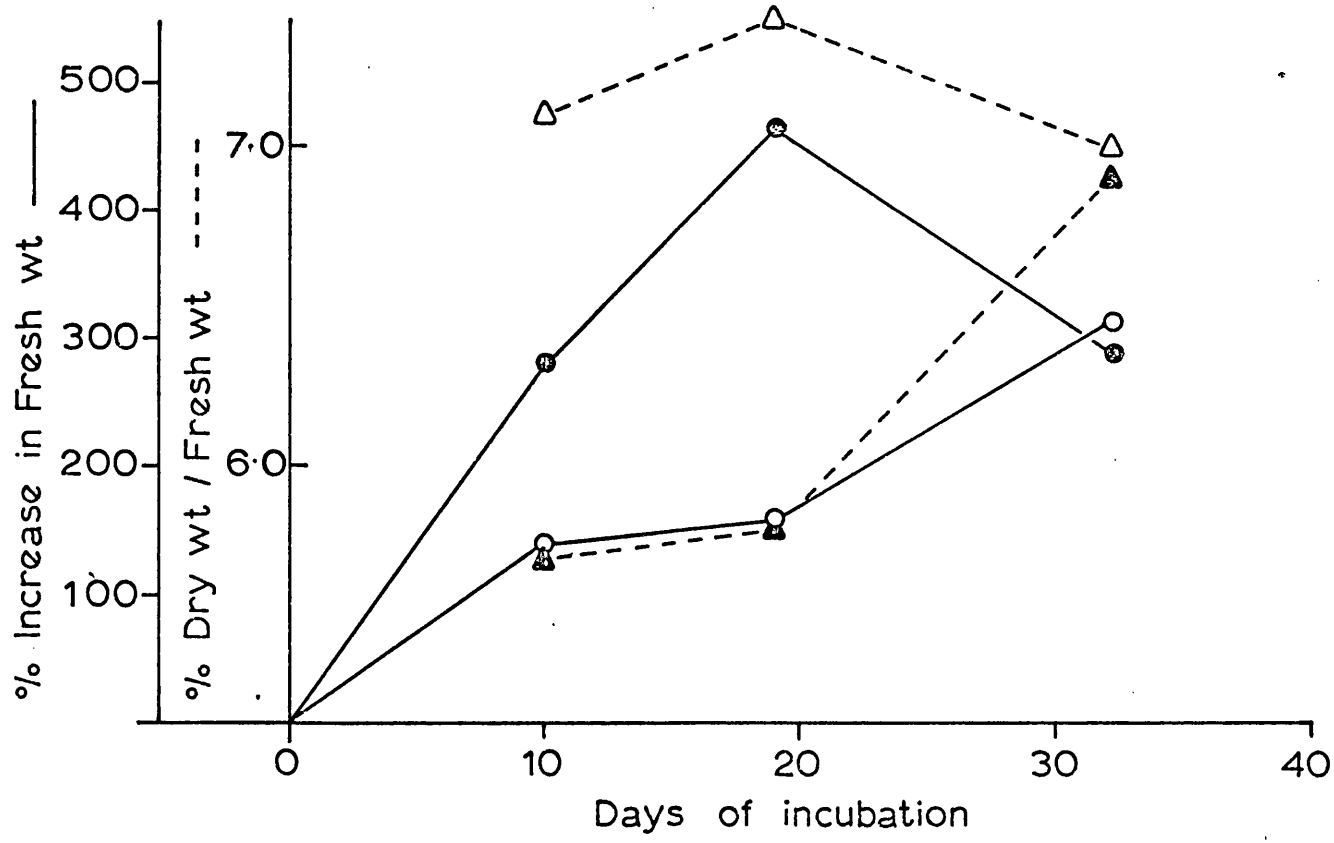
●——● % increase in fresh weight of "S" callus growth

Δ - - - - Δ Dry weight as % fresh weight of "A" callus growth

▲ - - - - ▲ Dry weight as % fresh weight of "S" callus growth

Each measurement recorded represents the mean of ten replicate callus cultures. Both "A" and "S" callus tissues were cultured on the medium of Murashige and Skoog with 2 mg/l 2,4-D and 0.5 mg/l kinetin, at a temperature of 30°.

Fig 3.3


% Increase in Fresh wt ——
% Dry wt / Fresh wt -----
500
400
300
200
100
7·0
6·0
0
10
20
30
40
Days of incubation

Certain callus cultures which require a supply of auxin for growth when first isolated can, under the influence of a high auxin level and an appropriate incubation temperature, develop the capacity for indefinite growth in the absence of external auxin. (Gautheret, 1946.) These "habituated" tissues may differ from the original callus in having a higher growth rate, in being more translucent, in having a reduced compactness to the cell mass (they are more friable) and in their reduced capacity to initiate organs and show internal differentiation. It is possible to speculate that the cells of the "A" callus tissue came under the influence of higher auxin levels in liquid medium than on semi-solid medium due to more direct contact with external auxin under the conditions encountered in such agitated liquid medium. However, it was not possible to maintain the growth of "S" callus tissues indefinitely in the absence of 2,4-D and after one passage the tissues appeared moribund. Thus the changes in callus morphology and growth rate described, although similar to those occurring in tissues which have become "habituated", were not attributed to this phenomenon. (However, see General Discussion).

The effect of light/dark and temperature variations was tested on "S" callus tissue in an identical experiment to that which was carried out using "A" callus tissue (Section 2). No significant differences could be demonstrated when comparing any of the four treatments but when the results for the light and dark treatments at 25° were pooled and compared with the pooled results for the light and dark treatments at 30°, a significant difference was observed (see Table 3.5).

TABLE 3.5

The effect of temperature variation on the growth of "S" callus tissue

Temp.	$\bar{x}$ Initial Fresh wt.	$\bar{x}$ Final Fresh wt.	$\bar{x}$ % Fresh wt.increase	S.D. of Mean
25°	979	2047	100	12
30°	861	2687	225	27

A statistical comparison of the results was made using a modified "t"-test (d).

Comparing	d	°F	Probability	
25° and 30°	4.17	24	<0.001	*

* significant

Twenty replicate cultures were used per temperature.

These results indicate that the "S" callus cultures display the requirement for an incubation temperature of at least 30° for optimum growth that was also evident in the "A" callus cultures. The standard deviation of the mean for the percentage increase in fresh weight for ten cultures of "S" callus tissue at 30° in the dark was 39, while that for the "A" callus cultures was 64.1, this difference probably being a reflection of the greater uniformity of the "S" callus tissues used in the inoculations.

The factors responsible for effecting the transition from "A" to "S" callus growth are clearly mediated through the conditions which prevail in agitated liquid medium. Two working hypotheses were established as a basis for further investigation.

1. The conditions in agitated liquid medium preferentially

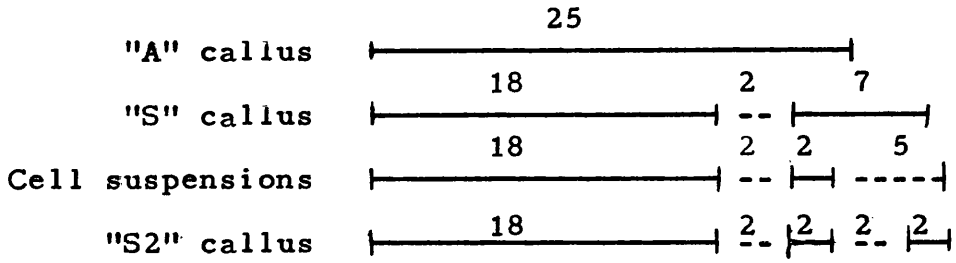
selected for a certain cell or tissue type.

2. The conditions in agitated liquid medium caused a stable change in cellular physiology probably brought about by a leaching-out of inhibitory metabolic products due to the presence of steeper concentration gradients and increased cell to medium contact.

As mentioned in Section 2 physical separation of the various tissue types by sterile dissection proved unsuccessful and the heterogenous "A" callus growth always became re-established. However, since growth of callus tissues in agitated liquid medium is generally enhanced (Caplin and Steward 1949, Venketeswaran 1962, Demoise and Partanen 1969) it may be that preferential stimulation of the actively growing regions of the "A" callus tissues coupled with the shearing forces associated with orbital shaking produced a more complete separation of the cell types than that which could be achieved by sterile dissection alone. Thus the re-establishment of the heterogenous "A" callus tissues after dissection could be attributable to an incomplete separation.

Cytological investigations were undertaken in order to determine whether the transition from "A" to "S" callus growth could be correlated with cytological changes in these tissues. The culture history of the "A" callus tissue used in these studies extended over 25 passages on solidified medium. The "S" callus tissues studied were obtained after 18 passages on agar medium, two "passages" in liquid medium and a further 7 passages on agar medium. Representative samples of cultured cells with two other pedigrees were included in these investigations.

1. Cell suspensions, which were derived from "S" callus tissues and with a culture history of 18 passages on agar, followed by two "passages" in liquid medium, then by two passages on agar and finally by 5 passages proper as a cell suspension. 2. Callus cultures designated "S2" which were obtained after 18 passages on agar, 2 "passages" in liquid medium, 2 passages on agar, 2 passages proper as a cell suspension, and finally two passages back on agar as callus tissues derived from the larger aggregates of these cell suspensions. The four cultural histories described above may be represented schematically.



key:- Agar (passage number) ——————

Agitated liquid medium (passage number) - - - - - - - - - - - - - - - -

In the case of "A" callus tissues the feulgen technique as described in the Materials and Methods section could not be used successfully to obtain metaphase preparations for chromosome counts or stained interphase nuclei for densitometry although many fixation, hydrolysis and staining schedules were tried. Hydrolysis over a range of 5 to 15 minutes failed to render the "A" callus tissues suitable for squashing and the intensity of the feulgen stain was always inadequate for the ready recognition of the nucleus within the cell. This situation could be improved upon by flooding the slide with 1% orcein in 45% propionic acid and after scanning many such

slides it had proved possible to count approximately 36 chromosomes in some twenty different nuclei indicating that at least some of the cells in "A" callus tissue retained their diploid status (2n = 36, Datta, 1967). No technical problems were encountered with the other tissues. Sixty metaphase plates were counted for the "S" callus cultures, and for the cell suspensions obtained from these cultures (see Figure 3.5). The absorptions of 200 randomly chosen interphase nuclei were measured densitometrically for "S" callus tissue, cell suspensions and "S2" callus tissue (Figure 3.4). Mitotic indices were also calculated from these slides by counting a thousand nuclei and expressing the number of recognisable mitotic figures in this total as a percentage.

TABLE 3.6

Culture	Mitotic index
"S" callus	1.7
Cell suspension	3.8
"S2" callus	0.7

From these cytological data it is clear that the great majority, if not all, of the cells comprising "S" callus tissue retains the diploid status, while polyploidy is evident in the cell suspension cultures and the "S2" callus tissues derived from these cultures. Since there is a higher mitotic index in the suspension cultures, this would indicate that at the time of sampling there were comparatively more dividing cells present in these cultures. While the mitotic index does not provide an absolute measure of the number of dividing

Explanation of Figure 3.4

DNA values (Feulgen microdensitometry) of the nuclei of cells comprising callus and cell suspension cultures of Hevea brasiliensis

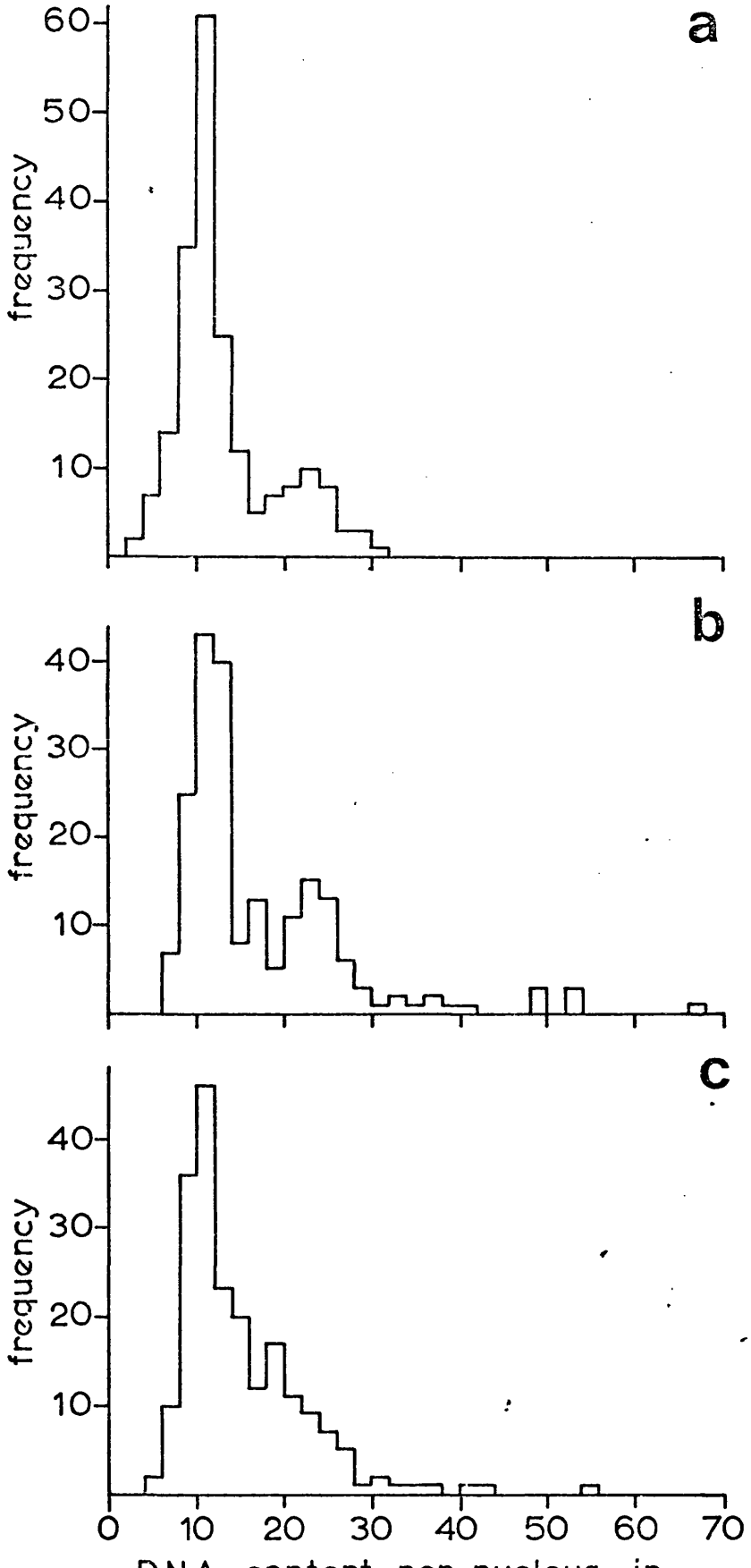
Histograms of the DNA values of 200 randomly selected nuclei.

Key:- a) "S" callus tissue.

b) Cell suspension.

c) "S2" callus tissue.

Fig 3.4


a
b
c
frequency
60
50
40
30
20
10
40
30
20
10
40
30
20
10
0
10
20
30
40
50
60
70
DNA content per nucleus in arbitrary units

Explanation of Figure 3.5

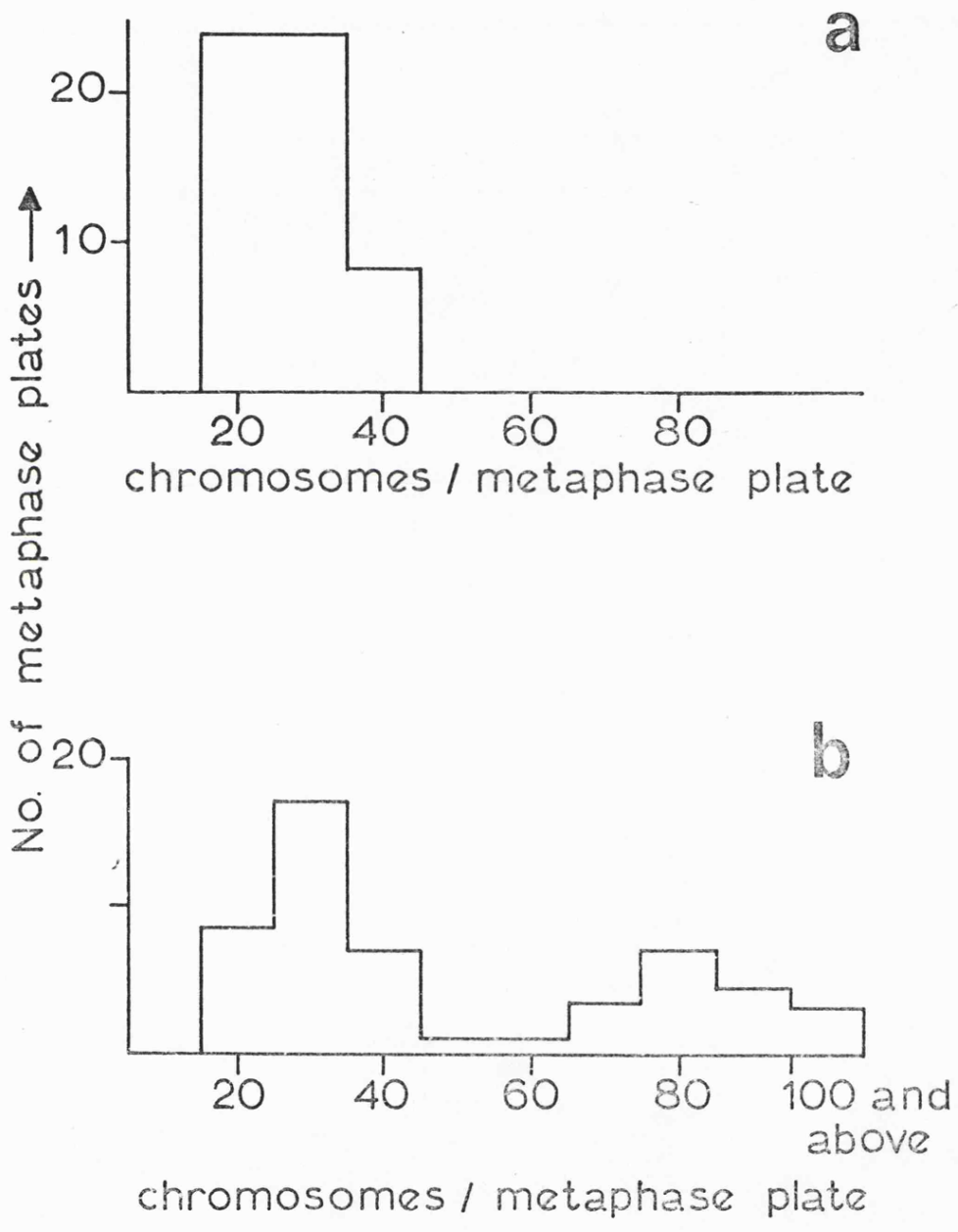
Chromosome numbers in "S" callus tissues and cell suspensions of Hevea brasiliensis

Key:- a) "S" callus tissues, with a culture history of 18 passages on agar, followed by 2 passages as "callus" in liquid medium, followed by 7 passages on agar.

b) Cell suspension, with a culture history of 18 passages as callus on agar, followed by 2 passages "callus" in liquid medium, followed by two passages back on agar and finally 5 passages as a cell suspension.

Each metaphase plate was counted three times and the mean of these counts included in the histograms.

Fig 3.5


a
20
10
No. of metaphase plates
20
40
60
80
chromosomes / metaphase plate
b
20
20
40
60
80
100 and above
chromosomes / metaphase plate

cells in these cultures, it does provide a strong, albeit circumstantial basis for comparison. Thus it is possible that the presence of polyploid cells in the suspension cultures, and consequently in the callus cultures derived from them, may be due to the fact that the rate of polyploid production is directly related to the rate at which these cells divide. It would only be possible directly to attribute the higher frequency of polyploid cells in Hevea suspension cultures to the physical conditions imposed upon the cultures through agitated liquid medium if it could be shown that there are similar numbers of cells dividing at similar rates in both suspension and callus cultures. Demoise and Partanen (1969), on the basis of similar results, suggested that the physical state of the medium exerts an effect on the rate of production of polyploids. For the reasons stated above it is at least as probable that the rate of production of polyploids is closely related to the rate at which the cells divide, and that under different physical conditions but with the same rate of cell division no effect on the rate of production of polyploids would be detectable.

Unfortunately it was not possible to obtain densitometry data for the "A" callus tissues. However, on the basis of the small number of metaphase plates that were counted for "A" callus tissue there is no reason to suppose that the transition from "A" to "S" callus tissue can be correlated with changes in the frequencies of the ploidy levels of their constituent, dividing cells.

The transformation of "A" callus tissue to "S" callus tissue through "passages" in liquid medium may have been

brought about by the presence of greater concentration gradients between the agitated liquid medium and the cells of the "A" callus tissue. The enhancement of these concentration gradients would facilitate a more efficient removal of inhibitory metabolic products than is possible when the callus tissue is grown on semi-solid medium. However, if this were the complete explanation then reversion back to the "A" callus type may be expected to occur when these tissues are allowed to remain on semi-solid medium for a prolonged period. After nine passages no reversion is evident. It may be that the behaviour of cells and their subsequent patterns of metabolism and growth can be modified by conditions operating during a period of cell division. The cells which remain active and dividing in the agitated liquid medium "passages" could be regarded as displaying a physiological adaptation which they retain when cultured on semi-solid medium and subsequently give rise to "S" callus tissues.

From "S" callus tissues it proved possible routinely to initiate cell suspensions and to subculture these cell suspensions indefinitely in the medium of Murashige and Skoog, shaking at 120 r.p.m., and incubated at 30°. The simple device of transferring callus tissues growing on agar medium to liquid medium for two or more"passages" and then returning the tissues to agar medium, provided the conditions necessary for the development of a friable callus tissue suitable for the initiation of cell suspensions. This device may be generally applicable to plant species which can be cultured *in vitro* as callus but which show recalcitrance in the formation of cell suspensions from these callus tissues.

SECTION 4

THE EFFECTS OF 2-CHLORO-ETHYLPHOSPHONIC ACID (2-CEPA) ON CALLUS AND CELL SUSPENSION CULTURES OF HEVEA BRASILIENSIS

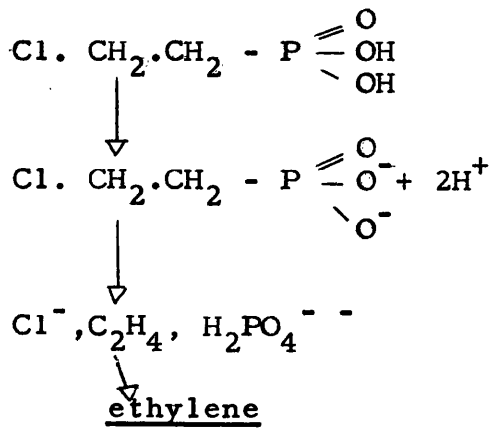
INTRODUCTION

2-chloroethylphosphonic acid (2-CEPA) is now well known as a plant growth regulator (De Wilde, 1971). Its effects on plants include induction of flowering (Cooke and Randall, 1968), maturation of fruits and abscission of leaves, (Morgan, 1969, Bukovac, Zucconii, Wittenbach, Flore and Inoue, 1971), seed germination (Chatterji, Harsh, Sankhla and Sankhla, 1971), and rubber latex yield-stimulation (Abraham, Wycherley and Pakianathan, 1968). In all cases its biological activity has been ascribed to the ethylene formed when it decomposes in aqueous solution above pH 3.5. (Cooke and Randall, 1968). The decomposition of 2-CEPA does not appear to be accompanied by the formation of metabolites (Weaver, Abdel-Gawad and Martin, 1972, Martin, Abdel-Gawad and Weaver, 1972, Yamaguchi, Chu and Yang, 1971) but a study of the decomposition of ^{14}C labelled 2-CEPA in stems and leaves of Hevea brasiliensis resulted in the detection of 13 labelled compounds in the leaves and 20 in the stem. (Archer, Audley and Mann, 1973). These results suggest that 2-CEPA is metabolised by the tissues of Hevea brasiliensis to a significant extent but it is possible that the compounds formed may have arisen in part from the metabolism of ethylene released by the non-enzymic decomposition of 2-CEPA. However, it is considered unlikely that most of the compounds observed were formed from ethylene since work carried out with a variety of plant tissues indicates that ethylene is poorly metabolised (Abeles, 1972).

The studies of Archer et al.,(1973) were made on the non-sterile stems and leaves of 2-year-old Hevea brasiliensis seedlings and thus it is possible to speculate that the conversion of ^{14}C-2-CEPA into a range of different compounds was mediated by micro-organisms. It was of interest, therefore, to incubate actively growing, sterile callus tissues with ^{14}C-2-CEPA to determine whether undifferentiated Hevea cells could metabolise ^{14}C-2-CEPA under conditions eliminating the possible role of micro-organisms in the work of Archer et al. (1973). Furthermore 2-CEPA is thought to act on plant tissues primarily through the intracellular release of ethylene (Warner and Leopold, 1969) and the inclusion of this compound in plant tissue culture systems affords an attractive method for the study of the effects of ethylene on growth and morphogenesis.

2-CEPA, stable in strongly acid conditions, rapidly decomposes with a resultant evolution of ethylene in alkaline solutions (Cooke and Randall, 1968).

2-CEPA.
(M.wt = 144.5)



Audley and Archer (1973) have shown that under "physiological" conditions of pH and temperature (citric acid - Na_2HPO_4 buffer over a pH range of 5.3-7.2 at 25°) 2-CEPA forms a small amount of 2-hydroxyethylphosphonic acid,

(2-HEPA) during its decomposition which is stable and does not evolve ethylene.

2-HEPA. $OH.CH_2.CH_2.-P(=O)(OH)(OH)$

While the main effects of 2-CEPA on plants are probably to be accounted for by the ethylene into which it decomposes, there is thus the possibility that 2-HEPA could also be formed and itself have physiological activity. The formation of this compound may account in part for the "metabolites" of 2-CEPA detected in the experiments of Yamaguchi et al. (1971) and Edgerton and Hatch (1972). The results of Audley et al. (1973) also show that any method for the assay of 2-CEPA which is based solely on the estimation of the ethylene formed on decomposition in alkali will give low values.

There are very few published reports on the effects of ethylene on plant tissues cultured in vitro. Bradley and Dahmen (1971) showed that 5 ppm ethylene in combination with other plant growth regulators decreases the incidence of specialised tissues inPeach mesocarp callus. Mansfield (1973) demonstrated that 2-5 ppm ethylene and 1% carbon dioxide had a greater stimulatory effect on growth from low densities of Acer pseudoplatanus suspension cultures than did 1% CO_2 alone. Huang (1971) was able to disrupt the organised growth of wild carrot callus tissue using ethylene which is in accordance with an earlier observation of a similar 2-CEPA effect noted by Wochok and Wetherell (1971). In this work 2-CEPA prevented the development of organisation in wild carrot callus tissue, an effect which can also be achieved by addition of 2,4-D and Huang (1971) was able to show the presence of higher levels of

naturally produced ethylene in auxin-grown tissue cultures than those grown on auxin-free medium. Wochok and Wetherell (1971) noted an abundance of single cells in the 2-CEPA medium and suggested that 2-CEPA disrupts cell association through the release of ethylene which may activate specific lytic enzymes resulting in cell wall degradation. Ridge (1972) showed that 2.0 mg/l and 20 mg/l of 2-CEPA greatly promoted the unorganised proliferation of cells from explants of Begonia x richmondensis. The growth habit of the callus tissue was very "smooth" due to the high tendency of the cells to dissociate, resulting from the breakdown of the middle lamellae and an increase in size of the separated cells. In the presence of 2-CEPA no differentiation of tracheidal structures occurred within the cell walls. The association of 2-CEPA with an enhanced latex flow from the rubber tree, and its effects on cell separation in tissue cultures prompted the work presented in this section on callus and suspension cultures of Hevea brasiliensis.

A. The Incubation of ^{14}C-2-CEPA with "S" callus tissues.

The gift of purified 2-CEPA and 1-2-^{14}C-2-CEPA from the biochemistry department of the Natural Rubber Producers Research Association is gratefully acknowledged. The 2-CEPA was initially obtained from Amchem Products (96% purity) and 1-2-^{14}C-2-CEPA prepared from this by a modification of the method of Maynard and Swan (1963) to a radiochemical purity of 99% (Audley and Archer, to be published). In order to measure radioactivity scintillation counting was used throughout, with a 0.9% 2-(4^{1}-t-butyl phenyl) 5-(4^{11}-biphenyl) - 1,3,4-oxadiazole (butyl-PBD) in toluene, and with Triton x 100 (2 : 1 v/v) asthe phosphor. Unless otherwise stated the

samples were counted with 14 ml of the scintillation cocktail described above.

Ten 100 ml Erlenmeyer flasks each containing 25 ml of semi-solid medium were each inoculated with 1 gm of actively growing "S" callus tissue. 6μC of ^{14}C-2-CEPA (specific activity 2.2 C/μMole. Total dps in 6μC = 222000) were dissolved in 1.67 ml of standard M + S medium, the pH checked at 5.6 and the solution filter-sterilised. 0.1 ml of this solution was inoculated into each of the ten "S" callus tissues and incubated at 30^{o} in the dark in a fume cupboard for 96 hours. After this time the total "S" callus tissues were removed from the agar and placed in a weighed conical flask, reweighed and then sealed and stored in a deep-freeze. The agar wastreated similarly.

Total weight of "S" callus tissues = 14.029 gm

Total weight of Agar medium = 201.500 gm

1. <u>"S" Callus Tissues</u>

After allowing to thaw, the "S" callus tissues were crudely"homogenised" by chopping them into small fragments and randomising the resultant fragmented mass. This was performed using a razor blade and chopping the "S" callus tissues on a glass plate. The mass of callus tissue was removed from the glass plate and weighed.

Weight of callus removed from glass plate = 12.270 gm. The fragments of tissue remaining on the plate and in the original weighed conical flask were washed with water into a weighed flask.

Wt. of residue + washings = 62.180 gm

The amount of radioactivity lost in this residue as a consequence of these preliminary manipulations = 2443.7 dps.

About one quarter of the callus tissue was taken and weighed (3.25 gm taken). The tissue was suspended in approximately 50 ml of 0.05 N.HCl and an homogenate prepared by disintegrating the tissue for 30 sec at full speed in a Whillems Polytron Type 20 homogeniser (Northern Media Supply Ltd.) Centrifugation followed and the supernatant was removed and placed in a 100 ml volumetric flask. This extraction procedure was repeated twice more on the tissue but with approximately 20 ml of 0.05 N.HCl the pooled supernatants collected and made up to 100 ml with 0.05N.HCl. After correcting for background and efficiency 75.3 dps/ml were counted. $\therefore$ in 100 ml = 75.3 x 100 = 7530 dps

$\therefore$ in 1 gm of callus tissue = $\frac{7530}{3.25}$ = 2317 dps/gm

A fourth re-extraction was carried out with 25 ml of 0.05 N.HCl and the supernatent counted but after correcting for background and efficiency only 1dps/ml was recorded. The re-extraction procedure was repeated using ethanol and 2:1 v/v chloroform/methanol but the number of counts in the fractions soluble in these solvents was not significantly above background.

Analysis of the 0.05 N.HCl extracts of "S" callus tissues after 90 hours of incubation with ^{14}C-2-CEPA

The pooled supernatents from the first 3 extractions with 0.05 N.HCl which were made up to 100 ml with 0.05 N.HCl were freeze-dried. The freeze-dried extract was then washed with 4 x 5 ml washings of water which were subsequently transferred to a 25 ml volumetric flask. 1 ml of Imidazole hydrochloride buffer pH 6.6 ($[Cl^-]$ = 0.25M) was added

and the pH of the solution adjusted to 6.6 with NaOH. A large amount of debris accumulated during the freeze-drying process and this was removed by low speed centrifugation. The volume was made up to 25 ml with extra washings and the pH rechecked, and 2 x 1 ml samples were taken for counting.

Cps/ml = 162.8

dps/ml = 277.5

$\therefore$ in remaining 23 ml total cps = 23 x 162.8 = 3744

$\therefore$ in remaining 23 ml total dps = 23 x 277.5 = 6383

In the original 100 ml from which the freeze-dried extract was obtained there were 7530 dps. Thus, 25 x 277.5 = 6938 dps were recovered after freeze-drying. The remaining 23 ml was them chromatographed.

Column Details

The 20 x 1.3 cm QAE Sephadex A25 Cl^- column was equilibrated with Imidazole hydrochloride buffer $[Cl^-]$ = 0.01 M pH 6.6 and the 23 ml of the extract loaded. (The buffer strength of the 23 ml is equal to 0.01 M Imidazole hydrochloride since 1 ml of 0.25 M Imidazole hydrochloride was contained in the original 25 ml of the extract, i.e. 1 ml of 0.25 M buffer = 25 ml of 0.01 M buffer.) Pressure was applied to the column under nitrogen set at a flow rate of approximately 3 ml/min and the effluent from the column collected. The column was loaded with 2 x 5 ml washings of Imidazole hydrochloride buffer $[Cl^-]$ = 0.01 M from the 25 ml volumetric flask and eluted with Imidazole hydrochloride buffer $[Cl^-]$ = 0.05 M pH 6.6. 90, 7 ml fractions were collected. Imidazole hydrochloride $[Cl^-]$ = 0.025 M pH 6.6

was then applied to the column and a further 10, 7 ml fractions collected. 1 ml of each fraction was taken for counting. Cps were corrected for background and the cps/ml plotted against the fraction number (see Figure 4.1). 2 x 2 ml samples of the bulked effluent and washings were also counted but no counts above background were detectable.

$\bar{x}$ cps/ml of fractions collected = 5.38

700 ml in total volume of fractions,

$\therefore$ 700 x 5.38 = 3766 cps

Total cps loaded on to the column = 3744

$\therefore$ % recovery from column = $\frac{3766}{3744}$ = 100.6%

As a check on the extraction procedure with 0.05 N.HCl, extraction with hot 10% Trichloroacetic acid (TCA) was performed. 0.93 gm of the "S" callus tissues were suspended in 10 ml of 10% TCA for 2 hours at approximately 20°. The suspended tissue was then incubated at 90° for 35 minutes. The volume was made up to 50 ml with washings of 10% TCA and 2, 1 ml samples counted. This extraction procedure recovered 2387 dps/gm and thus shows no significant improvement on the 2317 dps/gm extracted with 0.05 N.HCl.

2. Semi-solid Medium

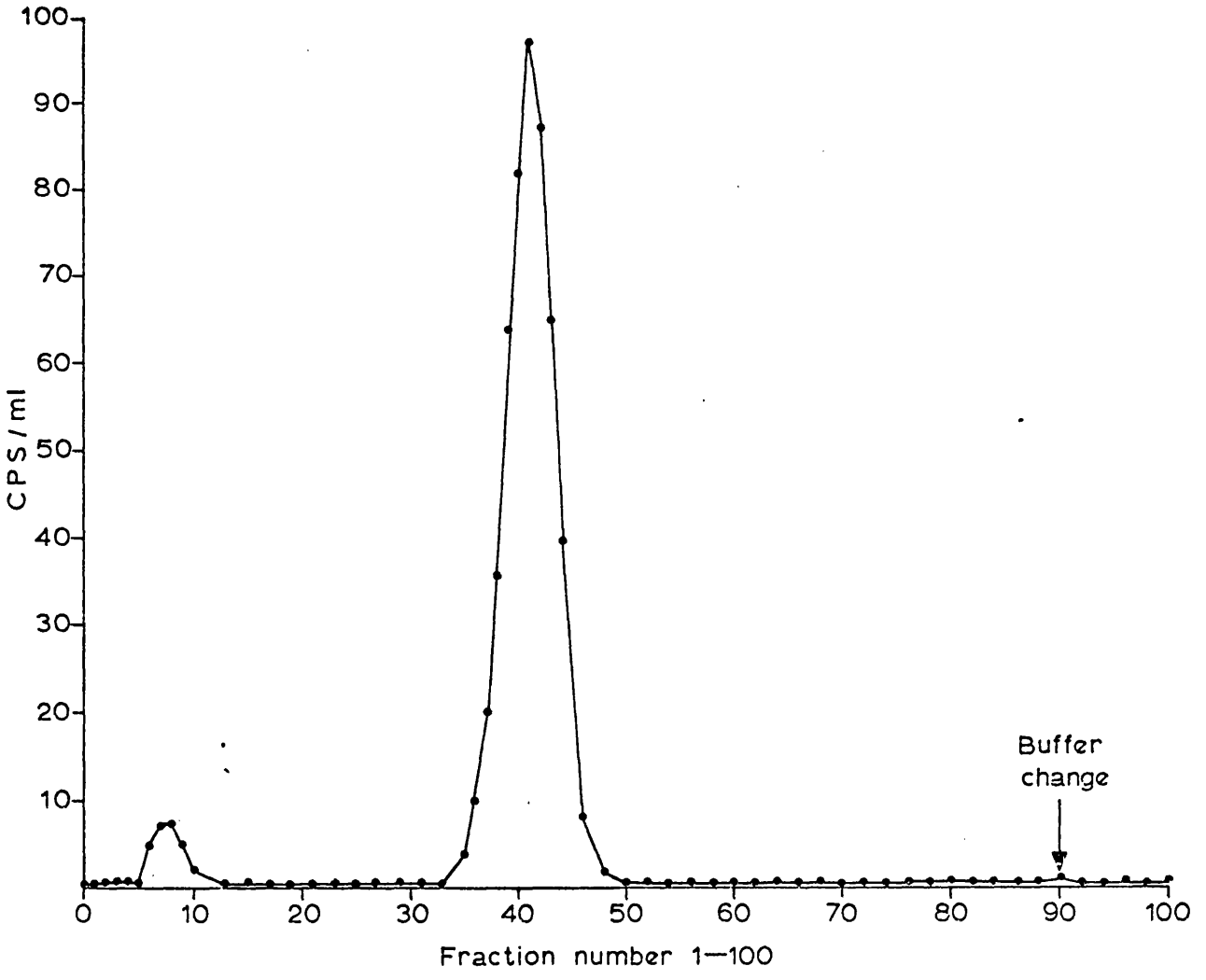
The agar medium from five flasks was transferred to a 250 ml centrifuge tube. The flasks were rinsed with distilled water and the washings plus the medium "homogenised" for 30 sec at full speed in a Whillems Polytron. The remaining five flasks were treated similarly. The total weight of the agar medium obtained was 201.50 gm. The two samples were mixed and "re-homogenised" using a Polytron. From this mixture two samples were each weighed into scintillation

Explanation of Figure 4.1

QAE Sephadex chromatography of the freeze-dried extract of "S" callus tissues which had been incubated with ^{14}C-2-CEPA

23 ml of sample was sorbed on to a column of QAE Sephadex A25Cl^- in Imidazole buffer pH 6.6 $[Cl^-]$= 0.01. The column was then washed with 60 ml of this buffer and eluted with 0.05 M Imidazole buffer. 100, 7 ml fractions were collected, the final 10 with 0.025 M imidazole buffer. A 1 ml sample from each fraction was taken, the radioactivity measured, and the count plotted against fraction number.

Fig 4.1


100
90
80
70
60
50
40
30
20
10
CPS / ml
Buffer
change
0
10
20
30
40
50
60
70
80
90
100
Fraction number 1—100

vials, another two samples were each weighed into 5 ml volumetric flasks and made up to volume with N.HCl. 1 ml of N.HCl was also added to each of the samples weighed into scintillation vials. The samples were counted and tabulated below.

TABLE 4.1

	Wt.in gm	Wt.counted	dps/gm of agar	Total dps
(1) weighed in vial	1.09	1.090	752	151,528
(2) weighed in flask	1.19	1.190	727	146,491
(3) weighed in flask	0.78	0.156	789	158,984
(4) weighed in flask	0.86	0.172	709	142,864

$\therefore \bar{x}$ of the Total dps in the agar medium = 149,967

A quantity of agar medium was weighed in a centrifuge tube (12.07 gm) and a volume of distilled water was added (14.32 ml). This was centrifuged and a supernatant of 13.5 ml was obtained. The pH of the supernatant was adjusted to 6.6 with 0.1 N. NaOH. 5 ml of Imidazole hydrochloride buffer $[Cl^-]$ = 0.05 M pH 6.6 was added and the total volume made up to 25 ml (giving Imidazole hydrochloride = 0.01 M). 10 ml were chromatographed and 2, 0.5 ml samples were counted. After correcting for background CPS/ml = 130.6. After correcting for efficiency dps/ml = 189.8

$\therefore$ in 10 ml loaded on to column there were

1306 cps and 1898 dps.

In 25 ml there are 189.8 x 25 = 4745 dps. These counts (4745 dps) are derived from the 13.5 ml centrifugate supernatant. Assuming the supernatant to be in equilibrium with

the agar after centrifugation then since

$$\frac{13.5}{12.07 + 14.32} = 51\%$$

∴ 49% of the counts remain in the agar.

Therefore the total counts in the 12.07 gm of agar originally weighed = $\frac{100}{49}$ x 4745

= 9684 dps = $\frac{9684}{12.07}$ = 802 dps/gm

To compare with this figure 2, 1 gm samples of agar were weighed into scintillation vials and counted. After correcting for efficiency dps/gm = 758. Thus it is clear that counts are not lost in the preparation of the supernatant sample for sorption on to the column.

Column details

The 20 x 0.5 cm QAE Sephadex A25 Cl^- column was equilibrated with Imidazole hydrochloride buffer $[Cl^-]$ = 0.01M pH 6.6 and then the 10 ml sample was sorbed on to the column. The column was washed with 0.01 M Imidazole buffer and the effluent and washings bulked and counted. No counts were detectable. Elution of the first 50 fractions was carried out with 0.05 M Imidazole buffer. 1 ml fractions were collected directly into scintillation vials. The last 10 fractions were eluted with 0.025 M Imidazole buffer. Unfortunately the ^{14}C-2-CEPA travelled down the column more slowly than was expected and thus the change of buffer strength from 0.05 M to 0.025 M Imidazole hydrochloride was made too soon. This resulted in an asymmetrical peak of ^{14}C-2-CEPA due to the rapid passage of the 0.025 M Imidazole buffer down the column causing a very high proportion of the remaining counts to be collected in fraction no. 52. However this did not affect the separation of ^{14}C-2-CEPA from any of the suspected

"metabolites" since these are generally detected in the early fraction numbers. Cps were corrected for background and the cps/ml plotted against the fraction number (see Figure 4.2).

Total cps recovered from chromatography = 991

Cps loaded on to the column = 1306

∴ % recovery after chromatography

$$= \frac{991}{1306} \times 100 = 76\%$$

The estimation of alkali-stable radioactivity in the main peak eluted from QAE Sephadex chromatography of the "S" callus tissue extract

It was assumed that the main peak in Figure 4.1 was composed of ^{14}C-2-CEPA but in order to confirm this an estimation of the alkali-stable radioactivity in the peak was obtained and a thin-layer chromatograph of the main peak prepared.

The remaining 6 ml of fractions 36-48 (Figure 4.1) were bulked, the volume accurately measured and 2, 1 ml samples counted.

Total Volume = 77 ml dps/ml = 67.6

∴ total dps in fractions 36-48 = 5229

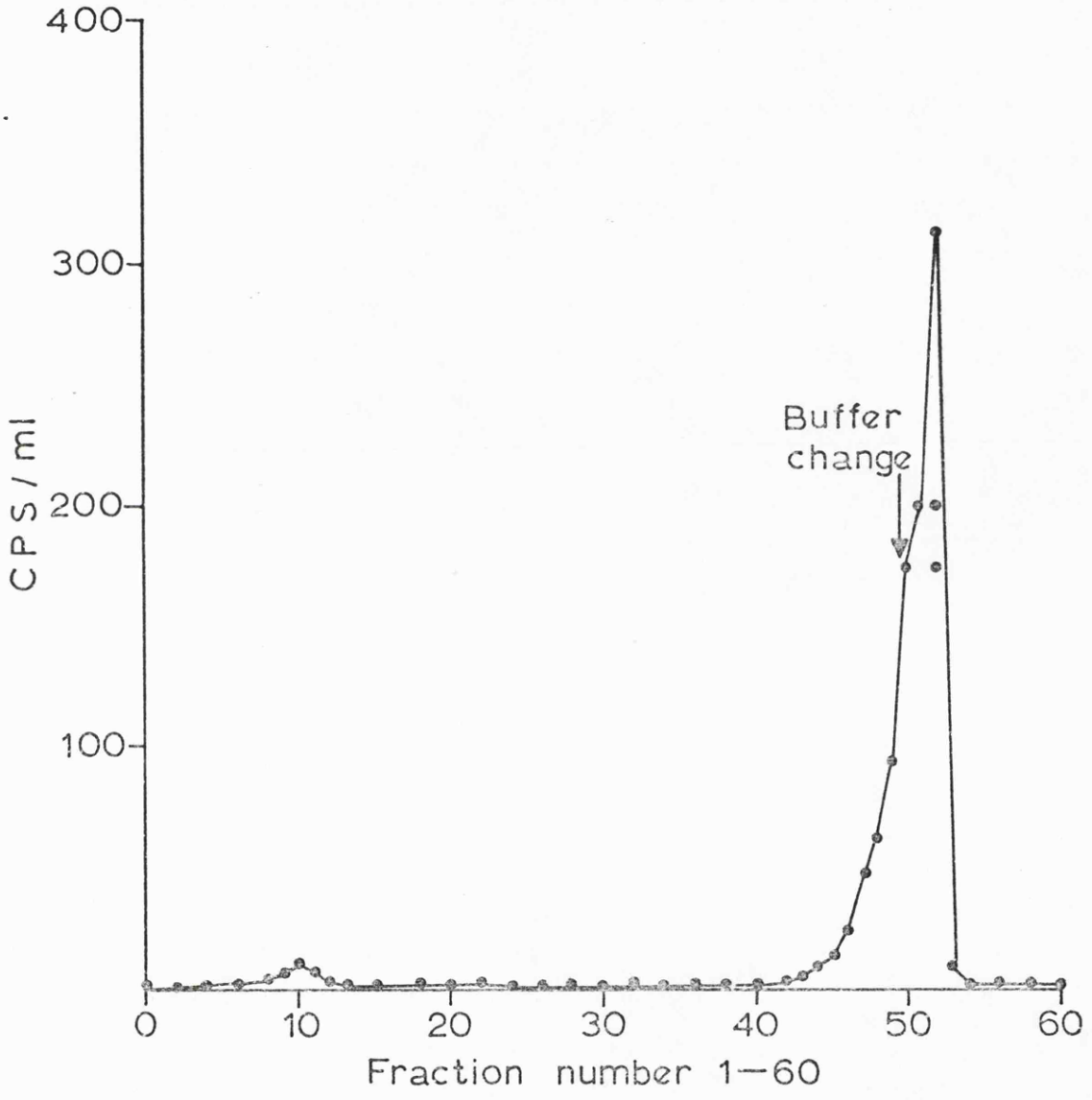
The bulked samples were passed down a BioRad AG50W-X12 H^+ column (3 x 1 cm) to remove the Imidazole and the resulting effluent was then freeze-dried. The residue was taken up in 10 ml of 80% ethanol and 2, 0.5 ml samples counted. After correcting for background and efficiency the total dps recovered from the cationic column was 3650. 2, 1 ml samples (365 dps per sample) were dried down in scintillation vials and 2 ml of 0.15 M glycine buffer pH 9.1

Explanation of Figure 4.2

QAE Sephadex chromatography of an extract of the agar medium which had supported the growth of the "S" callus tissues during the incubations with ^{14}C-2-CEPA

10 ml of sample was sorbed on to a column of QAE Sephadex A25Cl$^-$ in imidazole buffer pH 6.6 $[Cl^-] = 0.01$. The column was then washed with 10 ml of this buffer and eluted with 0.05 M Imidazole buffer. 60, 1 ml fractions were collected, the final 10 with 0.025 M imidazole buffer. The radioactivity was measured in each 1 ml fraction and the count plotted against fraction number.

Fig 4.2


400
300
200
100
CPS / ml
Buffer
change
0
10
20
30
40
50
60
Fraction number 1—60

added to each. The samples were then heated at 60° for 4 hours, cooled, two drops of N.HCl added and then counted. After correcting for background and efficiency and averaging the two samples 16 dps/ml remained.

$\therefore$ % of ^{14}C which is alkali stable

$$= \frac{16}{365} \times 100 = 4.4\%$$

This figure (4.4%) probably relates to the presence of ^{14}C-2-HEPA, with the main peak being ^{14}C-2-CEPA and shows that metabolites are absent within the limitations of this method.

Thin layer chromatography of the main peak eluted from QAE Sephadex chromatography of "S" callus tissue extract

The remainder of the 10 ml, in which the freeze-dried effluent from the Bio Rad column had been taken up, was blown to dryness and taken up in 0.15 ml 80% ethanol (total dps = 3650 - (2 x 365) = 2920 dps/0.15 M) 10 μl (195 dps) were run in Butanol-Acetic acid-water (12 : 3 : 5 v/v) together with 10 μl (1630 dps) of marker ^{14}C-2-CEPA. The T.L.C. was dried, marked with radioactive ink, wrapped in "Melanex" paper and exposed to Kodirex X-ray film for 2 weeks.

From the T.L.C. and the estimation of alkali-stable radioactivity it can be said that the main peak in Figure 4.1 is composed of unchanged ^{14}C-2-CEPA. The ^{14}C-2-CEPA used for the experiment was chromatographed on a 20 x 0.5 cm QAE Sephadex A25 Cl^- column and 1 ml fractions collected directly into scintillation vials.

Total number of dps chromatographed = 19675

dps in non-CEPA peak = 246

$\therefore$ % impurity = $\frac{246}{19675}$ x 100 = 1.25%

Thus the ^{14}C-2-CEPA used in the experiment was 98.75% pure. The percentages of non-CEPA in the agar medium and in the "S" callus tissues are calculated below.

Agar medium

Total dose = 6μC = 222000 dps

Total dps detected in agar = 150,000 dps

Total dps chromatographed from agar extract = 1898

dps in non-CEPA peak = 40

$\therefore$ % of non-CEPA in agar medium = $\frac{40}{1898}$ x 100 = 2.1%

(ignoring % recovery)

Thus % of non-CEPA on dose = 2.1 x $\frac{150,000}{222000}$ = 1.4%

"S"callus tissues

Total dose = 6μC = 222000 dps

Total dps extracted from 12.67 gm of "S" callus tissues

= 28,430

Total dps in "S" tissue extract chromatographed

= 6383

dps in non-CEPA peak = 280

$\therefore$ % of non-CEPA in "S" callus tissue

= $\frac{280}{6883}$ x 100 = 4.4%

Thus the % of non-CEPA on dose = 4.4 x $\frac{28430}{222000}$ = 0.56%

$\therefore$ Total non-CEPA on dose from experiment

= 1.4% + 0.56% = 1.96%

However, since 1.25% of non-CEPA is already present as impurity, the 1.96% non-CEPA detectable is clearly not significant within the limits of experimental error, and strongly

suggests that little or none of the ^{14}C-2-CEPA applied in the dose has been converted into non-volatile products by the "S" callus tissues. Of the total dose of 6μC (222000 dps) 150,000 dps was recovered from the agar medium and 28,480 dps from the "S" callus tissues. Therefore, 43,570 dps were not recovered and were presumably lost as ethylene, evolved during the decomposition of ^{14}C-2-CEPA which continued throughout the incubation period of the experiment. For this reason the percentage of non-CEPA must be calculated as a percentage of the dose rather than as a percentage of the total dps recovered.

As mentioned in the introduction to this section the work of Archer et al. (1973) clearly suggests that the decomposition of ^{14}C-2-CEPA in stems and leaves of 2-year-old Hevea seedlings is accompanied by a conversion of up to 39% of the ^{14}C-2-CEPA applied into a range of non-volatile products. This conversion was not evident when "S" callus tissues of Hevea brasiliensis derived from stem explants of approximately 1-year-old seedlings were incubated with ^{14}C-2-CEPA. There are three possible explanations for this lack of conversion. 1) The metabolic capabilities of mature leaves and pieces of green stem are different from those of "S" callus tissue. 2) The experimentally observed conversion of ^{14}C-2-CEPA into non-volatile products by mature leaves and pieces of green stem is mediated by micro-organisms which are not present in the sterile environment of the "S" callus tissues. 3) ^{14}C-2-CEPA was not taken up by the cells of the "S" callus tissues but was simply adsorbed on to cell walls and thus did not become involved in their metabolic processes.

However, this latter alternative does not preclude the possibility of the ethylene released from ^{14}C-2-CEPA having an effect on the growth and morphology of the cells of "S" callus tissue as is clearly suggested by data to be presented in the latter part of this section. The difficulty lies in establishing whether the effective ethylene release by 2-CEPA is an extra- or intra-cellular phenomenon or both. Warner and Leopold (1969) suggest that 2-CEPA is taken up in plants as is any weak aliphatic acid and subsequently breaks down at cytoplasmic pH with the formation of ethylene. However, there is a considerable release of ethylene from 2-CEPA at pH values above 4.1 (Cooke and Randall, 1968) and so 2-CEPA in a tissue culture medium of pH 5.6 will decompose to form ethylene in the absence of an intracellular environment. On the basis of the data to be presented it appears unlikely that no ^{14}C entered the cells of "S" callus tissues in the experiment described above, either in the form of ethylene or as undecomposed 2-CEPA. Failure to detect the incorporation of ^{14}C-ethylene has been commonly encountered, and even where incorporation is reported it always represents only a small percentage of the starting level which makes it reasonable to assume that the incorporation measured represents an artifact due to impurities in the ethylene used (Abeles, 1972). The mode of action of ethylene is unknown but Abeles (1972) suggests, simply on the basis of no contradictory evidence, that ethylene may be bound to its site of action by weak Van der Waal's forces and that the site may contain a metal.

The experiment described above was performed in order to eliminate the implication that the conversion of ^{14}C-2-CEPA

by non-sterile mature tissues of Hevea brasiliensis into a range of non-volatile products may involve contaminatory micro-organisms. However, the metabolic capabilities of the tissues of mature leaves and stem segments could be expected to be different from those of the undifferentiated "S" callus tissue and thus a failure to demonstrate a similar conversion of ^{14}C-2-CEPA in these tissues cannot be ascribed merely to its sterility. The experimental conditions used for the incubations of ^{14}C-2-CEPA in these tissues cannot be ascribed merely to its sterility. The experimental conditions used for the incubations of ^{14}C-2-CEPA with "S" callus tissues were based on the satisfactory results obtained for similar incubations with ^{14}C- Mevalonic acid (see Section 5). One practical drawback in the use of callus tissue for such experiments is that it is technically very difficult to "wash" the material free from any of the radioisotope adhering (in intercellular spaces or adsorbed on to the cell walls) to the cells. Cell suspension cultures appear more suitable from this viewpoint.

B. The effect of 2-CEPA on the level of dispersion and morphology of cells in liquid cultures of Hevea brasiliensis

The incorporation of 6 mg/l filter-sterilised 2-CEPA into semi-solid Murashige and Skoog medium pH 5.6 at a setting temperature of between 35°-40° had no detectable effect on the growth habit and friability of "A" callus tissue. The incorporation of 6 mg/l of filter-sterilised 2-CEPA into liquid Murashige and Skoog medium pH'd at 5.6 resulted in a marked increase in the level of cell dispersion in the medium supernatant using "S" callus tissues as the

inocula, this being apparent after 20 days (see Plate 5). Ten out of a total of fifteen replicate flasks showed this response. 35% of the cells in the medium supernatant of these cultures were either free or in aggregates of less than ten cells. When the 2-CEPA was autoclaved no difference in the levels of cell dispersion in the medium supernatant was detectable from that obtained in the absence of 2-CEPA.

The stability of 2-CEPA to autoclaving (15 psi at 121° C for 15 minutes) in the medium of Murashige and Skoog

Approximately 12 μC of ^{14}C-2-CEPA was dissolved in 4.9 ml of distilled water, and 0.1 ml of this solution diluted to 5 ml with N.HCl 2 x 1 ml samples were counted. After correcting for efficiency dps/ml = 135935. 0.25 ml of this solution ($\frac{135935}{4}$ = 0.918μC) was added to 10 ml of standard Murashige and Skoog medium pH 5.6 and the resulting solution weighed at 10.22 gm. The solution was then autoclaved, allowed to cool and reweighed at 9.84 gm. 2 x 2 ml samples were taken and counted. After correcting for efficiency 1712.5 dps were detected remaining in the weighed solution after autoclaving.

$\therefore$ percentage of dps recovered after autoclaving

$$= \frac{1712.5}{33984} \times 100 = 5\%$$

This material is probably 2-HEPA with all the ^{14}C-2-CEPA having decomposed (95% ethylene).

The specific activity of ^{14}C-2-CEPA = 2.2 μC/μMole

$$= 2.2\ \mu C/144.5\ \mu gm$$

$\therefore$ the concentration of 2-CEPA exposed to autoclaving

$$= \frac{0.918}{2.2} \times 144.5 = 60.3\ \mu g/10\ ml \text{ or } 6.03\ mg/l$$

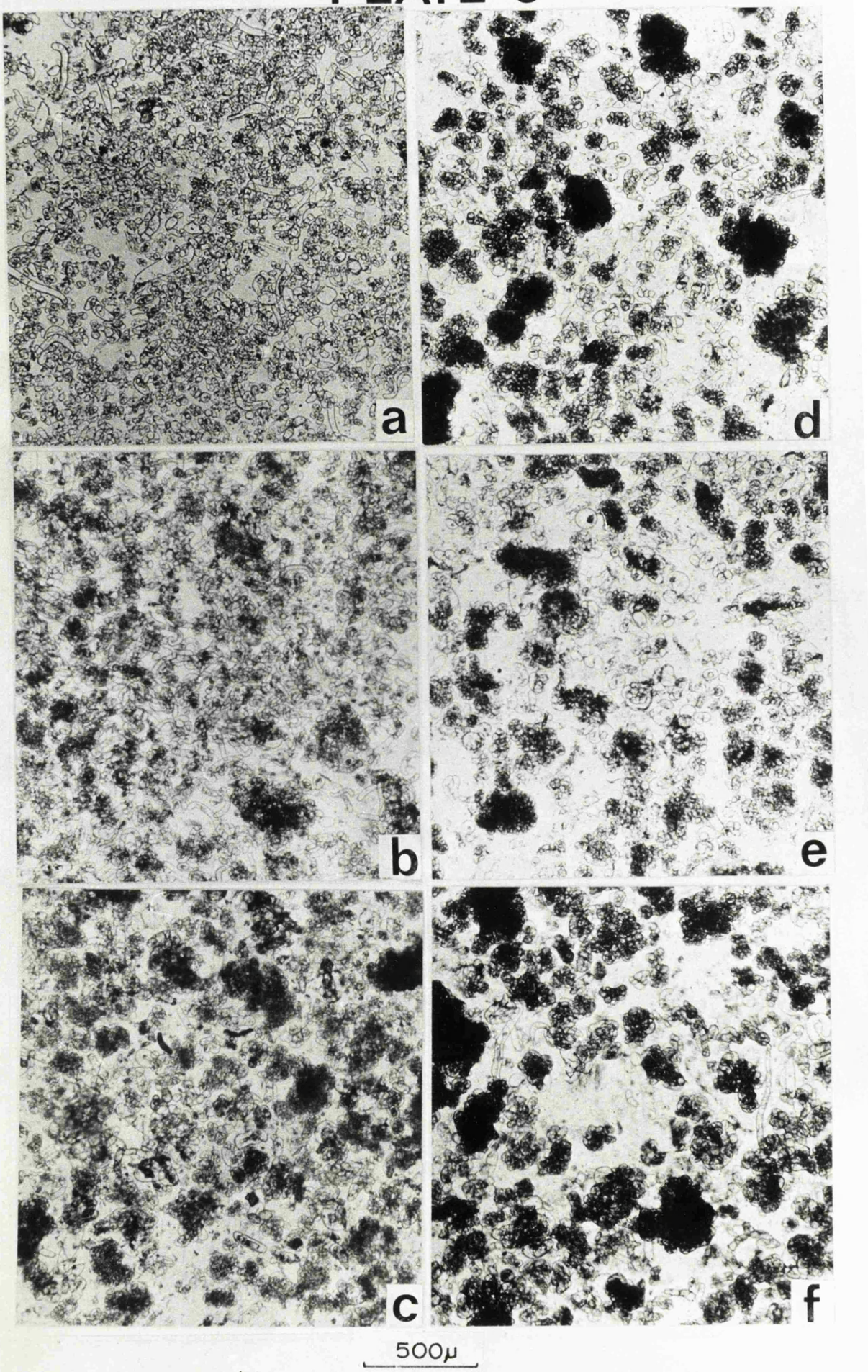
This is the concentration (6 mg/l) at which filter-sterilised 2-CEPA causes a marked increase in the levels of cell dis-

Explanation of PLATE 5

The effect of 6 mg/l 2 chloroethylphosphonic acid (2-CEPA) on the initiation of cell suspensions from "S" callus tissue inocula

a, b and c Day 1, 12 and 18 respectively of 1st passage cell suspensions. The parental suspension initiation cultures were treated with 6 mg/l 2-CEPA and subcultured after 30 days. In these 1st passage cultures cell aggregates are extremely rare for the first 10-12 days but after this time progressive aggregation occurs and on Day 18 the level of cell dispersion is comparable with that observed in the stock cell suspension cultures, (d, e and f, days 1, 12 and 18 respectively). 2-CEPA exerts an effect on cell morphology in the suspension initiation cultures which persists in the 1st passage cell suspension cultures for a similar period as the enhanced levels of dispersion, with the cells in a and b appearing elongated and thread-like.

PLATE 5



persion from "S" callus tissue inocula in agitated liquid medium and since the effect cannot be demonstrated with autoclaved 2-CEPA it is reasonable to attribute the phenomenon to the liberation of ethylene from the filter-sterilised 2-CEPA.

From Plate 5 a clear difference in the morphology of the cells growing in the presence of 2-CEPA is evident with many of these cells being elongated (hyphal-like) and intertwined to form loose "aggregates". The cellular morphology of Hevea suspension cultures normally observed takes the form of smaller rounded cells in aggregates ranging in size from tens to thousands of cells (Plate 5). The percentage of cells in aggregates of 10 or less is always below 20% The figure of 35% given for the 2-CEPA treated cells is probably a gross under estimate since many of the cells are in loose associations and thus would not pass through the 205μ filtration system used to obtain these measurements. It is important to observe that many of the cells in the aggregates occurring in the stock suspension cultures are contained within these aggregates, while in the 2-CEPA treated medium the "aggregation" is of a different nature since almost all the cells in "aggregates" are each in direct contact with the bathing medium. Thus the 2-CEPA treated cell suspensions approach the ideal of a single-cell suspension much more closely than the measured parameter suggests (Plate 5). The inocula of "S" callus tissues did not fragment or break up to any greater extent in the presence of 2-CEPA than is normally observed in the absence of the compound, thus the production of a finely dispersed cell suspension from these inocula appears to be related to the release of cells from the peripheral regions of the larger cell aggregates perhaps more properly considered

as large pieces of the "S" callus tissue inocula.

A further experiment was devised to test the effect of 6 mg/l of filter-sterilised and 6 mg/l of autoclaved 2-CEPA on the growth and dispersion of cell suspensions of Hevea brasiliensis. The five treatments tested are listed below and are subsequently referred to in the text by number alone.

1. Standard Murashige and Skoog medium.
2. Standard M + S + 6 mg/l filter sterilised 2-CEPA
3. Standard M + S,-2,4-D, + 6 mg/l autoclaved 2-CEPA
4. Standard M + S, + 6 mg/l autoclaved 2-CEPA
5. Standard M + S, + 6 mg/l autoclaved 2-CEPA

Treatments 1,2, 3 and 5 were inoculated with 10 ml of stock suspension culture assumed to be in stationary phase (i.e. 22 day-old), Treatment 4 was inoculated with 10 ml of the finely dispersed cell suspension obtained from the medium supernatant of a twenty-five day-old 2-CEPA-treated suspension initiation culture (inoculated with "S" callus tissue). Cell number /ml, dry weight in mg/ml, packed cell volume and the percentage of cells in aggregates of 10 or less were monitored for 17 days (Figure 4.3 to 4.5). Growth as measured by these parameters proceeded similarly in all treatments. The results for Treatment 4 reflected the progressive reaggregation of the cells from day 10 onwards, with the percentage of cells in aggregates of 10 or less dropping sharply (Figure 4.6). After 17 days these cultures were indistinguishable from those of the other treatments. In Treatment 3 the percentage of cells in aggregates of 10 or less declined to 3% which presented a clear difference when compared with the 10-20% measured in the other treatments. This increase in aggregation commonly occurs when 2,4-D is omitted from the standard Murashige and Skoog medium.

Explanation of Figures 4.3, 4.4, 4.5 and 4.6

The effect of 2-CEPA on the growth and dispersion of *Hevea brasiliensis* cell suspensions

TREATMENTS AND FOR Fig 4.6.

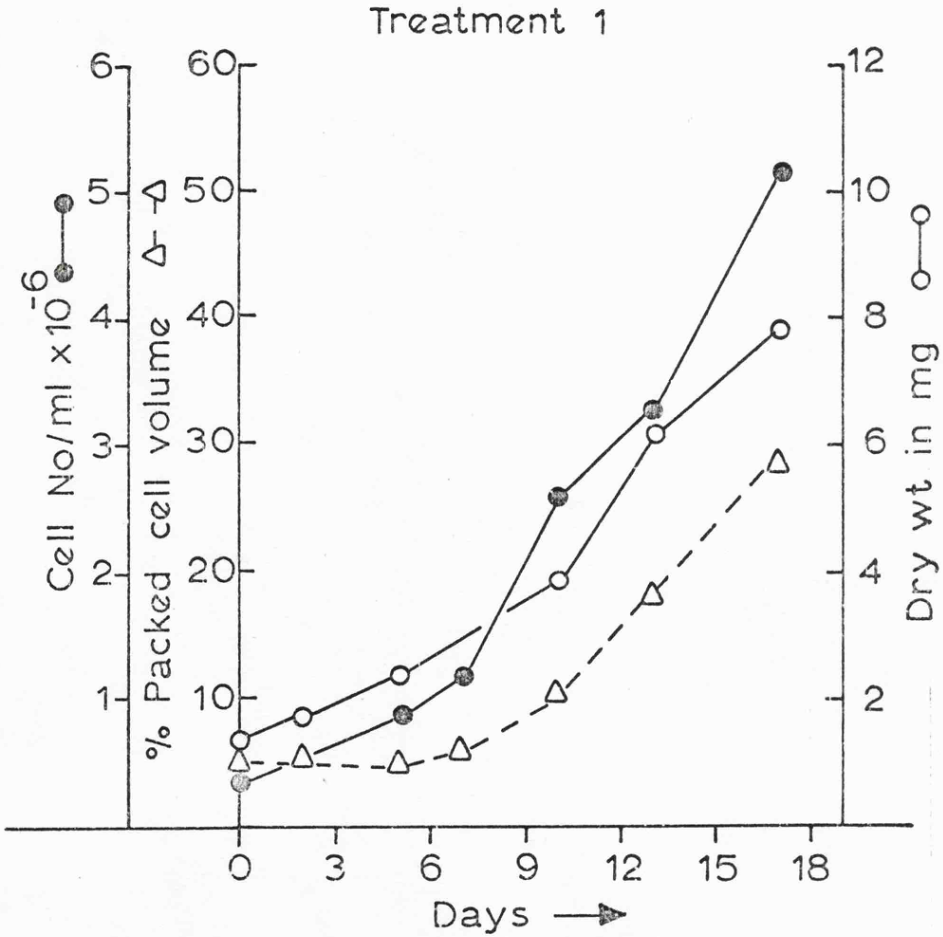
Key:-

1	●—●	Standard M + S
2	▲—▲	Standard M + S, with 6 mg/l filter-sterilised 2-CEPA
3	◓—◓	M + S minus 2,4-D with 6 mg/l autoclaved 2-CEPA
4	Δ—Δ	Standard M + S, with 6 mg/l autoclaved 2-CEPA
5	O—O	Standard M + S, with 6 mg/l autoclaved 2-CEPA

1, 2, 3 and 5 were inoculated with 10 ml of a stationary phase stock suspension culture. 4 was inoculated with 10 ml of cell suspension obtained from a 25 day-old 2-CEPA-treated suspension initiation culture.

The measurements recorded for cell number, dry weight, % packed cell volume and the estimates for the % of cells in aggregates of 10 or less, are the mean values for five replicate flasks.

Fig 4.3


Treatment 1
Cell No/ml x10^-6
% Packed cell volume Δ-Δ
Dry wt in mg
Days
0 3 6 9 12 15 18
1 2 3 4 5 6
10 20 30 40 50 60
2 4 6 8 10 12

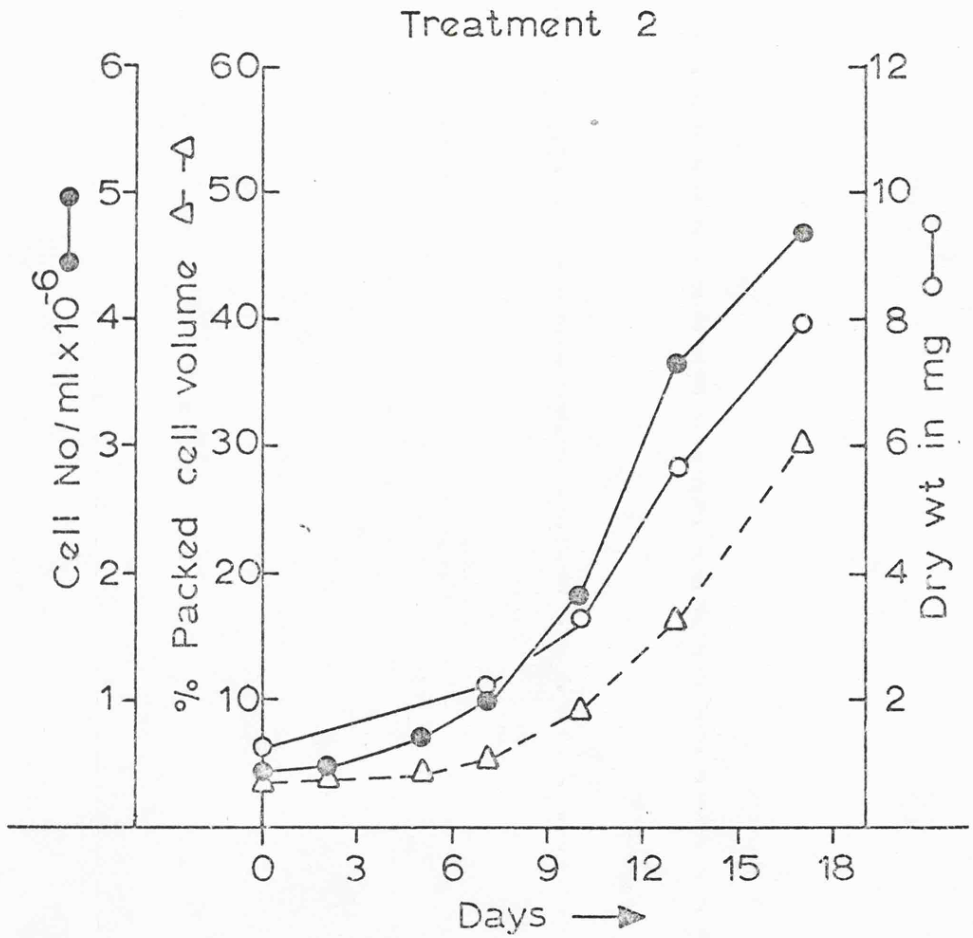

Treatment 2
Cell No/ml x10^-6
% Packed cell volume Δ-Δ
Dry wt in mg
Days
0 3 6 9 12 15 18
1 2 3 4 5 6
10 20 30 40 50 60
2 4 6 8 10 12

Fig 4.4

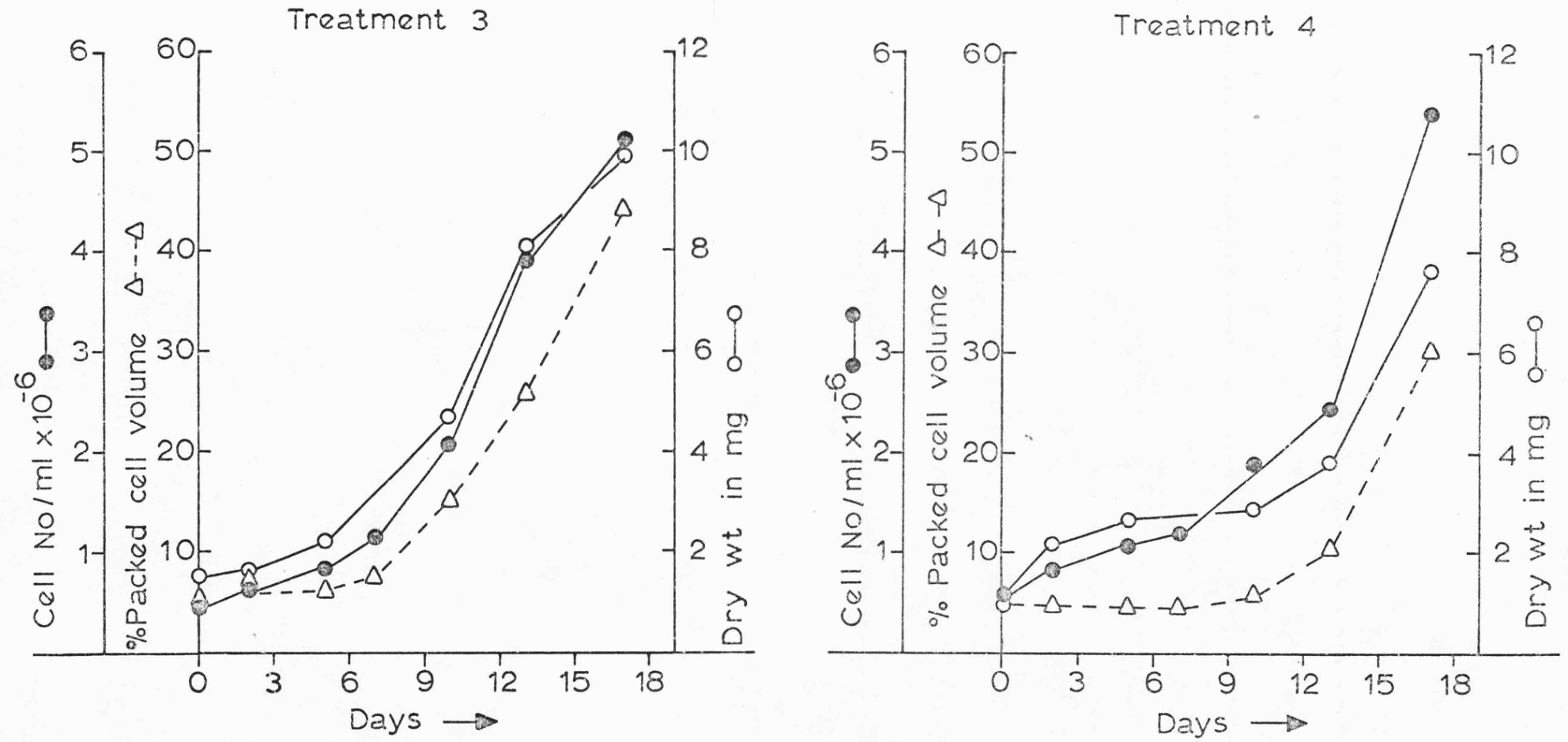

Treatment 3
Cell No/ml x10^-6
%Packed cell volume
Dry wt in mg
Days
Treatment 4
Cell No/ml x10^-6
% Packed cell volume
Dry wt in mg
Days

Fig 4.5

Treatment 5

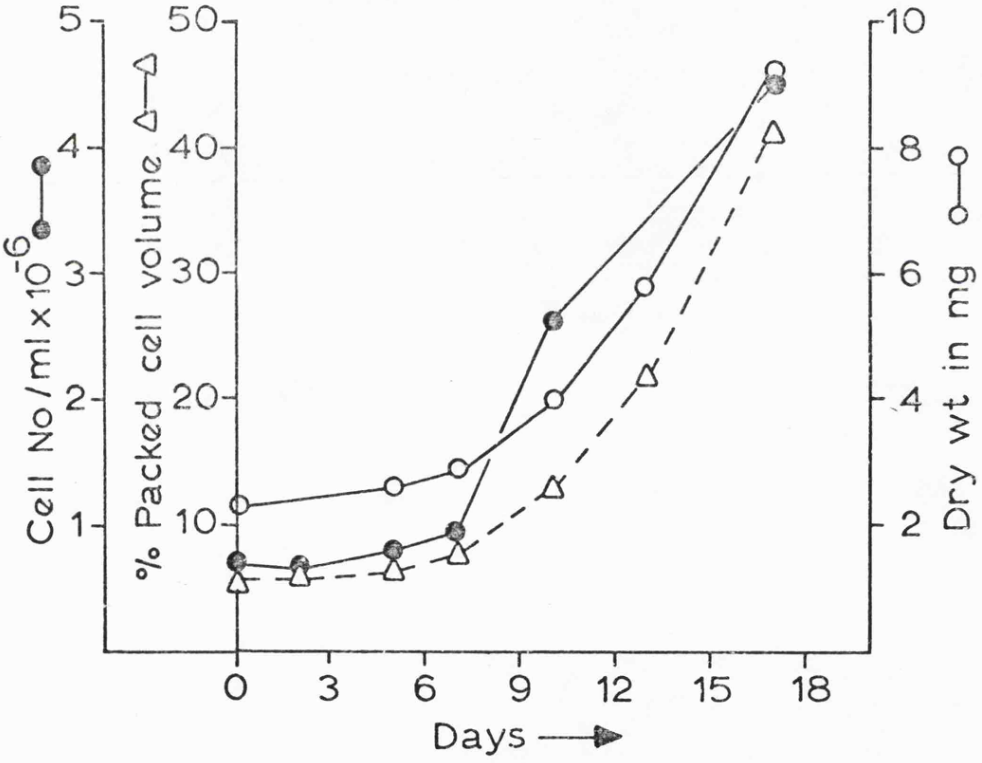
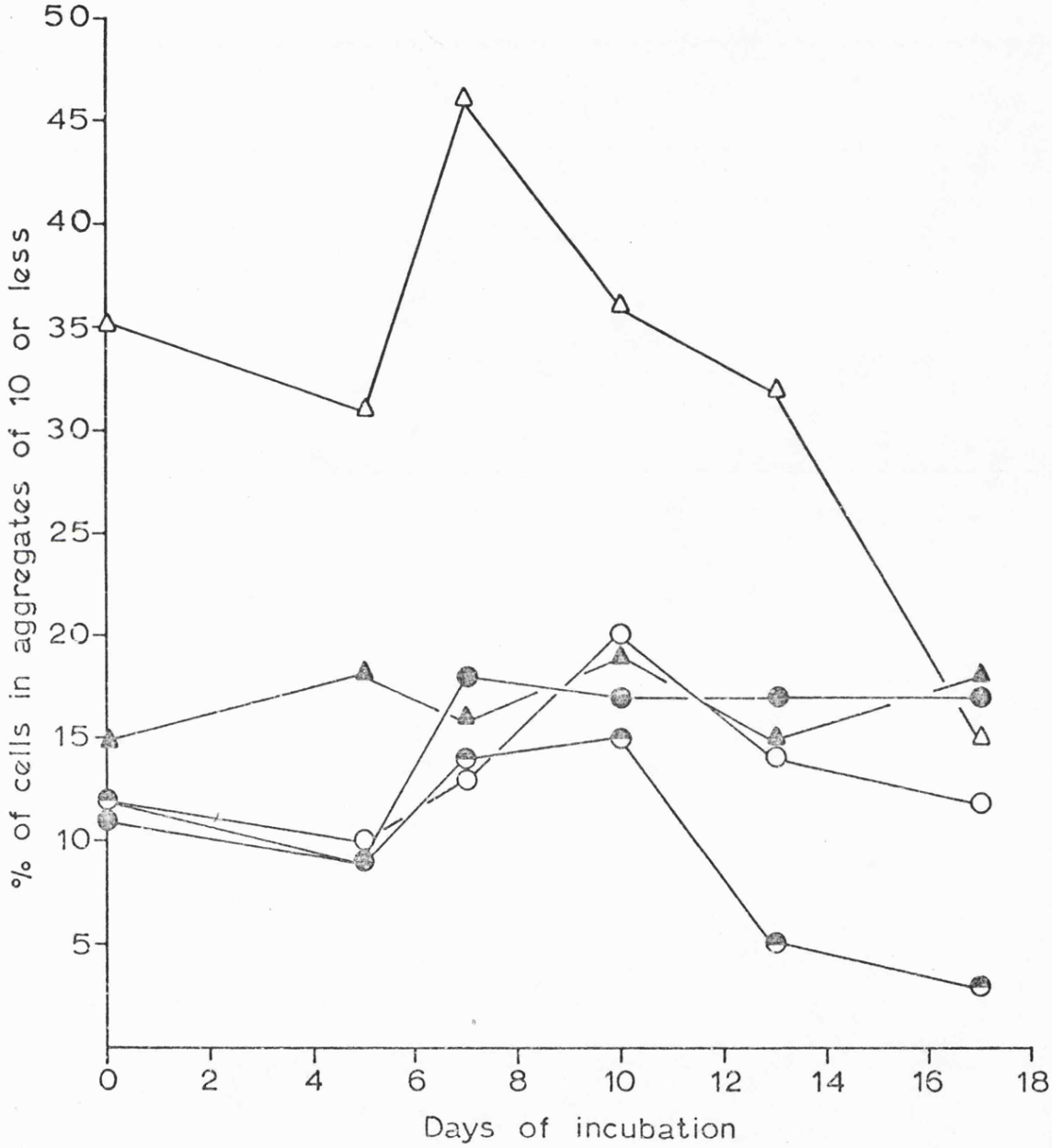


Fig 4.6


% of cells in aggregates of 10 or less
50
45
40
35
30
25
20
15
10
5
0
2
4
6
8
10
12
14
16
18
Days of incubation

Zimmerman and Wilcoxon (1935) first discovered that auxin increases ethylene production. Mackenzie and Street (1970) have clearly demonstrated a relationship between the presence of 2,4-D in the medium and endogenous ethylene production by Acer pseudoplatanus cell suspension cultures. In the absence of 2,4-D ethylene production is greatly reduced. Thus it is possible to speculate that the increase in aggregation of Hevea suspension cultures in the absence of 2,4-D is a direct consequence of a decrease in the production of endogenous ethylene by the cells. However, suspension cultures grown in the presence of 6 mg/l or 12 mg/l filter-sterilised 2-CEPA and in the absence of 2,4-D could not be maintained beyond one passage. The duration of growth for one passage in these circumstances can be attributed directly to the "carry-over" of 2,4-D from the previous passage. Therefore it appears that 2,4-D plays a more comprehensive role in the stimulation of cell growth and division than merely acting as the agent for endogenous ethylene production.

There appeared to be at least three possible reasons for the failure of 6 mg/l of filter-sterilised 2-CEPA to increase the levels of dispersion in Hevea cell suspension cultures.

1. More than one passage is required for the manifestation of the effect in these cultures.
2. The level of 2-CEPA applied was too low to effect an increase in cell dispersion in suspension cultures.
3. The physiological state of cells in suspension culture is very different from that of cells in callus cultures.

An experiment was set up to examine the effects of a second passage in 6 mg/l 2-CEPA and of increasing the level of 2-CEPA from 6 mg/l to 12 mg/l. These two 2-CEPA treatments

(6 mg/l and 12 mg/l) were inoculated with cell suspensions from Treatment 2 of the previous experiment. Ten replicate cultures were used for both treatments and a 10 ml inoculum size used for all cultures. The results (Figures 4.7 and 4.8) show that a second passage in 6 mg/l 2-CEPA and an increase in the level of 2-CEPA to 12 mg/l have no significant effect on growth and produce only a slight increase in the level of cell dispersion with the higher concentration of 2-CEPA being most effective in this respect.

It is possible that even higher levels of 2-CEPA may be required since it can be postulated that when "S" callus tissues are placed in agitated liquid medium large quantities of endogenous ethylene are generated due to increased stress (Meigh, Norris, Craft and Liberman, 1960) and it is this endogenous formation together with the contribution of 6 mg/l 2-CEPA which results in the production of the ethylene necessary to promote cell dispersion. The auto catalytic effect of ethylene on its own biosynthesis has been described by Burg and Dijkman (1967) and thus the addition of 6 mg/l 2-CEPA may itself promote the formation of endogenous ethylene.

A clear difference in the effects of the ethylene generated by 6 mg/l 2-CEPA on "S" callus tissue inocula in liquid medium, and cell suspensions derived from these inocula is evident. One explanation for this difference may be that the endogenous levels of ethylene produced by cells in suspension cultures are much higher than those in callus cultures, with the effects on cell dispersion and cell morphology being due to a rapid increase in the rate of ethylene production rather than to the total amount of ethylene produced. The ethylene contributed by the decomposition of 2-CEPA and

Explanation of Figures 4.7 and 4.8

The effect of 6 and 12 mg/l 2-CEPA on the growth and dispersion of cell suspensions of Hevea brasiliensis

The measurements recorded for a. Cell number and b. Packed cell volume (Figure 4.7), and for a. Dry wt. and b. Estimates of the % of cells in aggregates of 10 or less (Figure 4.8) are the mean values for five replicate flasks.

KEY :- ●——● 6 mg/l 2-CEPA

o - - - - - o 12 mg/l " "

▲——▲ Standard M+S

Fig 4.7

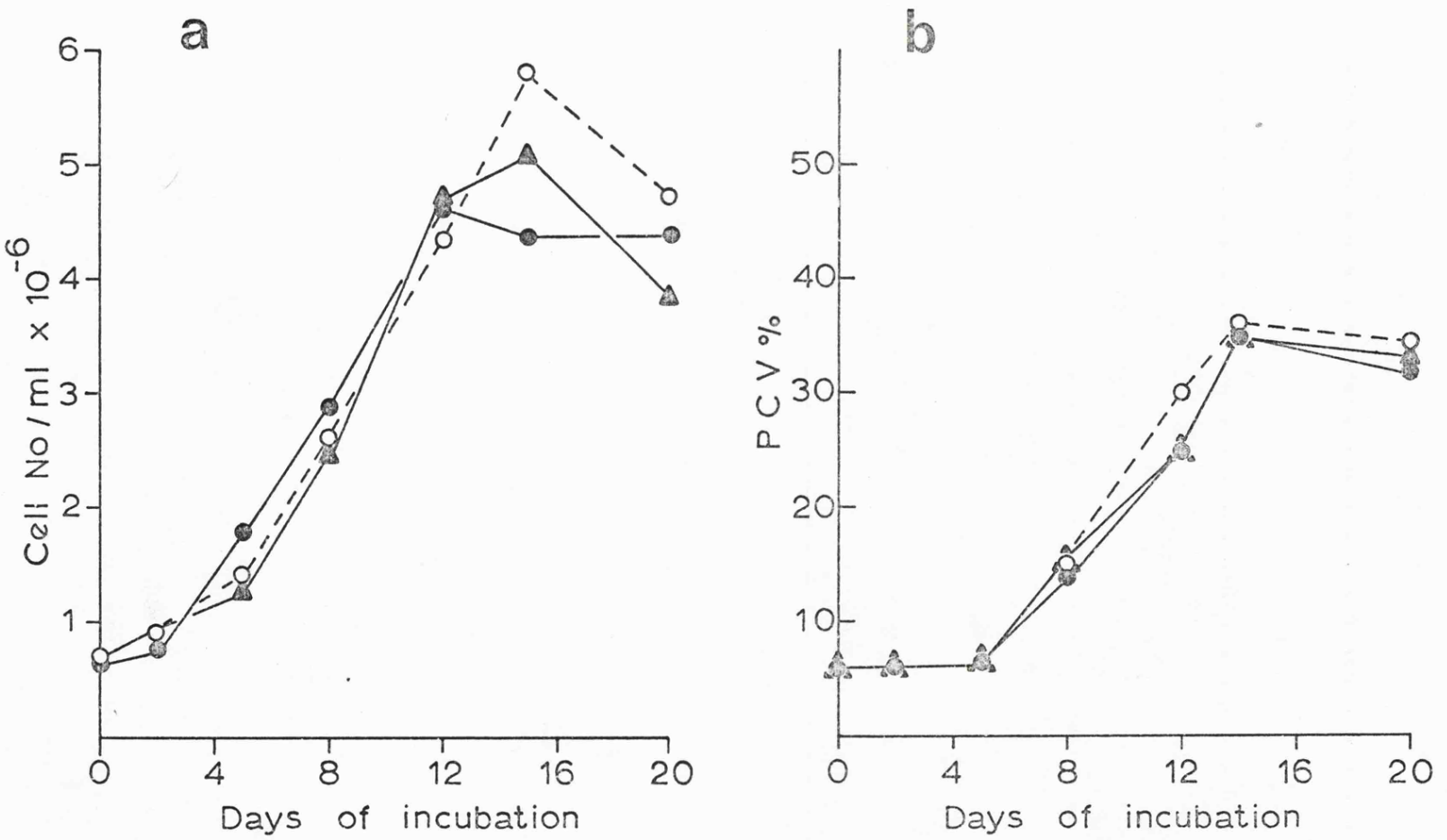
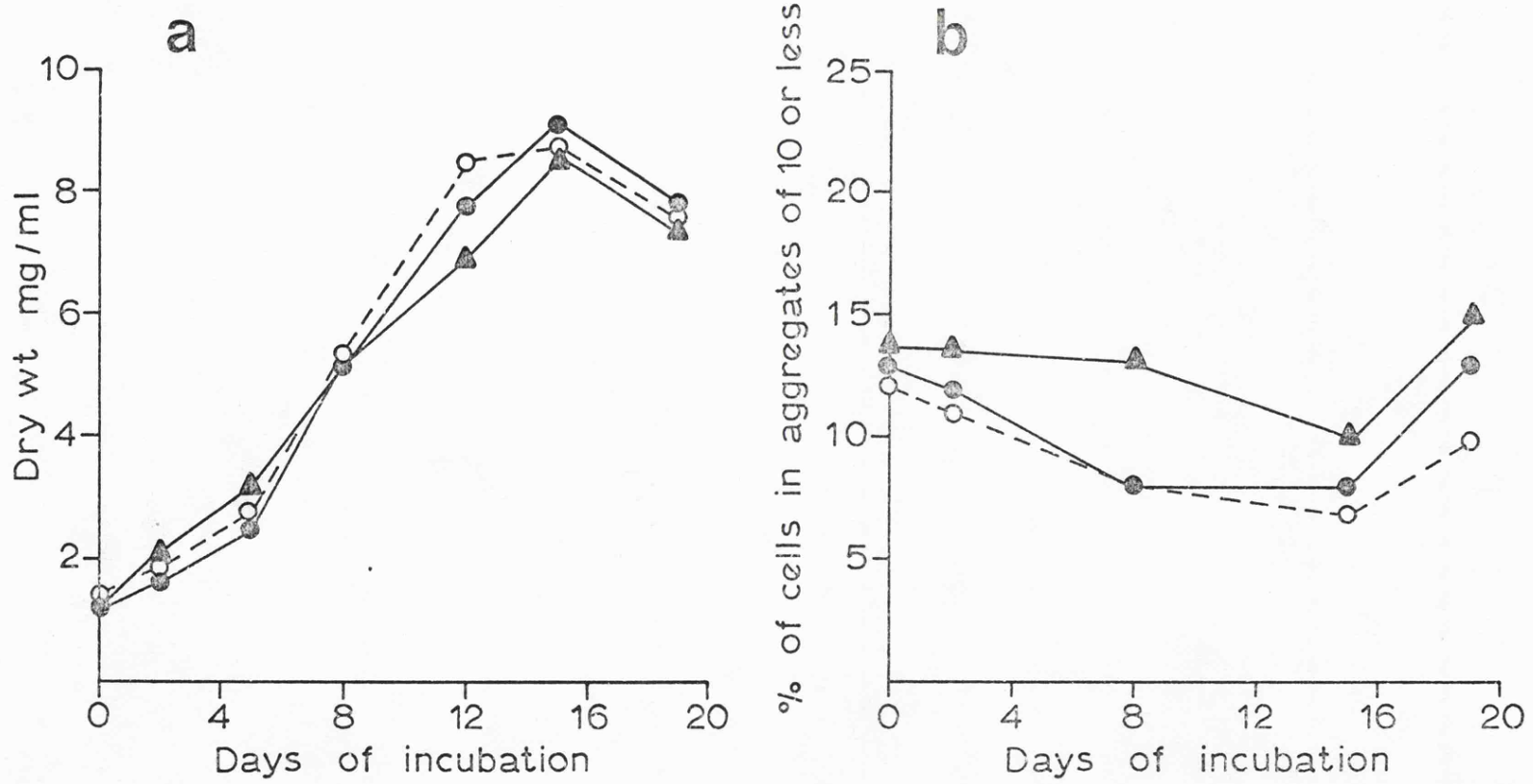

a
b
Cell No / ml x 10^{-6}
PCV %
Days of incubation
Days of incubation
0
4
8
12
16
20
1
2
3
4
5
6
10
20
30
40
50

Fig 4.8


a
Dry wt mg/ml
10
8
6
4
2
0
4
8
12
16
20
Days of incubation
b
% of cells in aggregates of 10 or less
25
20
15
10
5
0
4
8
12
16
20
Days of incubation

augmented by an autocatalytic effect of this ethylene on the rate of endogenous ethylene production, may together create the conditions in culture which result in the clear response of the cells from "S" callus inocula to the 6 mg/l 2-CEPA which is incorporated into the medium.

A high frequency of large, elongated cells was noted in enzyme-treated Acer pseudoplatanus suspension cultures and the presence of the enzyme cellulase was considered to be a prerequisite for the development of this type of cell (Mansfield, 1973). Abscission is known to involve the enzymatic dissolution of plant cell walls and Horton and Osborne (1967) found that the enzyme responsible for this dissolution was cellulase and that its synthesis was increased by ethylene. Abeles and Leather (1971) found that while cellulase synthesis followed the addition of ethylene, levels of cellulase remained constant after the gas was removed and they concluded that ethylene must be having another effect in addition to the regulation of enzyme synthesis. They presented evidence to show that this additional effect was the control of cellulase secretion. Thus ethylene also regulated the movement of cellulase from the cytoplasm to the cell wall through the membrane and once the gas was removed secretion of the cellulase stopped. The large, elongated cells which were noted in the presence of cellulase in Acer pseudoplatanus cell suspensions could be prevented from arising by the incorporation of 8% sorbitol into the growth medium (Mansfield, 1973). Here, cellulase is postulated to have a "softening" effect on the cell wall with cell expansion occurring as a consequence of the greater O.P. of the turgid Acer cells than that of the external medium. In view of the

above data it appears feasible to hypothesise that the effect of ethylene on the cells of "S" callus tissues is mediated through the stimulation of cellulase synthesis. However, until more data have been obtained most of the inferences drawn must remain speculative. One of the most obvious experiments that has not been carried out is to determine whether the degree of cell dispersion and the associated cell morphology can be maintained by a second passage in 6 mg/l filter-sterilised 2-CEPA.

Burg, Apelbaum, Eisinger and Kang (1971) state that whenever ethylene stimulates growth, the process influenced is cell expansion and not cell division. Steward and Bleichert (1970) found that the log phase of cell division in carrot cell suspensions was retarded by ethylene. From the growth data presented for cell suspensions of Hevea brasiliensis there is no suggestion that the ethylene generated by 6 mg/l and 12 mg/l CEPA is either inhibitory or stimulatory to growth.

If the ethylene effect could be shown to extend beyond the suspension initiation passage, then the cell suspensions of Hevea brasiliensis would present an attractive system for the study of ethylene action on plant cells.

SECTION 5

LATICIFERS IN CALLUS CULTURES OF HEVEA BRASILIENSIS

INTRODUCTION

Knudson and his co-workers (1950, 1955) attempted to exploit tissue cultures of laticiferous plants and to investigate their potentialities as rubber-producing systems but these attempts were unsuccessful. Bobilioff (1925) failed in his attempts to grow isolated laticifers of Hevea brasiliensis in culture and though many subsequent efforts have been made to study the formation of laticifers in cultured tissues these too have been largely unsuccessful. (Snyder, 1950). Structures approximating to laticifers have been seen to differentiate in cultures of Chicory (Gautheret, 1953) and Scorzonera (Gautheret, 1959). Arreguin and Bonner (1950) induced callus formation from stem segments of Guayule (Parthenium argentatum) and showed that these callus initiation cultures accumulate rubber. Acetate, acetone and B-methylcrotonic acid were effective in increasing rubber synthesis but since these guayule cultures were slow-growing and were not subcultured it is uncertain whether the rubber synthesising capacity would be maintained in the absence of the parental explant tissue. Mahlberg (1959, 1962) who worked with Euphorbia marginata did not see laticifer formation nor detect the presence of latex in cultured cells of this species. Bouychou (1953) reported that segments of articulated laticifers were formed by cambial activity when excised tissues from the stem of Hevea brasiliensis were maintained in vitro and that these laticifer segments were capable of producing rubber. He concluded that rubber was produced under

culture conditions without the presence of an active photosynthesis mechanism. However, the synthesis of rubber was detected only by staining with Sudan III which is not a selective stain for this substance. This work could not be repeated (see Section Two).

A young latex vessel is recognisably organised as a living cellular system in possession of nucleus, cytoplasm and associated characteristic organelles and membrane systems; as maturation proceeds, these features become progressively confined to the peripheral regions of the vessel, eventually to become vestigial, until at full maturity, latex as a cytoplasmic derivative occupies the major portion of the lumen. (Dickenson, 1968). The electron microscope studies of Dickenson (1963) confirmed the historical conception of the gross particulate structure of Hevea latex as consisting of three different particles. Rubber particles varying in size from 500 Å up to 1μ in diameter, Frey-Wyssling particles mainly composed of lipid material and of the same size order as the larger rubber particles, and lutoids of about 3μ in diameter. These latter particles are the most abundant of the larger particles and are spherical bodies bounded by an osmotically sensitive membrane and possessing a liquid interior (Ruinen, 1950).

It was of great interest to determine whether or not the cultured callus tissues obtained as described in Section Two retained the capacity to differentiate latex vessels and to synthesise rubber particles. The first part of Section Five describes the results of an histological examination of "A" callus tissues using both light and electron microscopical methods in order to accomplish this examination. The latter

part of the section describes a study of the uptake of ^{14}C-mevalonic acid by the "A" callus tissues of Hevea brasiliensis used in an attempt to demonstrate the active synthesis of rubber by this tissue.

Anatomical Studies

The cut surfaces of roots which were produced from "A" callus tissues (See Section Six) were seen to exude a white gelatinous substance identical in appearance and consistency to latex. The distribution of the putative latex vessels can be followed along the length of such roots. (Plate 6). Despite the poor quality of the sections obtained it is possible to observe that near the root-tip end the vessels are arranged in a circular fashion which persists along the length of the root until the callus tissue directly beneath the site of the emergent root is reached, where the organisation of vessels appears haphazard. The vessel-like entities depicted in the light micrographs in Plate 7 were observed in "A" callus tissues which had been subcultured continuously for some twenty passages and which showed no visible embryo- or organo-genetic features. The gross particulate structure of the vessel contents can be clearly seen. Some of this callus tissue was prepared for electron microscopical studies and from the many sections examined several structures were observed which could be tentatively identified as the vessel-like entities seen under light microscopy. (Plate 8). The main contribution to the gross particulate structure of the vessel contents as seen under the light micoscope is probably made by lutoid particles. The spaces seen within the vessel-like entities on the electron micrographs may well have once

Explanation of PLATE 6

The organisation of putative latex vessels within a root produced from "A" callus and within the callus directly beneath the emergence of the root from the callus mass

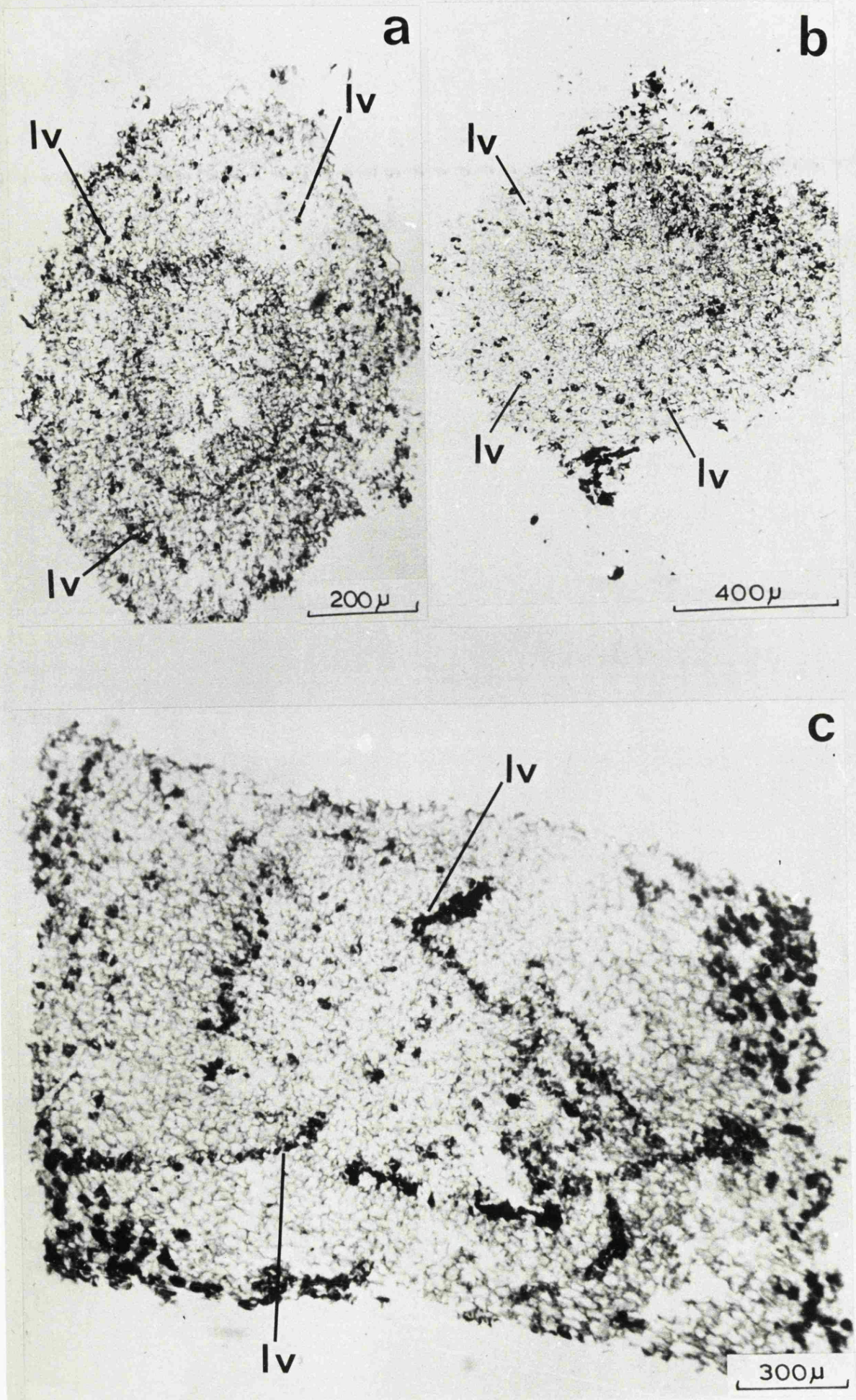
a Transverse section of a root produced from "A" callus, near to the root tip. Latex vessels (lv) appear to be present as a circular band in the cortex of the root.

b Transverse section of the same root at the juncture of the root and the callus mass from which it originated. The putative latex vessels (lv) retain their organisation as a circular band.

c Section (in the same plane as those of a and b) of the callus mass directly beneath the site of emergence of the root. The latex vessesls (lv) no longer retain an organised formation and the two vessels indicated in c appear to be sectioned longitudinally.

Osmium tetroxide (1%) was used both as a stain and fixative (see text) for these sections.

PLATE 6



Explanation of PLATE 7

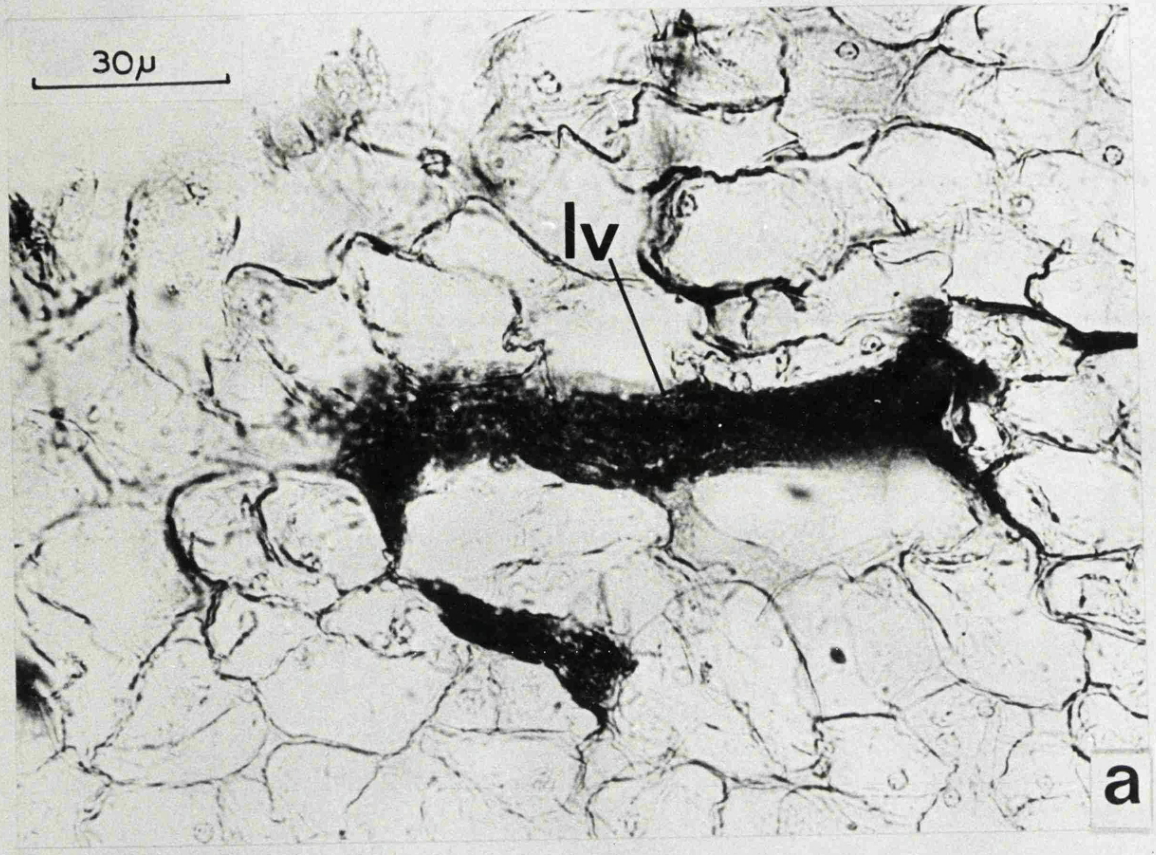
Light micrographs of putative latex vessels in 12th passage "A" callus tissues

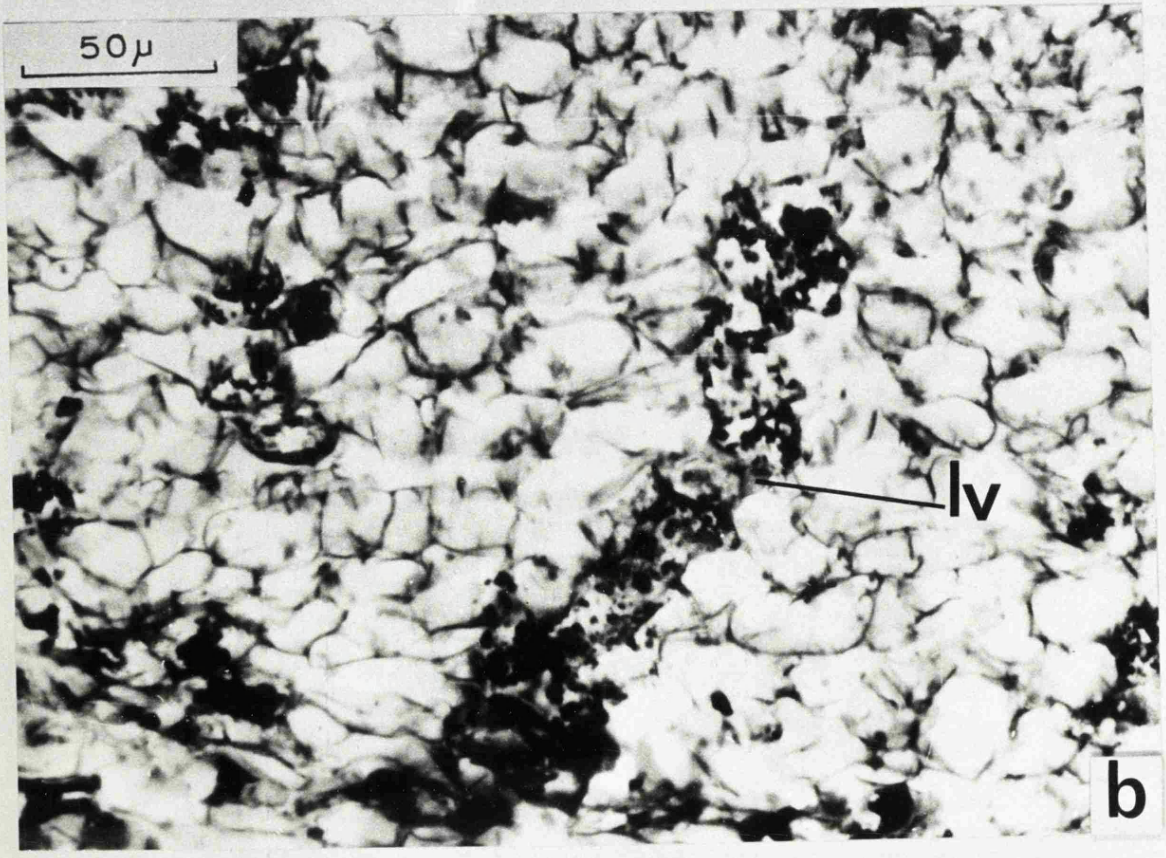
a Latex vessel (lv). Section stained with 1% w/v Nile Blue. The contents of the vessel appear particulate probably due to the presence of lutoid particles.

b Latex vessel (lv). Section fixed and stained with 1% osmium tetroxide. The particulate contents of the putative latex vessel can clearly be seen.

The "A" callus tissues used for both a and b were morphologically undifferentiated and had been so for the 12 passages in culture.

PLATE 7



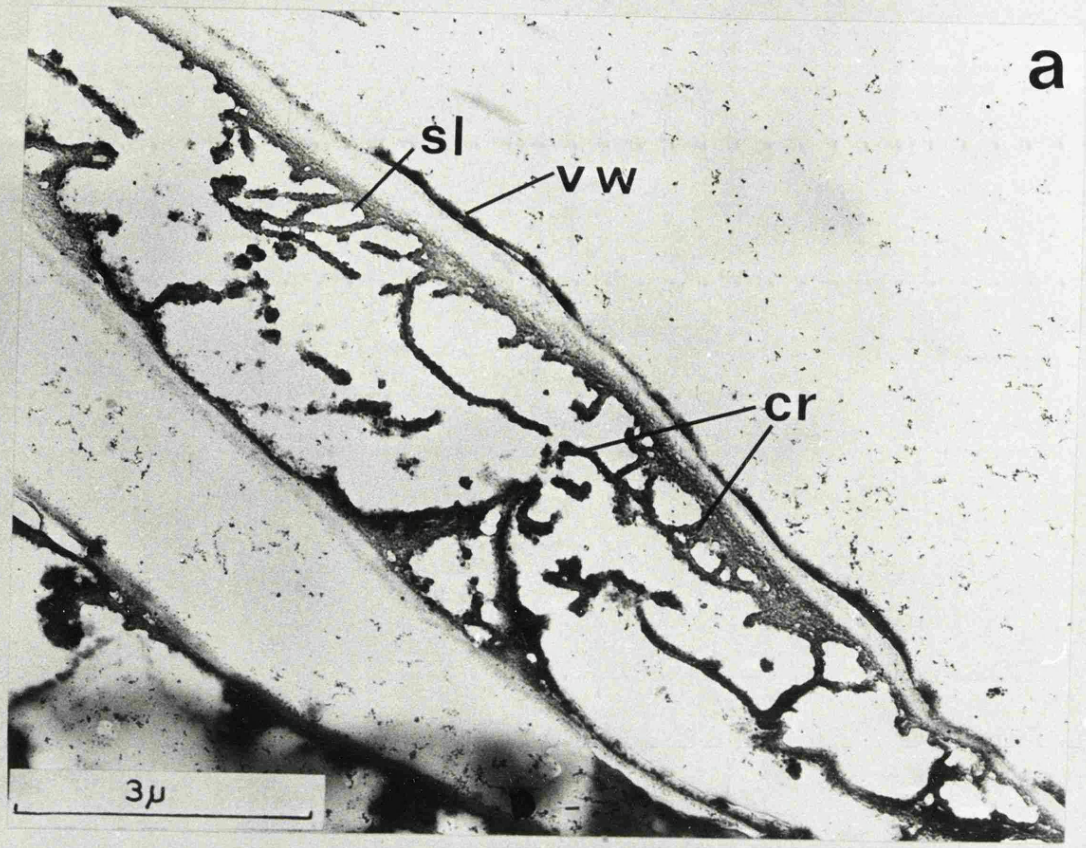


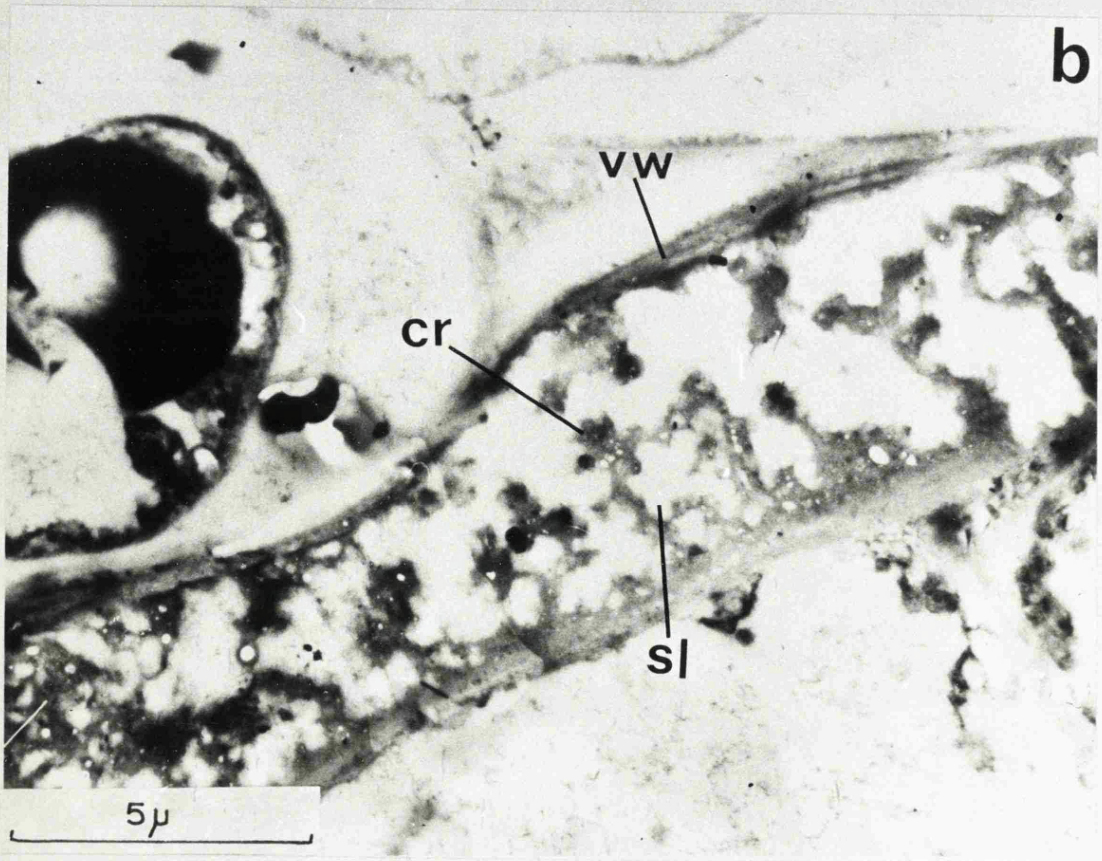
Explanation of PLATE 8

Electron micrographs of putative latex vessels in 12th passage "A" callus tissues

a, b Putative latex vessels showing a vessel wall (vw), the sites of ruptured lutoid particles (sl), and a mass of coagulated rubber particles (cr).

PLATE 8





been occupied by these lutoid particles which have ruptured during fixation and dehydration. The remaining material within such vessels would thus consist mainly of coagulated rubber particles. At the light and electron microscope level these vessel-like entities were usually located in the inner regions of the "A" callus tissues, and while neither of the sets of data is in any way conclusive, taken together they strongly suggest that latex vessels are present in this cultured tissue after twenty passages. This clearly excludes the possibility of these vessels being directly derived from those of the parental explant and thus they must have been differentiated in the cultured callus tissues.

Any additional data that would help to establish the synthesis of rubber particles by "A" callus tissues would be of significance and in view of this the following investigation of the uptake of ^{14}C-labelled mevalonic acid by "A" callus tissues was undertaken.

The uptake of ^{14}C-mevalonic acid by callus tissues of Hevea brasiliensis

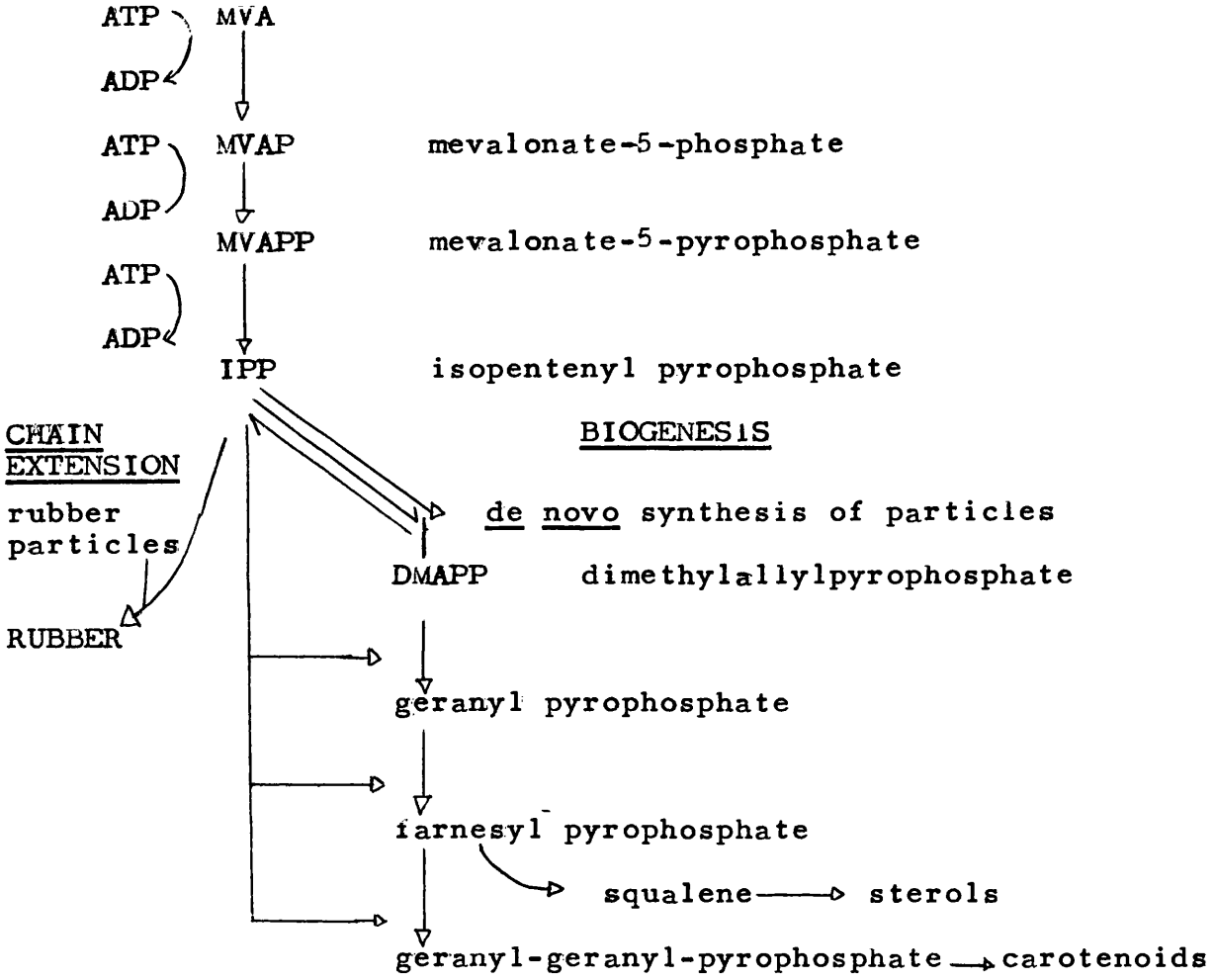
Introduction

In 1956 mevalonic acid was isolated as a precursor of sterol and terpenoid synthesis by Folkers et al., this discovery proving very important in relation to an understanding of isoprenoid biosynthesis including rubber. Park and Bonner (1958) showed that latex, when freshly tapped from Hevea brasiliensis incorporated 2% of the label from the added DL-^{14}C-mevalonic acid lactone. Edwin (unpublished) obtained incorporations of the active isomer approaching 100% efficiency by ensuring that all the precurosr was in the form of its salt, rather than the biologically inactive lactone. In latex, mevalonate is converted to isopentenyl pyrophosphate

via three A.T.P. dependent steps with mevalonate-5-phosphate and mevalonate-5-pyrophosphate being intermediates (Archer, Audley, Cockbain and McSweeney, 1963). This is the pathway followed by terpenoid biosynthesis in other systems (Popják and Cornforth, 1960). The biosynthesis of rubber from 1-^{14}C isopentenyl pyrophosphate was reported by Lynen and Hemming (1960) who also observed that its rate of incorporation was faster than that of mevalonate. The rapid formation of radioactive rubber in incubations of 1-^{14}C-isopentenyl pyrophosphate (IPP) with fresh latex was confirmed by Archer, Ayrey, Cockbain and McSweeney (1961) who obtained a 97% incorporation of the substrate into polyisoprene.

The final conversion of IPP to rubber takes place on the surfaces of existing rubber particles which are essential for the *in vitro* formation of polyisoprene. The rate of incorporation of IPP is proportional to the rubber content of the latex for a given particle size distribution, and over a range of particle sizes the rate is proportional to the surface area of the particles. The surface reaction appears to be predominantly a chain extension process involving existing rubber chains. Although it has never been demonstrated *in vitro* the *de novo* formation of rubber particles in a rubber-free system must occur since there is no evidence for the formation of new particles by the fission of existing ones. (Archer and Audley, 1967).

Rubber formation from mevalonic acid (MVA)



Experiments carried out by Barnard et al. (unpublished) indicated that radioactive mevalonate was not taken up into the rubber particles of the latex vessels in stem and petiole segments of the whole plant, and therefore this investigation was also of interest from the aspect of comparison between whole plant tissues and callus tissues as material for the study of rubber metabolism

The preparation of mevalonic acid from the DL mevalonic acid 2-^{14}C lactone received from the Amersham Radiochemical Centre

DL-mevalonic acid 2-^{14}C lactone (MVAL) was supplied from stocks held at room temperature as a benzene solution sealed

under nitrogen in borosilicate glass ampoules. 50μC (specific activity 12.9 μC/μMole) of 2-^{14}C MVAL was recovered from the benzene solution as follows. The benzene was evaporated in a nitrogen stream, 0.5 ml of 0.4% $NaHCO_3$ was added and the solution heated at 70° for 20 min. The mixture was cooled and then 185 μl of N/10 HCl was added. The pH was checked at between 5.6 and 6.0 and with washings, a final volume of approximately 1 ml was obtained.

Application of the 2-^{14}C-Mevalonate to "A" callus tissue

Firstly the volume of radioactive solution was filter-sterilised and made up to a volume of 5 ml with washings of sterile distilled water. Two methods of application were used.

a) Five, 250 ml flasks, each containing approximately 100 ml of semi-solid M + S medium pH 5.6 were inoculated each with approximately 4 gm (fresh weight) of "A" callus tissue. 0.5 ml of the radioactive solution was directly applied to each of the callus inocula by injection with a sterile, disposable syringe.

b) 0.5 ml of the radioactive solution was added to each of the five, 250 ml flasks containing approximately 100 ml of of molten M + S medium pH 5.6 at a temperature of about 38°. The flasks were then gently shaken to ensure an even distribution of the radioactivity. Once the medium had cooled and set to form a semi-solid substance, approximately 4 gm (fresh weight) of "A" callus tissue were inoculated into each flask.

The callus was harvested in method a) after 14 days incubation at 30° in the dark and in method b) after 21 days.

As well as following an extraction procedure for rubber from the "A" callus tissues, an extract of lipid material was also made to ensure that in the event of an absence of labelled rubber formation it would be possible to determine whether or not this was due to the failure of the "A" callus tissue to utilise 2-^{14}C mevalonic acid in metabolism.

Procedure for the extraction of rubber from "A" callus tissue

Application methods a) and b) were treated separately. The callus tissues were removed from the flasks (5 per method) and weighed. The total callus tissue was then chopped into small fragments on a glass plate and about one fifth removed for the lipid extraction. This was stored in a deep-freeze. The remainder was homogenised with a Whillems Polytron (type 20) in 100 ml of distilled water and a 1 ml sample of the resultant slurry taken for counting (see Section 4). The slurry was freeze-dried and then resuspended in 50 ml of 1% trichloroacetic acid in benzene. This was left to stir for 3½ hours just below boiling point. At this stage 40 mg of carrier rubber was added as a 0.5% solution in benzene and stirring continued for a further half-hour. The slurry was centrifuged, the supernatant collected and the residue washed with benzene (2 x 10 ml). The extracts and washings were pooled and a 1 ml sample counted. The solution was filtered, and reduced to 8 ml, firstly by rotary evaporation *in vacuo* and finally under nitrogen. This solution was then poured into 16 ml of 5% acetic acid in ethanol and left at 4° for 3-4 hours to precipitate the rubber. The precipitated rubber coagulum was washed twice with 5% acetic acid in ethanol and

once in ethanol, gathered with a fine glass rod and heated with 30 ml of N. KOH at 100°. The coagulum was then washed with water (three times) and alcohol (once), extracted overnight with alcohol, dried at 80° for 30 minutes and weighed. It was then dissolved in 3 ml of 0.4% trichloroacetic acid in toluene and the solution counted. The total number of counts in the rubber was estimated after allowing for the partial recovery of rubber on precipitation.

The extraction of lipid material

The chopped-up callus tissue sample was homogenised with the Polytron in 80 ml of 2 : 1 chloroform/methanol (Folch et al. 1951). After centrifugation the supernatant was transferred to a 500 ml separating funnel, and 200 ml of distilled water added and shaken with the supernatant. The resultant emulsion was broken by centrifugation, the upper layers removed and the lower layers (chloroform based) returned to the separating funnel for two further washes (centrifuging between each wash). This volume was reduced to approximately 10 ml by rotary evaporation *in vacuo*. The lipid extract was then blown to dryness under nitrogen, residual solvent removed in a vacuum desiccator, and weighed.

Results

The total number of counts present in each treatment of 5 flasks = 885,560 dps. However, 2-^{14}C-mevalonate is in the form of the DL-isomer and only the L-isomer is utilisable for biosynthesis ∴ 442,780 dps is the actual dose.

TABLE 5.1

Method of Application a)

	Sample	dps	Volume counted	Total volume of extract	Total dps in 18.6 gm callus tissue
rubber extracts	Aqueous extract of callus	436	0.5 ml	100 ml	113,360
	Benzene-TCA[1] extract	1075	0.5	75	209,625

1 this extract would contain unused mevalonate.

Rubber extraction

40 mg of carrier rubber was added to the homogenate of "A" callus tissues.

Wt. of rubber recovered = 31.2 mg dps = 253

dps/mg = 0.81

However, since only 31.2 mg of rubber were recovered,

total dps = $\frac{40}{31.2}$ x 25.3 = 32.4 dps

This count is too low to be acceptable as definitive proof that radioactive rubber has been recovered from the calus tissues and probably results from the presence of labelled impurities. As a test of this interpretation, the rubber was reprecipitated, re-extracted and counted again, as follows. The 15 ml volume in the scintillation vial was reduced to 5 ml under nitrogen and then poured into 10 ml of 5% acetic acid in ethanol. This solution was left to incubate at 4° for two days and the resulting coagulum re-extracted with ethanol.

Wt. of rubber recovered from 31.2 mg

= 24.2 mg dps = 15.6

dps/mg = 0.645

However, since only 24.2 mg of rubber were reovered,

total dps = $\frac{31.2}{24.2}$ x 15.6 = 20.3 dps

Since reprecipitation increases the purity of the extracted rubber, the decrease in specific activity suggests that impurities are the most probable source of any counts.

TABLE 5.2

Method of application b)

Sample	dps	Volume counted	Total volume of extract	Total dps
Aqueous extract of callus	134	0.5 ml	100 ml	33,500
Benzene-TCA extract[1]	365	0.5	65	59,250

1 Since the precursor was added to the medium in this method, these extracts cannot contain a large amount of unused mevalonate.

Rubber extraction

40 mg of carrier rubber was added to the homogenate of "A" callus tissues.

Wt. of rubber recovered = 27.7 mg dps = 14.2

dps/mg = 0,513

However, since there were only 27.7 mg recovered

total dps = $\frac{40}{27.7}$ x 14.2 = 20.5 dps

As with the results obtained in method a) this count is probably the result of impurities in the extracted rubber.

Lipid extraction

Method a)

Weight of lipid extracted from 4.2 gm callus tissue = 9.5 mg

Total weight of callus = 18.6 gm

$\therefore$ total lipid in callus $= \frac{18.6}{4.2} \times 9.4 = 41.6$ mg

% lipid of fresh weight $= \frac{9.4 \text{ mg}}{4.2 \text{ gm}} \times 100 = 0.22\%$

Sample	dps/9.4 mg lipid	Total dps in lipid	% incorporation of L-mevalonate into lipid
9.4 mg	25,200	111,500	26.4

Two-dimensional thin layer chromatography (2DTLC) of the Lipid Extract

The 20 x 20 cm plates were spread with Silica gel G. at a layer thickness of 0.4 mm, drying down to between 0.25 and 0.3 mm. 9.4 mg of lipid was dissolved in 120 μl of chloroform and 10 μl spotted. (2100 dps). The plates were run firstly in chloroform in the coating direction and then at right angles in 60-80° petrol/di-isopropyl ether 4/1 (v/v). (after Pennock, Hemming and Kerr, 1964). After drying the plates were marked with radioactive ink, wrapped in "Melanex" and placed on a Kodirex X-ray film for 3-4 days.

No attempt was made to identify further the separated spots, but with the aid of chromatograms of authentic lipid mixtures (Dunphy, 1966) it is possible to suggest that substances separated by the 2DTLC of the lipid extract include sterols, e.g. lanosterol and β-sitosterol placed near the origin and hydrocarbons, e.g. squalene furthest from the origin (Figure 5.1). Thus it may be concluded that

Explanation of Figure 5.1

a) Two dimensional thin layer chromatograph of a lipid extract of "A" callus tissues after incubation with ^{14}C-mevalonic acid for 14 days.

b) Two dimensional thin layer chromatograph of an authentic mixture of lipids (Dunphy 1966).

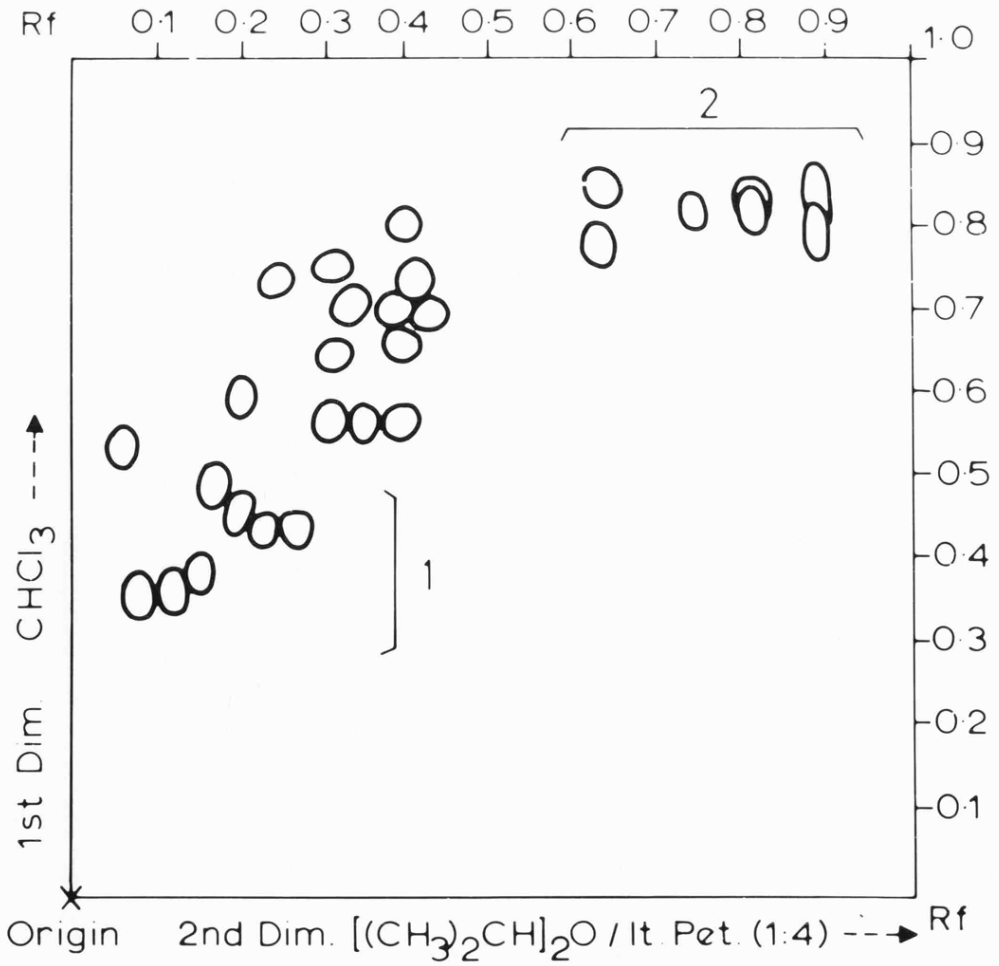
Key:- 1 Region in which compounds with sterolic properties are found.

2 Region in which compounds with predominantly hydrocarbon-like properties are found.

Fig. 5.1

a

b


Rf
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
2
1
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
1st Dim. CHCl3 ---→
Origin
2nd Dim. [(CH3)2CH]2O / lt. Pet. (1:4) ---→
Rf

2-^{14}C-mevalonate becomes extensively involved in the isoprene biosynthesis and lipid metabolism in general of "A" callus tissues.

Lipid extraction

Method of application b)

For this extraction an individual "A" callus was taken rather than a representative sample from the total amount.

6.5 mg of lipid extracted from 3.2 gm callus

$$\text{Total lipid in callus} = \frac{\text{Total wt of callus}}{\text{wt of callus sample}} \times 6.5 \text{ mg}$$

$$= \frac{16.2}{3.2} \times 6.5 = 32.9 \text{ mg}$$

$$\% \text{ lipid of fresh wt} = \frac{6.5 \text{ mg}}{3.2 \text{ gm}} \times 100 = 0.20\%$$

Sample	dps/6.5 mg lipid	Total dps in lipid	% incorporation of L-mevalonate into lipid
6.5 mg	8,100	41,000	9.3

The percentage incorporation of L-mevalonate into the lipid of "A" callus tissues is thus almost 3 times greater when the precursor is applied directly to the tissues than when it is added to the medium (26.4% compared with 9.3%). Two dimensional TLC plates were run as previously described and the resultant autoradiographs showed no discernible differences except that since there was less activity spotted at the origin (948 dps), the spots obtained on separation were less intense.

Summary

2^{14}C-mevalonate becomes extensively involved in isoprene biosynthesis and lipid metabolism of "A" callus tissues of

Hevea brasiliensis. The efficiency of incorporation is related to the method of administering the precursor, and from the experiments described, direct application to the callus tissues is more satisfactory than the initial incorporation of the 2^{14}C-mevalonate in the growth-supporting medium. Direct application was achieved by the injection of the precursor into the centre of the "A" callus tissue mass. This method can probably be further improved by the application of the precursor in smaller volumes (e.g. 0.1 ml) since a considerable proportion of the 0.5 ml applied to each of the "A" callus tissues escaped from their immediate vicinity. The superiority of directly applying the precursor to the callus tissues, rather than its initial incorporation into the growth medium, with regard to the efficiency of uptake, is probably a consequence of the steeper concentration gradients encountered in the former method.

By light and electron-microscopy, latex vessels have been tentatively identified in "A" callus tissues with no visually obvious embyro- or organo-genetic capacity, but the synthesis of rubber could not be demonstrated in these cultures by the uptake of the labelled precursor 2^{14}C-mevalonic acid. However, no successful incorporation of 2^{14}C-mevalonic acid into rubber has been achieved even when using stem segments as the test tissue (Barnard, unpublished) and so the results from these experiments cannot be regarded as desmonstrating the absence of rubber synthesis in "A" callus tissue of Hevea brasiliensis.

SECTION 6

EMBRYO AND ORGAN FORMATION IN CELL AND TISSUE CULTURES OF HEVEA BRASILIENSIS

Introduction

There are only a few reports of the initiation of organs from cultured callus tissues of woody species and these show no uniformity in their inductive conditions. In most of these reports organ initiation, usually root initiation, has been observed to occur freely from newly initiated callus still attached to the original parental explant or during the first few subcultures, but as subculture proceeds the initiation of organs no longer occurs (Jacquiot, 1955). The initiation of roots is far more frequently reported than that of shoots or embryos from callus tissues of woody plants (Gautheret, 1966). The paucity of reports of morphogenesis in cell cultures of woody species (e.g. Durzan and Steward, 1970) is probably related to the comparative difficulty encountered in establishing such cultures.

Jacquiot (1964) demonstrated shoot initiation in callus cultures of Betula but was unable to define the inductive conditions. Wolter (1968) initiated shoots from callus tissues of Populus tremuloides by the addition of 0.14 mg/l benzyladenine purine (B.A.P.) to a medium lacking auxin. Root initiation was achieved by omitting the cytokinin from the medium and using NAA as the auxin source. Winton (1968) found that a Populus tremuloides callus culture was stimulated to regenerate roots in the presence of a low concentration of 2,4-D (0.04 mg/l) or a high concentration of Kinetin (1.0 mg/l) while shoot formation was induced on media

containing 0.05-2.0 mg/l B.A.P. Wolter (1968) succeeded in rooting shoots after they were excised from the callus tissues but none elongated more than a few centimetres. Winton (1970) however, succeeded in raising trees from callus cultures of Populus tremuloides by this technique. Anther cultures of the hybrid Populus sieboldii x P. grandidentata (Sato, 1972) have given rise to shoot production in the presence of certain concentrations of NAA and BAP but the ploidy of the derived plantlets has yet to be determined. The formation of roots from callus cultures of Hevea brasiliensis has been reported by Chua (1966). This occurred spontaneously and after 5-6 months without subculture. There are no other reports of organ or embryo formation from callus tissues or cell suspensions of Hevea brasiliensis.

Rao (1964) has briefly outlined the early stages of the in vivo embryo development of Hevea brasiliensis. Here the zygote divides only after the formation of many endosperm nuclei, the first division of the zygote is transverse to form apical and basal cells and then the apical cell divides vertically and the basal cell horizontally to the plane of the first division to form a tetrad. Most of the embryonal parts are organised by derivatives of the apical cell and those of the basal cell form a short spread-out suspensor. Muzik (1954, 1956) has described four recognisable stages in the in vivo embryo development of Hevea brasiliensis. These are:- 1) Ovoid; 2) Heart-shaped; 3) Spatulate, in which the cotyledons elongate; and 4) the mature or broadly spatulate shape. In the ovoid and heart-shaped stages the embryo lies

within a liquid to gelatinous endosperm which is replaced later by the persistent nucellus (perisperm) which forms the bulk of the tissue of the mature seed. Bouharmont (1962) has also described the development of the in vivo embryo and a photograph of a median section through a 100-day-old embryo is reproduced from his work in PLATE 12. This stage would appear to correspond with that described by Muzik (1954) as (3) in which the embryo is spatulate with elongated cotyledons. A distinct embryo epidermis can be seen and the embryo is approximately 4 mm in length.

The time taken from fertilisation of the egg cell to production of the mature embryo and seed is between 3 and 4 months, and thus it may be anticipated that embryogenesis in vitro will extend over a similar period.

Since cultured tissues continuously change the external medium by a process of conditioning, the extent of this conditioning will be dependent upon the length of time which the tissues and medium are in contact. The culture requirements of developing embryos may change temporally. Hence it is likely that successful embryogenesis from cultured tissues of species which require relatively lengthy periods for the completion of embryo development in vivo will present special problems. Another consequence of the changing culture conditions and tissue requirements is that the conditions conducive to the formation of embryos may differ very significantly from the initial medium composition. In these circumstances once morphogenesis has been observed it should be possible to reduce the length of time required for the manifestation of embryogenesis by appropriate modification of the initial medium.

Experimental

1. Root formation

Although of relatively rare occurrence, the formation of roots was noted in the early passages of "A" callus tissues. (PLATE 9). Cut surfaces of these roots exuded a white viscous substance identical in appearance and consistency to latex. The cultures bearing these roots were generally those which had been left undisturbed beyond the normal time for transference to fresh medium. Root formation was never observed with callus tissues (including "S" tissues) which had been in culture for over ten passages. Reinert (1959) found that root development from carrot callus only took place when the callus tissue was allowed to deplete the auxin in the medium by prolonged growth without subculture or transfer to an auxin-free medium. Street (1955) working with cultured roots of Lycopersicum esculentum showed 1-naphthoxyacetic acid (1-NOA) to be a powerful anti-auxin and when Thomas and Street (1970) incorporated this substance into the auxin-free growth medium of Atropa belladonna suspension cultures, root development was very significantly enhanced. Further, in the presence of levels of NAA which normally suppress organogenesis 1-NOA was still effective in promoting root initiation. In view of these data, the following treatments were tested on "A" callus tissues which had been in culture for 18 passages.

Explanation of Plate 9

Root formation in the initial passages of "A" callus growth

a Root formation in an initiation passage after 21 days.

b Root formation from 2nd passage "A" callus tissues, 2 months over the normal time of transference to fresh medium.

c Root formation from 4th passage "A" callus tissues, three months over the normal time of transference to fresh medium.

PLATE 9

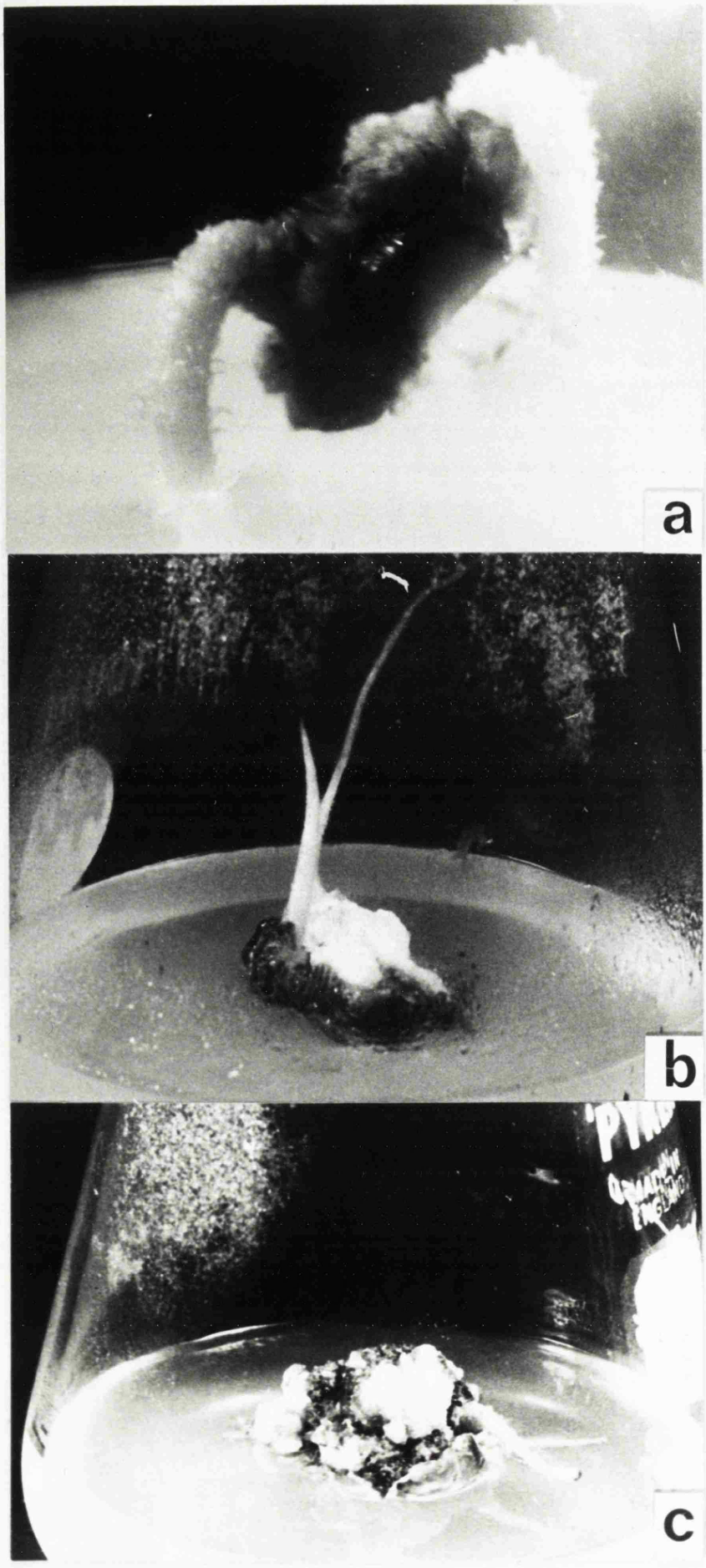


Table 6.1

Medium	1-NOA mg/l	IAA mg/l	% Sucrose
1	2	-	3 and 6
2	-	-	" " "
3	-	2	" " "
4	-	4	" " "
5	-	10	" " "
6	2	2	" " "

0.5 mg/l kinetin was present in all treatments but no 2,4-D. 12 treatments were tested in all with 10 replicate flasks used per treatment. Approximately 0.5 gm fresh weight of "A" callus tissue was inoculated per flask, and the pH of all media adjusted to 5.6. However, no root formation was noted in any of the cultures.

Various concentrations of IAA, kinetin and sucrose were also tested in the medium of Murashige and Skoog in the absence of 2,4-D for their effect on the induction of root formation. Since root formation had never been observed from tissues which had been in culture for more than ten passages the following treatments were tested on "A" callus tissues which had been in culture for 2 and 20 passages.

Table 6.2

Medium	IAA mg/l	Kinetin mg/l	% sucrose
1	1	0.02	3 and 10
2	2	0.02	" " "
3	5	0.02	" " "
4	1	0.2	" " "
5	2	0.2	" " "
6	5	0.2	" " "

24 treatments were tested in all, with 10 replicate flasks per treatment. Approximately 0.5 gm fresh weight of "A" callus tissue was inoculated per flask and the pH of all media adjusted to 5.6. However, here again no root formation was noted in any of the cultures. Root formation had been noted during the initiation trials (Section 2) on the medium of Linsmaier and Skoog but this could not be repeated.

The conditions conducive to root formation could, therefore, not be defined.

2. The formation of structures other than roots

Both "A" and "S" callus tissues appear to have the capacity to undergo different kinds of morphogenesis in the standard liquid medium of Murashige and Skoog. Typically, growth in these cultures is slow and is confined to large aggregates which show very little tendency to become dispersed. Morphogenesis becomes evident only when the tissues have remained in liquid medium for 4-6 months. This appeared to follow two distinct patterns.

A. Outgrowths, visible to the naked eye, developed from the very large aggregates which formed when both "A" and "S" callus tissues were used as inocula. (PLATE 10). These structures did not appear root-like in external morphology. The tissues bearing these structures were recovered from liquid medium and subcultured on to a variety of semi-solid media in attempts to promote further organised development.

Table 6.3

Medium	2,4-D mg/l	kinetin mg/l	½ normal/normal strength M + S
1	-	-	½ normal
2	-	0.5	normal
3	2.0	-	"
4	2.0	0.5	"
5	-	-	"

10 replicate flasks were inoculated per treatment and the pH of all media was adjusted to 5.6.

On media 3 and 4 most of the structures became obscured by proliferations of the surrounding callus tissues, while on media 1, 2 and 5 the callus tissues became moribund without displaying further organised growth. Some of the callus tissues cultured on media 3 and 4 showed neither growth nor necrosis and these were placed on to media containing lower 2,4-D concentrations (i.e. 1.0 mg/l and 0.5 mg/l) as well as being exposed to a variety of environmental conditions, i.e. 25^0 light (200 lux), 30° light (200 lux), 25° and 30° in the dark. However, attempts to promote further organised development of these structures were unsuccessful. The cultures either became moribund or proliferated callus

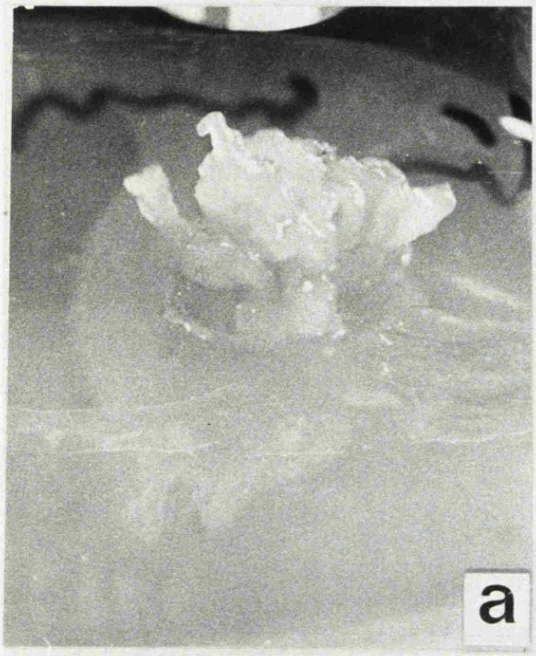
Explanation of PLATE 10

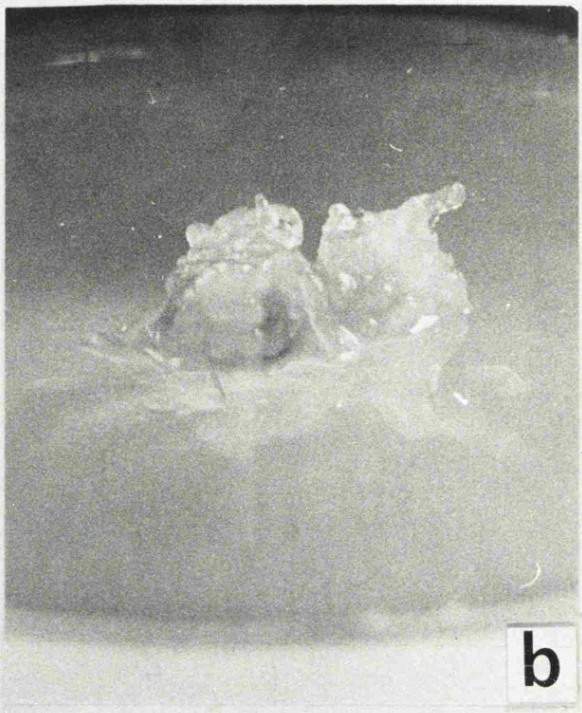
The formation of structures other than roots

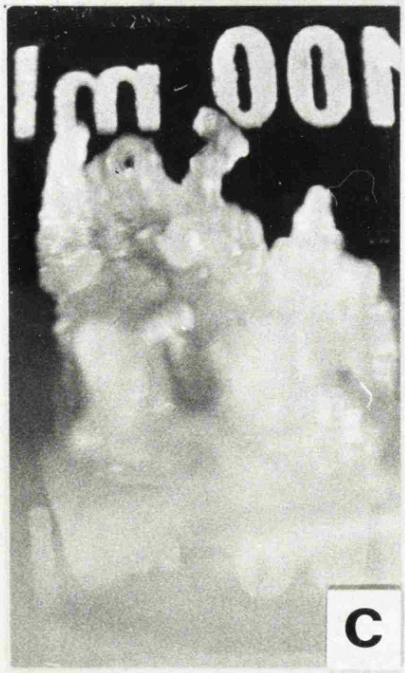
a "S" callus tissue, b and c "A" callus tissues, all recovered from agitated liquid medium after 4 months incubation. The callus tissues display a type of morphological differentiation which does not appear root-like.

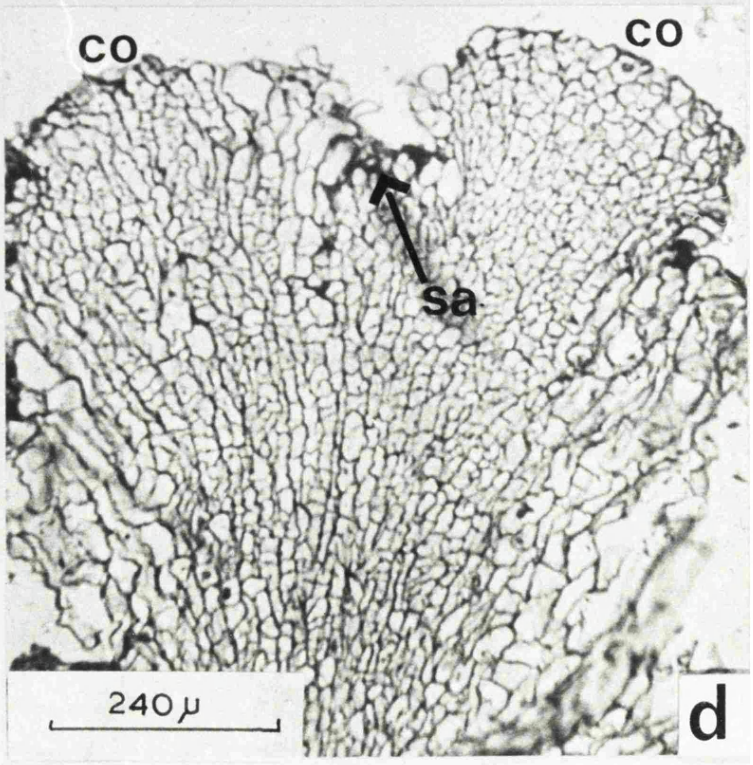
d Section through such a structure (on "A" callus) at a comparatively early stage of development. The structure appears shoot-like with possibly a shoot apex (sa) and two cotyledons (co) present.

PLATE 10









tissues to the extent of obscuring the original structures. Anatomically (PLATE 10), the structures were highly suggestive of shoot primordia, but despite this subsequent development into a more clearly recognisable shoot did not take place under the cultural conditions described.

B. The second pattern of differentiation produced more easily distinguishable structures, clearly not root-like (PLATE 11). These structures were commonly encountered in tightly packed clusters radiating from a common core. These clusters could be readily teased apart into a number of single structures (PLATE 11) since they were only loosely connected at their common core. In external appearance the cluster of structures resembled a compact mass of callus tissue and would have been regarded as such but for the fact that one or two of the member structures were markedly more elongated than their neighbours thus protruding conspicuously. This pattern of differentiation was detected only when the tissues were becoming moribund and thus no attempt was made to subculture them on to semi-solid media. The individual structures appeared embryo-like with partially extended cotyledons at their free (apical) ends. (PLATE 11). Development appears to have ceased at this stage probably indicating the abnormality of the embryo-like structures or the inadequacy of the culture environment to support their further growth. Examination of sections of these structures revealed a certain degree of vascularisation; protoxylem was frequently observed in the central axis of structures such as that depicted in PLATE 12.

Roots, shoot- and embryo-like structures are thus produced in cell and tissue cultures of Hevea brasiliensis

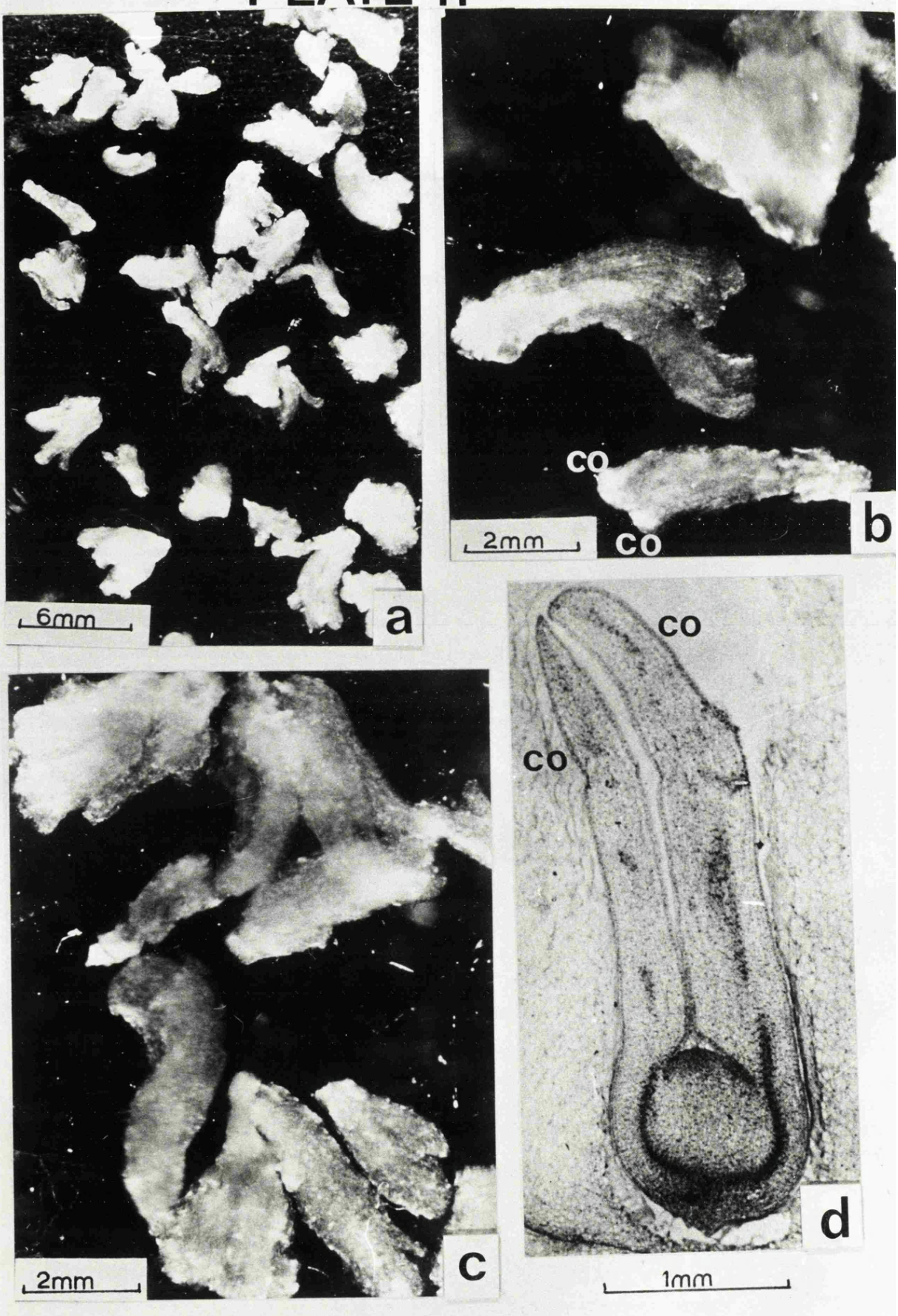
Explanation of PLATE 11

The formation of embryo-like structures

The structures depicted in a, b and c were formed in agitated liquid medium which had been inoculated with "A" callus tissues and allowed to incubate for 3 months. In these cultures the embryo-like structures were found in clusters radiating from a common core, and have been teased apart with a needle for the purposes of photography.

d *In vivo* embryo approximately 100 days-old (after Bouharmont). The cotyledons (co) are indicated in d as are those parts of the *in vitro* embryo-like structures which most resemble cotyledons (co) (in b).

PLATE 11

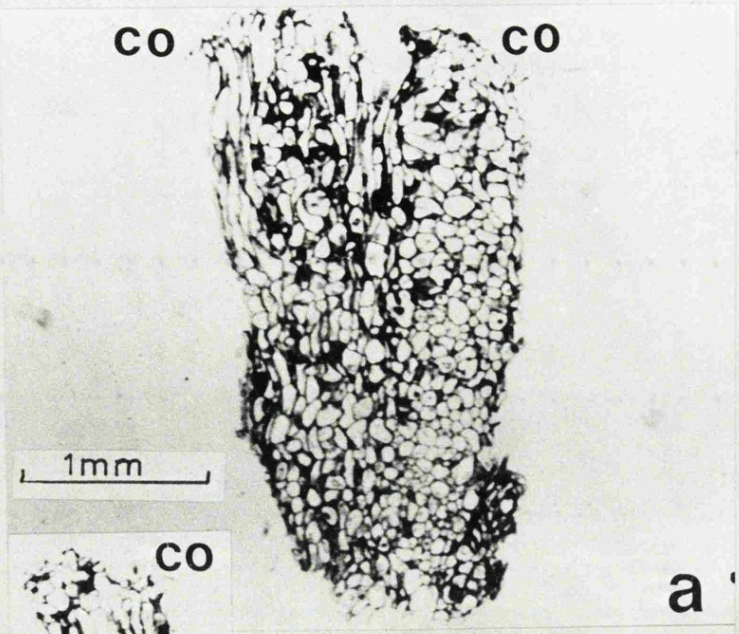


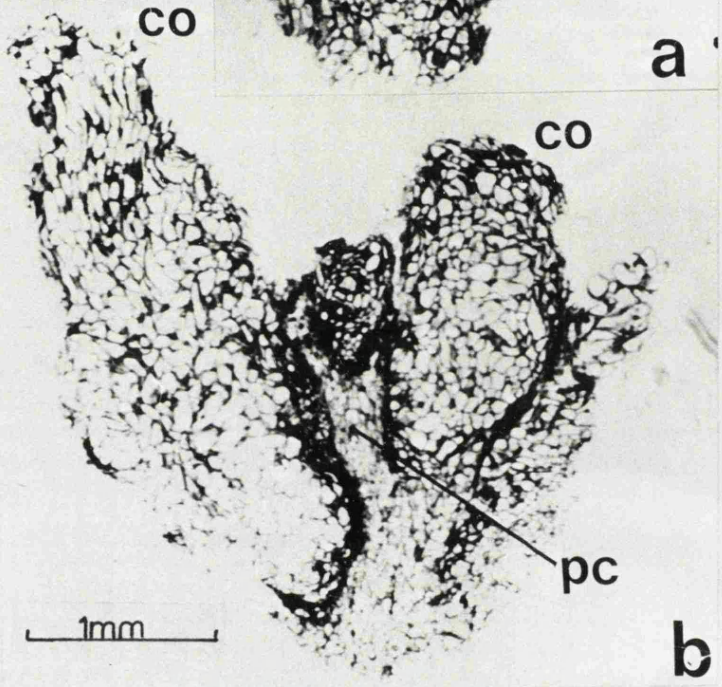
Explanation of PLATE 12

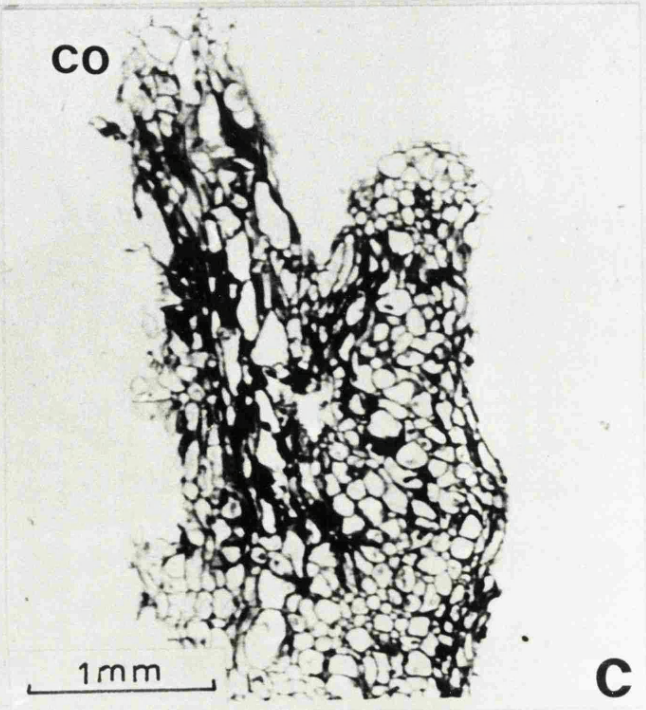
Anatomy of the embryo-like structures recovered from agitated liquid medium

The sections in a, b and c were fixed in 1% chrome acetic acid and stained with toluidine blue. Structures resembling cotyledons (co are indicated in a, b and c. None of the sections is median. A procambial system (pc) is possibly present in the structure sectioned in b.

PLATE 12







apparently primarily as a result of prolonging the passage length. Differentiation has never been observed in tissues which have been regularly subcultured. Conditioning or depletion of the medium appears, therefore, to be an important factor for the onset of organised growth. It is known that cells cultured in suspension release amino-acids into the culture medium (Tulecke and Rutner 1965) and thus an attempt was made to extract and analyse the amino acids in the liquid medium which had supported the patterns of differentiation described above. The incorporation of the appropriate quantities of such amino acids into the culture medium might then perhaps reconstitute conditions conducive to the observed differentiation.

4-6 month-old cultures (in which differentiation was evident) were taken, the differentiated structures and large aggregates removed, the medium pooled and filtered free of cells The subsequent treatment of the medium for the detection and estimation of amino acids is fully described in Section 1. Table 6.4 shows the quantities of the amino acids which were identified (see also Figure 6.1).

Explanation of Figure 6.1

Constitutents present in "desalted" conditioned medium as separated on the Auto analyser

Key	Constituent
1	Cysteic Acid
2	Urea
3	Unknown
4	"
5	Aspartic Acid
6	Threonine
7	Serine
8	Glutamic Acid
9	Proline
10	Citrulline
11	Glycine
12	Alanine
13	Valine
14	Isoleucine
15	Leucine
16	Norleucine (Internal Standard)
17	Tyrosine
18	Phenylalanine
19	Unknown
20	Ammonia
21	Unknown
22	Lysine
23	Histidine
24	Arginine

Fig 6.1

Elution diagram showing the separation of constituents present in "desalted" conditioned medium on the Technicon automatic amino acid analyser

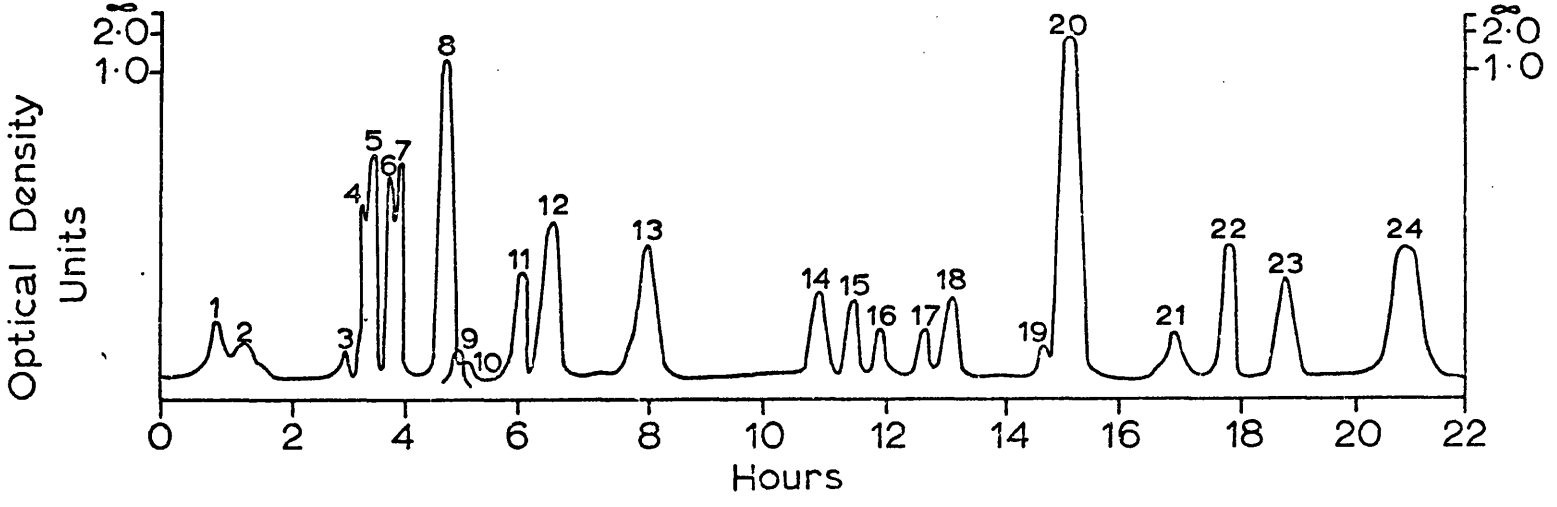


Table 6.4

The Amino Acids which were identified and quantified from four representative samples of "conditioned" medium

Amino acid	mg/l
Glutamic	54
Arginine	47
Proline	13
Lysine	13
Aspartic	12
Serine	10
Threonine	10
Histidine	8
Valine	8
Alanine	8
Phenylalanine	5.5
Isoleucine	4
Leucine	4
Glycine	3
Tyrosine	2.5
Cysteic	1.5
Citrulline	1
(Urea)	0.3

The quantities given for serine and threonine may be overestimates since the respective presences of glutamine and asparagine would contribute to these peaks. In order to test for these amides it is necessary to hydrolyse the sample

thus effecting conversion to glutamic acid and aspartic acid and consequently enhancing these peaks. However, this was not done and thus no estimate of these amides was obtained. This mixture of amino acids (plus urea) was incorporated into the media tabulated in Table 6.5.

Table 6.5

Treatment	2,4-D mg/l	kinetin mg/l	½ normal/normal strength M + S
1	-	-	½ normal
2	-	0.5	normal
3	2.0	-	"
4	2.0	0.5	"
5	-	-	"

Ten flasks were inoculated per treatment, 5 with "A" callus tissues and 5 with "S" callus tissues. The cultures were incubated at 30° in the dark, shaking at 120 r.p.m. The pH of all media was adjusted to 5.6. After one month some of the callus tissues (both "A" and "S") in treatments 1, 2 and 5 had developed numerous globular swellings. These tissues were removed from the liquid medium and placed on semi-solid normal strength Murashige and Skoog's medium minus 2,4-D and kinetin, but supplemented with the amino acid mixture described in Table 6.4. 2 mg/l 1γγdimethylallyl 6 amino purine (DAP) which has been shown to have a pronounced stimulatory effect on bud formation in various tissue cultures (Hamzi and Skoog, 1964) was also incorporated in the culture medium.

In the presence of 2,4-D (treatments 3 and 4 of Table 6.5) no differentiation in the form of globular swellings was evident, and growth in the cultures inoculated with "S" callus tissues was mainly confined to cells and cell aggregates in suspension. The observed structures (treatments 1, 2 and 5 of Table 6.5) did not develop further to result in recognisable embryos or shoots (after 6 months in culture). Since, however, distinctive structures such as those shown in PLATE 10 were formed on "A" and "S" callus tissues after 3 months incubation without subculture from their original liquid medium, there is thus the suggestion that the time taken for this pattern of differentiation to manifest istself in standard M + S medium (up to 6 months) is reduced in the presence of the amino acid mixture, but the data are insufficient for this to be regarded as unequivocal.

3. The influence of the amino acids identified and quantified in "conditioned" medium on the growth of cell suspensions

In order to test the effect of the amino-acid mixture on the growth of cell suspensions of Hevea brasiliensis the following treatments were set up.

Table 6.6

Medium	2,4-D mg/l	kinetin mg/l	Amino Acid mixture
1	2.0	0.5	-
2	2.0	0.5	+
3	-	-	+
4	-	-	-

The pH of all media was adjusted to 5.6. 5 ml of stock cell suspension were used to inoculate 50 ml of fresh medium and the parameters of cell number/ml x 10^{-6}, % packed cell volume and dry wt./ml of culture measured over the period of growth. The results shown in Figures 6.2 and 6.3 indicate that the presence of the amino acid mixture has no significant effect on the growth of the cell suspensions as measured by these parameters. On Day 26 the percentage of cells in aggregates of 10 or less was estimated for the four treatments.

Table 6.7

Treatment	% of cells in aggregates of 10 or less
1	10
2	15
3	4
4	4

In treatments 3 and 4 only large aggregates remained after 26 days and a large amount of cell debris was evident. The presence of this debris means that a large number of cells have released their contents, obviously including quantities of amino acids, into the culture medium and thus the results of a comparison of growth in the presence and absence of the amino-acid mixture under these conditions may be misleading. In treatments 1 and 2, however, the quantity of cell debris was comparatively small and the presence of the amino acid mixture appeared to exert no significant effect on the growth of these cell suspensions.

Explanation of Figures 6.2 and 6.3

The effect of the amino acids detected in "conditioned" medium on the growth of cell suspensions of Hevea brasiliensis

Key:- O—O Standard M + S

●—● Standard M + S, with the amino-acid mixture.

△—△ M + S minus 2,4-D, minus kinetin

▲—▲ M + S minus 2,4-D, minus kinetin with the amino-acid mixture

The measurements recorded for cell number, dry weight and % packed cell volume each represent the mean values for five replicate flasks.

Fig 6.2

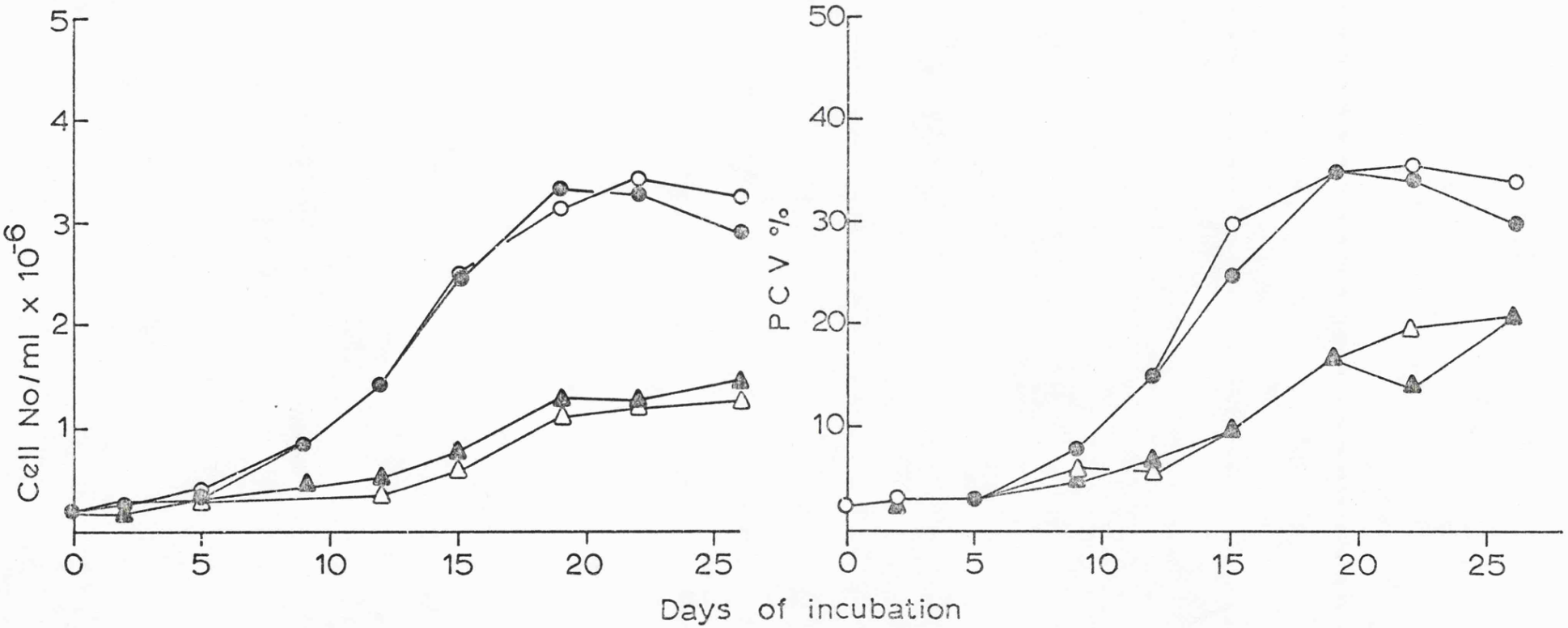
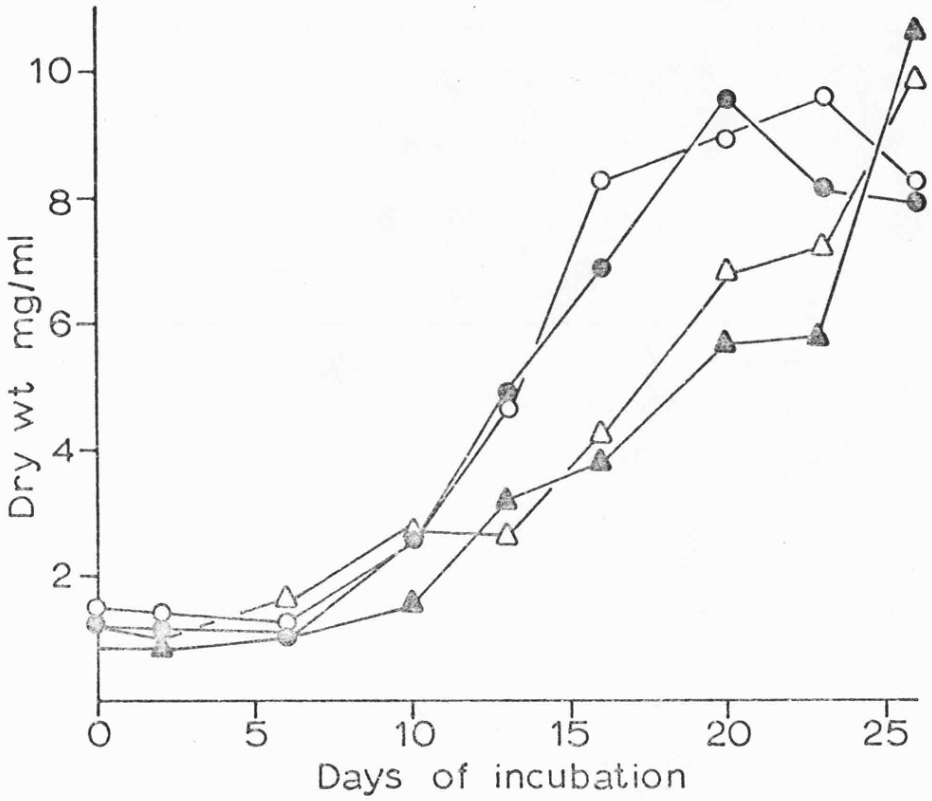

Cell No/ml x 10^{-6}
PCV %
Days of incubation
0
5
10
15
20
25
1
2
3
4
5
10
20
30
40
50

Fig 6.3



Growth in the presence and absence of 2,4-D and kinetin, on the other hand, was markedly different when measured by % P.C.V. And cell number/ml x 10^{-6}. Dry weight in mg/ml, however, was not similarly responsive resulting in a situation where after 26 days the cell number/ml achieved in the presence of 2,4-D and kinetin was almost 3 times that achieved in their absence, while the dry weights recorded for these treatments were very similar (Table 6.8).

Table 6.8

Treatment	Cell No. 10^{-6}/ml x 10^{-6}	Dry wt in mg/ml	Mg Dry wt/ 10^6 cells
M+S, plus amino acids, plus 2,4-D, plus kinetin	3.25	8	2.46
M+S, plus amino acids, minus 2,4-D, minus kinetin	1.25	10	8.0

Thus it is possible to conclude that in the absence of 2,4-D and kinetin, cells in suspension cultures of Hevea brasiliensis slow down in growth and fail to divide but continue to accumulate dry weight at a similar rate to cells in the presence of 2,4-D and kinetin. The experiment was restricted to one passage length since on transfer to a second passage in the absence of 2,4-D and kinetin the cultures became moribund within one week.

Another clear distinction between cell suspensions grown in the presence and absence of 2,4-D and kinetin is found in the level of aggregation. Attention was drawn to this

phenomenon in Section 4. Growth as measured by cell division and % P.C.V. was not retarded in the absence of 2,4-D and kinetin in the first passage of the experiment described in Section 4, presumably because an inoculum of 10 ml was used, resulting in a carry-over effect as sufficiently pronounced as to allow the cells to continue to divide at similar rates. There are at least two distinct effects on cell suspensions of Hevea brasiliensis directly attributable to the omission of 2,4-D and kinetin from the medium. 1) Cells accumulate more biomass on a per cell basis (and probably on a per culture basis) and there is a decrease in the number of cell divisions particularly towards the end of the passage when any effects from carry-over will be negligible (Figures 6.2 and 6.3). 2) A marked increase in the level of cell aggregation occurs (Table 6.7) and it is from some of these larger aggregates that numerous globular swellings arise. However, differentiation does not proceed further and the general appearance of the cultures becomes necrotic.

From these data it would appear that the conditions which stimulate morphogenesis in cell cultures of Hevea brasiliensis remain to be identified, but that these conditions arise (probably as a consequence of prolonged tissue to medium contact) is clearly indicated by the formation of embryo-like and shoot-like structures from such cultures. Preliminary results suggest that incorporation of an amino-acid mixture based on those detected and estimated in the medium bathing differentiating tissues may aid a more rapid achievement in the culture medium of conditions which stimulate the initiation of such differentiation. Clearly, however, conditions which enable the structures to continue their development have not been achieved.

SECTION 7

GENERAL DISCUSSION

The work of Riker and Hildebrandt (1958) suggests that optimum temperatures for the growth of plant tissue cultures are similar to those observed for the whole plants from which the cultures are derived. Since the native habitat of Hevea brasiliensis is tropical it was to be anticipated that an incubation temperature of 30° or above would be required for the in vitro culture of this species.

The application of standard tissue culture techniques resulted in the development of the morphologically heterogenous, compact and slow-growing "A" callus tissues. The homogenous, friable and relatively rapidly growing "S" callus tissues were fortuitously obtained as a consequence of culturing the "A" callus tissues in liquid medium for six to eight weeks. The resemblance of the "S" callus growth to that of habituated tissue was mentioned in Section 3 but the fact that "S" callus retains a requirement for an exogenous auxin supply indicates that it cannot be regarded as fully habituated. Yeoman (1973) points out that the change in auxin synthesising capacity characteristic of habituated tissue is probably quantitative rather than qualitative since normal tissues produce a certain amount of auxin. (Dannenburg and Liverman, 1957). Lescure (1970) treated a sycamore suspension with nitroso-guanidine and by subsequent plating isolated two strains capable of growth in the absence of 2,4-D. One strain showed enhanced friability and evidence was obtained to show that IAA oxidase activity was three

times greater in the parent cultures than in the friable strain with no auxin requirement. In this work it would seem possible to hypothesise that a decreased breakdown of endogenous auxin was at least in part responsible for the production of habituation.

Apart from the dependence of the "S" callus tissues on an exogenous auxin supply the extent of the transformation from "A" to "S" callus growth closely resembles that from normal to habituated tissue. The suggestion of Yeoman (1973) that the change in auxin synthesising capacity may be quantitative rather than qualitative raises the possibility that the "S" callus tissues are incompletely or partially habituated. In this event, regardless of whether the loss of an auxin requirement is a consequence of diminished auxin oxidase activity or of enhanced auxin synthesis or both, it may be expected that the "S" callus tissues would grow at an optimal rate on a lower auxin concentration than the "A" callus tissues. This, however, remains to be tested. There is also evidence that stable changes affecting other aspects of metabolism can occur in cultured tissues (Gautheret 1950, 1955) and Street (1969) considers that the phenomenon of habituation should not be conceived of as restricted to auxin metabolism.

While Fox (1963) found that habituated cultures of tobacco had higher chromosome numbers than those of normal callus cultures, Sacristan and Melchers (1969), and Melchers (1971) have been unable to establish any direct relationship between chromosome number and habituation in their tissue cultures. There is no evidence to suggest that the transition from "A" to "S" callus tissues is associated with a change in chromosome

number.

The transition from "A" to "S" callus growth probably represents a permanent change in the physiology of cultured Hevea brasiliensis cells. The reproducibility of this change seems to preclude somatic mutation, while its stability suggests involvement at the level of gene expression thus invoking the concept of enzymatic adaptation as proposed by Gautheret (1955) to account for the phenomenon of habituation. Braun and Lipetz (1966) subscribe to this proposal and suggest that an habituated or tumorous cell may be regarded as permanently trapped in a division orientated pattern of metabolism by the constitutive synthesis of hormones which in turn determine permanent excessive production of compounds that normal tissue cultures require from the medium. To consider the transition from "A" to "S" callus growth in these terms it is necessary to postulate that the change in culture conditions from agar to liquid medium effected a sudden increase in the level of intracellular auxin (and/or some other substance(s)). This increase would then cause a set of genes to display a novel range of expressions, one of which would be the enhanced production of endogenous auxin, thus establishing a new and stable pattern of metabolism.

It would be of interest to determine if, and in what quantities, ethylene is produced from the "A" and "S" callus cultures of Hevea brasiliensis. Zimmerman and Wilcoxon (1935) first discovered that the application of auxin increased the production of ethylene from plant tissues. Abeles (1972) considers that since auxin promotes ethylene production this means that the quantities of ethylene

produced by plant tissues will give an estimate of their endogenous auxin levels. Mackenzie and Street (1971) have shown that in cell suspensions of Acer pseudoplatanus ethylene is produced in greatest quantity by dividing cells and in smallest quantity by cells in the lag and staionary phases of growth. They also observed that the maximum ethylene level achieved coincided with initiation of the phase of cell separation. It has been shown that in certain circumstances applications of ethylene markedly enhance the friability of Begonia x richmondensis callus tissues (Ringe, 1972) and increase the level of cell dispersion in Daucus carota (Wochok and Wetherell, 1971) and in Hevea brasiliensis (Section 4). Ethylene treatment, at least in the latter case, did not appear to affect the rate of growth in these cultures and taking into account the suggestion of Abeles (1972) outlined above, and the observation of Burg, Apelbaum, Eisinger and Kang (1971) that ethylene stimulates cell expansion and not cell division, it is tempting to speculate that the friability of plant callus cultures is dependent solely on the quantity of (intracellular) ethylene present, which in turn is determined by the level of endogenous auxin. Thus the enhanced friability of habituated tissues when compared with the normal tissues from which they are derived would be an expected consequence of the higher endogenous auxin levels observed in these habituated tissues.

Grant and Fuller (1968) compared the chemical composition of friable with non-friable callus tissues of Vicia faba, and showed that non-friable callus has a greater total amount of cell wall polysaccharides, more of each particular cell

wall fraction per unit dry weight, but a decreased percentage of cellulose compared with pectic substances and hemicelluloses. Anatomically the differences between friable and non-friable callus tissues are here described as an absence of islands of small, closely packed cells in the non-friable callus tissues (although some cells of this type are present) and the presence of a greater proportion of large vacuolated cells. The friable tissues grew at a significantly higher rate than the non-friable tissues and it is possible to hypothesise that growth rates are dependant on the relative proportions of the small and large cells in these tissues. If this situation obtains, then it may be misleading to regard friable tissues as uniformly different in metabolism from non-friable tissues or to regard transition from one to the other as irreversible. The friable callus tissues and finely dispersed cell suspensions of Hevea brasiliensis were not produced merely by addition of the appropriate amounts of 2,4-D and 2-CEPA to the culture medium. It was the introduction of callus into agitated liquid medium and its subsequent recovery as callus which appeared to be of crucial importance in determining the extent of friability. These same physical traumatas also appear to be the most conducive, over a prolonged period, to the formation of shoot- and embryo-like structures.

The previous cultural history must also be taken into account when assessing the capacity of cultured tissues to undergo morphogenesis as well as the immediate physical conditions and medium constituents. Syono (1965) observed that prolonged subculture caused a diminution in competence,

while Gautheret (1946) noted that habituated tissues (arising in the presence of auxin) subsequently have a reduced capacity to intiate organs or show internal differentiation.

Diminished competence of cultured tissues (including those which are habituated) may be a consequence of the presence of comparatively high levels of ethylene since Wochok and Wetherell (1971) have shown that 2-CEPA suppresses organised growth in cultured wild carrot tissues. Similarly Ringe (1972) has shown that the morphogenesis during neoformation on stem segments from partial inflorescences of Begonia x richmondensis is inhibited in the presence of 2-CEPA suggesting an active role for ethylene in disrupting organised growth. Thus tissues producing comparatively large quantities of ethylene may be expected to show reduced morphogenetic capacity. The relationship between the administering of auxin and production of ethylene from plant tissues first discovered by Zimmerman and Wilcoxon (1935) appears to have wide ranging applications in interpreting the responses of cultured plant cells to variations in the growth hormone constituents of their culture medium. Thus it is generally necessary either to reduce the level of auxin (Halperin and Wetherell, 1964) or remove it from the medium (Reinert, 1959) to stimulate a morphogenetic response. Wochok and Wetherell (1971) specifically mention that the effect of 2-CEPA on organised growth is similar to that of 2,4-D. Reinhard, Gorduan and Volk (1968) reported that addition of 2,4-D increased the friability of Ruta graveolens callus clumps, while Torrey and Reinert (1961) found a drastic reduction in the number of free cells following omission of 2,4-D from

the medium. A similar observation was made when 2,4-D was omitted from the medium of cell suspensions of Hevea brasiliensis (Section 6). Thus the effects of ethylene on friability and cell dispersion of plant cultures, already considered in some detail, are closely paralleled by the effects of auxin on these cultures.

The literature relevant to the relationship between auxin and ethylene in their effects on plant tissue is extensive and it is not appropriate that it be reviewed here. It is sufficient to suggest that at least some of the effects of high auxin levels on cultured plant cells can be attributed to an enhanced production of ethylene by these cells, and that once the ethylene pathway has been activated it may persist in the absence of the inducing stimulus.

A range of morphogenetic responses was displayed by the callus tissues and cell suspensions of Hevea brasiliensis. Most easily recognisable was the formation of roots although as in all the morphogenetic events observed, the inductive conditions could not be identified. The factor which unified all forms of differentiation was the requirement of the tissues for a prolonged period in contact with one batch of culture medium suggesting that conditioning processes are of particular importance in the stimulation of morphogenesis. Indeed, preliminary analysis of this "conditioned" medium revealed the presence of relatively high concentrations of amino acids which were not among the original constitutents of the culture medium. Attempts at reconstituting a medium conducive to a more rapid manifestation of differentiation yielded equivocal results and it may be that specific,

unknown phytohormones are involved and will only be identified following exhaustive biochemical analysis of the conditioned culture medium.

Primordia as shown in PLATE 10 displayed a definite shoot-like appearance but these could not be induced to develop into leafy shoots. To interpret the formation of the embryo-like structures shown in PLATE 11 as evidence of *in vitro* embryogenesis would be controversial. Steward, for example, considers that *in vitro* embryogenesis is demonstrated only by the formation of embryos which have their origin in free floating cells. This view is supported by the observation of free cells, twin cells and aggregates of very few cells, free-floating globular and more advanced embryoids in cell suspension cultures of *Daucus carota* (Steward, 1964). In the original suspension cultures of *Hevea brasiliensis* the callus inoculum did not disperse finely in the agitated liquid medium. Thus it is likely that the early divisions of the cells from which these structures were produced took place in the peripheral regions of the callus clumps and were eventually shaken free of these parental aggregates on achievement of a substantial and relatively discrete tissue mass. Homés and Guillaume (1967) have criticised the interpretation of the few-celled aggregates as intermediate stages between free-floating single cells and clearly formed embryoids on the basis that carrot embryos in the early stages of development are composed of small densely cytoplasmic cells containing starch, whereas the clusters figured by Steward and co-workers consist of larger, vacuolated cells. Halperin and Wetherell (1964), and Halperin (1967) suggest that

embryoids arise from the surface layers of the larger cellular aggregates which are always present in embryogenic carrot suspension cultures. The superficially initiated embryoids are thought to be released at varying stages in their development as free-floating structures and which, if sufficiently advanced, are able to complete their development in isolation from the parent callus clump. Recently McWilliam, Smith and Street (1973) presented data to support this interpretation, obtaining evidence to suggest that individual embryoids have their origin in single superficial cells of the clumps of an embryogenic carrot suspension culture.

The origin of embryoids from single cells in embryogenic clumps is also implicated in the formation of embryos from cell suspension cultures of Atropa belladonna (Konar, Thomas and Street, 1972) and callus cultures of Ranunculus sceleratus (Konar and Nataraja, 1965). In the latter study the data indicated that individual cells about to embark upon embryogeny and even the 2 or 3 celled proembryos remain in protoplasmic continuity via plasmodesmata with the adjacent cells of the aggregate within which they are embedded. This had led Street (1974) to suggest that while the young embryoid is delineated from adjacent cells by a severing of cytoplasmic continuity and cutinisation of the boundary cell walls, isolation per se may not be a prerequisite for the initiation of embryogeny. Clearly, from this viewpoint, the suggestion that incipient embryogenesis has taken place in suspension cultures of Hevea brasiliensis can be regarded as more probable.

The embryo-like structures of Hevea brasiliensis were observed in tightly packed clusters radiating from a common core, suggesting some form of relationship or common origin. Amongst possible explanations may be the formation of a number of embryoids from a small, free floating, embryogenic cell aggregate, or from a free-floating embryoid itself, by adventive polyembryony. There are no data on which to decide between these alternatives since anatomical studies were not carried out on the smaller cellular aggregates. However, no isolated embryoids of an equally advanced state to those occurring in clusters were observed. Embryoids developed from somatic embryogenic clumps appear to be uniformly polarised (Halperin and Wetherell 1964, Konar et al. 1972, McWilliam et al. 1973) with the root pole directed towards the centre of the cell aggregate and the shoot towards the outside. The clusters of embryo-like structures in cultures of Hevea brasiliensis were similarly polarised (Section 6).

An acceptance of the view that embryo formation is initiated in the surface layers of the larger cellular aggregates poses further problems in identifying the conditions which enable this embryogenesis to occur. The cells surrounding and below those in which embryogenesis is to be initiated must fundamentally modify the micro environment (physical and chemical) of the embryogenic cells. The fact that embryoids developed from such embryogenic cell aggregates display a uniform polarity suggests the presence of determinative chemical gradients within the aggregate. The release of intracellular compounds into the external medium either by secretion or as a consequence of cell rupture,

or both, complicates the situation further resulting in complexity even when using a defined culture medium.

Halperin (1967) has suggested that the conditions under which callus induction takes place may be of crucial importance in the subsequent achievement of embryogenic competence by the cultured tissues. He considered the possibility that these primary cultures may contain inducible and non-inducible cells and active proliferation of the inducible cells to be essential for the retention of embryogenic capacity. Thus, failure to obtain embryogenesis in culture would be a consequence of inappropriate conditions of callus induction which failed to achieve the "dedifferentiation" necessary to restore to somatic cells the competence of the zygote. The persistently distinct and characteristic physiological features of callus cultures derived from adult versus juvenile phases of Hedera helix (Stoutmeyer and Britt, 1965) provides experimental support for this interpretation, with the induction of callus growth failing to delete the adult character of the cells. Foskett and Torrey (1969), in studies on xylem differentiation have shown that the cultural conditions operating during the induction of cell division can exert a decisive influence on the nature of any subsequent differentiation. Such observations are clearly relevant to the stimulation of cyto differentiation and embryogenesis from relatively recalcitrant tissues such as those of Hevea brasiliensis. Similarly the approach of Wetmore and Rier (1963) and Jeffs and Northcote (1967), who successfully differentiated xylem and phloem from parenchymatous callus masses by using a V-shaped incision in the callus and supplying auxin and

sugar at the incision, may be applicable to the study of laticifer formation in callus cultures of Hevea brasiliensis. Thus, while at present the cultured tissues of Hevea brasiliensis cannot be reproducibly manipulated in a manner satisfactory for experimental or commercial purposes, there are obvious possibilities still to be explored which may contribute to this objective.

In the introduction to this thesis three main objectives were formulated for the exploitation of plant tissue culture techniques to achieve increased yields from the rubber tree Hevea brasiliensis. The major objective of establishing an in vitro system for the production of large numbers of normal Hevea plantlets remains to be achieved. To estimate the extent of present progress and to evaluate the chances of future success, it is necessary to consider contemporary problems and perspectives in plant tissue culture as an applied science.

The establishment of callus and cell suspension cultures of many plant species has been achieved since the first plant tissue culture was reported upon by Gautheret in 1939. In vitro formation of roots of many species has been observed from tissue cultures particularly when they are first established. Reports of shoot and embryo formation with subsequent regeneration of the plant are, however, limited to a comparatively small number of species. The number of amenable species in certain plant families (e.g. the Solanaceae) is relatively large compared with that in certain other families (e.g. the Graminiae). Monocotyledonous or woody species are generally recalcitrant to culture and subsequent morphogenesis,

but the production of plants from cultures of Asparagus officionalis (Wilmar and Hellendoorn, 1968) and Populus tremuloides (Winton, 1968) suggests that there are no fundamental obstacles to the expression of totipotency in cultures of any plant species. The spectra of culture conditions inducing morphogenesis are clearly broader in some species than in others, resulting in plant cultures of certain species displaying qualitatively similar responses to similar variations in the medium constituents, e.g. the activity of kinetin in bud formation from callus cultures of Nicotiana tabacum (Skoog and Miller, 1957) and from root cultures of Convolvulus (Bonnett and Torrey, 1963). However, the anticipation of revealing a general common mechanism for the regulation of organogenesis following the work of Skoog and Miller (1957) appears precipitate in retrospect. In the face of this impasse, Street (1973) has suggested the need for more intensive studies of the endogenous phytohormone levels in plant cultures, the identification of the chemical nature of auxin, cytokinin and gibberellin in each particular tissue and a recognition that there may well be new phytohormones to be discovered.

Once the conditions required for the regeneration of plants from cultured tissues have been identified, then tissue culture techniques become relevant to the problems of propagation encountered with certain commercially important species such as the Coconut, Date and Oil Palms which have to be propagated from seed. If then under suitable cultural conditions uniformity of propagants is realised, it should be possible to produce large numbers of plants with commercially

desirable characteristics more rapidly. However, genetical uniformity of the propagant plants cannot be anticipated with any certainty under the culture conditions currently employed. Thus, Sacristan and Melchers (1969) have shown that abnormal plants with an aneuploid complement of chromosomes were regenerated from the callus tissues of Nicotiana tabacum after eight years in culture. Persistent subculturing of callus tissues leads to morphological, cytological and biochemical variation. There is thus a need for an adequate preservation method if cells are to be maintained in a genetically unaltered state whilst in culture. A freezing technique for the storage of embryogenic cell lines of domestic and wild carrot for long periods at liquid nitrogen temperatures has recently been developed by Nag and Street (1973). The fact that morphogenic potential is unimpaired by this freezing preservation technique suggests that it may be possible to establish stable cell and tissue banks of particular plant genotypes.

The induction of haploid plants from pollen grains (Guha and Maheshwari, 1964) provides a means of rapidly achieving homozygosity in plant breeding schemes. Colchicine, applied at early stages in the development of haploid embryos can effect a doubling in chromosome number and thus bring about the production of homozygous diploid plants. Pollen can also be induced to give rise to haploid callus tissue by the incorporation of appropriate levels of combinations of IAA, 2,4-D and kinetin into the culture medium. Here spontaneous chromosome doubling frequently occurs and while with repeated subculture polyploid cells begin to

accumulate, homozygous diploid plants may still be regenerated from these cultures. The production of homozygous diploid plants will surely aid the analysis and breeding of genetically complex species such as the Brassicas, Sugar beet and certain cereals. Palms have to be propagated from seeds of very diverse genetic origin and thus the plantations carry trees of very mixed quality. The production of many different homozygous varieties from the genetically heterogenous pollen of the Palms would be an aid to the improvement of these crops, though probably of less immediate value than true vegetative propagation through tissue culture for selected high quality trees. The vigour of dioecious species such as Asparagus could be enhanced by interbreeding doubled pollen plants, with the parents of the high-yielding hybrids being maintained vegetatively.

Work aimed at obtaining highly dispersed cell suspensions with a view to using these suspensions together with the plating technique of Bergmann (1960) to isolate single cell clones has been carried out in the laboratory of Street. (Street, King and Mansfield 1971, Mansfield 1973). Progress in this sphere, allied to the culture of callus tissues and cell suspensions derived from haploid plants will offer unique opportunities for the study of mutation in higher plants. The isolation of biochemical mutants from cultured plant cells has been reported by Carlson (1970), Binding (1972), and Maliga, Breznovits and Márton (1973). The latter authors have succeeded in obtaining streptomycin resistant plants from haploid callus cultures of Nicotiana tabacum and preliminary results suggest that this character is maternally

inherited. The number of streptomycin resistant callus colonies which arose in the cultures was dependent only on the initial cell number suggesting that every resistant colony can be regarded as the product of a discrete event (In this case with a frequency of occurrence equal to 1×10^{-6}). This promising work indicates that it is possible to select for a particular trait at the level of the cell in culture and for this trait to become incorporated into the genome of the regenerated plant. The establishment of the required culture conditions for the selection of a certain genetic trait may be regarded as a major problem and may present as many difficulties as those currently encountered in the induction of morphogenesis from recalcitrant plant tissues or tissues new to culture.

Attention has already been drawn to the role played by the culture medium as an agent of selection in the work of Carlson, Smith and Dearing (1972). Here, the isolated protoplasts of Nicotiana glauca and N. langsdorffii were induced to form fusion protoplasts, the products to regenerate callus and the callus tissues to differentiate shoots ultimately resulting in the production of hybrid plants. The production of interspecific plant hybrids by this method could obviate the limitations of sexual processes. It may be that a new plant breeding technique will develop centred upon this combination of protoplast and tissue and cell culture techniques.

The establishment of the large scale production of plant cells in suspension raises the possibility of using the primary culture product of cell mass as a food stuff.

Mandels, Jeffers and El-Bisi (1968), have studied the production of cell mass by tissue cultures derived from a number of edible plants and calculated that the present levels of yield in culture are of the same order of magnitude as crops grown conventionally. They consider that the yield from cultured plant cells must be increased tenfold if it is to become a practical proposition. The synthesis of appreciable amounts of secondary plant products by tissue and cell cultures in the absence of differentiation has been only rarely reported. Heble, Narayanaswami and Chada (1968) reported that tissue cultures of Solanum xanthocarpum yield β-sitosterol and diosgenin in quantities much higher than those obtained from the growing plant, but with the vast majority of tissue cultures of plant species with characteristic secondary products such a phenomenon remains to be demonstrated. Thus the possibility of using tissue cultures for the laboratory preparation of rubber, let alone production on a large scale must be regarded at this stage as improbable. Nevertheless the ultimate use of tissue culture to study the pathway of rubber biosynthesis must be regarded as a possible future development of the studies now reported.

In conclusion it should be stated that the problems which remain to be overcome before plant tissue culture techniques can be comprehensively applied to crop improvement are manifold and formidable. However, substantial progress has been made over the last two decades and if the momentum of advance is maintained then it is feasible to expect the eventual widespread use of tissue culture techniques in this field.

Appendix

Media : Constituents and Preparation

1 Murashige and Skoog

Constituent	Final Conc. in mg/l	
KNO_3=	1900	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium)
NH_4NO_3	1650	
$CaCl_2.2H_2O$	440	
$KH_2PO_4.H_2O$	170	
$MnSO.4H_2O$	22.3	STOCK B (Stock strength = x 20)
$ZnSO_4.7H_2O$	11.5	
H_3BO_3	6.2	
KI	0.83	
$Na_2MoO_4.2H_2O$	0.25	
$CuSO_4.5H_2O$	0.025	
$CoCl_2.6H_2O$	0.025	
$FeSO_4.7H_2O$	27.84	STOCK C (Stock strength = x 50 i.e. 20 ml per litre of final medium)
Na_2EDTA	37.84	
$MgSO_4.7H_2O$	370	STOCK D (Stock strength = x 50)
Glycine	2	STOCK E (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Nicotinic Acid	0.5	
Pyridoxine HCl	0.5	
Thiamine HCl	0.1	

Constituent	Final conc. in mg/l
Meso-inositol	100
Sucrose	30,000
* 2,4-D	2
* Kinetin	0.5

* unless otherwise stated in the text

Stocks A, B, C and D (inorganic salts) were stored at 4°. Stock E (amino acids, vitamins) was stored at -20°. Stock D ($MgSO_4.7H_2O$) was added finally and in the largest possible quantity of water to minimise the chances of precipitation. The medium pH was adjusted to 5.6 and, unless otherwise stated in the text, the details of preparation and storage described above were applied to all media included in this Appendix. For Agar media, "Ionagar no. 2" (7 gm/litre) was added in every case.

2 Linsmaier and Skoog

Stocks A, B, C and D are identical to those used for Murashige and Skoog. The remaining constituents are:-

Constituent	Final Conc. in mg/l
Thiamine HCl	0.4
Casein Hydrolysate	1000
Meso-inositol	100
Sucrose	30,000
* IAA	2
* Kinetin	0.03

* unless otherwise stated in the text

3 White

Constituent	Final Conc. in mg/l	
$Ca(NO_3)_2.4H_2O$	300	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium)
Na_2SO_4	200	
KNO_3	80	
KCl	65	
$NaH_2PO_4.H_2O$	16.5	
$MnSO_4.4H_2O$	7	STOCK B (Stock strength = x 20)
$ZnSO_4.7H_2O$	3	
H_3BO_3	1.5	
KI	0.75	
$Fe_2(SO_4)_3$	2.5	
$MgSO_4.7H_2O$	720	STOCK C (Stock strength = x 20)
Glycine	3	STOCK D (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Nicotinic Acid	1	
Thiamine HCl	0.1	
Pyridoxine HCl	0.1	
Cysteine	1	
Ca.D-Panto-thenic acid	1	
Sucrose	20,000	
* 2,4-D	2	
* Kinetin	0.5	

* unless otherwise stated in the text

4 White with the supplement of Wood and Braun

Constituent	Final Conc. in mg/l	
KCl	845	STOCK A (stock strength = x 20 i.e. 50 ml per litre of final medium)
$(NH_4)_2SO_4$	790	
$NaNO_3$	1800	
$NaH_2PO_4.H_2O$	300	
KNO_3	80	
Meso-inositol	100	

Stocks B, C, D and all other constituents identical to those detailed for the medium of White

5 Chua

Constituent	Final Conc. in mg/l	
Na_2SO_4	800	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium
$Ca(NO_3)_2.4H_2O$	400	
KNO_3	80	
KCl	65	
$NaH_2PO_4.H_2O$	33	
$MnSO_4.4H_2O$	4.5	STOCK B (Stock strength = x 20)
$ZnSO_4.7H_2O$	6.0	
H_3BO_3	0.375	
KI	3.0	

Constituent	Final Conc. in mg/l	
$FeSO_4.7H_2O$	27.84	STOCK C (Stock Strength = x 50 i.e. 20 ml per litre of final medium
Na_2EDTA	37.84	
Mg $SO_4.7H_2O$	180	STOCK D (Stock strength = x 50
Glycine	12	STOCK E (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Cysteine	20	
Tryptophan	10	
Phenylalanine	10	
Tyrosine	10	
Leucine	20	
Thiamine HCl	2	STOCK F (Stock strength = x 100)
Pyridoxine HCl	2	
Nicotinic Acid	20	
Ca.D-Pantothenic acid	2	
Biotin	2	
Vit B. 12	2	
Ascorbic Acid	20	
Citric Acid	20	
Casein Hydrolysate	5	
Meso-inositol	100	
Coconut milk (by volume)	10%	
Sucrose	100,000	
Yeast extract	1	

Constituent	Final Conc. in mg/l
* Kinetin	1
* IAA	1
* 2,4-D	1

* Unless otherwise stated in the text

6 Modified Chua

Stocks A, B, C, D, E and F as above. The remaining constituents are:-

Constituent	Final Conc. in mg/l
Casein Hydrolysate	1000
Meso-inositol	100
Coconut milk (by volume)	10%
Sucrose	40000
Yeast extract	1
Kinetin	0.5
* IAA or 2,4-D	2.0

* unless otherwise stated in the text

7 Heller

Constituent	Final Conc. in mg/l	
KCl	750	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium)
$NaNO_3$	600	
$NaH_2PO_4.H_2O$	125	
$CaCl_2.2H_2O$	75	

Constituent	Final Conc. in mg/l	
$MgSO_4.7H_2O$	250	STOCK B (Stock strength = x 50 i.e. 20 ml per litre of final medium)
$ZnSO_4.7H_2O$	1	STOCK C (Stock strength x 20 i.e. 50 ml per litre of final medium
H_3BO_3	1	
$NiCl_2.6H_2O$	0.03	
$CuSO_4.5H_2O$	0.03	
KI	0.01	
$MnSO_4.4H_2O$	0.01	
$AlCl_3$	0.03	
$FeCl_3.6H_2O$	1	STOCK D (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Thiamine HCl	1	
Sucrose	20000	
* 2,4-D	2	
* Kinetin	0.5	

* Unless stated otherwise in the text

8 Gautheret

Constituent	Final Conc. in mg/l	
$Ca(NO_3)_2.4H_2O$	500	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium)
KH_2PO_4	125	
KNO_3	125	

Constituent	Final Conc. in mg/l	
$MnSO_4.4H_2O$	3	STOCK B (Stock strength = x 20)
H_2SO_4	1	
KI	0.5	
$Ti(SO_4)_3$	0.2	
$ZnSO_4.7H_2O$	0.18	
$BeSO_4$	0.1	
$CuSO_4.5H_2O$	0.05	
H_3BO_3	0.05	
$NiSO_4$	0.05	
$MgSO_4.7H_2O$	125	STOCK C (Stock strength = x 50 i.e. 20 ml per litre of final medium)
$FeSO_4.7H_2O$	0.05	STOCK D (Stock strength = x 50
Glycine	3	STOCK E (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Cysteine HCl	10	
Thiamine HCl	0.1	
Pyridoxine HCl	0.1	
Nicotinic Acid	0.5	
Sucrose	30,000	
* 2,4-D	2	
* Kinetin	0.5	

* Unless stated otherwise in the text

9 Miller

Constituent	Final Conc. in mg/l	
KNO_3	1000	STOCK A (Stock strength = x 20 i.e. 50 ml per litre of final medium)
NH_4NO_3	1000	
$Ca(NO_3)_2 \cdot 4H_2O$	500	
KH_2PO_4	300	
KCl	65	
$MnSO_4 \cdot 4H_2O$	14	STOCK B (Stock strength = x 20
$ZnSO_4 \cdot 7H_2O$	3.8	
H_zBO_3	1.6	
KI	0.8	
$Cu(NO_3)_2 \cdot 3H_2O$	0.35	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1	
$MgSO_4 \cdot 7H_2O$	71.5	STOCK C (Stock strength = x 20)
Na'e EDTA	13.2	STOCK D (Stock strength = x 20)
Nicontinic Acid	0.5	STOCK E (Stock strength = x 100 i.e. 10 ml per litre of final medium)
Pyridoxine HCl	0.1	
Thiamine HCl	0.1	
Sucrose	30,000	
* NAA	2	
* Kinetin	0.5	

* Unless stated otherwise in the text

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