ALKALOID PRODUCTION IN CULTURED TISSUES AND ORGANS OF *LYCOPERSICUM ESCULENTUM* MILL.

by

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GENERAL INTRODUCTION.

Much current interest is focussed on the capacities of cultured plant tissues and organs to synthesise secondary products such as alkaloids, steroids and isoprenoids. Not only are the biosynthetic pathways of many of these compounds obscure, but their significance in cell metabolism and in the life of the organism as a whole, has not yet been satisfactorily explained.

These gaps in our knowledge are, to a large extent, the result of inherent limitations of intact plant studies e.g. difficulties in labelled precursor incorporation, presence of transportation systems, lack of control of environmental factors, etc. Adoption of cell, tissue and organ culture techniques not only eliminates such problems, but also offers spectra of morphological organisation which are convenient for investigations of the relationships between biosynthetic and morphogenetic potentials.

Various workers (e.g. Routien and Nickell, 1956; Tulecke and Nickell, 1960; Klein, 1960; Tulecke, 1961; Nickell, 1962; Street et al., 1965; Furuya, 1968.) have drawn attention to the possibility of culturing plant cells on a large scale for production of important compounds. The levels of certain secondary metabolites have been increased by modifying cultural conditions but, in many cases, accompanying changes in morphological organisation also took place. Where morphological changes did not occur only relatively small increases have been observed. A system in which cultured plant cells or tissues could be manipulated so as to elaborate significant amounts of certain secondary products without giving rise to organised

structures, would not only be of academic interest but also possess much commercial potential.

A number of species of Solanum and Lycopersicum are known to synthesise the steroidal alkaloid* tomatine. Biochemical and physiological studies of tomatine using intact plants have revealed this compound to possess a number of interesting properties (see part I, section 3). However, very little is known about tomatine synthesis in vitro. Hence the aim of this research to investigate the capacities of cultured tissues and organs derived from tomato to synthesise tomatine.

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*It is commonly held that 'true' alkaloids are ultimately derived from amino-acids and that plants elaborating them usually contain a number of alkaloids biogenetically related to each other (Swan, 1967). Hegnauer (1964) has therefore proposed that steroidal alkaloids be considered as 'pseudo' alkaloids, as they are usually derived from nitrogen-free constituents into which nitrogen has been incorporated at some later stage. Furthermore, most plants synthesising steroidal alkaloids only contain one type of alkaloid rather than a range of closely related structures. He suggests that such compounds would be more accurately referred to as "basic or nitrogenous steroids". However, the term 'steroidal alkaloid' is now generally accepted by chemists and biologists and has been employed throughout this work.

PART I

REVIEW OF LITERATURE.

Because tomatine possesses properties of both an alkaloid and a steroidal glycoside, this review considers the production of both these classes of compounds by cultured plant tissues and organs.

SECTION 1. ALKALOIDS.

About 2000 alkaloids have been isolated from plants (Swan, 1967), but some have been studied more thoroughly than others. Especially well documented are those found in Solanaceous plants and notably the Nicotiana and tropane alkaloids, which are employed as insecticides and medicinal drugs respectively. The current interest which centres on the alkaloids of Catharanthus roseus and other members of the Apocynaceae, derives from reports that a number of Catharanthus alkaloids possess oncolytic (anti-tumour) activity (Johnson et al., 1963; Neuss et al., 1964).

These groups of alkaloids have been the choice of a large number of workers interested in the alkaloid biosynthetic capacities of plant tissue cultures. However, in vitro studies have also been made of various less well known alkaloids from widely differing taxonomic groups.

Nicotiana alkaloids.

Studies by Dawson (1942) have shown that cultured excised roots of Nicotiana tabacum var. Turkish synthesise nicotine. However, most of the nicotine synthesised was found to be excreted into the

culture medium. Dawson subsequently concluded that the root apex is the main site of nicotine synthesis and that the preponderence of this alkaloid in aerial parts of the plant is due to translocation. Extracts of cultured roots from other Nicotiana species have been found to contain not only nicotine but also nornicotine and anabasine (Schröter and Engelbrecht, 1957). Investigations of the relationship between nicotine production and growth of cultured tobacco (var. Turkish) roots have shown that alkaloid synthesis is associated with dry weight and elongation of the root (Solt, 1957). Suppression of elongation by indole-3-acetic acid (IAA) resulted in a decrease in the rate of nicotine synthesis. The finding that nicotine levels increased only in root tips and not in mature root tissues, was considered further evidence that nicotine biosynthesis is unique to actively growing cells. Analyses of cultured roots of Nicotiana glauca have revealed the presence of nicotine and anabasine (Solt et al., 1960). The association between nicotine production and root growth was confirmed, but synthesis of anabasine, which was present in larger amounts than nicotine, was found to be confined to mature root tissues and was not related to root growth.

Nicotine has been detected in Nicotiana glutinosa stem callus (Dawson, 1960). Alkaloid levels were highest during the first passage but decreased with time, and in established callus no nicotine could be detected. Benveniste et al. (1966) were also unable to detect nicotine in tobacco (var. P-19) stem callus and Krikorian and Steward (1969) observed an alkaloid- positive reaction in only one of a

number of callus cultures derived from different Nicotiana species. On the other hand, nicotine has been detected in callus tissues derived from root, stem and leaf of N.tabacum var. Virginica after 23 passages (Speake et al., 1964). Nicotine and anatabine have been reported present in tobacco (var. Bright Yellow) stem callus (Furuya et al., 1966), and nicotine in cell cultures initiated from Nicotiana rustica embryos (Krikorian and Steward, 1969).

The predominance of nicotine synthesis in the root has not yet been explained, but there is some evidence that production of this alkaloid is influenced by the degree of morphological organisation. Cultured excised tobacco roots exhibit higher nicotine levels than callus or cell cultures of root origin (Speake et al., 1964). Furuya (1968) reports that other Japanese workers have found higher concentrations of nicotine in tobacco stem callus bearing root and leaf structures than in undifferentiated callus. The greater ability of cell cultures of embryo origin than of pith origin to elaborate nicotine has been attributed to their greater potential totipotency (Krikorian and Steward, 1969).

The biosynthesis of Nicotiana alkaloids has not been studied in detail using cultured tissues and organs but some precursor work has been done. Ornithine and lysine labelled with ^{14}C are reported to be incorporated into nicotine and anabasine respectively when 'fed' to cultured roots of N.glauca (Solt et al.,1960). These authors were unable to increase alkaloid yields or alter the relative amounts of each alkaloid by adding precursors such as nicotinic acid, either

alone or in combination with ornithine or lysine. However, Furuya (1968) has reported increases of more than 100 per cent in nicotine levels in tobacco callus grown on a medium supplemented with nicotinic acid.

Relatively large amounts of nicotine have been detected in stem callus derived from N.tabacum var. Bright Yellow grown on a medium containing IAA, but no alkaloid could be found when the callus was grown on a similar medium but with 2,4-dichlorophenoxyacetic acid (2,4-D) as auxin (Furuya et al., 1967). However, when alkaloid-free callus, previously grown on a 2,4-D medium, was transferred to one containing IAA, nicotine could be detected (Furuya, 1968).

Catharanthus alkaloids.

Extracts of seeds and seed callus of C.roseus have been found to show similar patterns of alkaloids on thin-layer chromatography plates, but impurities in the extracts hindered identification (Babcock and Carew, 1962). Tissue from C.roseus has been grown in suspension culture and qualitative analyses of cells and 'spent' culture medium have revealed alkaloids to be present in both, although none were identified (Carew, 1966). Harris et al. (1964) have tentatively identified vindoline in C.roseus callus tissue and this finding has been confirmed by Richter et al. (1965), who also detected vindolinine in stem and leaf callus of C.roseus. Neither alkaloid was found to be present in root callus. However, Langkamp et al. (1966), although unable to detect vindoline in root and stem callus

of C.roseus, did find ajmalicine, vindolinine and other unidentified alkaloids. Both C.roseus crown-gall cells grown in suspension culture and the 'spent' culture medium have been found to contain small amounts of alkaloids (Boder et al.,1964). The principal alkaloid present was identified as vindoline and a second constituent thought to be vincoline. These authors also found that the suspension cultures could effect transformation of added alkaloids. Addition of vindoline-HCl to the culture medium resulted in the appearance of desacetyl-vindoline and other oxidation products of this alkaloid. Krikorian and Steward (1965) have grown callus and suspension cultures from different organs of a number of C.roseus cultivars, and have detected ceric ammonium sulphate-positive spots in extracts of a number of these cultures. Four of these spots proved to be alkaloids and one was identified as ajmalicine, a major alkaloid in the plant stem. None resembled those with oncolytic activity. When the culture medium auxin, 2,4-D, was substituted by IAA changes in the alkaloid complement of the cultured cells were observed.

Cultures have been initiated from other members of the Apocynaceae and some of these analysed for alkaloids. Mitra and Kaul (1964) have reported finding traces of the indole alkaloid reserpine in root callus of Rauvolfia serpentina. Three types of callus were used which differed in visual appearance, friability, degree of cell differentiation and growth rate. It was stated that these three types of callus differed in their ability to elaborate

reserpine but no details were given. Reserpine has also been detected in seed callus of Alstonia constricta (Harris et al., 1964; Carew, 1965). The use by the latter author of two year old callus suggests de novo alkaloid synthesis rather than 'carry-over' from the original explant.

Tropane alkaloids.

Atropine has been detected in cultured excised roots of Atropa belladonna (Reinouts van Haga, 1957; West and Mika, 1957). The former author reported that alkaloid levels were significantly increased when the precursors arginine or ornithine were present in the culture medium. The latter workers also found atropine in Belladonna root callus but could not detect it in stem or leaf callus. Furthermore, the amount of alkaloid in root callus was slightly higher than that in cultured roots.

In contrast, Raj Bhandary et al. (1969), working with A.belladonna and A.belladonna cultivar lutea, were unable to detect atropine in root callus. Suspension cultures derived from seed, leaf and stem callus were also devoid of alkaloid. However, a number of alkaloids viz. atropine, hyoscine, tropine and cuscohygrine were found in cultured roots derived from seedlings and alkaloid-free seed callus. These authors have suggested that synthesis of Belladonna alkaloids is associated with processes peculiar to the normally organised root, and that the presence of atropine in the root callus of West and Mika could have been due to use of a culture medium conducive to root development.

Although devoid of tropane alkaloids, cells of A.belladonna grown in liquid suspension have been reported capable of absorbing and accumulating exogenously applied alkaloids such as atropine, hyoscyamine, hyoscine and scopoline (Raj Bhandary et al.,1969). There was no evidence of alkaloid degradation, and growth was unaffected by alkaloid concentrations as high as 250 mg l^{-1}. Absorption and accumulation of atropine has also been observed in cultured cells of Datura tatula and Datura metel (Elze and Teuscher, 1967).

Tropane alkaloids have been detected in cultured excised roots of Hyoscyamus niger (Telle and Gautheret, 1947) and Datura stramonium (Stienstra, 1954). In each case the authors concluded that cultured roots contained less alkaloid than intact roots. Hyoscyamine and hyoscine have been found in cultured roots of D.stramonium var. tatula, but levels of these alkaloids decreased when glutamic acid was present in the culture medium at concentrations greater than 0.2 per cent (French and Gibson, 1957). Working with cultured roots of the same variety, Gibson and Abbott (1963) found that more hyoscine than hyoscyamine was synthesised when proline was added to the culture medium.

Work by Staba and Jindra (1968) has drawn attention to the importance of certain ionic constituents of the culture medium in alkaloid biosynthesis. Suspension cultures derived from seed callus of D.stramonium, which normally produced only choline, elaborated,

in addition, cuscohygrine and pseudotropine when manganese levels were increased. In contrast to the findings of Chan and Staba (1965) hyoscine was not detected, and alkaloid concentrations were unaffected by ornithine or phenylalanine in the culture medium.

Krikorian and Steward (1969) have analysed cultured tissues derived from various species of Atropa, Hyoscyamus and Datura. Weak Dragendorff-positive spots were observed, but there was no evidence of accumulation of alkaloids such as atropine or hyoscine in either the cells or culture medium.

Callus tissue derived from rhizomes of Scopolia japonica has been analysed for tropane alkaloids (Konosima et al., 1967). Intact rhizomes contained hyoscine and atropine, but callus tissue elaborated only hyoscine and three unidentified Dragendorff-positive compounds which were not present in the intact organ. The alkaloid content of callus tissue was found to vary according to the auxin/kinetin ratio, with highest levels at 0.22 ppm 2,4-D and 2.0 ppm kinetin. Addition of yeast extract, casein hydrolysate or peptone to the culture medium resulted in increases in concentrations of callus alkaloids although callus growth was increased, unaffected and decreased respectively.

'Miscellaneous' alkaloids.

Callus tissues derived from petiole, leaf and stem of Macleaya cordata (Papaveraceae) have been found to synthesise protopine, sanguinarine and allocryptine (Neumann and Müller, 1967). Light-

microscopic studies revealed that alkaloid synthesis occurred only in certain cells which were anatomically indistinguishable from other callus cells. Examination of these cells by electron microscopy and micro-autoradiography further revealed the alkaloids to be localised in the vacuole as a thin layer bordering the tonoplast.

Furuya (1968) has also isolated sanguinarine, but from callus tissue derived from the opium poppy (Papaver somniferum). Cultured stem tissue from this species has been used by Grützmann and Schröter (1966) to study thebaine transformation. Cells grown in liquid suspension were unable to synthesise thebaine but when grown in its presence morphine and codeine could be detected.

Carew and Schwarting (1958) have grown rye embryo callus in association with the fungus Claviceps purpurea but were unable to detect ergot alkaloids. However, Chang and Carew (1968) later established that alkaloids could be produced by this host-parasite combination. The alkaloids agroclavine and elymoclavine were identified and a third compound was tentatively identified as chanoclavine. These alkaloids were synthesised regardless of whether the host tissue was in the form of callus or cell suspension. The authors attributed their success, and the lack of it experienced by earlier workers, to the greater amount of information on strain selection, growth, nutrition and alkaloid biosynthesis by Claviceps spp. which is now available.

The steroidal alkaloid tomatine has been isolated from cultured excised roots of Lycopersicum esculentum (Sander, 1956) and Lycopersicum

pimpinellifolium (Bruske, 1966). Neither author detected the alkaloid in the culture medium. The latter author reported that both growth and tomatine levels were higher in roots cultured in an agitated nutrient medium than in a 'static' one. Alkaloid concentrations in agitated roots increased initially by a factor of almost three and then decreased to their original levels. Similar changes in alkaloid concentration also occurred in roots grown in 'static' culture. Another steroidal alkaloid, solasonine, has been identified in extracts of stem callus from Solanum xanthocarpum (Heble et al., 1968a).

Excised embryos and cotyledons of Ricinus communis (Euphorbiaceae) have been grown under sterile conditions to study ricinine biosynthesis (Waller and Nakazawa, 1963). Labelled (^{14}C) nicotinic acid was incorporated into the alkaloid in both sterile cultures and the intact plant but, whereas addition of this precursor resulted in increases in ricinine in cultured organs, it had no effect on alkaloid levels in the intact plant. Ricinine was degraded by cultured cotyledons but this loss of alkaloid could be offset by the presence of nicotinic acid in the culture medium.

A further study of ricinine biosynthesis has been carried out by Hadwiger and Waller (1964) using cultured excised roots of R.communis. Ricinine was synthesised by cultured roots and at a rate which was correlated with that of root growth. However, the finding that alkaloid concentration in the culture medium increased while that in the root decreased, suggested that excretion of the alkaloid was taking place.

Although exogenously applied nicotinic acid was incorporated into ricinine, alkaloid levels did not increase as had been observed with cultured embryos and cotyledons.

Callus tissue has been obtained from stem explants of Peganum harmala (Zygophyllaceae) and analysed for alkaloids (Reinhard et al., 1968). Whereas the aerial parts of the intact plant elaborate vasicine and harmine (the former being the principal alkaloid), only the latter alkaloid was detected in callus. Nevertheless, the concentration of harmine in the callus was found to be almost six times that in the intact plant.

A study of the alkaloid-synthesising ability of callus tissue derived from certain members of the Convolvulaceae, which elaborate hallucinogenic indole alkaloids, has been carried out by Staba and Laursen (1966). Cultures were initiated from seed of Rivea corymbosa and from three varieties of Ipomoea violacea. Trace amounts of alkaloids were reported to be present in the callus tissues and culture media of two of the Morning Glory varieties and also in Rivea callus.

Thin-layer chromatographic analyses of eight-week old cultured excised roots of Delphinium elatum (Ranunculaceae) have revealed a number of alkaloid spots, the largest of which was identified as methyllycaconitine(Strzelecka, 1966). This alkaloid was also found in roots and green parts of eight-week old aseptically-grown plants.

Callus cultures of Conium maculatum (Umbelliferae) grown either on solid or in liquid medium have been found to synthesise α-coniceine

and N-methylconiine, as well as other unidentified alkaloids (Mahrenholz and Carew, 1966).

Callus tissue obtained from seeds of Hippeastrum vittatum (Amaryllidaceae) has been reported to be devoid of the alkaloids hippeastrine and lycorine which are found in the seed (Suhadolnik, 1964). The one unidentified alkaloid which was detected in callus tissue did not correspond to any in the seed.

Steinhart (1962) has claimed that the enzymes involved in methylation reactions leading to candicine synthesis are retained in stem callus of the cactus Trichocereus spachianus. However, the ability of such callus to elaborate this alkaloid has not been conclusively established.

SECTION 2. STEROIDAL GLYCOSIDES.

The two most important (and best documented) types of steroidal glycoside are the cardiac glycosides and saponins. The former play an important role in drug therapy and the latter are widely used for synthesis of steroidal hormones. Consequently, the majority of plant tissue culture studies of steroidal glycosides have been concerned with compounds from these two groups.

Cardiac glycosides.

Callus and suspension cultures from germinated seeds of Digitalis lanata and Digitalis purpurea have been examined for cardiac glycosides

(Büchner and Staba, 1964). Kedde-positive substances were detected in all cultures but none were identified. The authors, nevertheless, concluded that the cultures synthesised cardenolides (C-23 cardiac glycosides) different from those of the intact plant.

The abilities of seed callus of D.purpurea, D.lanata and Digitalis mertonensis and root cultures of D.lanata to transform digitoxin, digitoxigenin and other related compounds have been investigated by Stohs and Staba (1965). No transformation products could be detected after two days incubation, but Kedde-positive compounds appeared in D.lanata callus and cultured roots after seven days, and in D.purpurea callus after 16 days. However, the only information given on the nature of these transformation products was that extra keto groups were thought to be present in digitoxigenin. Graves and Smith (1967) have also analysed callus and suspension cells of Digitalis origin but have failed to detect cardiac glycosides. Nor did addition to the culture medium of pregn-5-en-3β-ol-20-one or progesterone, compounds which they showed could be metabolised and transformed by cell cultures from a number of dicot species, stimulate synthesis of cardenolides. Sterols in various forms (mixture, chemically modified, pure) have been added to callus cultures of D.mertonensis derived from germinated seed and the subsequent pattern of Baljet-positive compounds examined in both cells and culture medium (Medora et al., 1967). In some cases there was an increase in the number of Baljet-positive substances but the fact that none of these compounds were Kedde-positive casts doubt on their being cardiac glycosides.

Saponins.

The steroidal sapogenin, hecogenin, which is found in leaves of Agave spp., has not been detected in leaf callus of Agave toumeyana (Weinstein et al., 1959).

Diosgenin has been isolated from stem callus of Solanum xanthocarpum and chemically characterised (Heble et al., 1968b). The amount of diosgenin in the callus was found to be about eight times greater than that in berries. It is probably of significance that β-sitosterol levels were also higher in callus than in berries. Synthesis of diosgenin has been studied in root callus and suspension cultures from Dioscorea deltoidea using both undifferentiated callus and callus bearing organised structures (Kaul and Staba, 1968). Diosgenin was found to be present in all cultures but, whereas undifferentiated tissue produced significant amounts (1 per cent), 'organised' cultures contained only trace quantities. This finding is of interest since it is the opposite of the situation found in other tissue culture studies of secondary products. Further studies of diosgenin biosynthesis in undifferentiated and 'organised' suspension cultures derived from roots of D.deltoidea have been carried out using radioactive precursors (Stohs et al., 1969). When ^{14}C-labelled cholesterol was added to undifferentiated cultures a relatively high proportion of the label was incorporated into diosgenin. In 'organised' cultures there was little or no incorporation into the sapogenin. Centrifugation of cell homogenates revealed that most of the radioactivity was localised in the

microsomal, mitochondrial and cytoplasmic fractions, with relatively low amounts in the cell wall and nuclear fractions. The authors suggest that the low incorporation into diosgenin in 'organised' cultures may be due to the loss or reduction in numbers of mitochondria which accompany cell differentiation.

Tomita et al. (1970) have found three steroidal sapogenins in one-year old seedling callus of Dioscorea tokoro. These were diosgenin, yonogenin and tokorogenin, the last being the most abundant. These compounds were also found in seeds and seedlings. The ratio of amounts of sapogenins in seeds and callus were similar but seedlings exhibited a different pattern due to another sapogenin, isodiotigenin, being the most abundant. A fifth sapogenin, kogagenin, which was present in trace amounts in seeds and seedlings, was absent in callus tissue.

SECTION 3. TOMATINE.

The following review of tomatine has been included for two reasons. Firstly, to illustrate the properties which render this alkaloid of interest, and secondly, because no comprehensive review of tomatine has yet been compiled.

Discovery.

From expressed juice of tomato plants (Lycopersicum esculentum var. Pan America) Irving et al. (1945) obtained a preparation which, even in impure form, inhibited growth of Fusarium oxysporum f. lycopersici

The active principle, which they found to be present throughout the plant, was designated 'lycopersicin'. However, 'lycopersicin' was later altered (Irving et al., 1946) to 'tomatin' as the former term had already been employed as a synonym of lycopene (Duggar, 1913). Leaves of Lycopersicum pimpinellifolium were found to be a more abundant source of tomatin (Fontaine et al.,1947) and from this material Fontaine et al. (1948) first isolated and crystallised the antifungal principle. The purified inhibitor was called'tomatine' to distinguish it from the crude or partially purified tomatin.

Structure.

The first attempts to chemically characterise tomatine were made by Fontaine et al. (1948) who suggested it to be a "glycosidal alkaloid" consisting of an aglycone fraction, tomatidine, and a tetrasaccharide moiety.

An empirical formula for tomatidine of $C_{27}H_{43}O_2N$ was suggested by Kuhn et al. (1950) but this was not confirmed by later workers (Fontaine et al., 1951; Sato et al., 1951) who calculated the empirical formulae of tomatidine and tomatine to be $C_{27}H_{45}NO_2$ and $C_{50}H_{83}NO_{21}$ respectively. Infra-red spectroscopy and other studies suggested that tomatidine was a steroidal secondary amine (Fontaine et al., 1951). The steroid nature of the aglycone was confirmed by degradation to allopregnenolone (Sato et al., 1951). Nuclear magnetic resonance spectroscopy (Toldy and Radics, 1966) and X-ray diffraction

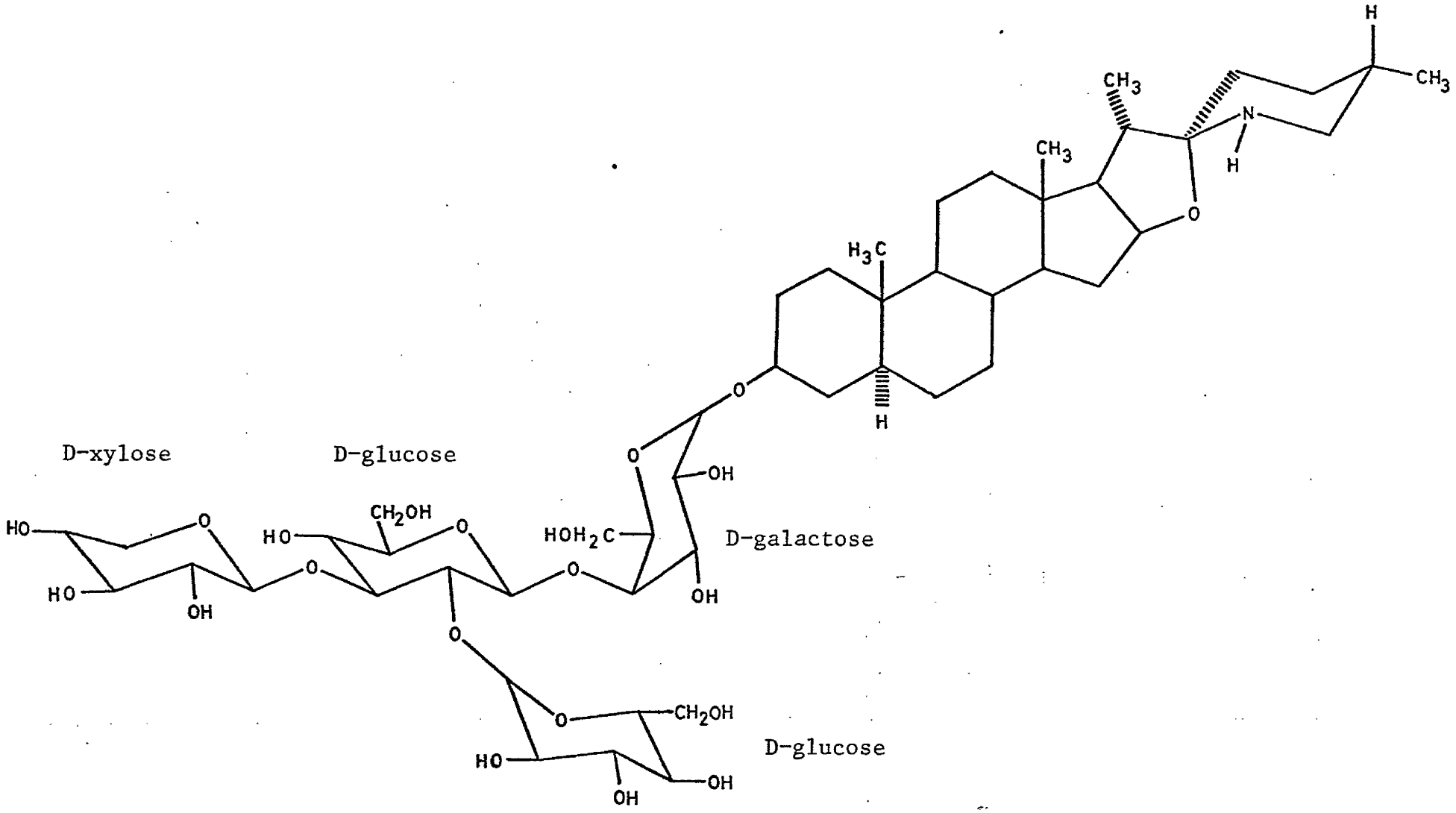

Fig.1. Structure of α-tomatine

(Höhne et al., 1967) have been employed to verify the steric configuration of tomatidine which, according to Schreiber (1968) is (25S)-5α, 22βN-spirosolan-3β-ol (fig.1).

Analyses of the sugars released on acid hydrolysis of tomatine revealed the tetrasaccharide to consist of two molecules of glucose and one each of galactose and xylose (Ma and Fontaine, 1950). The tetrasaccharide moiety of tomatine (or β-lycotetraose as it is generally called) has since been accurately characterised as O-β-D-glucopyranosyl-(1→2 glu)-O-β-D-xylopyranosyl-(1→3 glu)-O-β-D-glucopyranosyl-(1→4 gal)-β-D-galactopyranose (Schreiber, 1968), (fig. 1).

Modified forms of tomatine differing in their sugar moieties are known to exist naturally. It has been suggested that the form whose sugar moiety is the tetrasaccharide be called α-tomatine, and that those forms lacking xylose, lacking one glucose and lacking xylose and one glucose be called β_1-, β_2- and γ-tomatine respectively (Kuhn et al., 1957). According to this nomenclature, β_2-tomatine has been found in tomato plants infected with Septoria lycopersici (Arneson and Durbin, 1967), and β_1-tomatine in certain varieties and mutants of L.esculentum and L.pimpinellifolium (Schreiber et al., 1961).

Occurrence of α-tomatine (and modified forms) in Solanaceous plants.

Genus Solanum

S.acaule BITT.

S.boerhaavii THELL.

S.demissum LINDL.

S.depexum JUZ.

S.polyadenium GREENM*.

S.punae JUZ.

S.rantonnetii CARR

S.schreiteri BUK.

S.simplicifolium BITT.

S.stoloniferum SCHLECHT. et BOUCHÉ

*also contains polyanine (tomatidine + 2 xylose + glucose).

Genus Lycopersicum

L.cheesmannii RILEY

L.chilense DUN.

L.esculentum MILL.

L.esculentum MILL.
var. esculentum MILL.*

L.esculentum MILL.
mutant*

L.esculentum MILL.
mut. exilis**

L.esculentum MILL.
mut.prunoidea**

L.glandulosum MILL.

L.hirsutum HUMB. et BONPL.

L.peruvianum (L.) MILL.

L.pimpinellifolium (JUSL.) MILL.*

L.pimpinellifolium (JUSL.) MILL.
var. ribesiodes (A.VOSS) LEHM.

L.pimpinellifolium (JUSL.) MILL.
mutant*

* also contains β_1-tomatine.

** contains β_1-tomatine only.

Distribution in the plant.

Tomatine is not present in dormant seeds but first appears during the early stages of germination, especially in the tip of the young radicle (Sander et al., 1961). As the plant develops, tomatine appears in other organs (Sander, 1956). Leaves and buds of

L.esculentum show high tomatine concentrations, but highest levels are found in fully expanded flowers (Tukalo 1958). The shoot is recognised as being the main site of tomatine accumulation but the alkaloid is also found in the root although it does not accumulate to the same degree. The presence of tomatine in cultured excised roots and its absence from exuded sap from decapitated plants indicates that de novo tomatine synthesis occurs in both root and shoot (Sander, 1956). In this respect tomatine differs from alkaloids such as nicotine and hyoscyamine both of which are synthesised principally in the roots of their respective species but accumulate in the shoot as a result of transportation.

After pollination the ovaries of tomato flowers are particularly rich in tomatine and may contain as much as 1.5 per cent compared with 0.15 per cent in the rest of the plant (Heftmann, 1965). Young green tomato fruits are also rich in tomatine but as fruit development proceeds alkaloid levels decrease. By injecting tomatine into isolated tomato fruits, Sander (1956) demonstrated that disappearance of tomatine during fruit set is due to degradation. Later studies by Sander and Angermann (1961) revealed that degradation of tomatine begins at the aglycone with separation of the nitrogen-containing group preceding hydrolytic changes in the carbohydrate moiety. Further, from studies of tomatine degradation in ripening fruits of a number of Lycopersicum species which exhibit different degrees of reddening, Sander (1958a) suggested that the isoprenoid nucleus of tomatine might be utilised in

lycopene synthesis. This could account for the discrepancy between reports that ripe tomato fruits are tomatine-free (Heftmann, 1965), and that small amounts of alkaloid are present (Kajderowicz-Jarosinska 1965). Plants from which fruits are removed tend to accumulate tomatine, apparently due to loss of organs of alkaloid breakdown. In keeping with this finding, tomatine levels in long-day vegetative plants of Lycopersicum glandulosum were found to be almost five times as high as those in short-day flowering plants (Sander, 1958b).

The enzyme(s) responsible for tomatine degradation in ripening fruits has (have) not been isolated, but an enzyme has been detected in tomato leaves which hydrolyses tomatine (Prokoshev et al.,1956). This enzyme, called tomatinase, exhibits a certain degree of substrate specificity, as it does not hydrolyse other closely related steroidal alkaloids such as solanine.

Neither the distribution nor the site of synthesis of tomatine in the cell is known with certainty.

Biosynthesis.

Studies of the biosynthesis of tomatine have concentrated almost entirely on the aglycone moiety, and there exists almost no information on synthesis of the tetrasaccharide moiety or the mechanisms which lead to condensation of the two moieties.

The first tracer studies of the biogenesis of tomatidine were made by Sander and Grisebach (1958) who obtained labelled tomatine

from seedlings of L.pimpinellifolium grown in the presence of acetate -1-^{14}C. Solasodine, a steroidal alkaloid very similar to tomatidine, has been isolated, radio-isotopically labelled, from plants of Solanum laciniatum ('S.aviculare') which had been grown in the presence of ^{14}C-labelled acetate or mevalonate (Guseva and Paseshnichenko,1962). Degradation of the extracted solasodine revealed a distribution of the label which was consistent with the aglycone having been synthesised via cyclisation of squalene.

The stages following cyclisation are not completely understood but various authors (Ehrhardt et al., 1965; Benveniste et al., 1964 ; Aexel et al., 1967) have drawn attention to the widespread distribution in the plant kingdom of the pentacyclic triterpene cycloartenol, and the general absence of lanosterol. It has been suggested that the former may be the most important intermediate in steroid biosynthesis in higher plants.

Application of ^{14}C-labelled cholesterol to leaf axils of L.esculentum has resulted in incorporation of the label into tomatidine (Tschesche and Hulpke, 1966). Labelling of the aglycone was also achieved by Heftmann et al. (1967) when cholesterol-4-^{14}C was applied to young leaves, flower buds and flowers of L.pimpinellifolium. These reports suggest that cholesterol, or a closely related phytosterol, might occupy an intermediate position between the first product of cyclisation and the completed aglycone.

Plants which elaborate steroidal alkaloids frequently contain steroidal sapogenins (Schreiber, 1968). The structural similarities

between these compounds render it probable that their biosyntheses and metabolism are closely related. Nevertheless the constancy of sapogenin levels in developing fruits of Solanum dulcamara suggests that there is no direct conversion from alkaloid to sapogenin (Sander, 1963). The sapogenins present in L.esculentum are neotigogenin (Sander, 1961) and tigogenin (Fayez and Saleh, 1967). Tschesche (1955) has suggested that both steroidal sapogenins and steroidal alkaloids are formed by further cyclisation of the common intermediate, 16-dihydrokryptogenin, although, in the case of alkaloid biosynthesis, nitrogen is believed to be introduced by transamination prior to the final cyclisation (Schreiber, 1968). However, the origin of the nitrogen and the form in which it is incorporated into the steroid framework still await elucidation. Structurally, 16-dihydrokryptogenin constitutes a feasible intermediate between phytosterols and steroidal sapogenins or alkaloids.

The final stages in tomatidine biosynthesis as suggested by the above information are shown in fig.2.

Biological activity.

Studies of the biological activity of tomatine were initiated by the finding that a crude extract of tomatine inhibited the growth of F.oxysporum f. lycopersici (Irving et al., 1945). Further work by these authors (Irving et al., 1946) showed that impure tomatine also inhibited growth of a number of bacteria and plant- and animal-

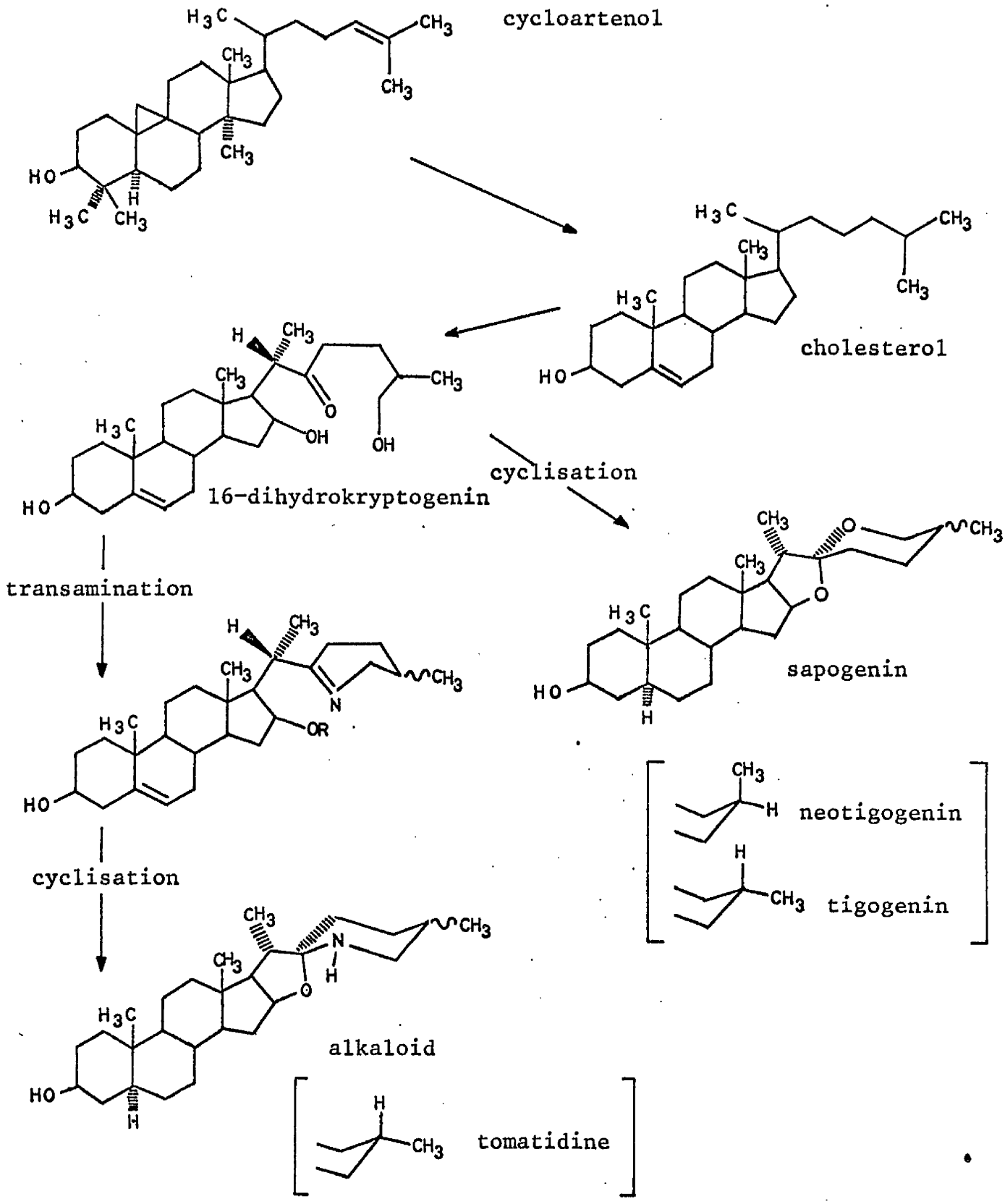

Fig.2. Possible route for the final stages of biosyntheis of steroidal alkaloids and sapogenins.

pathogenic fungi, although no inhibition of Escherichia coli or Pencillium notatum was observed. However, it was later reported (Fontaine et al., 1947) that low concentrations of partially purified tomatine did inhibit E.coli and P.notatum but, inexplicably, higher concentrations did not. Partially purified alkaloid also proved highly effective in vitro against certain human dermatophytic fungi and various yeast-like forms causing internal disease; slightly effective against gram-positive and gram-negative bacteria, certain fungi and plant-pathogenic Actinomyces; but without effect on human-pathogenic Actinomyces. First tests of the antibiotic spectrum of purified tomatine indicated that its antibacterial activity was not as great as that of the partially purified form and that E.coli was unaffected by concentrations as high as 1 mg cm^{-3} (Fontaine et al., 1948). However, it was still capable of inhibiting plant- and animal-pathogenic fungi, although it proved more effective against fungi associated with human disease than against F.oxysporum f. lycopersici.

Despite the variable reports of the effect of tomatine on E.coli, Sicho and Mrhova (1961) have presented evidence that tomatine inhibits oxygen metabolism in this bacterium under aerobic conditions. Inhibition was found to be greater with lactic acid as carbon source than with glucose, suggesting that the alkaloid might be inhibiting the enzyme malic dehydrogenase.

Other workers studying the effect of tomatine on micro-organisms (Wolters, 1966, 1968; Sackmann et al., 1959; McKee, 1961; Arneson and Durbin, 1968a) have confirmed its non-specific antibiotic activity.

Compared with other antibiotics, inhibition by tomatine tended to be weak and often varied with such factors as the strain of micro-organism used and the nature, both chemical and physical, of the culture medium.

The only investigation of the effect of tomatine on plant material is that conducted by Vendrig (1964) who reported that tomatine exhibits growth regulating activity in the Avena coleoptile test, although no details of this activity were given. However, a certain amount of doubt has been cast on the validity of this report by the inability of Borris et al. (1965) to confirm the growth regulating properties attributed to digitonin in the same paper.

The effect of tomatine on the feeding habits and mortality rates of the Colorado beetle (Leptinotarsa decemlineata) and its larvae has been examined by infiltrating leaves of Solanum tuberosum with the alkaloid (Kuhn and Löw, 1961). Tomatine concentrations of 3 mM kg^{-1} of leaves were sufficient to cause 100 per cent larval mortality. However, tomatine has no repellent effect on two other potato beetles — Epilachna sparsa and Epilachna niponica (Buhr, 1961). Working with nymphs of the Potato Leafhopper (Empoasca fabae), Dahlman and Hibbs (1967) found that, of various steroidal alkaloids tested, tomatine elicited the most sharply defined reactions. Imbibition was completely restricted by 10^{-2}M tomatine and with increasing tomatine concentrations nymphal mortality also increased.

Preliminary toxicity tests of partially purified tomatine were carried out on guinea pigs by Fontaine et al. (1947). Administration of 10 mg of tomatin preparation by various methods (subcutaneous,

intravenous and intraperitoneal), in almost all cases, elicited undesirable responses. However, the authors pointed out that some of these effects might have been due to impurities in the preparation.

A comprehensive study of the pharmacological and toxicological properties of tomatine has been made by Wilson et al. (1961). A single oral dose of 1g kg^{-1} body weight proved fatal to rats and the LD_{50} for mice, to which tomatine was administered intravenously, was about 18mg kg^{-1} body weight. Subcutaneous introduction of sub-lethal doses of tomatine usually resulted in abscess formation, but intravenous administration caused a temporary drop in blood pressure and fluctuations in respiratory rate. Large intravenous administrations of alkaloid caused haemolysis in certain organs. The minimum amount of tomatine to induce lysis in 100 cm^3 of diluted blood was 0.15 mg. Tomatine caused conjunctivitis when applied as a 5 per cent ointment to the eye but did not produce irritation when applied to the skin.

Tomatine has been reported to destroy serum cholinesterase (Pokrovskii, 1956; Orgell, 1963)but Wilson et al. (1961) state that this enzyme-destroying ability in no way accounts for its observed pharmacological activity.

Extracts of tomato crown-gall tumours have been found to exhibit anti-histaminic activity in isolated guinea pig ileum (Kovacs et al., 1964; Calam and Callow, 1964). Analyses of the extracts revealed the active principle to be tomatine and, in fact, intraperitoneal injection of tomatine protected experimental animals from the lethal effects of a histamine aerosol.

Tomatine can also prove toxic to larger domestic mammals. Acute illness and death has been observed in pigs which had eaten the alkaloid-rich green parts of tomato plants (Forsyth, 1968).

Role in phytopathology.

The in vitro toxicity of tomatine to a number of bacteria, fungi and insects has resulted in further studies to decide if tomatine is implicated in resistance mechanisms of the plant to certain pathogens and predators.

In view of tomatine inhibition of F.oxysporum f. lycopersici it was suggested that this alkaloid might contribute to the resistance of certain tomato varieties to Fusarium wilt (Irving, 1947). The author claimed that resistance depended, not on the absolute levels of alkaloid, but on the rate at which it was synthesised. The lack of correlation between tomatine levels and degree of wilt resistance in a number of tomato varieties was later confirmed by Kern (1952). McKee (1961) is of the opinion that, although steroidal alkaloids may prove toxic to a particular pathogen in vitro, they do not play an active part in resistance to infection in vivo. The amounts of tomatine in roots and stems of tomato plants have been reported to be too low to effectively inhibit F.oxysporum f. lycopersici (Kern, 1952), but other workers (Arneson and Durbin, 1967) have suggested that, since tomato leaves are richer in tomatine than roots or stems (Sander, 1956), the alkaloid may only be effective against leaf-infecting fungi.

Assuming uniform distribution, the concentration of tomatine in leaves has been estimated to be 10^{-3}M (Heftmann, 1967). However, Arneson and Durbin (1968a) have pointed out that localisation of tomatine within certain cells or tissues could result in sufficiently high concentrations to inhibit fungal growth.

Septoria lycopersici, a leaf-infecting fungal parasite of tomato, is capable of detoxifying tomatine in vivo by enzymatically hydrolysing one glucose unit from the molecule to yield β_2-tomatine (Arneson and Durbin, 1967). As a result of this finding, further investigations have been made of the effect of tomatine on other tomato fungal pathogens, on other plant fungal pathogens and on common saprophytic fungi (Arneson and Durbin, 1968a). The tomato pathogens proved to be least sensitive to tomatine, but it was not known whether this was due to detoxification of the alkaloid or whether other mechanisms were involved.

Various authors (Schreiber, 1957; Fraenkel, 1959) hold that the toxic nature of steroidal alkaloids renders the plant unattractive to various insect predators. Schreiber (1957) has reported that Colorado beetles and their larvae die of alkaloid poisoning soon after devouring foliage of Solanum auriculatum, but Stürckow (1959) believes that alkaloids such as tomatine act as repellents rather than as toxins. Whereas tomatine is toxic to the Colorado beetle (Kuhn and Löw, 1961), the potato alkaloids, solanine and chaconine,

are apparently without effect on this insect (Fraenkel, 1959). Tomatine is also more toxic to the potato leafhopper than is solanine (Dahlman and Hibbs, 1967). In view of the well-established insect-attractant properties of various isoprenoids, it has been questioned whether it is any less likely that certain secondary products exist solely to repel insect predators (Fraenkel, 1959).

Mode of toxic action.

The toxicity of tomatine to a wide range of organisms is well documented, but the actual mechanism of toxicity is not yet understood. The antibiotic properties of tomatine have been attributed to the tomatidine moiety (Fontaine et al., 1948) and, in fact, the aglycone has been found to be more toxic than the glycoside to certain fungi (Wolters, 1964) and to mice, when administered intravenously (Wilson, et al., 1961). Conversely, tomatidine, unlike tomatine, does not affect imbibition by, or survival of the potato leafhopper (Dahlman and Hibbs, 1967). Tomatidine has also been reported to be less inhibitory than tomatine to three test fungi (Arneson and Durbin, 1968b). The detoxification of tomatine by Septoria lycopersici has stimulated further research into the contribution of the sugar moiety to the toxicity of the glycoside.

Due to a hydrophobic steroid moiety at one end of the molecule and a hydrophilic sugar moiety at the other, tomatine possesses the surfactant properties characteristic of a saponin. It has been suggested that such properties account for the fungitoxicity of the

alkaloid (McKee, 1959). However, although the less toxic hydrolysis products of tomatine possess less surface activity, it is thought that the toxicity of tomatine is greater than can be accounted for by its surfactant properties alone (Arneson and Durbin, 1968b).

Steroids having a free 3β-hydroxyl group are known to form a stable 1:1 complex with tomatine (Schulz and Sander, 1957), and Schreiber (1957) has proposed that the toxicity of tomatine is due to its complexing with membrane sterols and altering or destroying membrane permeability. A certain amount of evidence exists which supports this hypothesis. Large oral doses of tomatine induce haemolysis in laboratory rodents (Wilson et al., 1961) and leakage of anthocyanin occurs in beetroot discs incubated in tomatine solutions (unpublished observation by this author). Species of Pythium and Phytophthora which are insensitive to the sterol-complexing antibiotic Nystatin (Fowlks et al., 1967) are also unaffected by tomatine (Arneson 1967). Tomatidine, β_1-tomatine, β_2-tomatine and the protonated form of tomatine which exists at low pH do not form complexes with sterols—observations which are consistent with their much reduced toxicities (Arneson and Durbin, 1968b). The fact that these compounds, although unable to complex with sterols, are slightly toxic in varying degrees to different organisms suggests there may be more than one mechanism of toxic action.

Applications

As a result of its fungistatic properties, it was suggested that tomatine might be of value as a therapeutic agent (Irving et al.,1946).

However, subsequent toxicity tests have ruled out parenteral administration and restricted its possible clinical use to topical or oral application. Tomatine has been produced commercially for therapeutic purposes and recommended for the treatment of certain forms of dermatitis (Schreiber, 1968). A South American company has described a tomatine ointment for skin application, but no details of its clinical efficacy are available (Wilson et al., 1961).

Since the finding that tomatine forms a molecular complex with 3β-hydroxy steroids, other workers (Rinehart et al., 1962; Huang et al., 1963) have demonstrated that this technique can be employed for cholesterol determination.

Certain steroidal alkaloids, including tomatine, can be used as starting materials for the synthesis of steroidal hormones (Fontaine et al., 1955), due to their ability to be degraded to intermediates in steroid hormone synthesis such as 3β-hydroxy-pregn-5,16-dien-20-one (Sato et al., 1951; Kuhn and Löw, 1952; Schreiber and Aurich, 1966). Pregnenolone, obtained by degradation of tomatidine has been used for the partial synthesis of progesterone (Tuzson, 1961). In certain countries a modified form of tomatine, tomatid-5-en-3β-ol, is commonly employed for synthesis of steroidal hormones (Schreiber, 1968).

PART II

MATERIALS AND METHODS

SECTION 1. TISSUE AND ORGAN CULTURE TECHNIQUES.

Culture vessels.

All glassware used as culture vessels and for preparation of culture medium was of 'Pyrex' brand. Before use, glassware was cleaned in a strong 'Pyroneg' solution for at least 24 hours, followed by six rinses with tap water and two final rinses with glass-distilled water. Excised roots were grown in $100cm^3$ and $250cm^3$ wide-mouth Erlenmeyer flasks and in 1ℓ penicillin culture flasks. Similar $100cm^3$ and $250cm^3$ flasks were used as culture vessels for callus tissue and cell suspensions respectively. Culture flasks were closed with plugs of white, non-absorbent cotton wool wrapped in white muslin cloth. Plugs were protected from dust, and drips in the autoclave by covering with aluminium caps or aluminium foil.

Constituents of culture media.

Chemicals and solvents used in the preparation of culture media were, unless otherwise stated, obtained from BDH Chemicals Ltd., Poole, and were, where possible, of 'AnalaR' grade. Single glass-distilled water was used at all times. Stock solutions were stored in a refrigerator at $4^{\circ}C$ except where stated otherwise.

The constituents of the nutrient medium for growth of excised roots are shown in table 1. The inorganic elements were those of White (1943) as modified by Street and McGregor (1952), but without ferric chloride.

TABLE 1. Constituents of excised root culture medium

	Anhydrous weight per litre of culture medium
Sucrose	20 g
Aneurin hydrochloride	0.1 mg
Pyridoxine hydrochloride	0.1 mg
Nicotinic acid	0.5 mg
Glycine	3.0 mg
Fe-EDTA (calculated as Fe)	1.03 mg
Calcium nitrate, $Ca(NO_3)_2 . 4H_2O$	200 mg
Potassium nitrate, KNO_3	80 mg
Potassium chloride, KCl	65 mg
Sodium dihydrogen orthophosphate, $NaH_2PO_4 . 2H_2O$	16.5 mg
Manganese chloride, $MnCl_2 . 4H_2O$	4.5 mg
Zinc sulphate, $ZnSO_4 . 7H_2O$	1.5 mg
Potassium iodide, KI	0.75 mg
Sodium sulphate, $Na_2SO_4 . 10H_2O$	200 mg
Magnesium sulphate, $MgSO_4 . 7H_2O$	360 mg
Boric acid, H_3BO_3	1.5 mg
Molybdic acid, H_2MoO_4	0.0017 mg
Copper sulphate, $CuSO_4 . 5H_2O$	0.013 mg
Meso-inositol	50 mg
Glass-distilled water to 1ℓ	

These were prepared as a 10X strength stock solution. As recommended by Street et al. (1952), iron was supplied in chelate form using di-sodium ethylenediamine tetra-acetate (EDTA). The iron source was a 10 per cent solution of 'Specpure' ferric chloride (Johnson Matthey Chemicals Ltd., London). The Fe-EDTA mixture was prepared by the method of Sheat et al. (1959) as a 100X strength stock solution. Vitamins, also those recommended by White (1943), were prepared as a 100X strength stock solution and stored at -15°C. Sucrose was used as carbohydrate source and was added in crystalline form. Meso-inositol was also added in crystalline form. This has been shown to stimulate growth of excised tomato roots in culture (Sinha, 1968).

Various types of culture media were employed for growth of callus tissue (table 2) but all contained sucrose, vitamins, Fe-EDTA, inorganic salts and meso-inositol as above. Auxin was supplied in all cases either a 2,4-dichlorophenoxyacetic acid (2,4-D) or as α-naphthalene acetic acid (NAA). Stock solutions were prepared by dissolving auxin in the minimum volume of 0.1N sodium hydroxide (NaOH), adding distilled water, adjusting pH to 5.5 with 0.1N hydrochloric acid (HCl) and making to the desired volume with distilled water. Certain callus culture media contained coconut-'milk' which had been obtained from undamaged coconuts, filtered and stored at -15°C. In chemically-defined culture medium coconut-'milk' was substituted by supplementary inorganic salts (Braun and Wood, 1962) and 6-furfurylaminopurine (kinetin). The latter was obtained from Koch-Light Laboratories, Colnbrook, Bucks. Supplementary

TABLE 2. Constituents of callus and suspension culture media.

Chemically-defined medium	'Coconut-milk' medium
Sucrose, Vitamins, Fe-EDTA, Inorganic salts	as in table 1
*Potassium chloride, KCl (845 mg)	
*Sodium nitrate, $NaNO_3$ (1800 mg)	
*Sodium dihydrogen orthophosphate, $NaH_2PO_4 . 2H_2O$ (230.7 mg)	
*Ammonium sulphate, $(NH_4)_2SO_4$ (790 mg)	
Kinetin (see part IV)	Coconut-'milk' (150 cm^3)
2,4-D or NAA (see part IV)	2,4-D (6 mg)
Meso-inositol (see part IV)	
Glass-distilled water to 1ℓ	
Agar (7 g, in callus culture medium only)	

*anhydrous weight per litre of culture medium.

inorganic salts were prepared as a 10X strength stock solution. A kinetin stock solution was prepared as for auxin, except that the roles of NaOH and HCl were interchanged. The nutrient medium was solidified with 'Oxoid' agar No.3. (Oxoid Ltd., London).

Culture media for growth of cell suspensions were similar to certain callus culture media except that agar was omitted.

Preparation of culture media.

The various constituents of the culture media were added, where appropriate, in the following order:- sucrose, vitamins, Fe-EDTA, distilled water, White's inorganic salts, coconut-'milk' or supplementary inorganic salts plus kinetin, auxin and meso-inositol. Solutions were made to their final volume with distilled water and their pH then adjusted (4.8 for root culture medium, 5.5 for callus and suspension culture medium) with a few drops of 0.1N NaOH. Where required, agar was then added.

Root culture medium was dispensed in $50cm^3$, $100cm^3$ or $500cm^3$ aliquots into $100cm^3$, $250cm^3$ or 1ℓ culture flasks respectively. Suspension culture medium was dispensed in $100cm^3$ aliquots into $250cm^3$ flasks. After sealing, flasks were autoclaved at 103.4 kN m^{-2} (120^oC) for ten minutes.

After addition of agar, callus culture medium, still in the preparation flask, was heated in the autoclave to dissolve the agar. Aliquots of $50cm^3$ were then dispensed into $100cm^3$ flasks and the medium sterilised as above.

Heat-labile compounds to be added to the culture medium were sterilised by passing through a 'Millipore' filter (pore size 0.45 μm) and aseptically added to cool sterilised medium.

Initiation and maintenance of cultures.

The plant material from which cultures were derived was Lycopersicum esculentum MILL. var. Suttons Best of All. Seeds were obtained from Sutton and Sons Ltd., Reading.

All aseptic transfers and manipulations were carried out in an inoculating room fitted with an ultra-violet lamp.

Excised root cultures. Tomato seeds were wetted for five minutes in a 1 per cent 'Lissapol' solution and then sterilised by immersion in 0.5 per cent bromine water for three minutes. The bromine water was decanted and, after washing four times with sterile distilled water, the seeds placed in sterile petri-dishes containing two Whatman No.1 filter papers moistened with $5cm^3$ of sterile distilled water. After five days incubation in the dark at $25^{o}C$, 10mm seedling radicle tips were excised and placed individually in culture flasks. After a further seven days growth, one root was chosen from which to initiate a clone. The main axis of the root was cut into sectors each bearing four or five lateral roots. These 'sector cultures' were grown for seven days after which more 'sector cultures' were initiated from similar sectors of the primary lateral roots. At the same time, 'tip cultures' were initiated using the 10mm apex of the primary laterals. The clone was maintained

as an equal number of 'tip' and 'sector' cultures each being grown in $100cm^3$ culture flasks.

Cultures for experimental work were initiated from 10mm tips of the primary lateral roots of seven-day old 'sector cultures'. For growth experiments roots were grown individually in $100cm^3$ culture flasks for seven days. For qualitative tomatine analysis 1ℓ penicillin culture flasks were inoculated with 15 root tips and incubated for 28 days. Quantitative alkaloid analyses were conducted using five roots grown in a $250cm^3$ culture flask for ten days. (In later quantitative work these conditions are referred to as 'standard culture conditions').

All excised root cultures were incubated in the dark at 25^oC.

The stock root clone was subcultured every seven days by using 'tip cultures' as a source of sectors and vice-versa. A little sterile culture medium was poured into a dry, heat-sterilised (160^oC for two hours) petri-dish and a single cultured root transferred to the petri-dish using a platinum loop. Tips about 10mm long, or sectors bearing at least five lateral roots were excised using 'Mayo' scissors and placed in fresh culture medium using the platinum loop. No more than five inocula were taken from any one culture. Where five or more roots were grown in the same culture vessel, tips (five from each root) were transferred to fresh medium together rather than individually.

Callus cultures. A small, undifferentiated outgrowth of cells on the hypocotyl of a sterile tomato seedling grown on agar-solidified basal medium (sucrose, Fe-EDTA, vitamins and White's salts) was excised

and placed on 'coconut-milk' medium (see p. 80) where it proliferated to produce a hypocotyl callus.

Callus tissue which had developed at the cut end of a cultured excised root was removed and, on also being incubated on 'coconut-milk' medium, gave rise to root callus.

The above two cultures are referred to later in the text as 'spontaneous' cultures.

Tomato seeds were germinated in petri-dishes as previously mentioned (p. 36). After seven days growth, 10mm segments of the cotyledon, radicle apex and middle region of the hypocotyl were removed from each seedling and placed on chemically-defined culture medium (see p. 86). Flasks were incubated in the dark at 25°C for 56 days during which time callus tissue developed on all excised segments. Callus cultures were obtained by transferring the callus to fresh culture medium.

Callus cultures were grown in a constant temperature room at 25°C under low intensity illumination (c.500 lx) supplied by 'Warm White' fluorescent lamps. Chlorophyllous callus (see p. 80) was grown under illumination of c.2000 lx.

Callus tissues were subcultured at 56-day intervals by removing, with forceps, a piece of tissue (c.200 mg fresh weight) from the surface of the callus and placing on fresh medium. No more than five inocula were taken from any one callus.

Suspension cultures. Cultures of cells in liquid suspension were initiated by transferring a piece of callus tissue (c.2g fresh weight)

into an identical, but liquid, medium and agitating on a horizontal, rotary shaker (Mk II B from L.H.Engineering Ltd., Stoke Poges, Bucks.) at 200 r.p.m. Horizontal displacement was 50 mm.

The shaker was sited in a constant temperature room at 25°C. Illumination of c.500 lx was supplied by 'Warm White' fluorescent lamps.

Suspension cultures were subcultured every 28 days. Using a cotton wool-plugged sterile pipette with a 5 mm diameter nozzle, $20cm^3$ volumes of suspension were withdrawn and transferred to fresh culture medium. A maximum of four inocula was taken from each flask.

Unless otherwise stated, callus and suspension cultures were harvested for experimental work after 56 and 28 days growth respectively.

Growth measurements.

In certain experiments, excised root growth was measured by length of the main axis (LMA), number of lateral roots (LN) and total length of all the lateral roots (TLL). A little culture medium in which the root had been growing was poured into a petri-dish and the root placed in this. LMA was measured (in mm) using a ruler, the number of lateral roots visible to the naked eye was recorded, and TLL was obtained by summing the estimated length (in mm) of each lateral root. The constancy of the TLL/LN ratio indicated this method to afford a valid measure of TLL.

Fresh and dry weights were employed to measure growth in excised root, callus and suspension cultures.

Excised roots were rinsed with distilled water, spread out between two sheets of 'general purpose' filter paper and blotted for exactly ten seconds. After weighing in pre-weighed aluminium foil 'cups', roots were dried at 80°C for 24 hours and reweighed.

Callus tissue was carefully removed from the agar medium and immediately weighed in foil 'cups'. Dry weight measurements were made as for excised roots.

Suspension cultures were filtered using 'general purpose' filter paper and the cell residue washed three times with distilled water. When the last of the washings had filtered through, the cells were transferred to foil 'cups' and fresh and dry weights determined as above.

Extractions for tomatine were carried out immediately after determination of fresh weight. Use of freeze-dried material was not considered for timetable reasons. However, in order to be able to calculate tomatine content on a dry weight basis, parallel experiments were carried out to determine fresh weight/dry weight ratios of tissues or organs. Standardisation of weight-determination techniques reduced variation in this ratio in replicate flasks to insignificant levels.

SECTION 2. TECHNIQUES FOR EXTRACTION, IDENTIFICATION AND ASSAY OF TOMATINE.

Extraction of tomatine.

Unless otherwise stated, all chemicals used in experimental work were obtained from BDH Chemicals Ltd., Poole, and were, where possible, of 'AnalaR' grade.

The extraction techniques were modifications of that used by Rooke et al. (1943) for extraction of solanine from potato.

Excised root cultures. For quantitative estimation of tomatine, roots were placed in a beaker and ground with a glass pestle in $1cm^3$ of a solution consisting of 94 per cent methanol, 4 per cent water and 2 per cent glacial acetic acid. A further $9cm^3$ of this solution were then added and the beaker left covered for 18 hours. The extract was filtered under vacuum, the residue of homogenised roots washed twice with $2cm^3$ of methanol, scraped off the filter paper (Whatman No.1) and re-extracted for five hours in $10cm^3$ of 64 per cent methanol in the same beaker. After the second filtration, a third extraction was carried out for two hours in $10cm^3$ of 64 per cent methanol. Beaker washings were used to wash the root residue after the third filtration. The bulked filtrates were transferred (with methanol washings) to a boiling flask and evaporated to dryness on a rotary evaporator at 45^oC. Flask contents were taken up three times in 0.5 cm^3 of hot methanol and the methanol extracts applied to a thin-layer chromatography (TLC) plate (see below).

In qualitative work where larger amounts of roots were extracted, a modified version of the above procedure was employed. Roots were homogenised using an MSE homogeniser and extracted as above except that $25cm^3$ volumes of extractant were used and washings were with 5 cm^3 of methanol. The extract was reduced to about $50cm^3$ under vacuum at 45^oC and, when cool, was transferred to a beaker along with 2 per cent acetic

acid washings. The pH was adjusted to 10.0 with ammonia solution (sp.gr.0.88) and, after covering, the beaker left in a refrigerator at 4°C overnight to allow the precipitate to develop. The extract was filtered using Whatman No.1 filter paper and the precipitate washed with 2 per cent ammonia solution. After drying at 40°C, the filter paper plus precipitate was extracted for 18 hours in 10cm^3 of methanol. The methanol extract was filtered through Whatman No.1 filter paper, the residue washed with methanol and the filtrate plus washings evaporated to dryness under vacuum at 35°C. Flask contents were taken up as above.

Callus and suspension cultures. Tomatine was extracted by the method employed for large amounts of cultured roots except that cells and tissues were homogenised in an MSE 'Ato-Mix' homogeniser at full speed for one minute and 1cm^3 of extractant per gram of fresh tissue was used.

Unless stated otherwise, callus cultures were analysed for tomatine only when they had become established on a particular culture medium.

TLC of extracts was carried out using glass plates coated with a 0.25 mm layer of Silica Gel G as devised by Stahl (Anderman and Co.Ltd., London). After coating and allowing to air-dry, plates were activated at 100°C for 45 minutes. Unless stated otherwise, excised root extracts were chromatographed on 50 x 200mm plates and callus and suspension culture extracts on 200 x 200 mm plates. In any one experiment, thin-layer plates of the same 'batch' were used throughout.

Application of substances to TLC plates by 'spotting' was carried

out using glass capillary tubes of 1mm bore. Where larger amounts of solutions or extracts were to be chromatographed, they were applied as a horizontal band not more than 5mm wide by means of a Pasteur pipette with a fine cotton wool 'wick'. For quantitative estimates of tomatine in cultured roots, extracts were applied as a band the full width (50mm) of the thin-layer plate, whereas in similar work with callus and suspension cultures the applied band was 180mm long.

The solvent systems used for TLC of culture extracts were devised during the course of this work and details are given in part III, section 2.

Developed chromatograms were dried using an air blower and specific compounds located on the plates by spraying with the appropriate locating reagent. The various locating reagents employed were prepared or obtained as shown in table 3. On application of Dragendorff reagent, alkaloids appeared as orange coloured zones, but with ninhydrin, ceric ammonium sulphate and sugar-locating reagent heating at 100°C for 15 minutes was necessary to make the substances visible. After locating with iodine vapour, the iodine was evaporated from the chromatogram by a current of air.

All chromatograms in this report are tracings of the originals.

For quantitative work, Dragendorff reagent proved to be the most suitable locating reagent for tomatine. Preliminary experiments showed that down to 0.7 µg of tomatine and 0.3 µg of tomatidine could be detected. However, Dragendorff reagent is not specific for alkaloids

TABLE 3. Locating reagents used in TLC

Modified Dragendorff reagent (Cromwell, 1955).

Solution A	Solution B
850 mg bismuth oxynitrate	8 g potassium iodide
40 cm^3 distilled water	20 cm^3 distilled water
10 cm^3 glacial acetic acid	

Mix 5 cm^3 of solution A with 5 cm^3 of solution B, add 20 cm^3 of glacial acetic acid and 100 cm^3 of distilled water.

Ceric ammonium sulphate.

Dissolve 1 g of ceric ammonium nitrate in 100 cm^3 of 10 per cent (v/v) sulphuric acid.

Sugar locating reagent.

Add 0.5 g of m-phenylenediamine and 1.2 g of stannous chloride to a mixture of 20 cm^3 of glacial acetic acid and 80 cm^3 of ethanol.

Ninhydrin.

Aerosol spray

Iodine vapour.

From heated iodine crystals.

but will give positive reactions with certain other non-nitrogenous organic compounds (Farnsworth et al., 1962). In fact, extracts of callus tissue often showed a number of Dragendorff-positive zones and in such cases the location of tomatine was checked using 50 per cent (v/v) sulphuric acid (see p.50).

A 10mm wide edge strip of the TLC plate was delimited by a vertical score in the gel and, after covering the rest of the TLC plate with a sheet of glass, sprayed with the locating reagent. This method of location results in loss of a certain amount of alkaloid, but total alkaloid was calculated by simple proportion as follows:-

$$\text{total tomatine} = \text{recovered tomatine} \times \frac{\text{length of applied band}}{\text{length of non-sprayed zone}}$$

The tomatine zone was scraped off the plate and placed in a 5 x 150mm glass column, the nozzle of which was plugged with methanol-extracted cotton wool. Tomatine was eluted from the gel with $5cm^3$ of methanol and the eluate collected in a test-tube. The eluant was evaporated off by placing the test-tube in a water bath at $80^{\circ}C$ for 45 minutes.

Where eluted tomatine was to be assayed, controls were obtained by similarly eluting an equal area of gel from a 'blank' plate. A preliminary experiment revealed that it was not necessary to obtain this gel from a region corresponding in Rf to tomatine on a developed 'blank' plate, but that gel from a dry, unused plate could be used.

Identification of extracted tomatine.

Various methods were employed to confirm the identity of tomatine

extracted from cultured tissues and organs. However, since they constitute part of the experimental work, they are only outlined here but detailed on the pages in parentheses. The methods included measurement of Rf of tomatine and its aglycone, tomatidine, released on acid hydrolysis of the glycoside (p. 49); colour reactions of the glycoside and aglycone on TLC after spraying with 50 per cent (v/v) sulphuric acid (p. 50); visible and ultra-violet spectroscopy of the sulphuric acid chromogen of tomatine using a Pye 'Unicam' SP 800 spectrophotometer (p. 51); mass spectroscopy of tomatidine using an AEI (MS 12) mass spectrometer (p. 59); analysis of the sugar moiety qualitatively by TLC (p. 59), and quantitatively using the anthrone technique (p. 60), galactose dehydrogenase (p. 60) and glucose-6-phosphate dehydrogenase (p. 60).

Tomatine assay

The assay method was based on the amount of absorbance of the sulphuric acid chromogen of tomatine at 325nm, as measured on a Pye 'Unicam' SP 800 spectrophotometer.

After elution of tomatine and evaporation of the eluant, 5cm^3 of concentrated sulphuric acid were pipetted into the test-tubes which were then incubated in a water bath at 40^{o}C for 24 hours. When cool, test-tube contents were transferred to 'Spectrosil' quartz cells (path length 10mm) and absorbance scanned (at fast scanning speed) between 550-250nm. Absorbance was measured at 325nm and amount of tomatine calculated by reference to a calibration graph. No results were taken

from treatments showing abnormal spectra.

The calibration graph was constructed using 'authentic' tomatine (Koch-Light Laboratories, Colnbrook, Bucks.). The amounts used were 10µg, 25µg, 50µg and 100µg. Regression analysis of the data afforded the graph shown in fig.3. The Beer-Lambert law applied between the limits used. The amount of tomatine was calculated from the equation of the regression line (absorbance value substituted for 'y').

SECTION 3. STATISTICAL METHODS.

Standard error.

Results are expressed as the mean of a number of replicates specified in the text followed by the standard error of the mean. The formula used for calculation of standard error was:-

$$\text{standard error} = \sqrt{\frac{S(x^2) - \frac{(Sx)^2}{n}}{n(n-1)}}$$

where $S(x^2)$ = sum of squares of each replicate value.

$(Sx)^2$ = square of sum of replicate values.

n = number of replicates.

't-test'

In certain cases a 't-test' was used to compare two mean values. The formula for calculation of 't' was:-

$$t_{d.f.} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left[\frac{S(x_1)^2 - \frac{(Sx_1)^2}{n_1} + S(x_2)^2 - \frac{(Sx_2)^2}{n_2}}{n_1+n_2-2}\right]\left[\frac{1}{n_1} + \frac{1}{n_2}\right]}}$$

where $\bar{x}$ = value of mean

d.f. = degrees of freedom (= n_1+n_2-2)

(other symbols as for standard error).

Regression analysis.

Regression analysis of data for construction of a calibration graph for tomatine assay was carried out according to the method of Snedecor (1965).

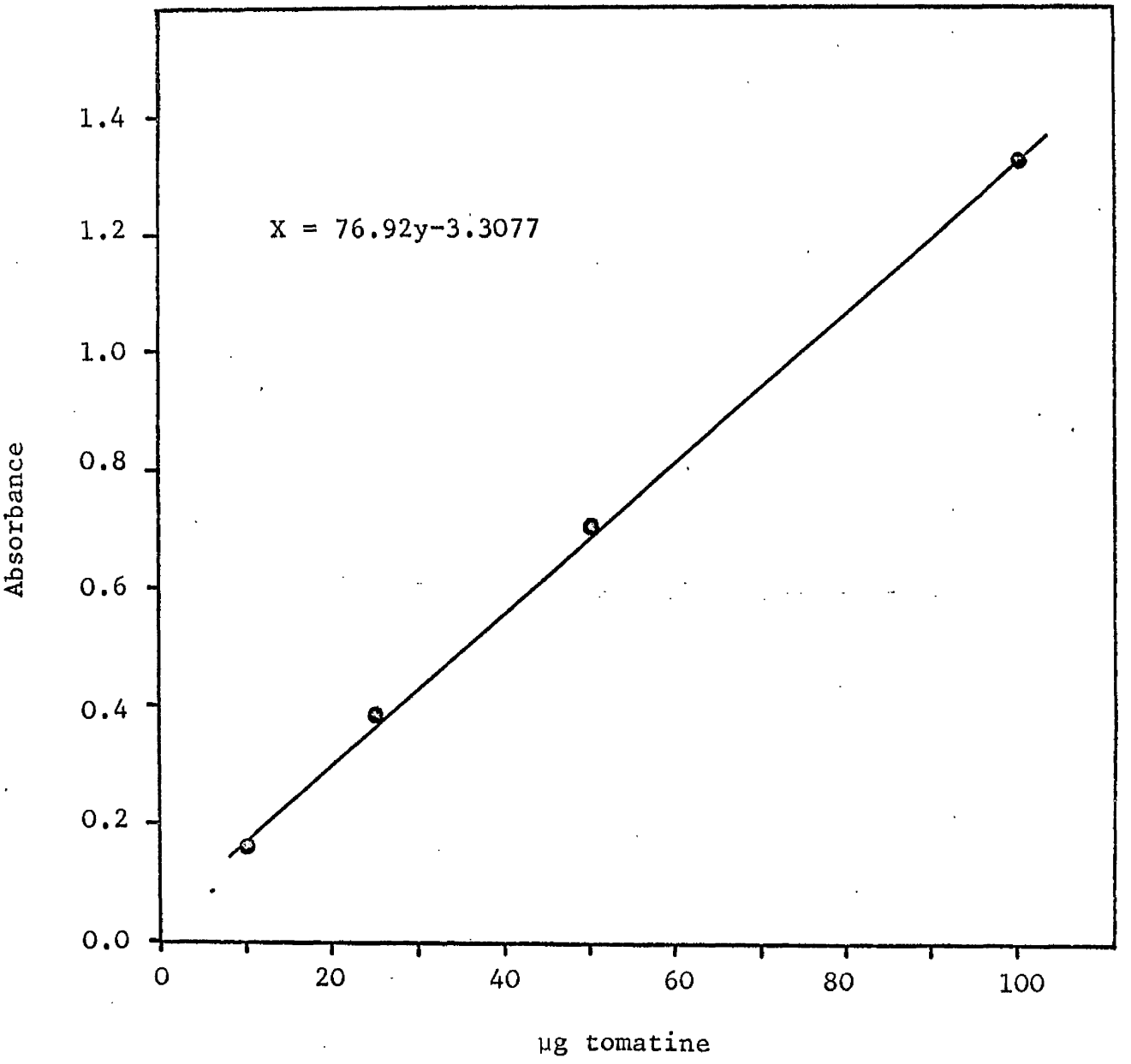

Fig.3. Calibration graph for tomatine assay. Absorbance of the sulphuric acid chromogen of tomatine at 325 nm.

PART III

STUDIES WITH EXCISED ROOT CULTURES.

SECTION 1. ANALYSIS OF CULTURED ROOTS FOR TOMATINE.

Extraction of cultured roots.

Cultured roots from nine penicillin flasks were harvested after 28 days growth and bulked to yield 20.9g fresh weight. Roots were extracted as reported on p. 41 and a spot of extract applied to a TLC plate. 'Authentic' tomatine and tomatidine (Koch-Light Laboratories, Colnbrook, Bucks.) were applied as one spot, and a third spot consisting of extract plus 'authentic' alkaloids was added. The chromatogram was developed in iso-propanol : formic acid : water (IFW, 73 : 3 : 24), and, when dry, sprayed with Dragendorff reagent. The chromatogram is shown in fig.4.

Of the three Dragendorff-positive spots in the extract, one had an Rf (0.73) close to that of 'authentic' tomatine (0.70). Co-chromatography of extract and 'authentic' alkaloids was consistent with this compound being tomatine.

The fact that forms of tomatine with modified sugar moieties exhibit different Rf's from that of α-tomatine (Arneson and Durbin, 1967) was a possible clue to the identities of the lower-Rf compounds in the extract.

Identification of the hydrolysis products of Dragendorff-positive compounds.

The basis of this experiment was that hydrolysis of tomatine or any of its modified forms results in liberation of the aglycone, tomatidine.

Half of the root extract was applied as a band to a 200 x 200 mm TLC plate and the chromatogram developed in IFW. After location of Dragendorff-positive zones, bands of gel, one for the 'high-Rf' compound

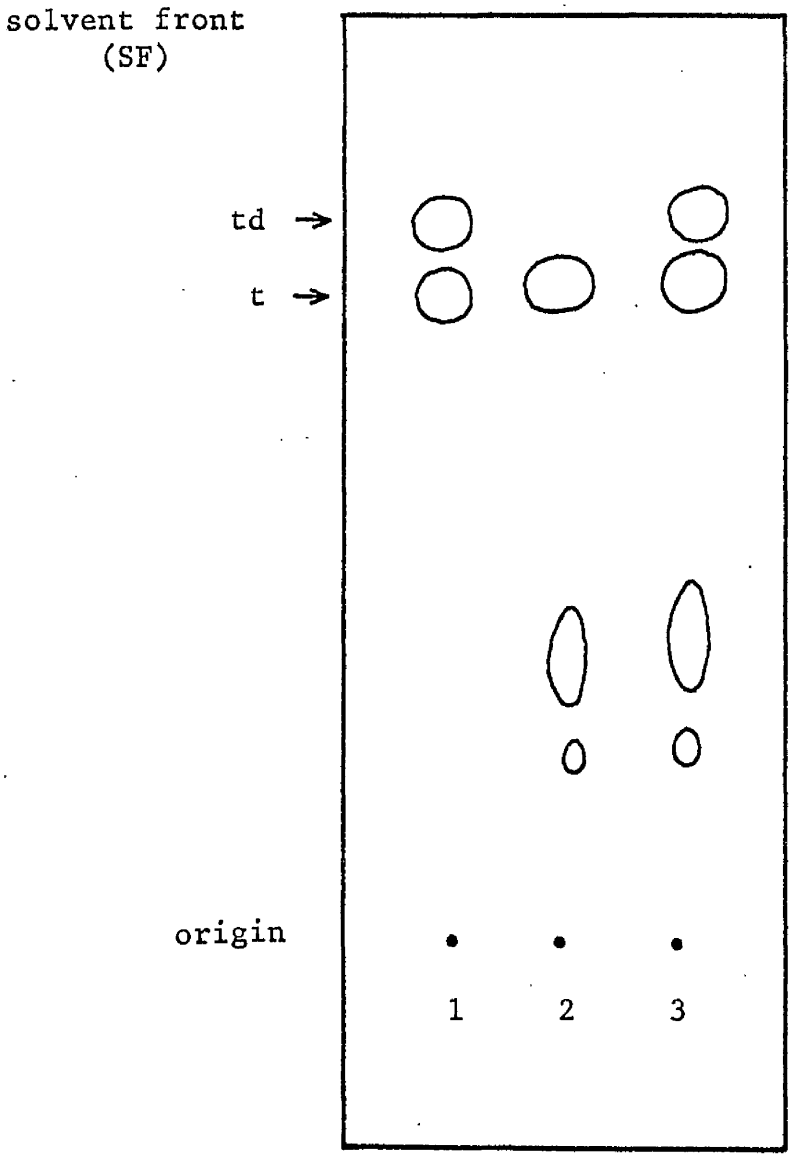

Fig.4. Chromatogram of cultured root extracted developed in IFW and sprayed with Dragendorff reagent.

1. 'authentic' tomatine (t) and tomatidine (td)
2. root extract
3. 1+2

and one including both 'low-Rf' compounds were scraped out and eluted. One half of each eluate was retained while the other half was evaporated to dryness on a rotary evaporator at 35°C.

'High-Rf' compound. After removal of the eluant, flask contents were boiled under reflux in 10 cm^3 of 1.0N HCl for one hour. The acid was removed on a rotary evaporator at 45°C and 10 cm^3 volumes of water added and evaporated off at 45°C until all the acid had been removed. Hydrolysed contents were taken up in a few cm^3 of hot methanol. Two chromatograms were prepared each having a spot of hydrolysed extract, a spot of 'authentic' alkaloids and a spot of extract plus alkaloids. One chromatogram was developed in IFW and the other in chloroform : methanol (94:6).

After spraying with Dragendorff reagent the former chromatogram showed two spots in the hydrolysed extract which corresponded in Rf to tomatine and tomatidine (fig. 5a). The presence of the original compound resembling tomatine indicated that complete hydrolysis had not occurred.

Two spots were also present in the hydrolysed extract in the chloroform/methanol-developed chromatogram (fig. 5b). The spot which moved from the origin had a lower Rf than tomatidine but co-chromatography revealed only one spot at the Rf of the aglycone.

These findings provide further evidence that tomatine is present in cultured tomato roots.

'Low-Rf' compounds. This extract was hydrolysed as above except that the process was carried out for nine hours. Spots of hydrolysed extract and 'authentic' alkaloids were applied, as above, to TLC plates which were then developed in the above two solvents.

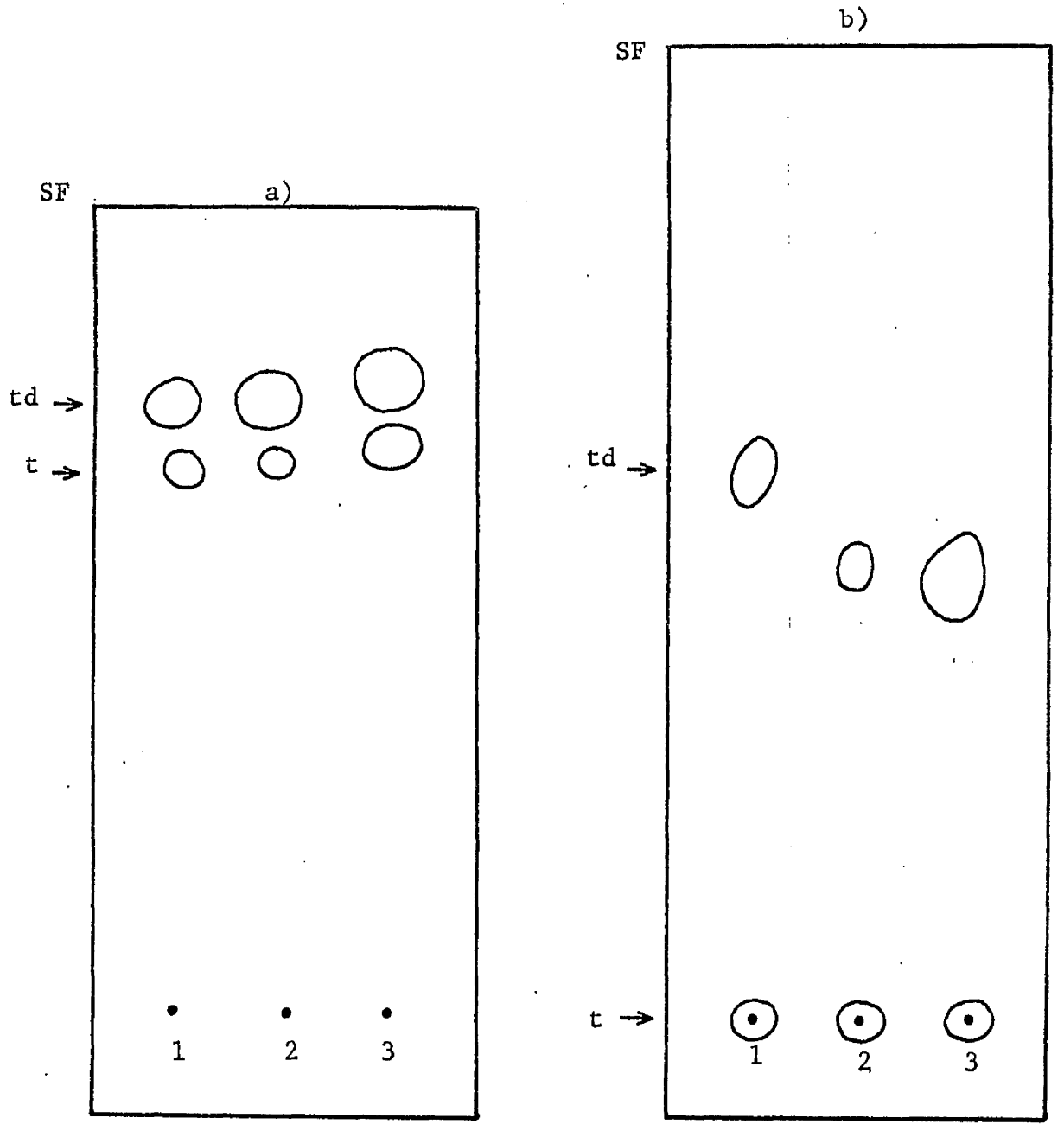

Fig.5. Chromatograms of the hydrolysed 'high-Rf' compound developed in a) IFW and b) chloroform/methanol and sprayed with Dragendorff reagent

1. 'authentic' tomatine (t) and tomatidine (td)
2. hydrolysed 'high-Rf' compound
3. 1+2

No compounds resembling either tomatine or tomatidine were apparent in the hydrolysed extract after spraying with Dragendorff reagent. (Later experiments showed that, as a result of hydrolysis, a compound was produced which was identical to choline in Rf (IFW solvent) and colour reaction with Dragendorff reagent.)

It was concluded that no modified forms of tomatine were present in the root extract.

Colorimetric identification of the 'high-Rf' compound.

The method employed was that of Heftmann et al. (1966) except that 50 per cent (v/v) sulphuric acid was used instead of 50 per cent (w/w) acid. These authors only reported initial and final colours of tomatidine, and so a preliminary experiment was carried out to observe colour changes in more detail in both the glycoside and aglycone.

'Authentic' tomatine and tomatidine were applied as separate spots to TLC plates which were then developed in IFW. When dry, plates were sprayed with the acid and heated in an oven at 100°C.

The colours of the spots were recorded in daylight over a period of 30 minutes, after which the chromatograms were examined under ultra-violet light of wavelength 254 nm. Results are shown in table 4.

This procedure was repeated using TLC plates to which had been applied 'authentic' alkaloids, hydrolysed extract and the original root extract.

Spots in the extracts which corresponded in Rf to tomatine and tomatidine showed colour changes identical to those observed in the

TABLE 4. Identification of tomatine and tomatidine by their colours on TLC plates, after spraying with 50 per cent sulphuric acid and heating. (Colours in daylight.)

	3 min	6 min	9 min	12 min	18 min	24 min	30 min	under UV (254 nm) 30 min
Tomatine	pink	no colour	green	purple green	purple	purple grey	purple black	brown
Tomatidine	pink	no colour	green	green	green	green	green	pale yellow

appropriate 'authentic' alkaloid.

Examination of the chromatogram after completion of observations revealed the presence of other compounds in the original root extract which did not show up with Dragendorff reagent (fig. 6). One of these compounds had an Rf only slightly lower than that of the 'high-Rf' compound. The implications of the position of this compound on the chromatogram are discussed below.

Spectroscopic identification of the 'high-Rf' compound.

Certain steroids can be identified by the absorption spectrum of their sulphuric acid chromogens (Diaz et al., 1952; Walens et al., 1954). The method employed here was based on this principle, but the acid concentrations and the lengths of the incubation period used by the above authors were modified.

The absorption spectra of 'authentic' tomatine and the 'high-Rf' compound were compared as follows:-

1 cm^3 of 'authentic' tomatine solution (1 mg in 10 cm^3 of methanol) was pipetted into each of three test-tubes and 1 cm^3 of 'high-Rf' extract into a further three test-tubes. A test-tube containing 1 cm^3 of methanol was used as the 'blank'. The methanol was evaporated off by immersion in a water bath at 80°C for 20 minutes. When cool, 5 cm^3 of concentrated sulphuric acid were added to each of the tubes, which were then incubated in a 40°C water bath for 24 hours. Absorbance was scanned between 550-250 nm.

The three 'authentic' tomatine replicates gave identical absorption

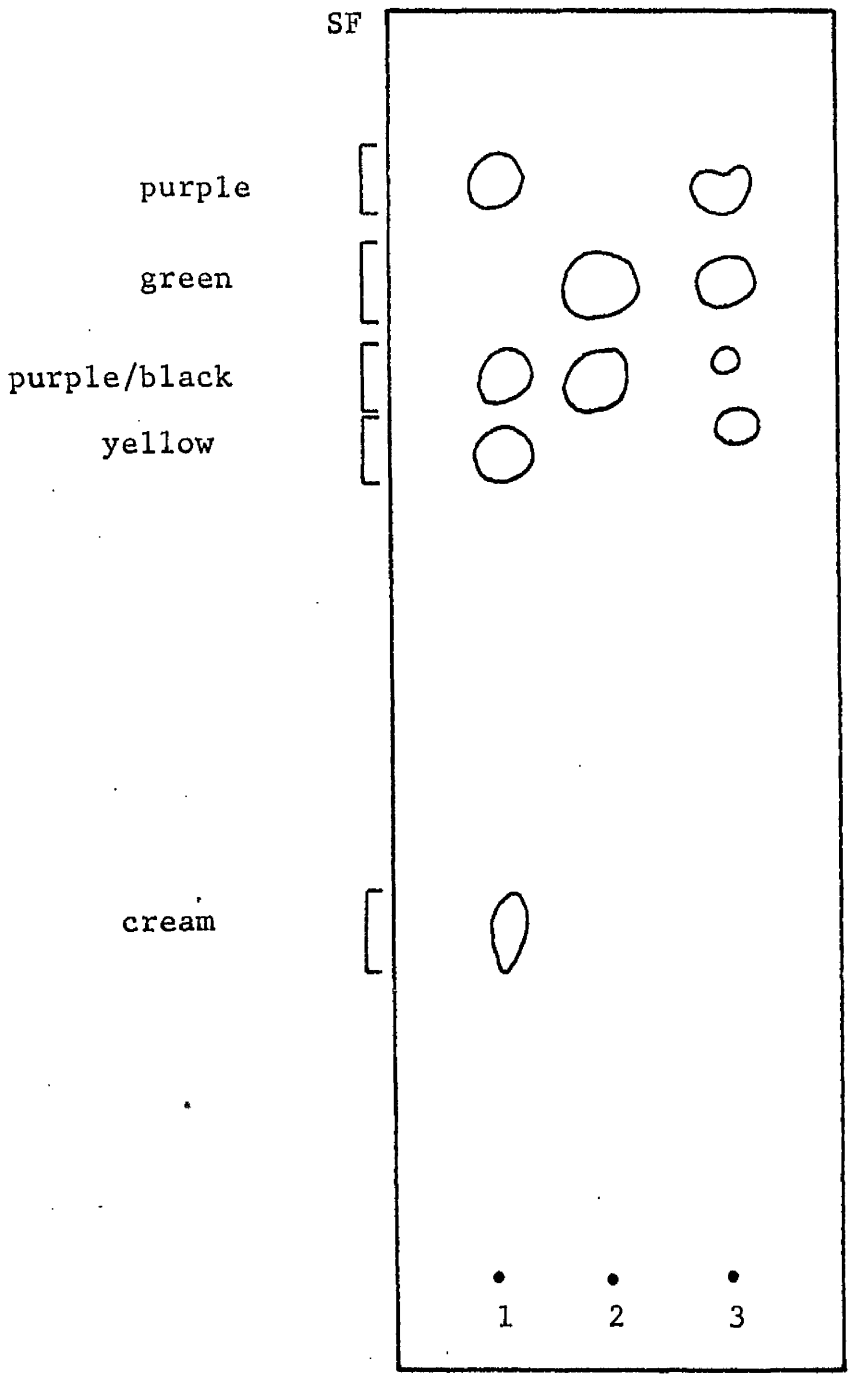

Fig.6. Chromatogram of cultured root extracts developed in IFW, sprayed with 50 per cent sulphuric acid and heated. (colours are those after 30 minutes)

1. original root extract
2. 'authentic' tomatine and tomatidine
3. hydrolysed root extract.

spectra, one of which is shown in fig. 7. The characteristic features of the spectrum are a λ_{max} at 322 nm, two smaller peaks at 257 nm and 410 nm, and a trough at 278 nm.

The absorption spectra of the three 'high-Rf' replicates were also identical to each other but showed slight differences from that of 'authentic' tomatine (fig. 7). The λ_{max} occurred at 325 nm, one of the smaller peaks was at 260 nm, and the trough at 278 nm was less well-defined. These differences could be due to impurities in the extract and this possibility is strengthened by the finding of a compound in the root extract which had an Rf very close to that of tomatine.

Nevertheless the absorption spectrum of the 'high-Rf' compound is sufficiently similar to that of 'authentic' tomatine to suggest it to be tomatine.

SECTION 2. DEVELOPMENT OF A TECHNIQUE FOR PURIFICATION OF TOMATINE.

The following experiments were carried out as a result of the finding in the previous section that TLC of root extracts using the IFW solvent system did not satisfactorily separate tomatine from certain other constituents of the extract. Where an assay technique involves non-specific reactions, quantitative work necessitates a high degree of purity in the compound to be assayed. In this case, such a requirement was emphasised by the presence of concentrated sulphuric acid in the assay mixture.

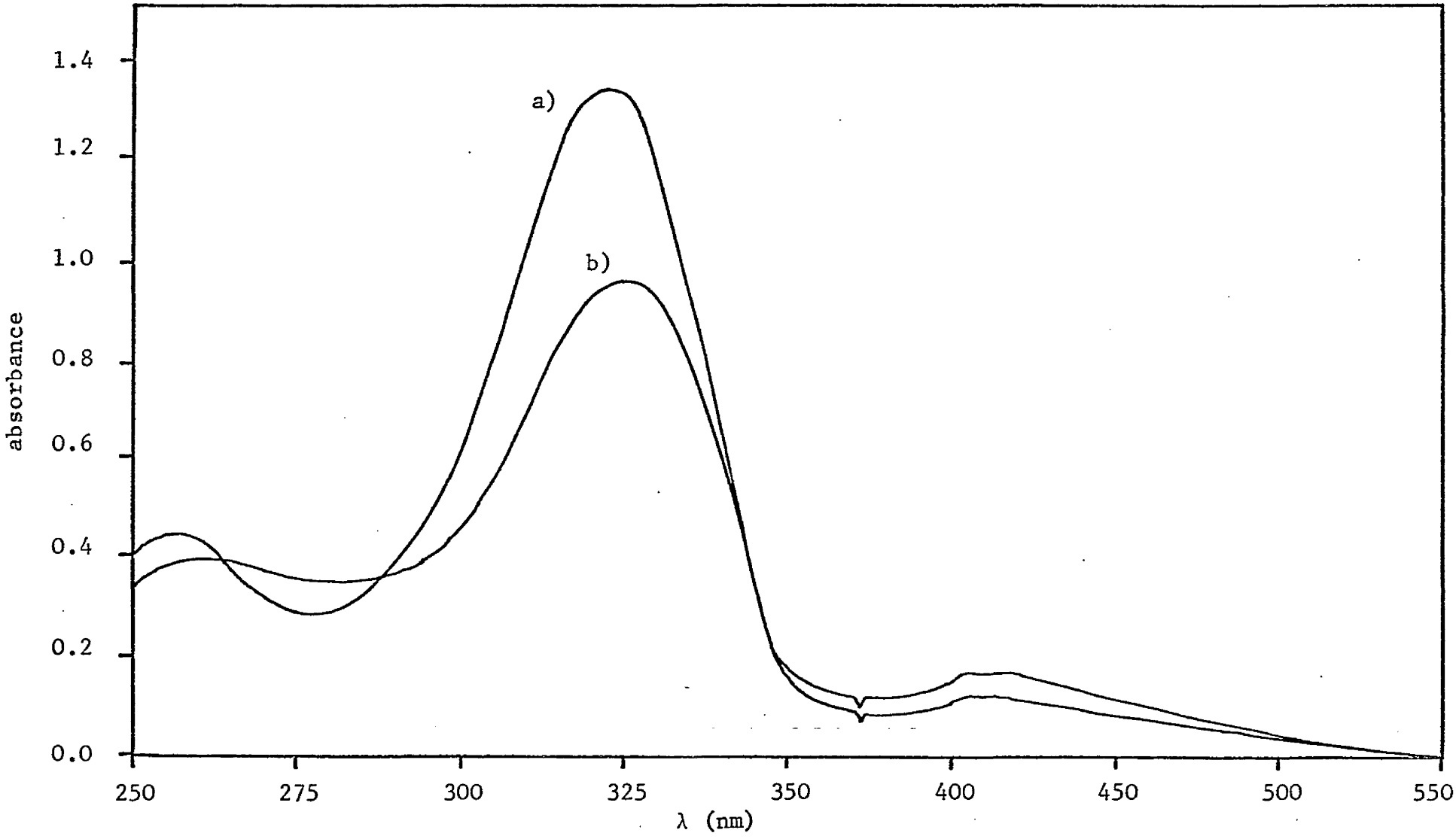

Fig.7. Absorption spectra of the sulphuric acid chromogen of a) 'authentic' tomatine and b) the 'high-Rf' compound.

Determination of a satisfactory solvent system.

Various possible solvent systems for separation of tomatine on TLC were tested using, initially, 'authentic' alkaloids to eliminate those which did not afford suitable Rf values.

Tomatine and tomatidine were applied as spots to TLC plates which, after development, were treated with Dragendorff reagent. Of the ten solvent systems tested, the two most suitable were found to be IFW (as before) and the upper phase of n-butanol : glacial acetic acid : water (BAW, 4:1:5). The Rf's of tomatine were 0.68 and 0.37 respectively and those of tomatidine 0.77 and 0.68 respectively.

Although alkaloids gave compact spots in both solvent systems, they were much less sensitive to Dragendorff reagent on plates developed in BAW, and the glycoside, in particular, was difficult to detect.

Further examination of BAW- and IFW-developed chromatograms.

To each of four TLC plates were applied one spot of original root extract and a second spot containing 'authentic' tomatine and tomatidine. Two of the plates were developed in IFW and two in BAW. One plate from each solvent was sprayed with ceric ammonium sulphate (CAS), and the two remaining plates with Dragendorff reagent.

Of the chromatograms developed in IFW, the Dragendorff-treated plate was essentially as previously observed (fig. 4). The chromatogram sprayed with CAS revealed compounds close to tomatine, the most distinctive being immediately below tomatine. Treatment of the BAW-developed chromatogram with Dragendorff reagent showed two other spots immediately above and

below tomatine. CAS treatment of the BAW-developed chromatogram confirmed that separation of tomatine from other root extract components was even less successful in this solvent than in IFW.

Although BAW was consequently rejected as a possible solvent system, adoption of IFW did not entirely eliminate the problem of contamination of tomatine, due to the presence of a compound with only a slightly lower Rf than tomatine.

Identification of the 'potential' contaminant of tomatine.

'Authentic' tomatine and root extract were applied to a TLC plate which was developed in IFW. Tomatine was located using iodine vapour, after which the chromatogram was sprayed with sugar-locating reagent (SLR).

The compound lying below tomatine was the only one in the extract to react with SLR, its colour being the golden-brown characteristic of sugars.

To decide if the Rf of this compound was also characteristic of sugars, a further chromatogram, to which had been applied 'authentic' tomatine; a solution consisting of glucose, fructose and sucrose; and alkaloid and sugars together, was developed in the same solvent system. Tomatine and sugars were detected as before.

The sugars did not separate in this solvent but appeared as a single, golden-brown spot in an identical position, in relation to tomatine, to the unknown contaminant. The 'potential' contaminant of tomatine was therefore concluded to be a sugar or sugars.

Separation of contaminating sugars from tomatine.

The method devised was based on the differential solubility of these two compounds in distilled water of pH 5.5. Sugars dissolve readily, whereas tomatine does not.

'Authentic' tomatine, the sugar solution used above, and tomatine plus sugars were applied as three separate spots to a TLC plate. The chromatogram was developed in distilled water (allowing the solvent front to reach the top of the plate), after which it was dried and re-developed in IFW. Tomatine was detected with iodine vapour and sugars with SLR.

Sugars were carried almost to the front of the distilled water solvent whereas the Rf of tomatine after development in IFW was similar to that previously observed.

TLC of root extracts using the two solvent-systems (I).

'Authentic' tomatine and original root extract were applied as separate spots to each of four TLC plates. The chromatograms were developed using the two solvent-systems devised above. One chromatogram was sprayed with Dragendorff reagent and a second with CAS. Tomatine was detected with iodine vapour on the two remaining chromatograms, after which one was sprayed with SLR and the other with ninhydrin.

The chromatogram sprayed with Dragendorff reagent appeared essentially as had been previously observed (fig. 8a). The chromatogram treated with CAS (fig. 8b) showed that the Rf of the unknown compound lying close to the IFW-solvent front was unaffected by initial development in distilled water. In the region between the two solvent fronts two spots were

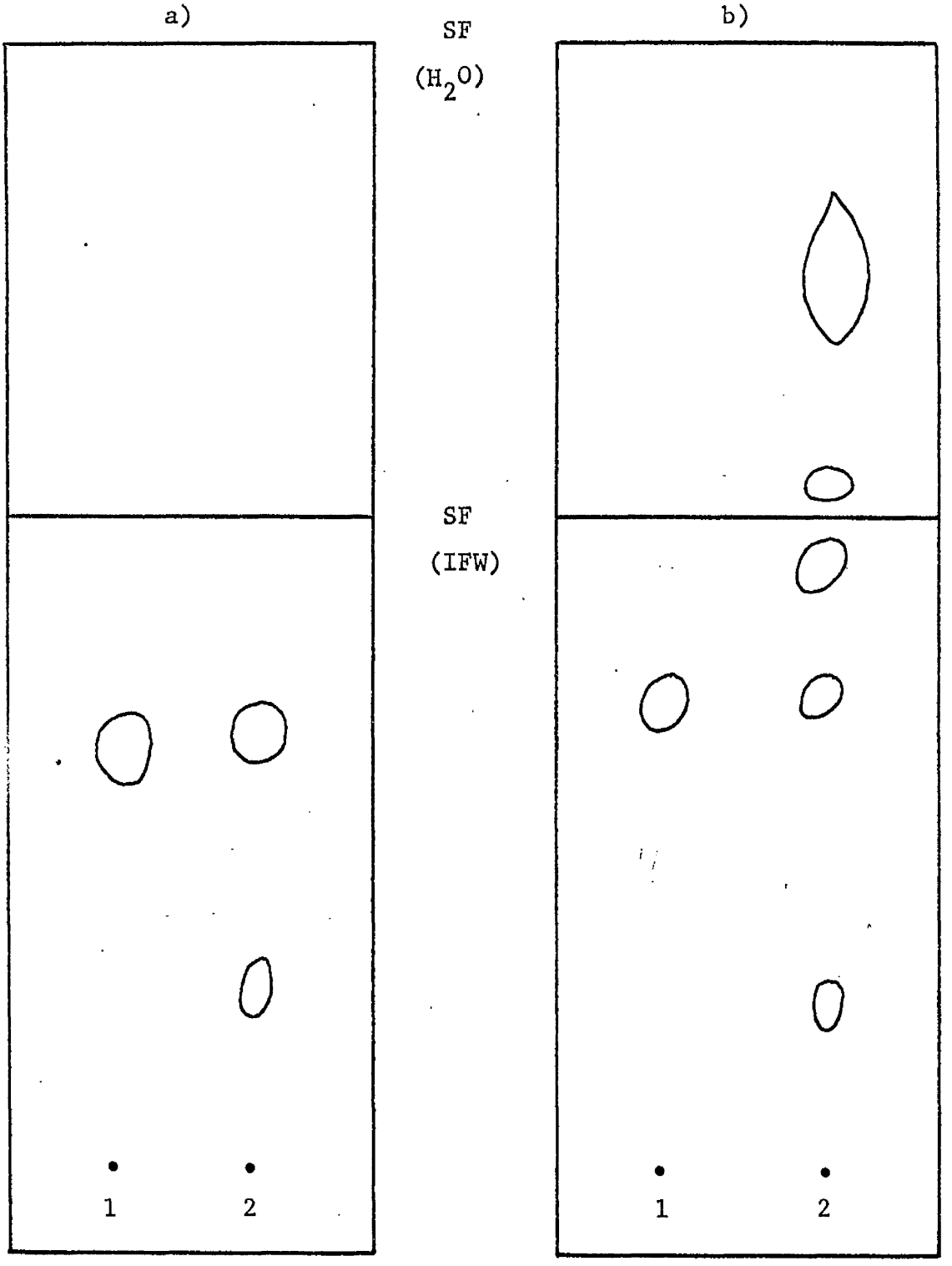

Fig.8. Chromatograms of cultured root extracts developed in the two solvent-systems and sprayed with a) Dragendorff reagent and b) CAS.

1. 'authentic' tomatine
2. root extract.

apparent. The larger (and blacker) was probably sugars but the identity of the smaller spot was not known.

Spraying with SLR indicated that use of these two solvent-systems also resulted in separation of tomatine and sugars in cultured root extracts (fig. 8c).

In the ninhydrin treated plate no such-positive compounds were present in the vicinity of tomatine (fig. 8d).

The only substance not removed from the vicinity of tomatine was the unknown which lay close to the IFW-solvent front. However, this compound was separated from tomatine by such a distance that the purity of eluted alkaloid was not jeopardised.

TLC of root extracts using the two solvent-systems (II).

Since only small amounts of root extract, applied to TLC plates as a spot, had so far been separated, it was necessary to test if a similar separation of extract components would be achieved when the whole extract was applied to the plate as a band.

Two flasks of roots grown under standard culture conditions were harvested and extracts were applied as bands to separate TLC plates. One chromatogram was developed in IFW only and the other using the two solvent-systems. Each chromatogram was divided into two equal halves by a vertical score in the gel, the left-hand side treated with CAS and the right-hand side with SLR.

The chromatograms (fig. 9) revealed that application of the 'entire' root extract as a band did not hinder separation of tomatine from other extract components.

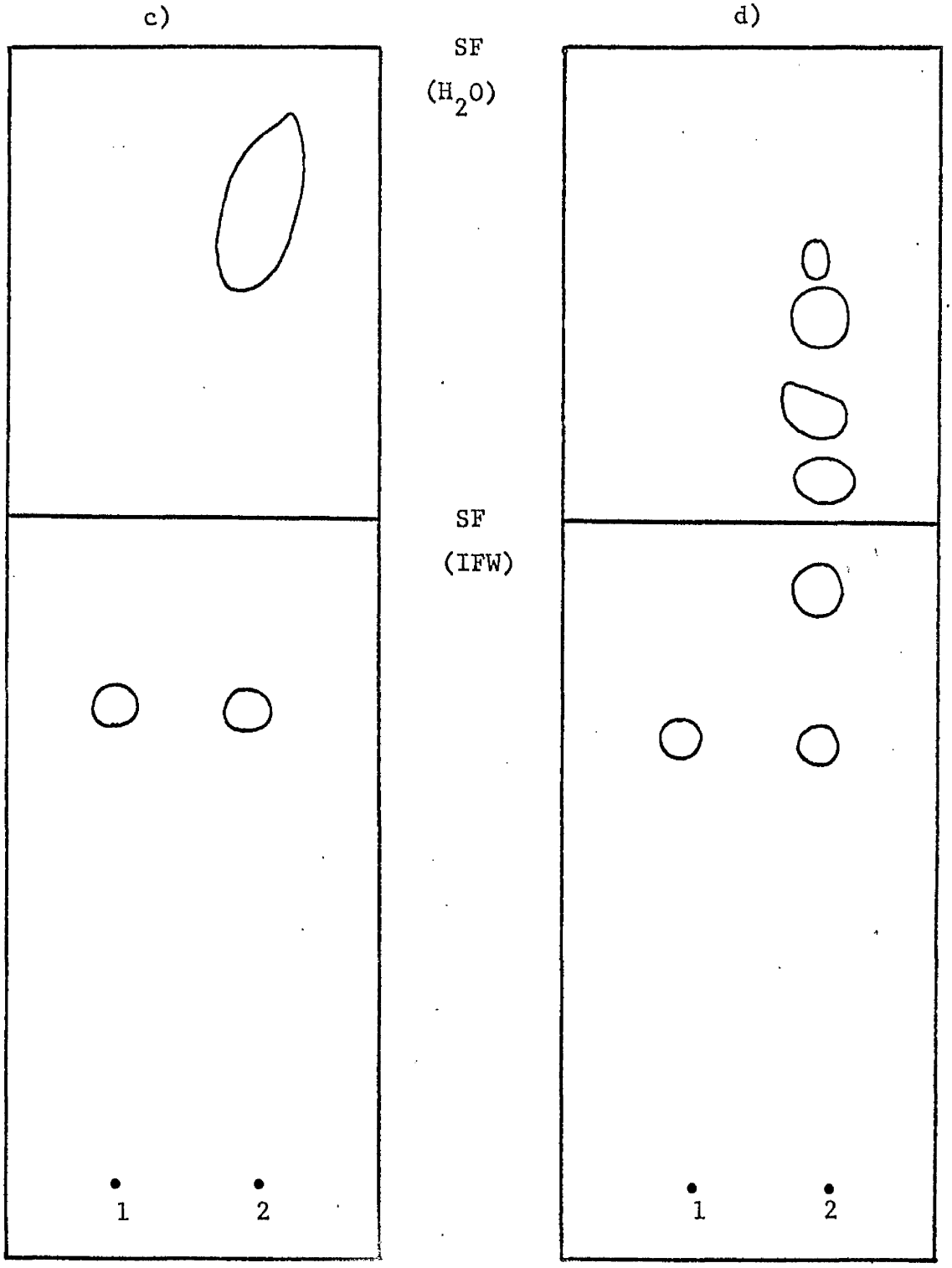

Fig.8. Chromatograms of cultured root extracts developed in the two solvent-systems and sprayed with c) SLR and d) ninhydrin. (Tomatine detected with iodine vapour.)

1. 'authentic' tomatine.

2. Root extract.

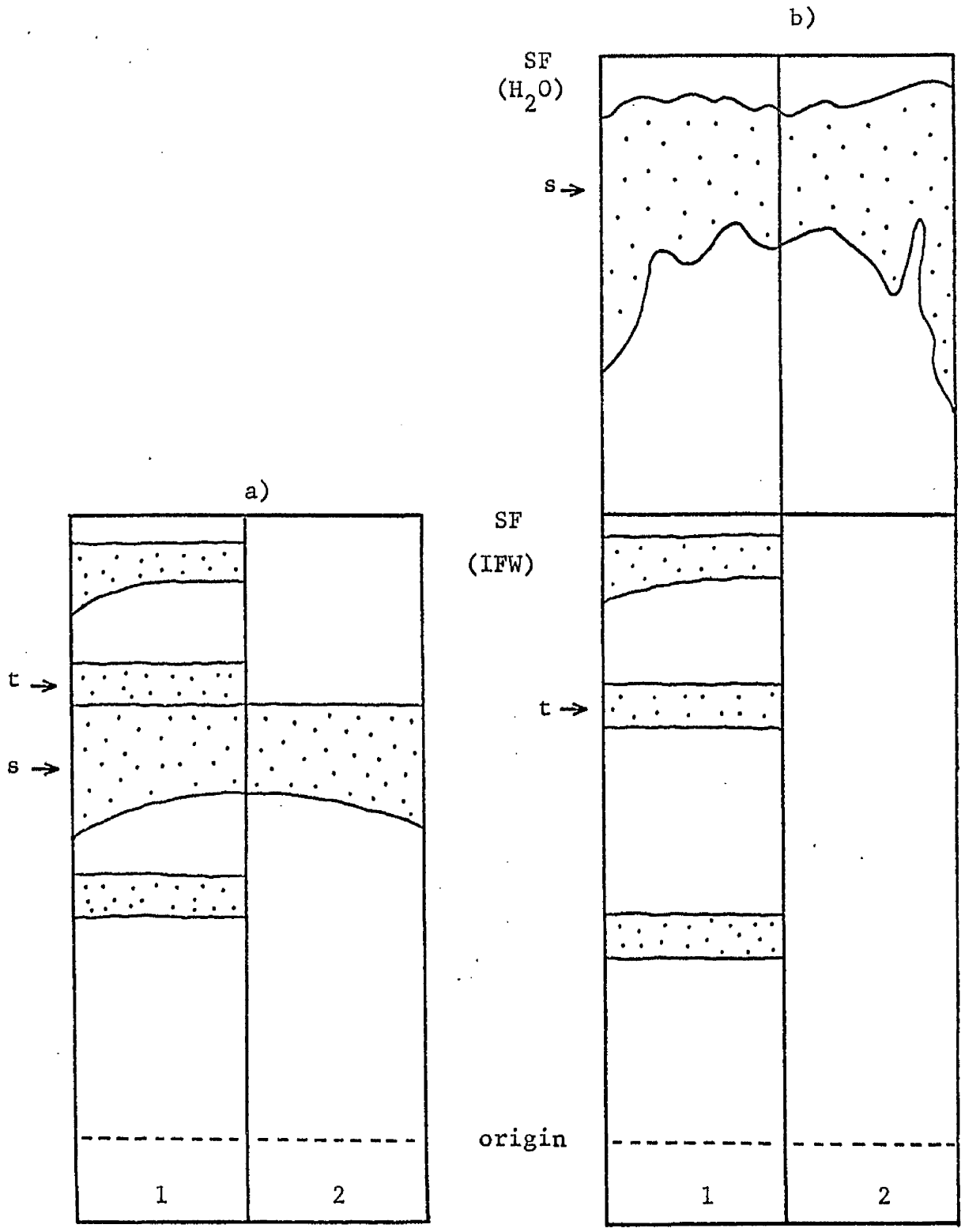

Fig.9. Chromatograms of extracts of cultured roots from one flask grown under standard culture conditions developed in a) IFW and b) the two solvent-systems. Region 1 sprayed with CAS. Region 2 sprayed with SLR. (t = tomatine, s = sugars)

Examination of tomatine purified by TLC using two solvent-systems.

The purity of tomatine, separated by TLC using the above solvent systems, was tested by the absorption spectrum (visible/ultra-violet) of its sulphuric acid chromogen.

Three flasks of cultured roots, grown under standard culture conditions, were harvested and the root extracts applied as bands to separate TLC plates. Chromatograms were developed using the two solvent-systems devised above. Tomatine was located, eluted, and the absorption spectrum of its acid chromogen scanned between 550-250 nm.

None of the discrepancies found in the previous absorption spectrum (fig. 7) of extracted tomatine were present. The positions of all peaks and troughs, as well as the relative depths of the latter, conformed accurately with those in the spectrum of 'authentic' tomatine.

It was therefore concluded that no other compounds in the root extract were present in the tomatine eluate. The compound which lay close to the IFW solvent front, far from contaminating tomatine, proved valuable in quantitative work. The fact that it was visible without spraying and that the tomatine zone always lay parallel to this zone, even on the rare occasions when 'drag' occurred at the edge of the chromatogram, meant that the tomatine zone could be accurately delimited after location of the alkaloid in a 10 mm edge strip.

Confirmation of the identity of extracted tomatine by analytical methods.

After weekly subculturing of the stock clone of excised roots and initiating cultures for experimental work, a number of root culture flasks

remained. These were harvested and the roots stored at -15°C. When sufficient roots had been obtained, they were extracted for tomatine by the method employed for larger amounts of roots and the extract chromatographed on 200 x 200 mm plates using the two solvent-systems. The eluate containing tomatine was divided into two halves, one of which was used to provide tomatidine, and the other to provide the tetra-saccharide moiety.

Analysis of tomatidine. Tomatine was hydrolysed by boiling under reflux in 15 cm^3 of 1.0N HCl for five hours. After cooling, pH was adjusted to 10.0 with ammonia solution (sp.gr. 0.88) and the extract left in a 4°C refrigerator overnight to allow precipitation of the aglycone. The extract was filtered through Whatman No.1 filter paper and the precipitate washed three times with 2 per cent ammonia solution. The filter paper containing the precipitate was dried at 40°C and extracted in a Soxhlet apparatus with diethyl ether for 48 hours. The ether extract was evaporated to dryness under vacuum at 30°C. Tomatidine was taken up three times in ether and the extracts applied (using a Pasteur pipette with an ether-extracted, cotton wool 'wick') to a 200 x 200 mm TLC plate. The chromatogram was developed in chloroform : methanol (94:6). Tomatidine was located using Dragendorff reagent and the aglycone zone eluted in a column (plugged with ether-extracted cotton wool) with 15 cm^3 of ether. The eluate was evaporated to dryness on a rotary evaporator at 30°C.

Tomatidine was taken up in a few cm^3 of ether, a spot of which was then applied to a TLC plate, as was a spot of 'authentic' tomatidine solution. The chromatogram was developed as above and sprayed with CAS.

No compounds other than tomatidine appeared on the plate. The remainder of the ether extract of tomatidine was dried by allowing the solvent to evaporate at room temperature.

Purified tomatidine was analysed by mass spectroscopy. As reference, a mass spectrum was also obtained for 'authentic' tomatidine. The line diagrams constructed from the spectra are shown in fig. 10.

Molecular weights were calculated from the position of the parent ion and found to be, in both cases, 415. The difference in the base (largest) peak, and other slight differences between the line diagrams of extracted and 'authentic' tomatidine can be attributed to the fact that the two analyses were carried out at different times. Nevertheless, the two line diagrams are sufficiently similar to conclude that tomatidine was present in the extract.

Qualitative analysis of the sugar moiety. The second half of the tomatine extract was hydrolysed as before. The acid was evaporated off under vacuum at 45°C, and 15 cm^3 volumes of distilled water added to the flask and evaporated off till all traces of the acid had been removed. Flask contents were taken up in 10 cm^3 of distilled water, filtered through Whatman No.1 filter paper, and the filtrate (containing sugars) evaporated to dryness at 45°C.

The extracted sugars were dissolved in 0.5 cm^3 distilled water and 5µl of this solution was applied as a single spot to a 100 x 200 mm TLC plate. Separate spots containing 2.5µg of 'authentic' glucose, galactose and xylose were also applied, as was a spot containing 2.5µg of each of these sugars. Chromatography plates were prepared with 0.1 N boric acid

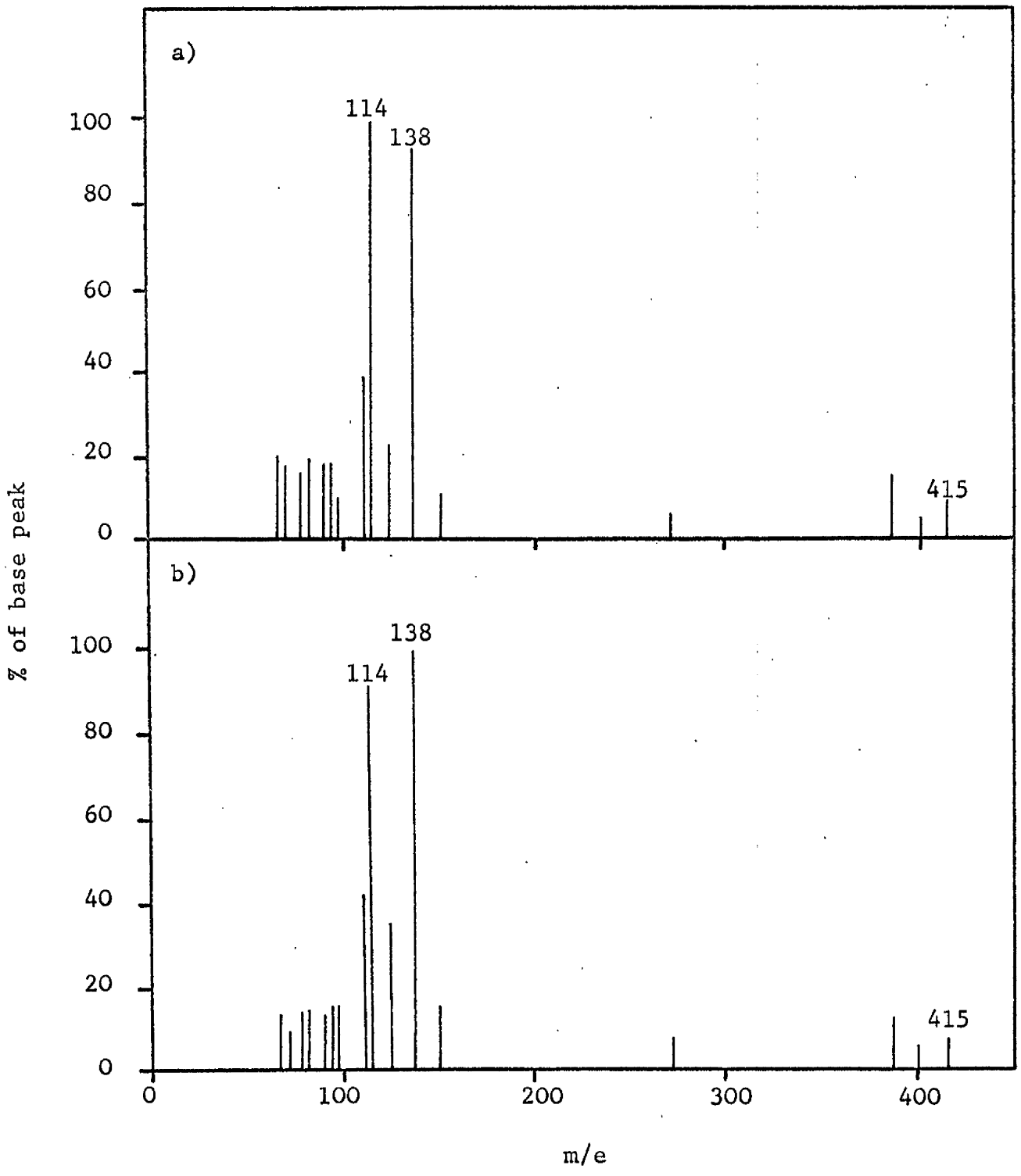

Fig.10. Line diagrams constructed from the mass-spectrum of a) tomatidine released on hydrolysis of extracted tomatine and b) 'authentic' tomatidine.

(Stahl, 1965); the solvent system was methyl ethyl ketone : glacial acetic acid : water (30:10:10); and the locating reagent CAS.

As shown in fig. 11 the sugars obtained from hydrolysed tomatine corresponded to glucose, galactose and xylose. The glucose spot was larger than either the galactose or xylose spots suggesting that relatively more of this sugar was present.

Estimation of total sugars. The total amount of sugar was calculated so that, on estimation of glucose and galactose (see below), xylose could be determined by subtraction. The method employed was adapted from the anthrone technique used by McCready et al., (1950). See appendix 1a. The sugar solution was made to 25 cm^3 with distilled water, but in order to preserve enough of the extract for determination of glucose and galactose only one calculation of total sugar (involving 5 cm^3 of extract) was possible (table 5).

Estimation of glucose and galactose. The remaining 20 cm^3 of extract was evaporated to dryness under vacuum at 45°C and the sugars then taken up in 0.5 cm^3 of distilled water. Use of such a small volume was necessary to obtain measurable concentrations of the hexose sugars, but as each test required 0.2 cm^3 of solution, only one determination of each sugar was possible.

Glucose and galactose were determined using 'Biochemica Test Combination' kits obtained from the Boehringer Corporation (see appendices 1b and 1c). Briefly, the methods involved oxidation of glucose-6-phosphate (G-6-P) by G-6-P dehydrogenase and subsequent reduction of NADP, and oxidation of galactose by galactose dehydrogenase and subsequent reduction

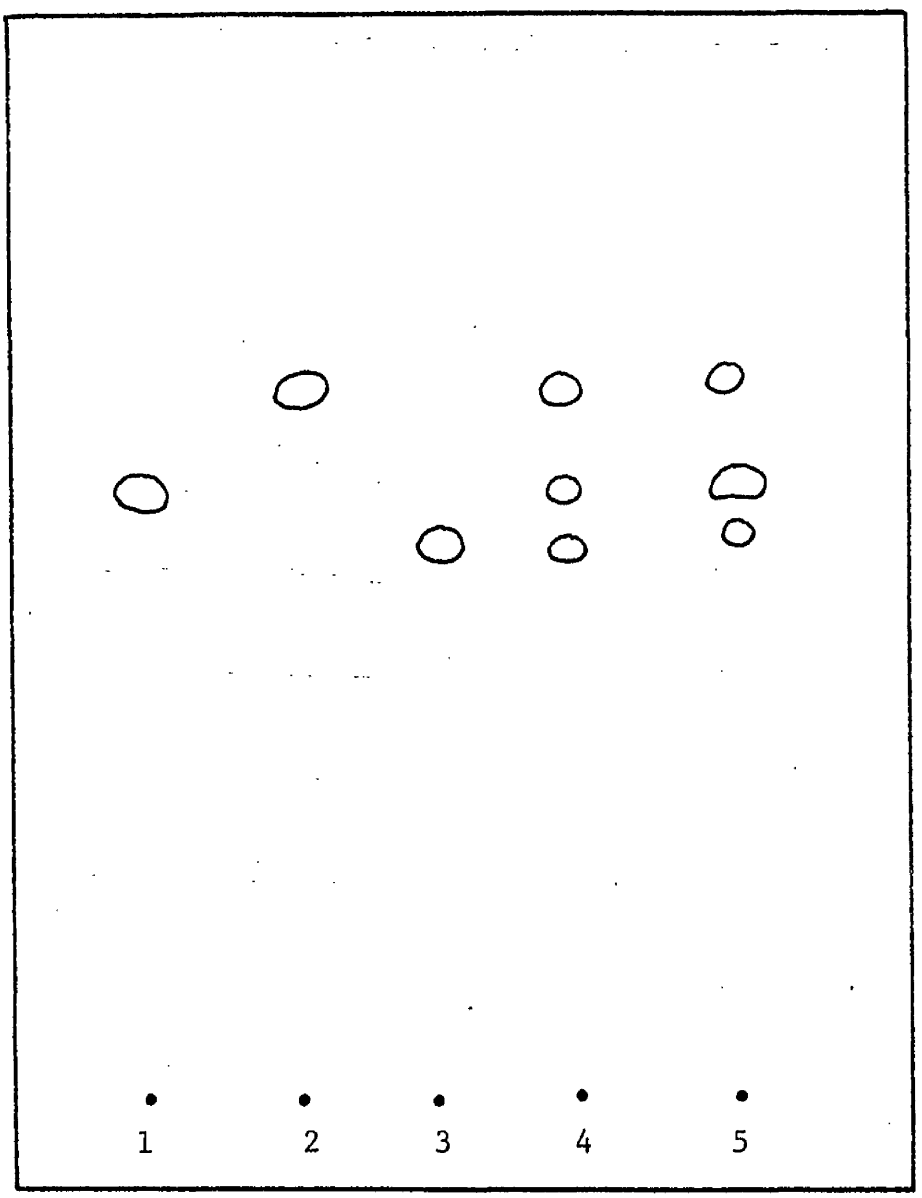

Fig.11. Chromatogram of sugars from hydrolysed tomatine developed in methyl ethyl ketone/glacial acetic acid/water.

1. 'authentic' glucose
2. " xylose
3. " galactose
4. 1+2+3.
5. sugars from hydrolysed tomatine.

TABLE 5. Data from sugar analyses of extracted tomatine.

Sugar	Method	Concentration (mg 100 cm^{-3})
Total sugar	Anthrone	20.00
Glucose	G-6-P dehydrogenase	9.18
Galactose	galactose dehydrogenase	5.04
Xylose	by subtraction	5.78

Galactose : glucose : xylose = 1.0 : 1.8 : 1.1

of NAD. The amount of absorbance by the reduced co-enzymes, as measured on a Pye 'Unicam' SP500 spectrophotometer, was proportional to the amount of sugar present.

The concentrations of glucose and galactose calculated and the amount of xylose present are shown in table 5.

Although no replicate determinations were conducted the ratio of sugars was sufficiently close to the expected 1:2:1 ratio to provide, in conjunction with the mass-spectroscopic data for the aglycone moiety, conclusive evidence that cultured excised tomato roots synthesise α-tomatine.

The efficiency of the extraction procedure for tomatine.

Before adoption of the extraction technique incorporating the TLC methods developed above, an experiment was carried out to investigate whether the amounts of tomatine revealed by the assay technique were an accurate indication of the amounts of alkaloid in the tissues.

In the following procedure each treatment was replicated five times.

Excised roots were harvested and, before homogenisation 0.03 cm^3 of an 'authentic' tomatine solution (10 mg in 10 cm^3 of methanol) were added to the beakers using an 'Agla' micrometer syringe. Similar volumes of the solution were dispensed into test-tubes. Controls consisted of harvested roots in beakers to which had been similarly dispensed 0.03 cm^3 of methanol and test-tubes containing the same volume of methanol.

Tomatine was extracted from the harvested roots and assayed. The mean amount of tomatine added to the five beakers containing roots was

calculated by assaying the tomatine in the test-tubes. The recovery of added alkaloid is shown in table 6.

The relatively high standard error for the mean recovery of tomatine was to be expected since replicate roots to which tomatine had been added were all assumed to contain the same level of endogenous alkaloid (0.5µg mg^{-1} fresh weight), which would not be the case. It is also appreciated that the added alkaloid would be extracted more easily than would be the endogenous alkaloid. Nevertheless, the results indicate that the efficiency of the extraction procedure was sufficiently high to allow its adoption for future quantitative studies.

SECTION 3. ROOT GROWTH AND TOMATINE PRODUCTION.

The following experiments were prompted by reports from various workers that some secondary products are synthesised only in actively growing tissues, whereas others are elaborated only in mature tissues in which growth has ceased.

Comparison of growth and tomatine production in seedling radicles and cultured excised roots of tomato.

After ten days growth under standard conditions cultured roots from seven flasks were harvested. Length of the main axis (LMA), lateral root number (LN) and total lateral length (TLL) were measured on roots from three of these flasks, while those from the remaining four were analysed for tomatine.

Tomato seeds were placed in petri-dishes (ten per dish) containing

TABLE 6. Efficiency of the extraction procedure for tomatine, as measured by recovery of added alkaloid.

No tomatine added	tomatine added				
tomatine ($\mu g\ mg^{-1}FW$)	'calculated' endogenous tomatine (μg)	total tomatine extracted (μg)	tomatine added (μg)	tomatine recovered (μg)	% recovery
0.50 $\pm$0.02	70.7 $\pm$4.4	96.9 $\pm$6.7	29.2 $\pm$0.8	26.2 $\pm$2.8	89.8 $\pm$9.7

Results are expressed on a per flask basis.

two sheets of Whatman No.1 filter paper and 5 cm^3 of distilled water, and left to germinate under low light at 25°C. Seedling growth was allowed to continue for ten days after development of a 10 mm long radicle. (The 10 mm radicle was held to be the equivalent of the 10 mm tip used as inoculum for cultured roots.) Seven days after commencement of the experiment a further 3 cm^3 of water were added to each petri-dish to prevent desiccation of the seedlings.

At the end of the growth period, radicles were excised and growth measurements and tomatine analyses conducted. The contents of two petri-dishes were used for each of the four replicate tomatine analyses and growth was measured in 20 radicles from two petri-dishes. Due to differences in the fresh weight/dry weight ratios of seedling radicles and cultured roots, results have been expressed on a dry-weight basis.

Although main axis length was identical in seedling radicles and cultured roots, extensive lateral root development occurred in the latter, whereas none were produced by the former (table 7a). In appearance, cultured roots were thicker and more robust than radicles and this was reflected in their higher (c. 10x) dry matter accumulation. However, despite their higher growth rates, cultured roots contained only about one third the amount of tomatine on a unit dry-weight basis found in seedling radicles (table 7b).

Tomatine production by cultured roots at different stages of growth.

Cultured roots were harvested at two-day intervals over a period of ten days, and also after 17 and 24 days. For alkaloid determination at

TABLE 7a. Growth of ten-day old cultured roots and seedling radicles.

	LMA (mm)	LN	TLL (mm)
cultured roots	128.7 ±3.4	30.4 ±2.0	385.8 ±39.7
seedling radicles	121.6 ±4.3	0	-

TABLE 7b. Tomatine content of ten-day old cultured roots and seedling radicles.

	dry weight (mg)	total tomatine (µg)	tomatine ($\mu g\ mg^{-1}$ DW)
cultured roots	2.67 ±0.20	11.97 ±1.32	4.44 ±0.20
seedling radicles	0.26 ±0.01	3.64 ±0.53	14.02 ±2.34

Results are expressed on a per root basis.

day 0, 10 mm inoculum tips were used. Four replicates, each consisting of a requisite number of flasks to provide sufficient material, were taken at each harvest, except at day 0 when three replicates of 200 root tips were used. After fresh weight determinations, tomatine was extracted and assayed.

Both total alkaloid and fresh weight showed similar increases (x48 and x51 respectively) over the first ten days (fig. 12a). Increases in amount of tomatine began to decline after this time and no net accumulation of alkaloid was observed after 17 days. The graph for fresh weight, on the other hand, showed no indication of entering the senescent phase even after 24 days. During the 24 day period, fresh weight and total tomatine increased by factors of 129 and 64 respectively.

When results were plotted on a two day incremental basis (over ten days only) the positive correlation* between growth rate and rate of tomatine production was more apparent (fig. 12b). The greatest increase in fresh weight occurred between 6-8 days when rate of tomatine synthesis was also at its maximum. A slight decrease in growth rate occurred between 4-6 days although the reason for this was not known. However, the fact that this was accompanied by a decrease in rate of tomatine synthesis was further evidence of a positive correlation between these two factors. Despite maintenance of the high growth rate between 8-10 days, rate of tomatine synthesis fell sharply.

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*The term 'positive correlation' is used here to denote a relationship between two factors in which increases or decreases in one are accompanied by increases or decreases respectively in the other. Direct proportionality between the two factors is not implied.

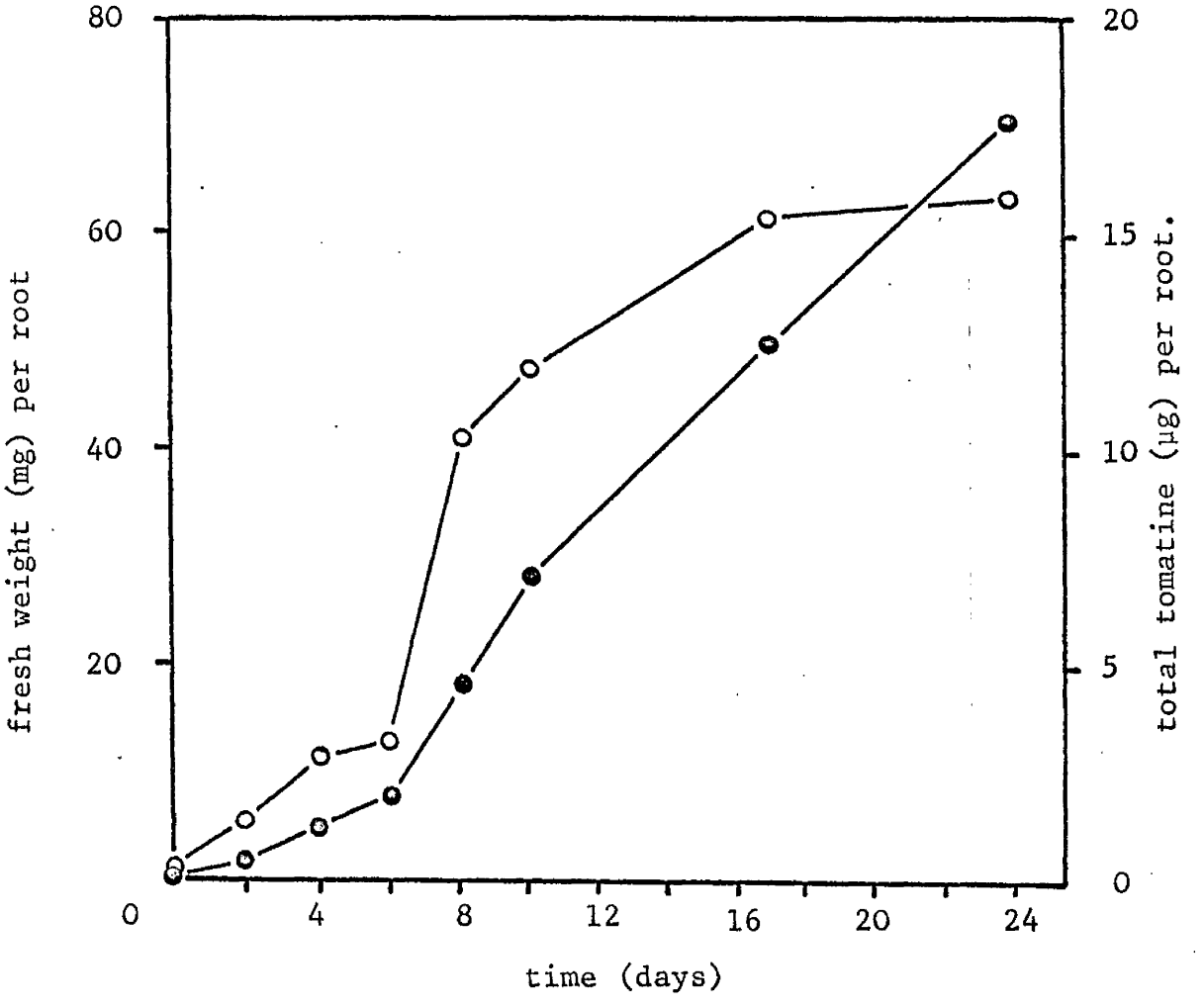

Fig. 12a. Tomatine production in cultured roots over a 24 day growth period. (Data in appendix 2.)

●——● fresh weight

○——○ total tomatine

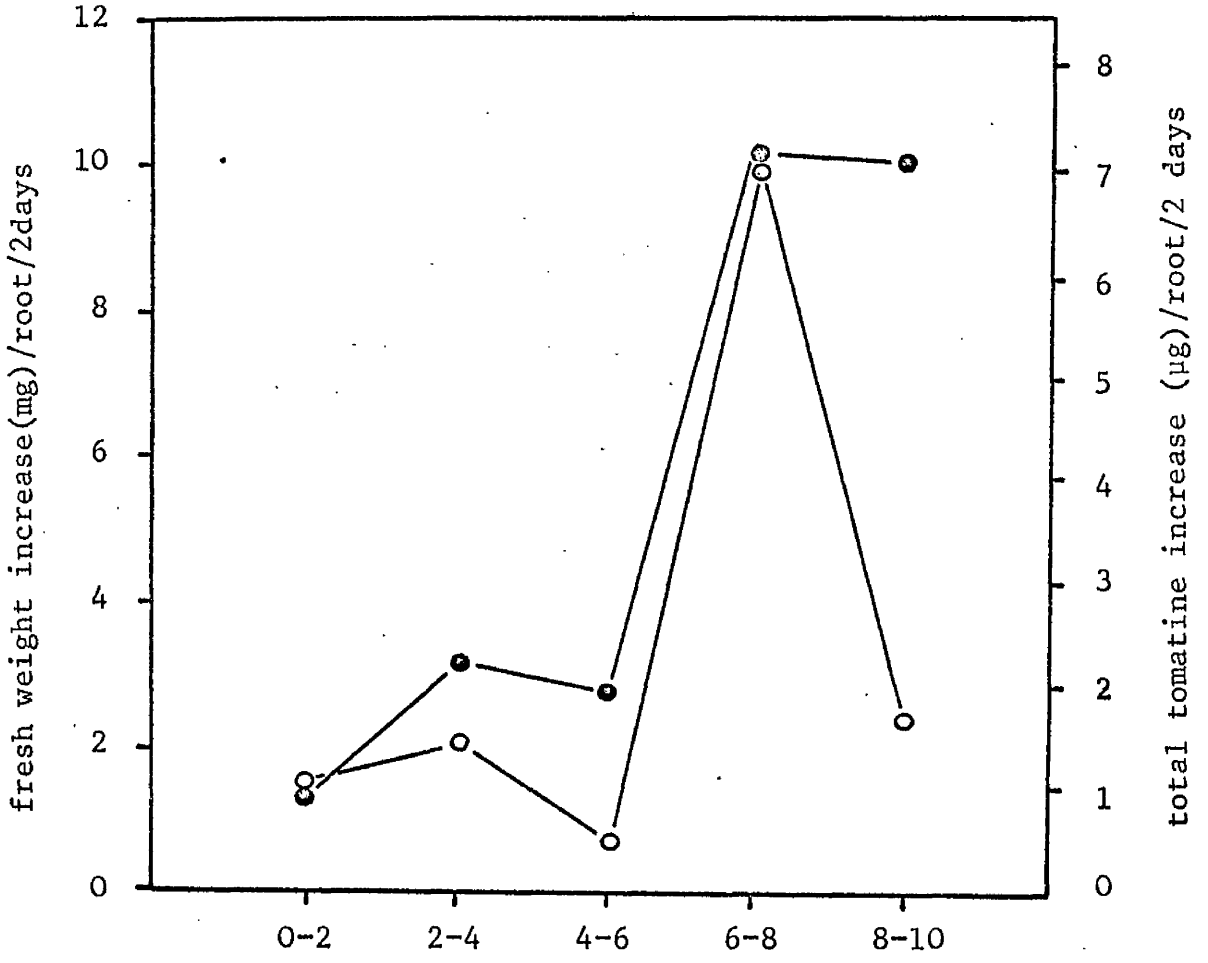

Fig.12b. Two-day increments in tomatine production in cultured roots during the first ten days of a 24 day growth period. (Data from appendix 2.)

●——● fresh weight increase

○——○ total tomatine increase

Changes in the amount of tomatine per unit of fresh weight are shown in fig. 12c. Alkaloid levels increased by 56 per cent during the first two days of growth but thereafter decreased. After 24 days, the concentration of tomatine was 51 per cent of that in the inoculum tips at day 0.

These results suggest that a positive correlation exists between synthesis of tomatine and growth of cultured roots, as measured by fresh weight, and that actively growing cells are the sites of tomatine biosynthesis.

Attempt to determine the site of tomatine synthesis in the tomato root.

Direct analysis of the apical region of the root at different times is hindered by the fact that, in such regions, cells are constantly 'moving out' in a basipetal direction. An indirect approach was therefore adopted in which non-meristematic basal regions of the cultured root were analysed for tomatine at different times.

Roots were harvested after three and ten days and the basal 30 mm of each root excised. Lateral roots were removed from these regions which were then extracted for tomatine. Each treatment consisted of four replicates and each replicate of 15 basal segments. Three- and ten-day old segments differed in fresh weight/dry weight ratio and therefore results have been expressed on a dry-weight basis.

Although ten-day old segments were almost double the weight of three-day old segments, no further accumulation of tomatine had taken place in the former in the intervening seven days (table 8). The nature of the

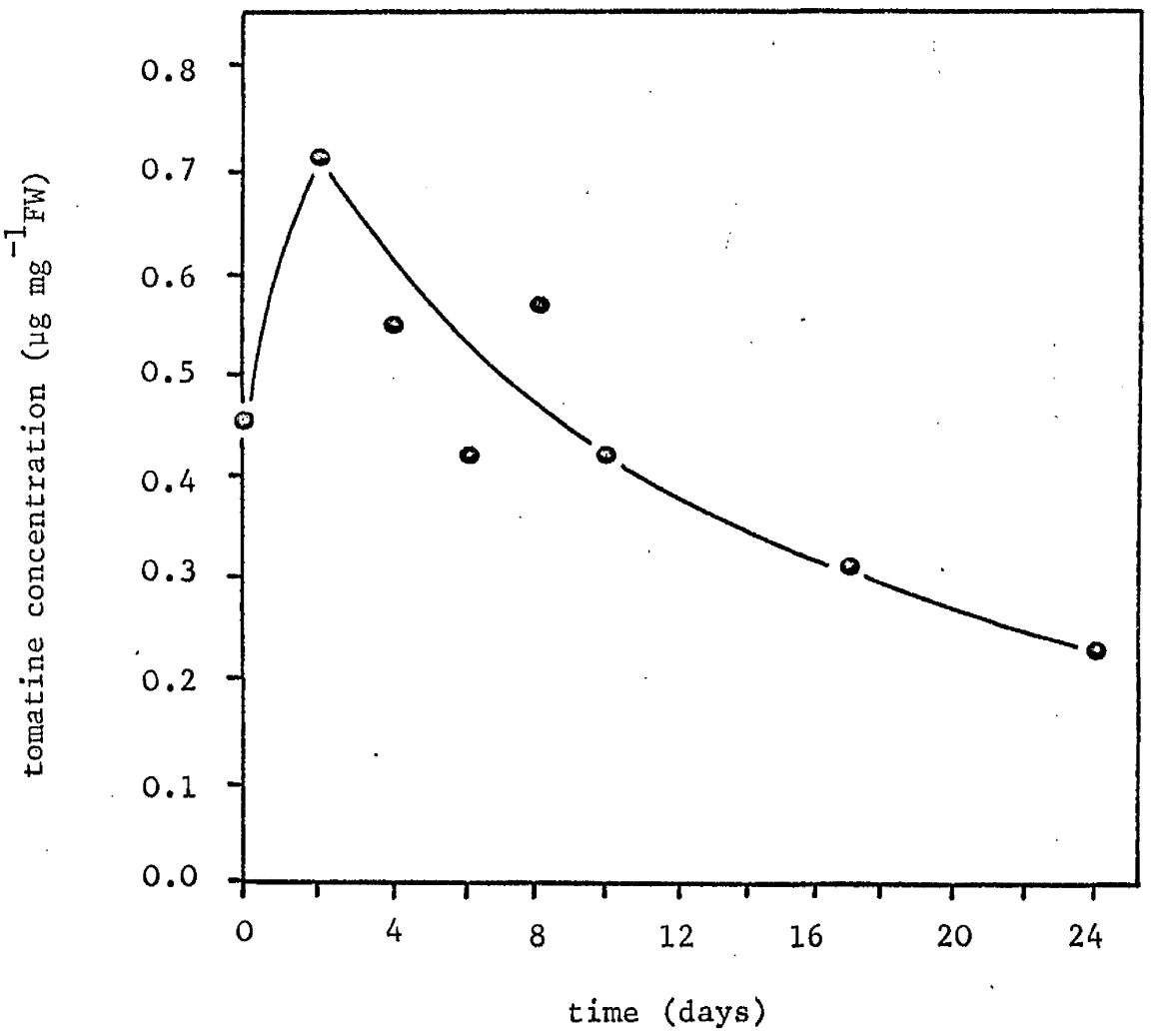

Fig.12$_c$. Concentration of tomatine in cultured roots over a 24 day growth period. (Data in appendix 2.)

TABLE 8. Tomatine content of three- and ten-day old basal root segments.

	dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1}_{DW})
3-day	0.27 ±0.02	2.09 ±0.77	7.74 ±3.04
10-day	0.47 ±0.02	1.85 ±0.47	3.94 ±1.12

Results are expressed on a per segment basis.

growth which had taken place was not known.

By indicating that tomatine production does not occur in relatively mature tissues, these results provide some evidence that apical regions are the sites of alkaloid biosynthesis.

Extraction of root culture medium for tomatine.

One possible explanation for the lack of further tomatine accumulation in roots older than 17 days was that the alkaloid was excreted into the culture medium. This possibility was tested as follows:-

Root cultures were grown under standard conditions for 30 days after which they were divided up to give two replicates, one of 12 flasks and one of 13 flasks. Roots were removed and discarded and the culture medium in each replicate was bulked (giving 1.2ℓ and 1.3ℓ respectively) and reduced to 50 cm^3 on a rotary evaporator at 45°C. The medium was extracted for tomatine using the method employed for large amounts of cultured roots. Extracts were applied, along with 'authentic' tomatine and tomatidine, to TLC plates which were developed using the two solvent-systems. Chromatograms were sprayed with Dragendorff reagent, inspected and then re-sprayed with CAS.

No Dragendorff-positive spots were present in the medium extract and CAS confirmed the absence of substances with Rf values corresponding to those of tomatine or tomatidine.

It was therefore concluded that lack of further tomatine accumulation in cultured roots older than 17 days was not due to excretion of the alkaloid into the culture medium.

Distribution of tomatine in cultured roots.

Since root apices appeared to be the sites of tomatine synthesis, an experiment was conducted to determine whether the alkaloid also accumulated in such regions or was evenly distributed throughout the root system.

Cultured roots were transferred to petri-dishes and separated carefully to avoid breakage of lateral roots, etc. Each root was then cut up to provide the following regions. Tip 5 mm of the main axis; tip 5 mm of lateral roots, including all laterals equal to or less than 5 mm; remainder of the lateral roots; apical, middle and basal third of the main axis after removal of lateral roots. Results recorded in table 9 are means of three replicates. Due to differences in the fresh weight/dry weight ratios of the three regions of the main axis (greater towards the apical end) results have been expressed on a dry-weight basis.

Tomatine was present in all regions of the cultured root. Alkaloid levels were similar in the different regions tested and there was no evidence of accumulation in apical regions. From these data it was calculated that 2.5 per cent of the total alkaloid in one cultured root is found in the tip 5 mm of the main axis, 38.5 per cent in the remainder of the main axis and 59.0 per cent in the lateral roots. These values are very close to the relative dry weights of these regions which were 2.5 per cent, 41.4 per cent and 56.1 per cent respectively.

TABLE 9. Tomatine content of different regions of the cultured root.

	number per replicate	dry weight (mg)	total tomatine (µg)	tomatine ($\mu g\ mg^{-1}_{DW}$)
Tip of main axis	150 tips	0.052 ±0.001	0.22 ±0.01	4.34 ±0.19
Apical $\frac{1}{3}$ of main axis	8 flasks	0.23 ±0.01	1.00 ±0.05	4.35 ±0.26
Middle $\frac{1}{3}$ of main axis	8 flasks	0.31 ±0.01	1.18 ±0.14	3.76 ±0.39
Basal $\frac{1}{3}$ of main axis	8 flasks	0.28 ±0.02	1.23 ±0.21	4.34 ±0.50
Tips of laterals	4 flasks	0.53 ±0.02	2.64 ±0.19	4.98 ±0.20
Remainder of laterals	4 flasks	0.58 ±0.01	2.59 ±0.11	4.44 ±0.15

Results refer to appropriate regions in one cultured root.

Effect of tomatine on the growth of cultured roots.

'Authentic' tomatine (100 mg) was dissolved in 500 cm^3 of distilled water at pH 3.0. The solution was serially diluted and the appropriate volume of each dilution added to root culture medium to give tomatine concentrations of 0.01, 0.1, 1.0, 10.0 and 100.0 ppm. The pH of the culture medium was then adjusted to 4.8 and the medium autoclaved. Growth was measured by LMA, LN and TLL. Each treatment was replicated ten times.

Exogenously applied tomatine, up to concentrations of 100 ppm, did not affect cultured root growth as measured by the above criteria (table 10).

Uptake of tomatine by cultured roots.

This experiment was an extension of the previous one and was carried out to decide if exogenous tomatine was absorbed and metabolised by cultured tomato roots. The method was based on estimation of tomatine in roots and in culture medium containing 'authentic' alkaloid after a suitable incubation period.

Tomatine was added to root culture medium at a concentration of 100 ppm. The three treatments were - 'normal' culture medium with excised roots, culture medium containing tomatine with excised roots, culture medium containing tomatine without excised roots. The last treatment was to ensure that any loss of tomatine from the culture medium was not due to chemical modification or degradation of the alkaloid under standard culture conditions. Each treatment consisted of four replicates.

TABLE 10. Effect of tomatine on the growth of cultured roots.

tomatine concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	132.4 ±5.0	55.2 ±2.2	257.0 ±19.7
0.01	139.0 ±4.6	53.9 ±3.7	220.5 ±15.9
0.1	136.2 ±4.5	52.1 ±2.7	241.0 ±14.5
1.0	134.6 ±5.6	50.7 ±2.7	216.0 ±17.5
10.0	146.6 ±5.5	54.5 ±2.8	224.5 ±19.9
100.0	135.2 ±8.7	51.2 ±4.1	200. ±20.5

Before measuring fresh weights, roots were washed in a glass column for 20 minutes with 10 cm^3 of distilled water to remove tomatine from the 'free space'. Washings were collected, their volumes measured, and, after measuring the volume of culture medium remaining in each flask, returned to the appropriate flask. From each flask, 1 cm^3 of medium was withdrawn and evaporated to dryness under vacuum at 45°C. The contents of this sample were taken up in methanol, chromatographed, and assayed as for cultured roots. Tomatine was extracted from cultured roots by the standard method.

Cultured roots grown in the presence of exogenous tomatine contained more alkaloid than those grown in 'normal' culture medium, the mean increase being 135µg (table 11a). The levels of tomatine in culture medium both with and without cultured roots were identical (table 11b). Due to the smallness of the alkaloid increase in cultured roots in relation to the amount of tomatine added to the culture medium, it was not possible to decide if tomatine had been absorbed by root cells. The higher tomatine levels could have been due to incomplete removal of exogenous alkaloid from the 'free space'.

Nevertheless, it could be concluded that large amounts of tomatine are not absorbed from the nutrient medium by cultured tomato roots. The 100 per cent recovery of tomatine from culture medium indicates that tomatine-degrading enzymes are not secreted by cultured roots into the medium.

TABLE 11a. Tomatine content of cultured roots grown in the presence of tomatine (100 ppm).

	fresh weight (mg)	total tomatine (µg)	tomatine ($\mu g\ mg^{-1}_{FW}$)
-tomatine	163.3 ±9.3	77.71 ±8.84	0.47 ±0.03
+tomatine	151.2 ±2.0	212.69 ±15.89	1.40 ±0.09

Results are expressed on a per flask basis.

TABLE 11b. Recovery of tomatine added to root culture medium at 100 ppm.

	volume of medium recovered (cm^3)	tomatine ($\mu g\ cm^{-3}$ of recovered medium)	tomatine ($mg\ 100\ cm^{-3}$ of original medium)
-roots	94.8 ±1.2	112.55 ±20.14	10.7 ±1.9
+roots	103.8* ±0.9	103.04 ±20.19	10.7 ±2.0

Results are expressed on a per flask basis.

* volume includes the 10 cm^3 root washing.

Effect of tomatine on the growth of tomato and lettuce seedlings.

Since tomatine did not influence growth of cultured tomato roots, the following experiment was conducted to study whether intact tomato seedling organs responded differently to tomatine. Lettuce was also used to further compare the growth responses of non-tomatine- and tomatine-producing species to exogenous alkaloid.

Tomatine solutions were prepared by dissolving 'authentic' alkaloid in distilled water at pH 3.0 and then re-adjusting pH to 5.5. The concentrations of solutions used were 0.1, 1.0, 10.0 and 100.0 ppm. Ten seeds of tomato (var. Suttons Best of All) or lettuce (var. Grand Rapids, obtained from Thompson and Morgan Ltd., Ipswich), were placed in petri-dishes containing two sheets of Whatman No.1 filter paper and 5 cm^3 of the appropriate tomatine solution. Four replicate petri-dishes were used for each treatment. When incubated at 25°C in the dark, lettuce seeds germinated after one day and tomato seeds after five days. Hypocotyl and radicle lengths were measured in lettuce and tomato seedlings after three and seven days respectively (i.e. two days after germination). Results are shown in table 12.

Tomatine did not affect elongation of either the radicle or hypocotyl in tomato seedlings. Tomatine was also without effect on the hypocotyl of lettuce seedlings, but at 100 ppm radicle growth was inhibited.

SECTION 4. THE EFFECTS OF SOME PHYSICAL FACTORS ON TOMATINE PRODUCTION.

Preliminary qualitative analyses of cultured roots had suggested

TABLE 12. Effect of tomatine on the growth of tomato and lettuce seedlings.

tomatine concentration (ppm)	Tomato		Lettuce	
	hypocotyl length (mm)	radicle length (mm)	hypocotyl length (mm)	radicle length (mm)
0	17.6	58.7	11.9	21.7
	±1.2	±3.7	±0.8	±1.6
0.1	18.5	64.9	9.3	21.5
	±1.7	±3.9	±0.9	±4.2
1.0	12.5	46.8	11.7	23.6
	±1.1	±2.9	±0.3	±1.6
10.0	13.8	53.7	10.5	23.4
	±1.1	±3.4	±0.8	±1.9
100.0	18.0	53.3	11.1	8.7
	±1.7	±3.7	±0.6	±0.6

that the amount of tomatine in light-grown roots was less than that in dark-grown roots. The possibility that light inhibits tomatine synthesis in cultured roots was studied by analysing for alkaloid, roots cultured under different light intensities. The effect of temperature on tomatine production was also investigated to determine if the previously observed correlation between root weight and tomatine production also existed under sub- and supra-optimal conditions.

Effect of light intensity on tomatine production.

Different light intensities were achieved by placing flasks at different distances from a bank of fluorescent lamps ('Warm White'). Light intensity was measured at a level corresponding to the surface of the culture medium in the flask using an 'EEL' electroselenium light meter. The light intensities used were 540, 1620 and 3240 lx. Control flasks were covered with aluminium foil to exclude light and each treatment was replicated four times. Two further flasks, one 'uncovered' and the other 'covered', and both lacking roots but containing a thermometer, were used for each treatment to detect temperature changes. Results are shown in table 13.

The only significant difference in root growth between 'light' and 'dark' treatments occurred under light intensity of 3240 lx. However, the decreased growth in flasks exposed to light could have been due to supra-optimal temperature (28°C) rather than to light intensity.

Tomatine concentrations in light-grown roots, in no cases, differed from those in dark-grown controls.

TABLE 13. Effect of light intensity on tomatine production in cultured roots.

light intensity (lx)	light treatment	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1}_{FW})	final culture medium temperature (^{o}C)
	light	119.0	47.45	0.41	24
540		±14.8	±2.83	±0.03	
	dark	115.3	58.51	0.51	24
		±12.3	±4.00	±0.02	
	light	121.2	49.79	0.41	25
1620		±10.1	±4.68	±0.01	
	dark	105.3	49.97	0.47	25
		±11.7	±7.80	±0.04	
	light	109.1	53.15	0.50	28
3240		±3.9	±7.66	±0.09	
	dark	165.2	72.11	0.44	25
		±5.0	±6.58	±0.03	

Results are expressed on a per flask basis.

Effect of temperature on tomatine production.

Excised root cultures were grown in dark incubators at 15, 20, 25, 30 and 35°C. Regular checks showed that these temperatures were maintained to within ±1°C over the ten-day growth period. Four replicate analyses were carried out for each treatment.

The optimum temperature for growth (as measured by fresh weight) was 25°C, with growth at the temperature extremes being c.3 per cent of that at 25°C. At 20°C, fresh weight was reduced by 66 per cent whereas at 30°C the reduction was 34 per cent.

The graph for total amount of tomatine followed that for fresh weight very closely except at 30°C (fig. 13). Between 25°C and 30°C fresh weight decreased by 34 per cent, whereas total alkaloid increased by 13 per cent. Nevertheless, the results still indicate a positive correlation between these two factors.

Changes in tomatine concentration approximately reciprocated those in growth. The concentration of tomatine in roots grown under the standard temperature of 25°C agreed closely with values obtained in previous experiments, but at sub- and supra-optimal temperature, tomatine concentrations were consistently higher by about 73 per cent.

SECTION 5. EFFECT OF ENRICHMENT OF THE CULTURE MEDIUM ON TOMATINE PRODUCTION.

In the following experiments, attempts were made to increase growth of cultured roots by supplementing the culture medium with various nitrogenous compounds. The object was to investigate whether alterations in

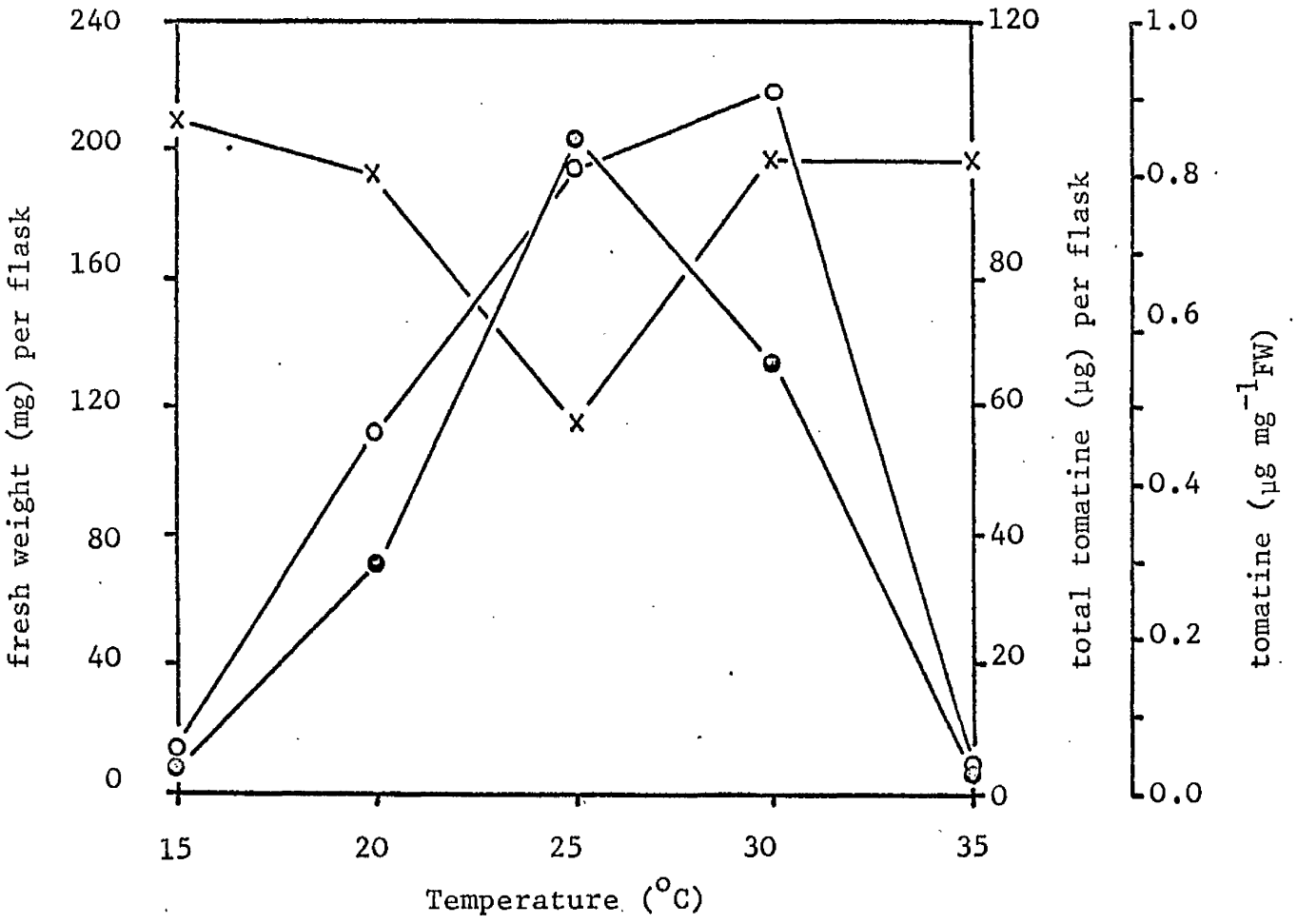

Fig.13. Effect of temperature on tomatine production in cultured roots. (Data in appendix 3.)

●——● fresh weight

○——○ total tomatine

×——× tomatine concentration

growth rates would be accompanied by changes in the total amount or concentration of tomatine in the roots. The three compounds used were ammonium sulphate (inorganic), urea (simple organic) and casamino acids (complex organic). In each case a second experiment was carried out to test the effects of these compounds on cultured root growth as measured by linear dimensions and lateral root number.

Effect of ammonium sulphate on root growth and tomatine production.

The concentrations of ammonium sulphate in the culture medium were 5.3, 10.7, 53.4 and 106.8 ppm. These values were respectively 0.025, 0.05, 0.25 and 0.50 of the standard concentration used by Sheat et al. (1959). The appropriate amount of ammonium sulphate was weighed out and added directly to the culture medium. For growth measurements, 12 replicate flasks were used per treatment, and for tomatine determination, four replicate flasks.

As shown in fig. 14a, neither the length of the main axis nor the number of lateral roots were affected by addition of ammonium sulphate, although a depression occurred in the latter curve at the lowest salt concentration. However, the total lengths of lateral roots were reduced to about 70 per cent of the control value at all concentrations.

Fresh weight tended to show a steady decline with increasing concentration of ammonium sulphate and total amount of tomatine also decreased in a similar manner (fig. 14b). At the highest salt concentration, fresh weight and total alkaloid showed decreases of 31 per cent and 57 per cent respectively. Depressions were again present in both curves at the lowest

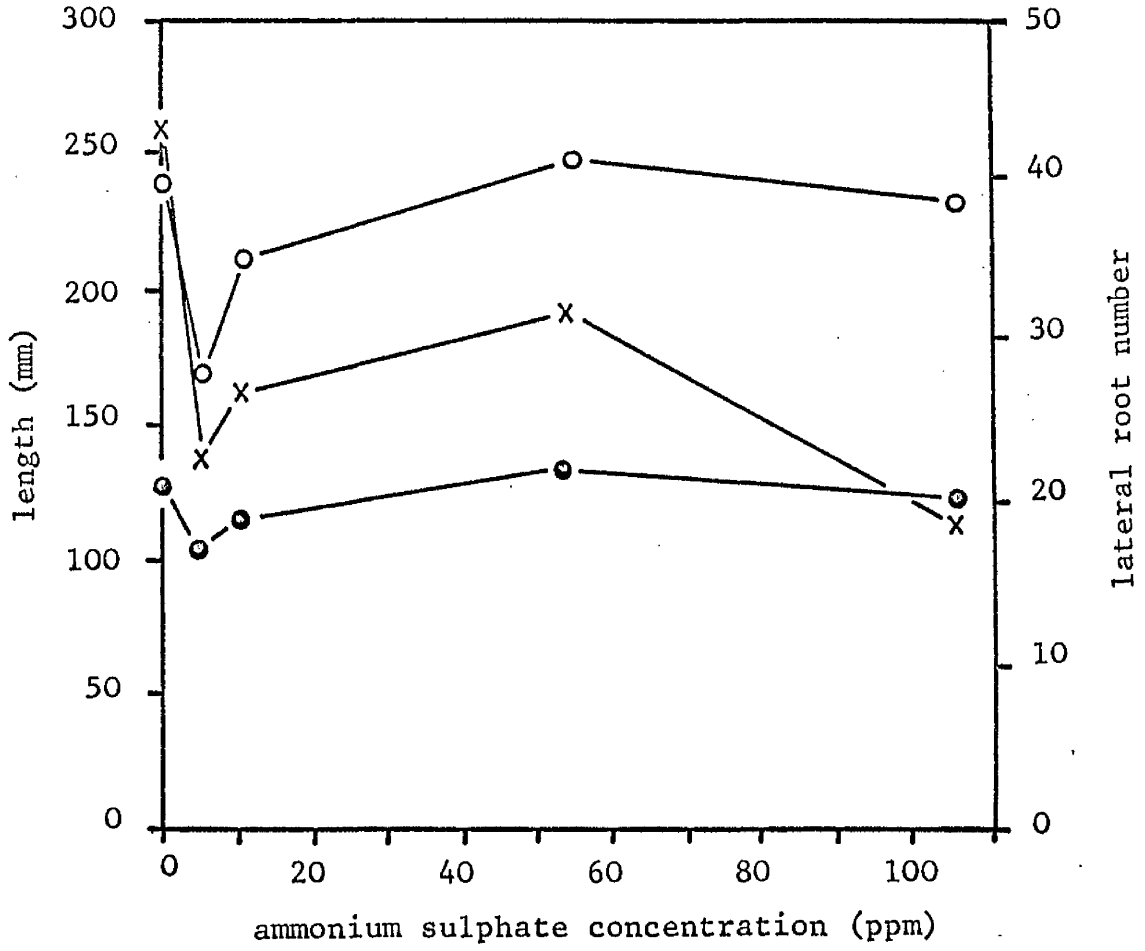

Fig.14a. Effect of ammonium sulphate on the growth of cultured roots. (Data in appendix 4a.)

●——● LMA

○——○ LN

x——x TLL

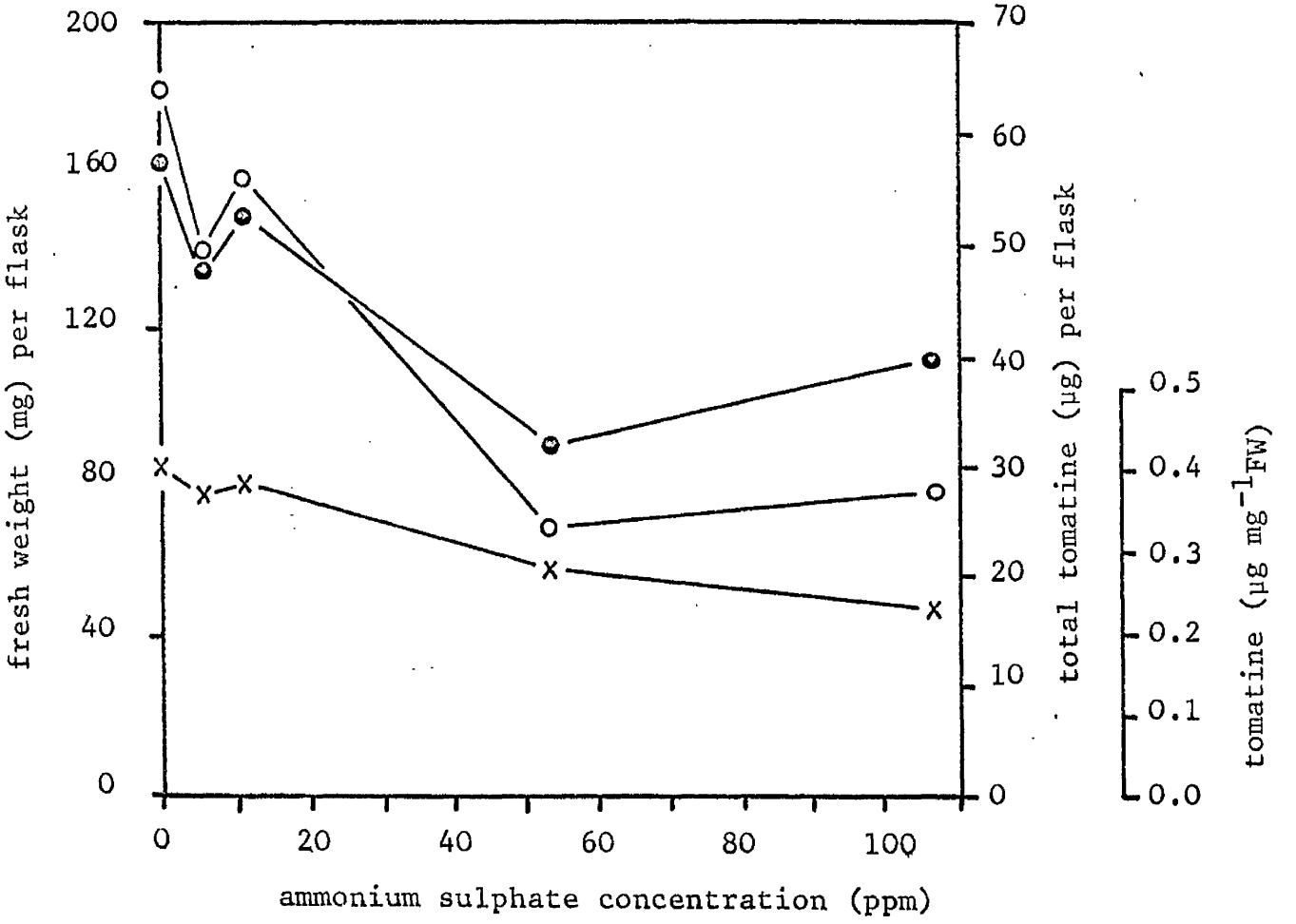

Fig.14b. Effect of ammonium sulphate on tomatine production in cultured roots. (Data in appendix 4b.)

●——● fresh weight

o——o total tomatine

x——x tomatine concentration

concentration of ammonium sulphate. Concentrations of tomatine also decreased with increasing levels of the salt, and at 106.8 ppm ammonium sulphate alkaloid levels were about 58 per cent of control values.

Effect of urea on root growth and tomatine production.

Stock solutions of urea were first prepared by transferring sterile 40 per cent urea from ampoules (Oxoid Ltd., London) to an appropriate volume of sterile distilled water. For growth experiments, 1 cm^3 aliquots of different urea solutions were aseptically added to 49 cm^3 of sterile culture medium, and for tomatine determination experiments, 2 cm^3 to 98 cm^3 of culture medium. The final concentrations of urea were 25, 50, 100 and 200 ppm.

Urea did not affect lateral root number but main axis length was slightly greater at 200 ppm urea and total lateral length tended to increase with increasing concentrations of the additive (fig. 15a).

Fresh weight and total tomatine were unaffected by 25 and 50 ppm urea but at 100 and 200 ppm both showed increases of about 22 per cent (fig. 15b). Relative changes in fresh weight and total tomatine were so similar that concentrations of tomatine remained unaltered throughout the experiment.

Effect of casamino acids on root growth and tomatine production.

Bacto-casamino acids (Difco Laboratories, Detroit, Mich., U.S.A.) were weighed out and added directly to the culture medium to give final concentrations of 50, 100, 250 and 500 ppm. Ten and four replicates per

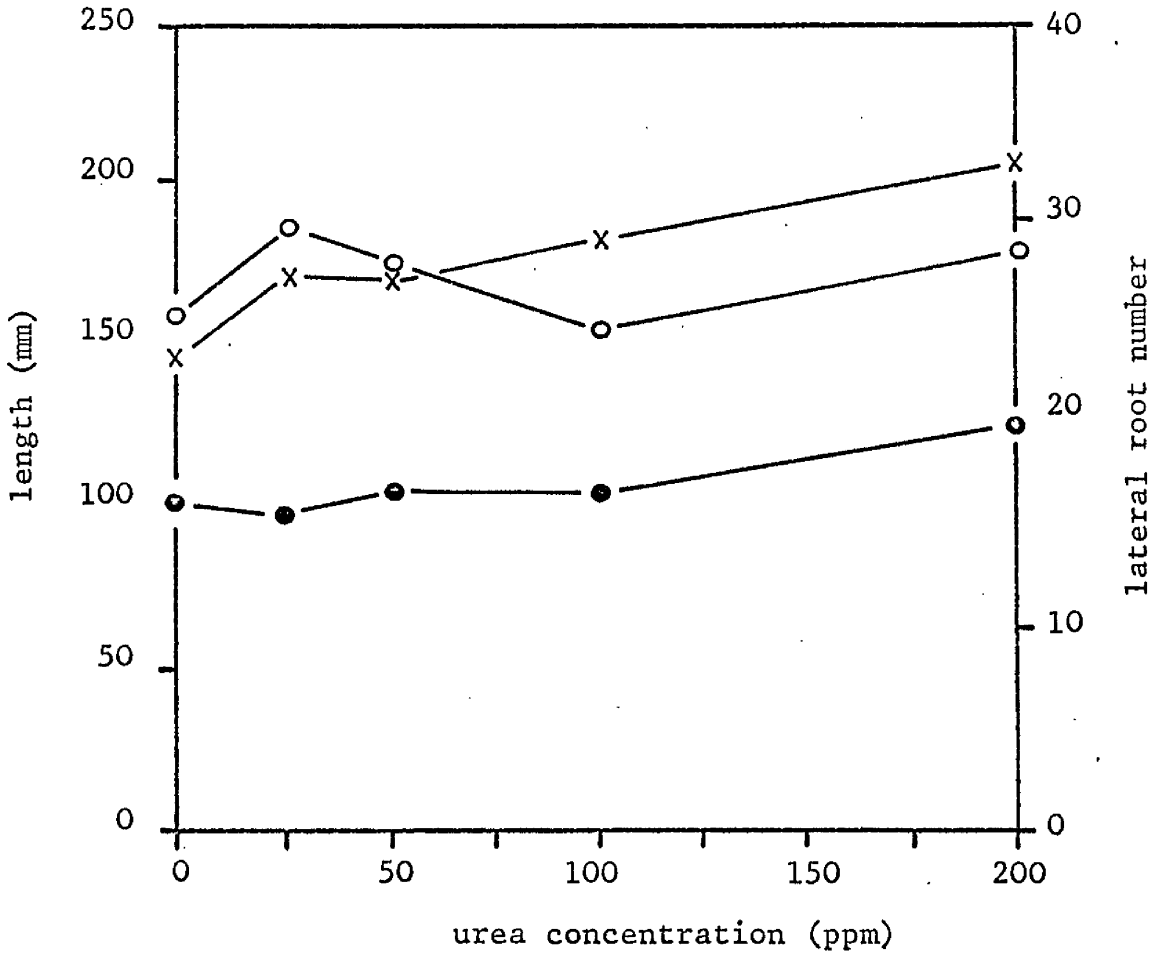

Fig.15a. Effect of urea on the growth of cultured roots.

(Data in appendix 4c.)

LMA

LN

TLL

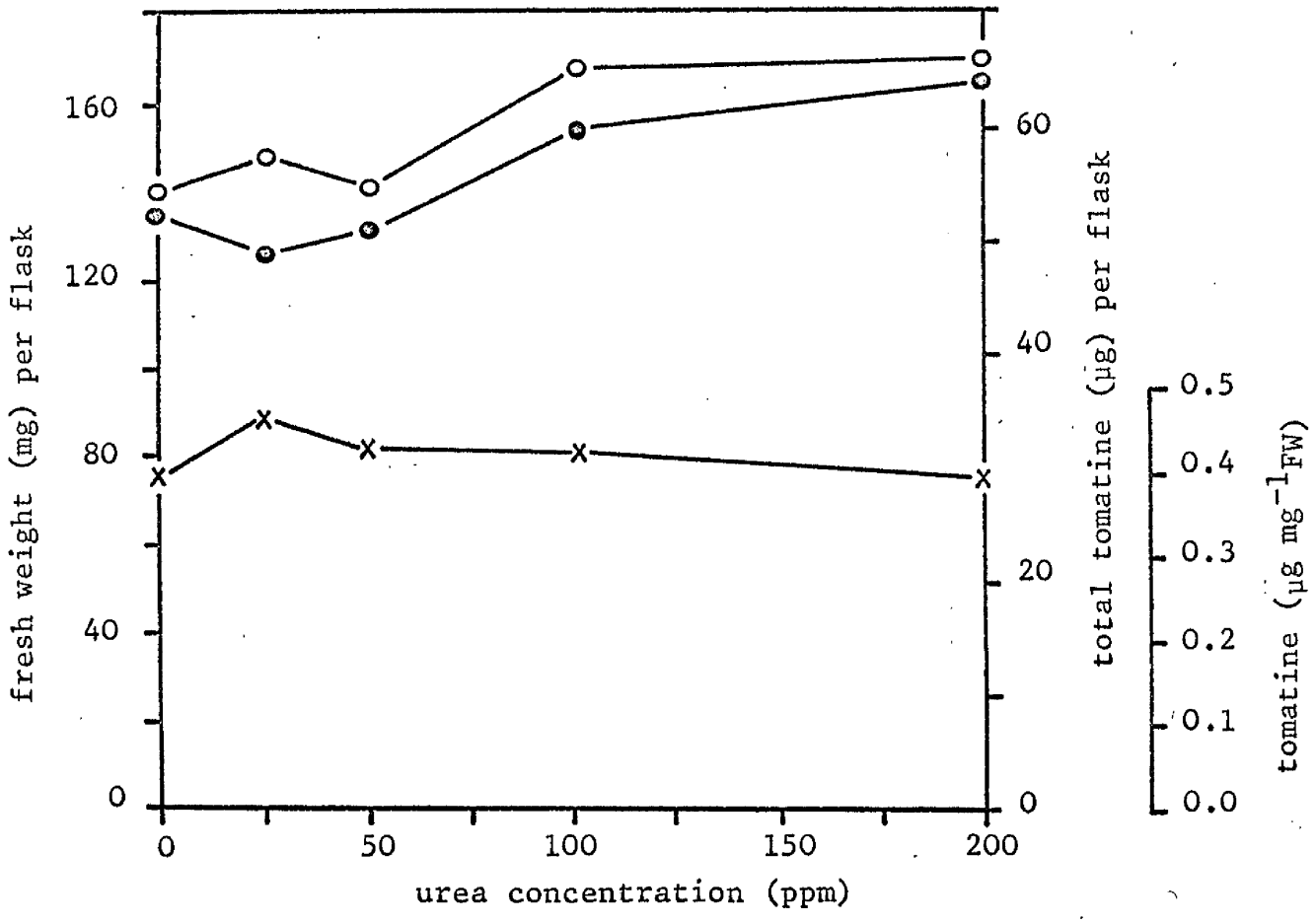

Fig.15b. Effect of urea on tomatine production in cultured roots. (Data in appendix 4d.)

⊙——⊙ fresh weight

○——○ total tomatine

×——× tomatine concentration.

treatment were used in growth experiments and tomatine determination experiments respectively.

Addition of casamino acids at concentrations up to 100 ppm resulted in increases in linear growth of the main axis and total length of lateral roots (fig. 16a). At 100 ppm, axis length was increased by 31 per cent and total lateral length by 66 per cent. Concentrations greater than 100 ppm proved to be supra-optimal, and 500 ppm casamino acids was inhibitory to all three growth parameters.

Addition of casamino acids to the culture medium tended to increase fresh weight although a depression was present at 100 ppm (fig. 16b). The curve for total tomatine was very similar to that for fresh weight and also showed a depression at 100 ppm. Tomatine concentration was little affected by addition of casamino acids.

Despite the fact that none of the above three compounds increased growth or alkaloid content of cultured roots to a large extent, the results obtained did confirm the previously observed correlation between growth of cultured roots, as measured by weight, and synthesis of tomatine. On the other hand, correlations between alkaloid production and other growth parameters were difficult to establish.

SECTION 6. EFFECTS OF STEROID PRECURSORS AND A STEROID INHIBITOR ON TOMATINE PRODUCTION.

The finding that tomatine levels were lower in cultured tomato roots than in seedling roots suggests that cultured contain precursors of the alkaloid

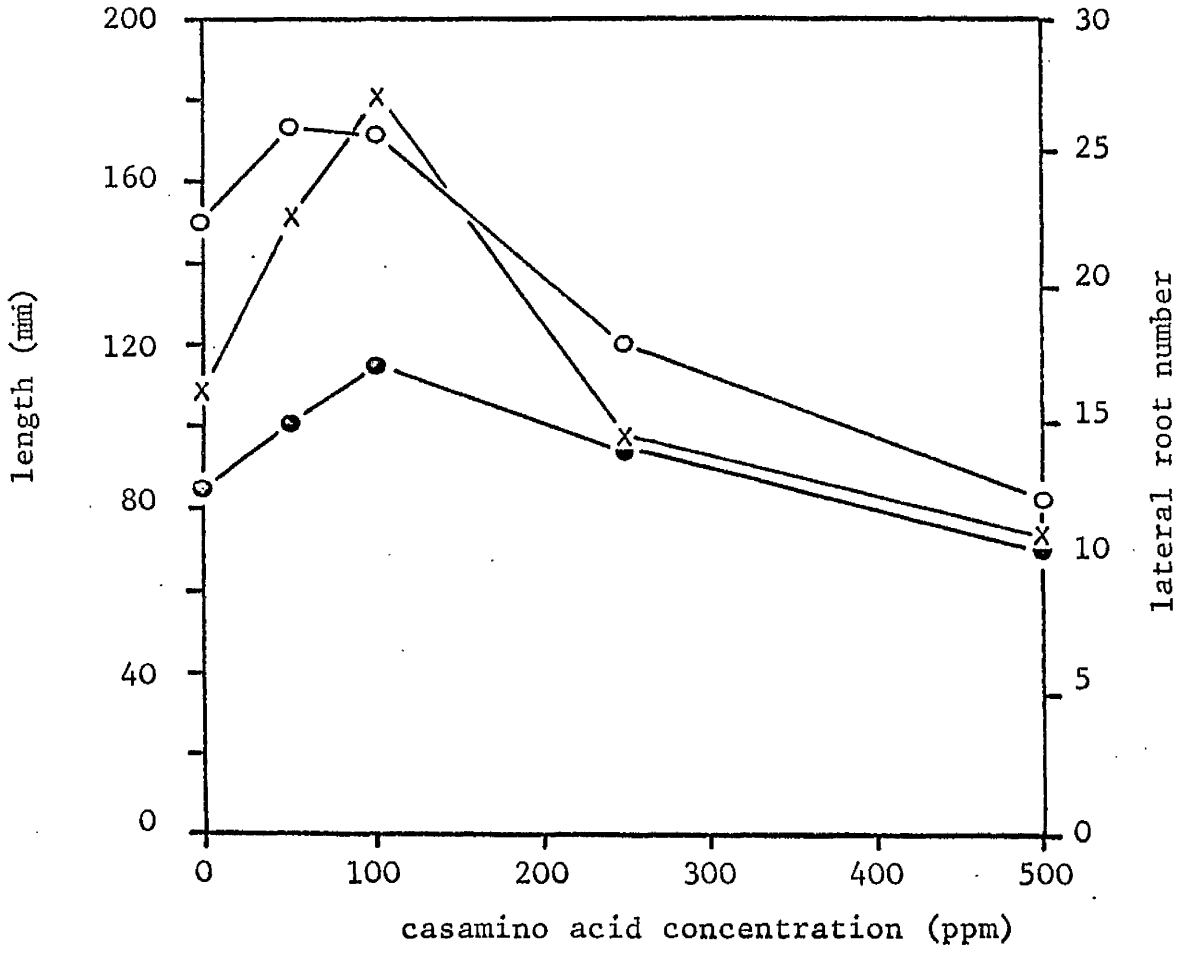

Fig.16a. Effect of casamino acids on the growth of cultured roots. (Data in appendix 4e.)

●——● LMA

○——○ LN

×——× TLL

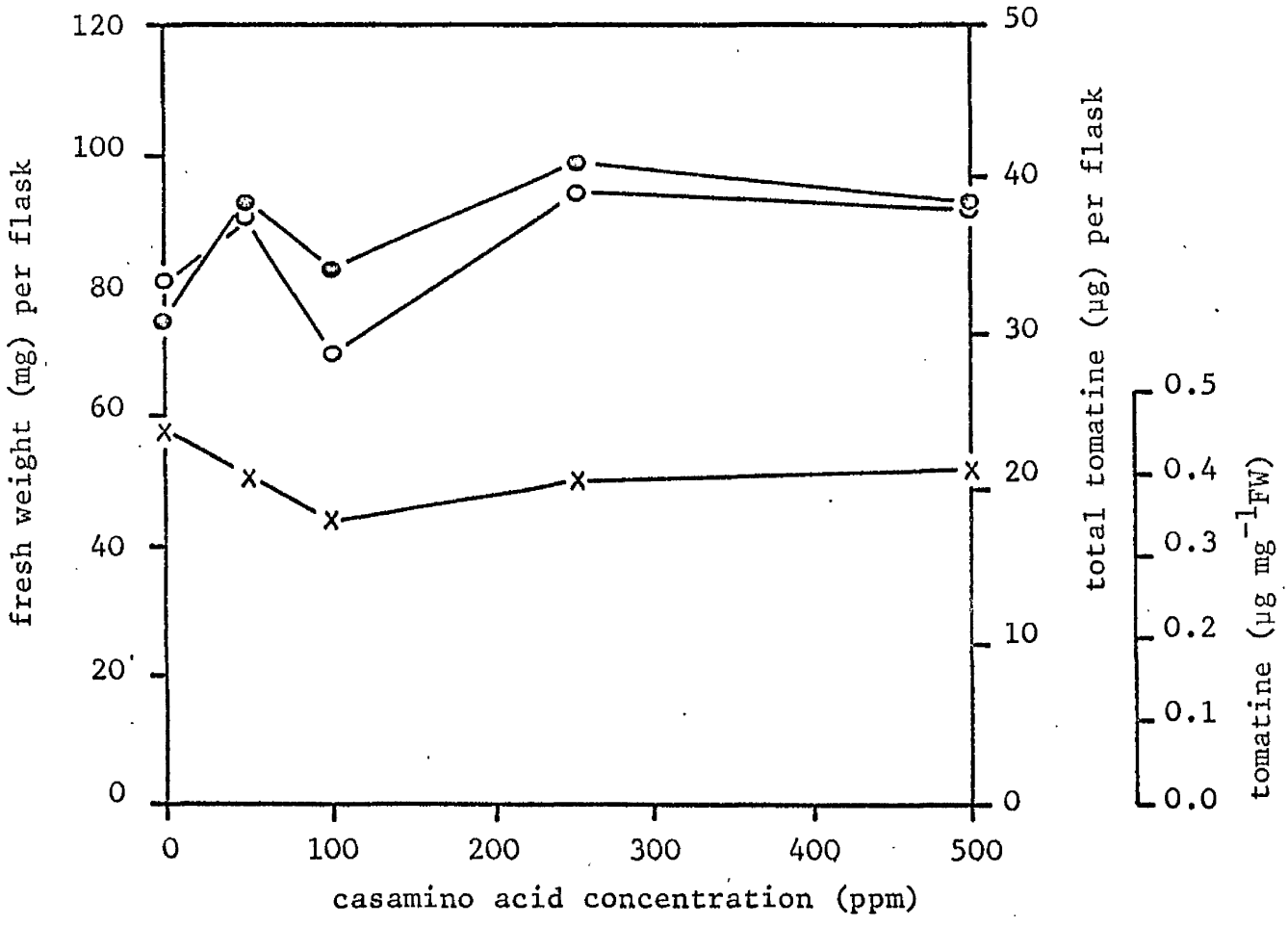

Fig.16b. Effect of casamino acids on tomatine production in cultured roots. (Data in appendix 4f.)

o——o fresh weight

o——o total tomatine

x——x tomatine concentration

might be limiting under cultural conditions. Since the major part of the alkaloid consists of a steroid nucleus, the effects of steroid precursors, such as acetate, mevalonate and cholesterol were tested. The effect of SKF 7997-A_3, an inhibitor of the steroid biosynthetic pathway, was also investigated. Experiments were also conducted, as in section 5, to study the effects of these compounds on other parameters of cultured root growth.

Effect of acetate on root growth and tomatine production.

Anhydrous sodium acetate was weighed out and added directly to the culture medium to give final concentrations of 1, 5, 10 and 20 ppm. Growth and tomatine determination experiments were conducted using ten and four replicates respectively.

With increasing concentrations of sodium acetate,decreases in all three growth parameters (LMA, LN, TLL) were observed (fig. 17a). At 20 ppm acetate, main axis length, lateral root number and total lateral length were respectively 75 per cent, 52 per cent and 42 per cent of their control values.

Total tomatine tended to decrease with increasing acetate levels as did fresh weight (fig. 17b). At the highest acetate concentration fresh weight was reduced by 19 per cent and total alkaloid by 31 per cent. Tomatine concentrations were unaffected by acetate addition.

Effect of mevalonic acid lactone on root growth and tomatine production.

Because of its viscous nature, mevalonic acid lactone (Sigma Chemical

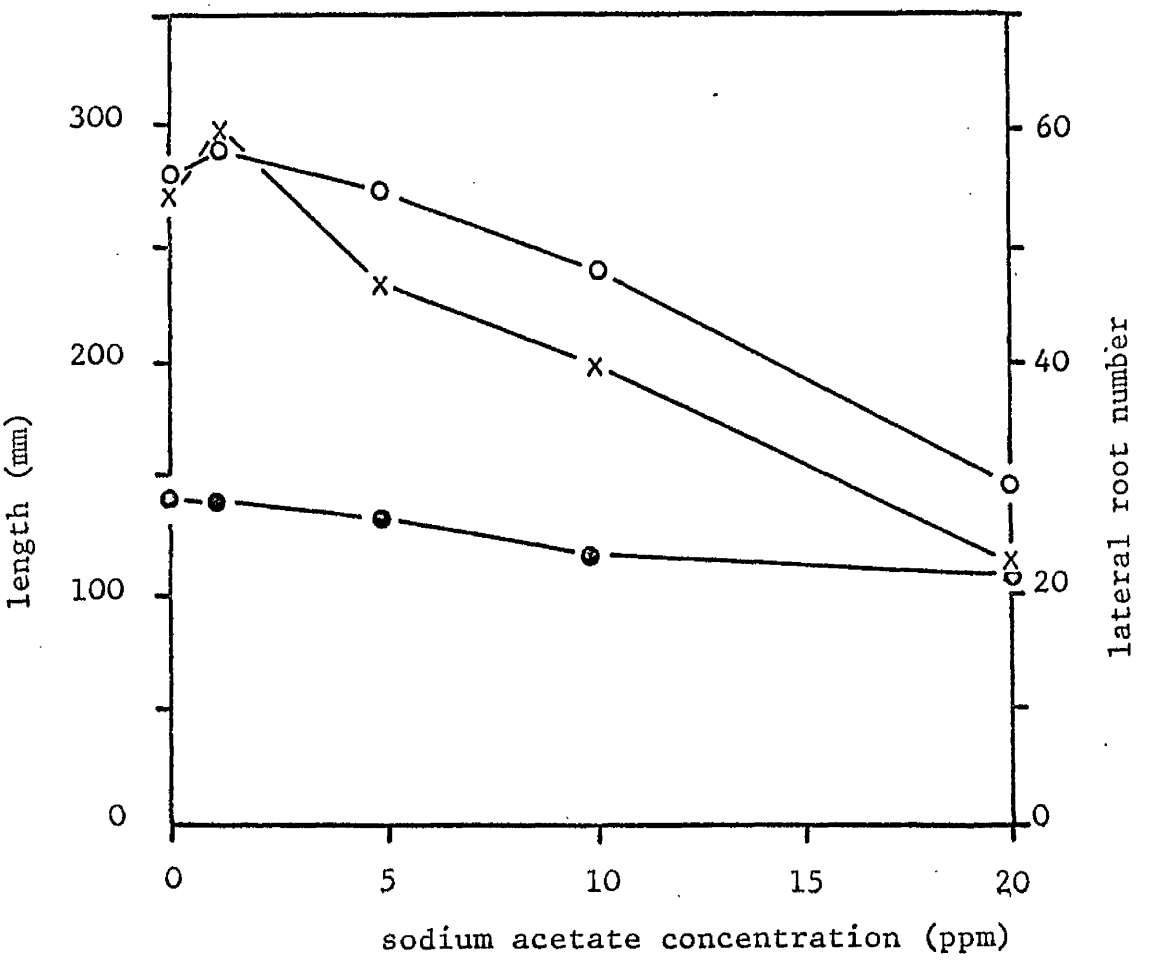

Fig.17a. Effect of sodium acetate on the growth of cultured roots. (Data in appendix 5a.)

●——● LMA

○——○ LN

×——× TLL

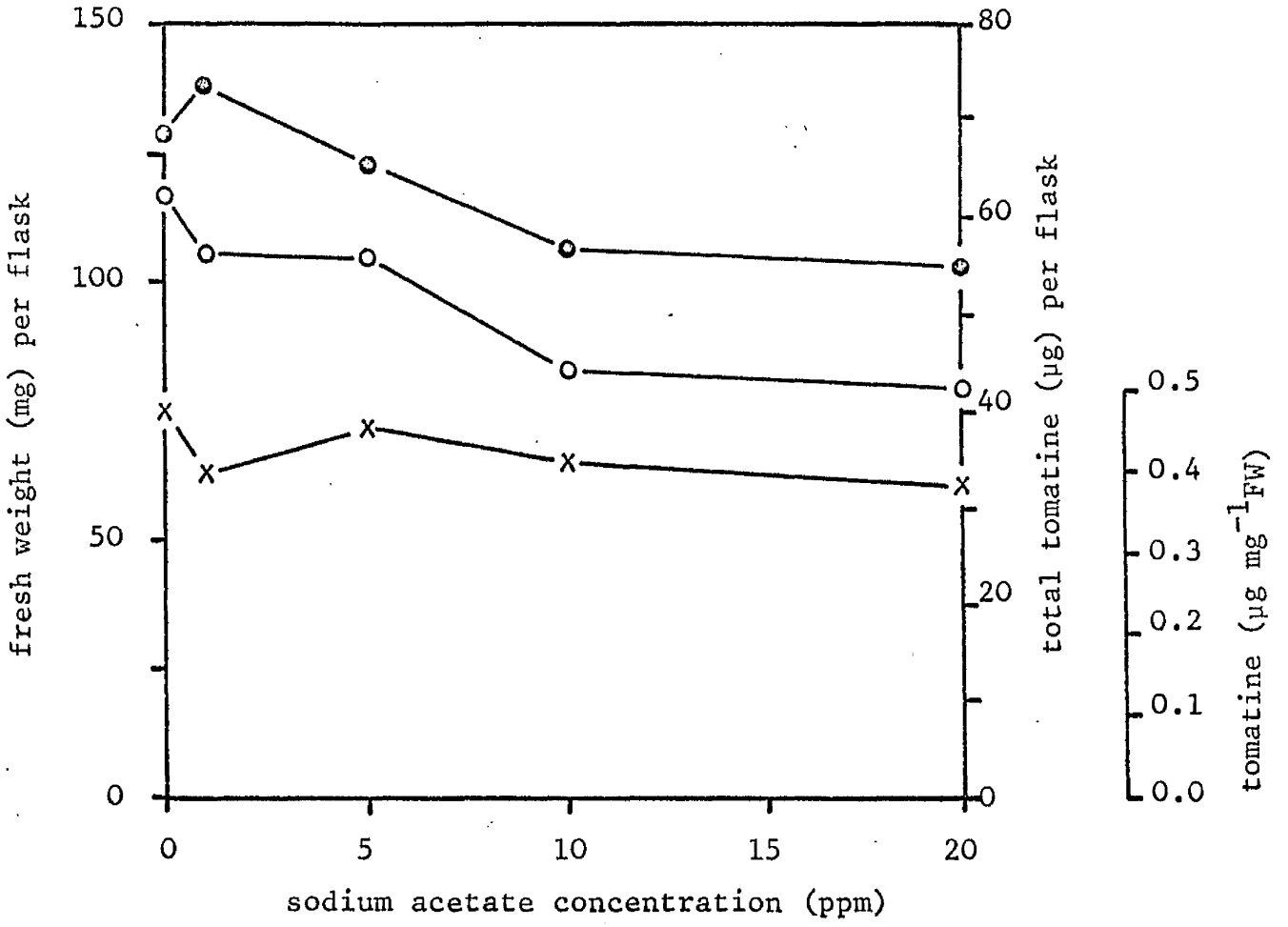

Fig.17b. Effect of sodium acetate on tomatine production in cultured roots. (Data in appendix 5b.)

●——● fresh weight

○——○ total tomatine

x——x tomatine concentration.

Co. Ltd., London) was dissolved in distilled water and volumes of this solution aseptically added to different volumes of sterile distilled water. Sterile mevalonate solution was then aseptically added to cool, sterile culture medium to give final concentrations of 5, 10, 25 and 50 ppm. In growth experiments, each of the treatments was replicated 12 times, and in tomatine determination experiments four replicates were used.

Growth of cultured roots, as measured by LMA, LN and TLL, was not affected by additions of mevalonate in concentrations up to 50 ppm (fig. 18a).

Fresh weight was unaffected by addition of mevalonate, but total tomatine decreased with mevalonate concentrations up to 10 ppm, after which no further changes occurred (fig. 18b). The decrease in amount of tomatine was of the order of 30 per cent. A similar pattern and level of decreases occurred in tomatine concentration.

Effect of cholesterol on root growth and tomatine production.

Stock solutions were first prepared by dissolving cholesterol (Sigma Chemical Co. Ltd., London) in 1 cm^3 of hot ethanol plus one drop of 1 per cent Tween 80, and making the volume to 100 cm^3 with distilled water. The appropriate stock solution was added to 900 cm^3 of culture medium so that the final sterol concentrations were 5, 10 and 25 ppm, and the alcohol concentration, 0.1 per cent. Culture medium so prepared had a faint odour of alcohol but this was not detectable after autoclaving. Preparation of culture solutions by this method ensured good dispersion

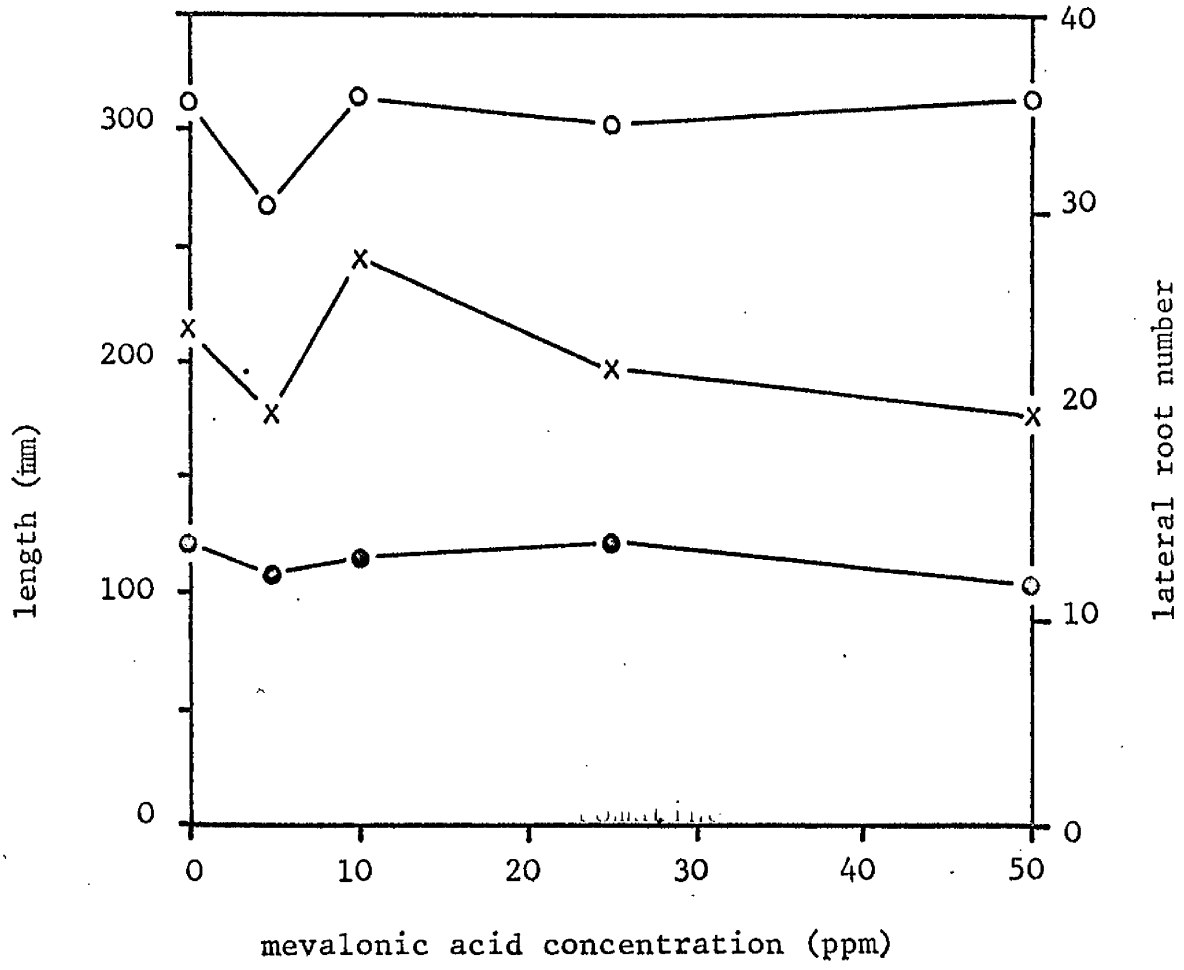

Fig.18a. Effect of mevalonic acid lactone on the growth of cultured roots. (Data in appendix 5c.)

o——o LMA

o——o LN

x——x TLL

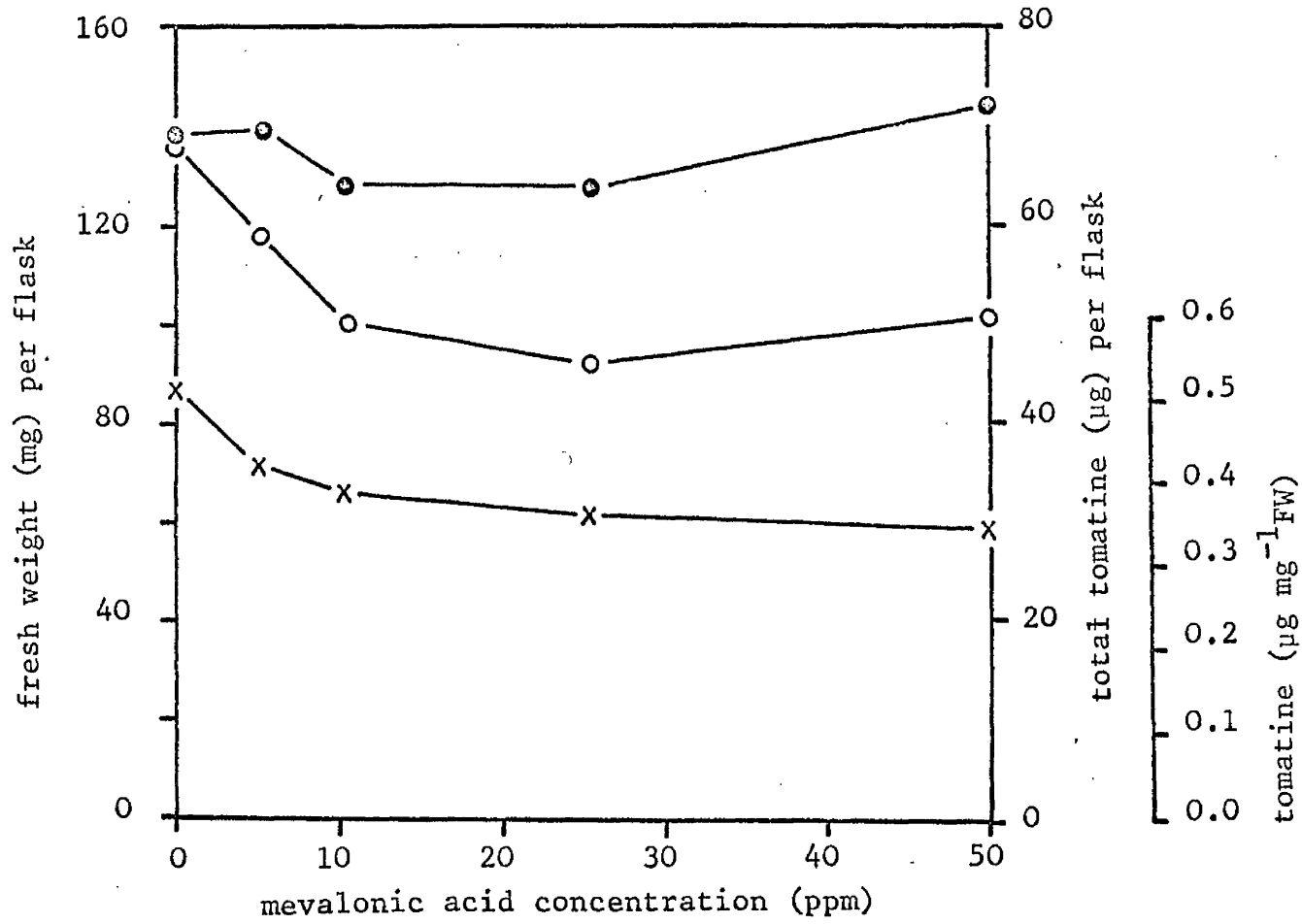

Fig.18b. Effect of mevalonic acid lactone on tomatine production in cultured roots. (Data in appendix 5d.)

o——o fresh weight

o——o total tomatine

x——x tomatine concentration

of cholesterol, which appeared as a very fine suspension. Four and 12 replicates were used in tomatine determination and growth experiments respectively.

As shown in fig. 19a, main axis length, lateral root number and total lateral length all decreased with increasing concentrations of cholesterol. At 25 ppm cholesterol the respective reductions were 35 per cent, 69 per cent and 72 per cent.

Fresh weight and total tomatine increased with increasing sterol levels by factors of about 30 per cent and 36 per cent respectively (fig. 19b). Concentrations of tomatine in cultured roots remained constant throughout the experiment.

Effect of SKF 7997-A_3 on root growth and tomatine production.

SKF 7997-A_3 [tris-(2-diethylaminoethyl)-phosphate trihydrochloride] causes inhibition of steroid biosynthesis by blocking conversion of lanosterol to zymosterol. The compound is produced by Smith, Kline and French Laboratories, Philadelphia, U.S.A.

Sterile stock solutions of SKF 7997-A_3 were prepared by aseptically transferring different volumes of inhibitor solution to sterile distilled water. The appropriate volume of stock solution was then aseptically added to sterile culture medium. Final concentrations of inhibitor were 1.0, 2.5, 5.0, 10.0 and 25.0 ppm. All five concentrations were used in the growth experiment but only the first three were employed for the tomatine determination experiment. The former experiment involved 12 replicates per treatment and the latter, four.

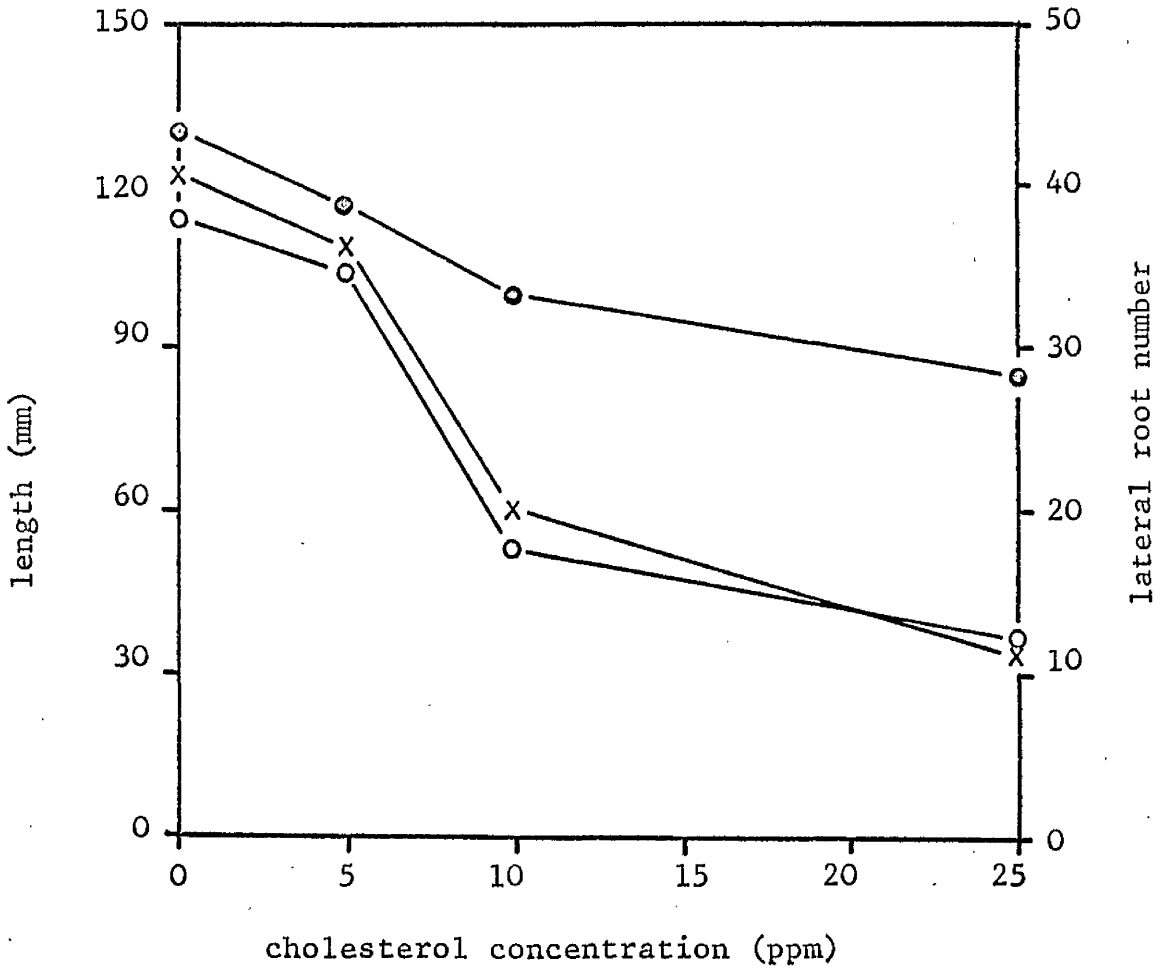

Fig.19a. Effect of cholesterol on the growth of cultured roots. (Data in appendix 5e.)

⊙——⊙ LMA

O——O LN

X——X TLL

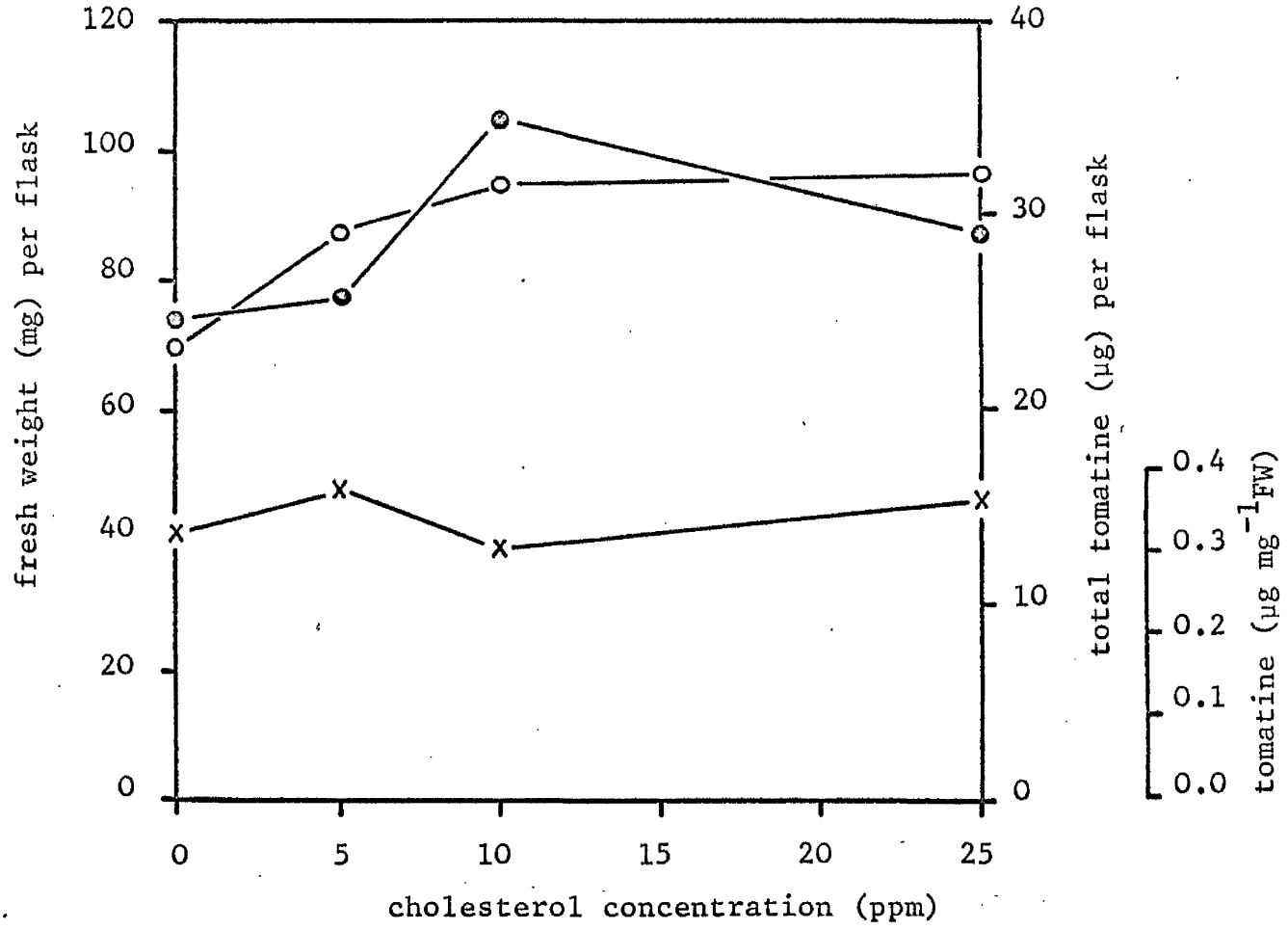

Fig.19b. Effect of cholesterol on tomatine production in cultured roots. (Data in appendix 5f.)

●——● fresh weight

○——○ total tomatine

×——× tomatine concentration

Main axis length, lateral root number and total lateral length were all reduced as a result of addition of SKF 7997-A_3 to the culture medium, although the first parameter was not so severely inhibited as the last two (fig. 20a). At 5 ppm inhibitor, the respective decreases were 46 per cent, 93 per cent and 93 per cent. At the highest concentration of SKF 7997-A_3, main axis length was reduced by 74 per cent and no lateral roots were produced.

With increasing concentrations of SKF 7997-A_3, both fresh weight and total tomatine decreased (fig. 20b). At 5 ppm inhibitor, the respective reductions were 76 per cent and 73 per cent. The decreases in these two factors followed such a similar course that concentrations of tomatine remained constant throughout the experiment.

Although addition of precursors did not result in increases in tomatine concentrations in cultured roots, these experiments, in general, again confirmed the correlation between weight of cultured roots and tomatine production. As before (section 5), correlations could not be established between other growth parameters and alkaloid production.

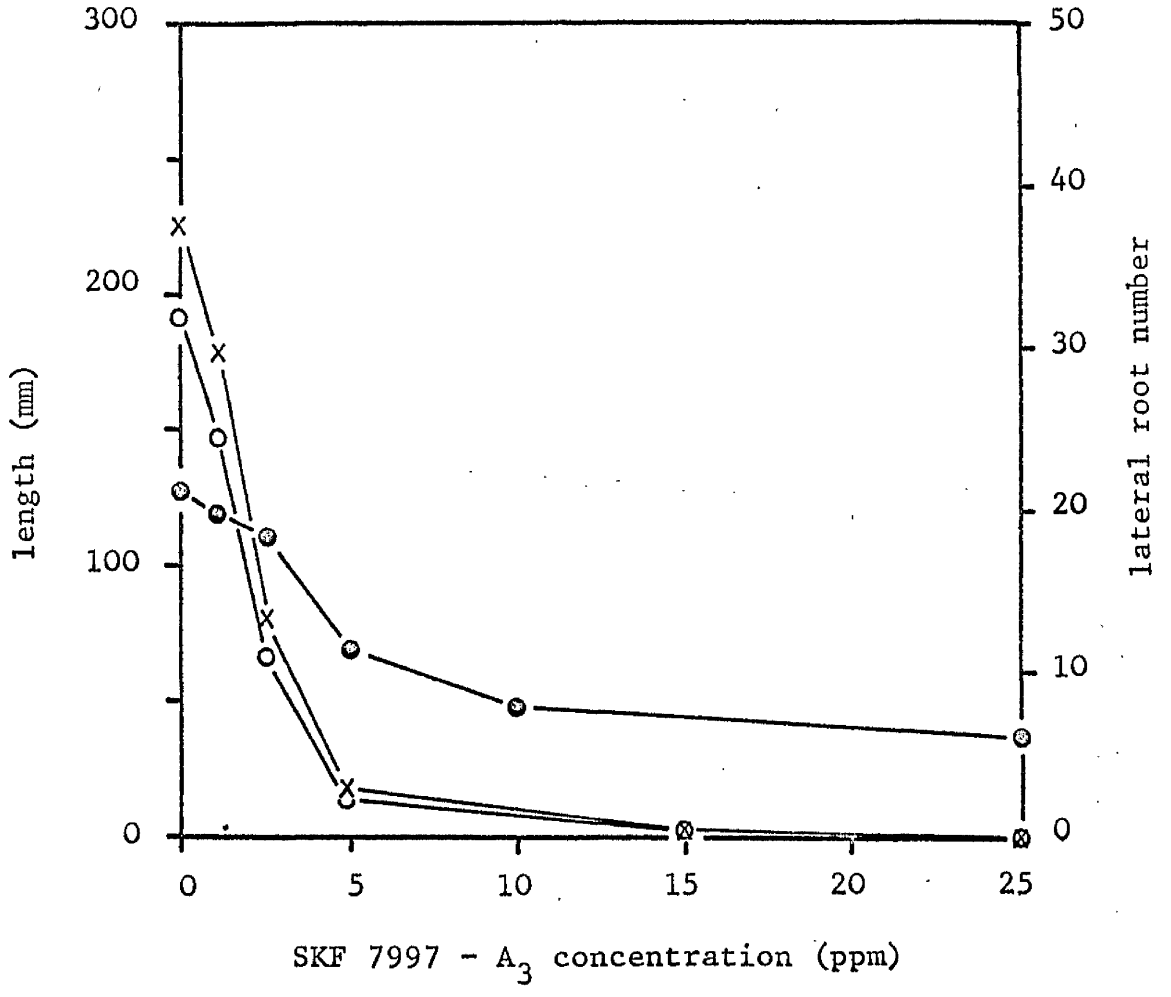

Fig.20a. Effect of SKF 7997-A_3 on the growth of cultured roots. (Data in appendix 5g.)

⊙——⊙ LMA

○——○ LN

×——× TLL

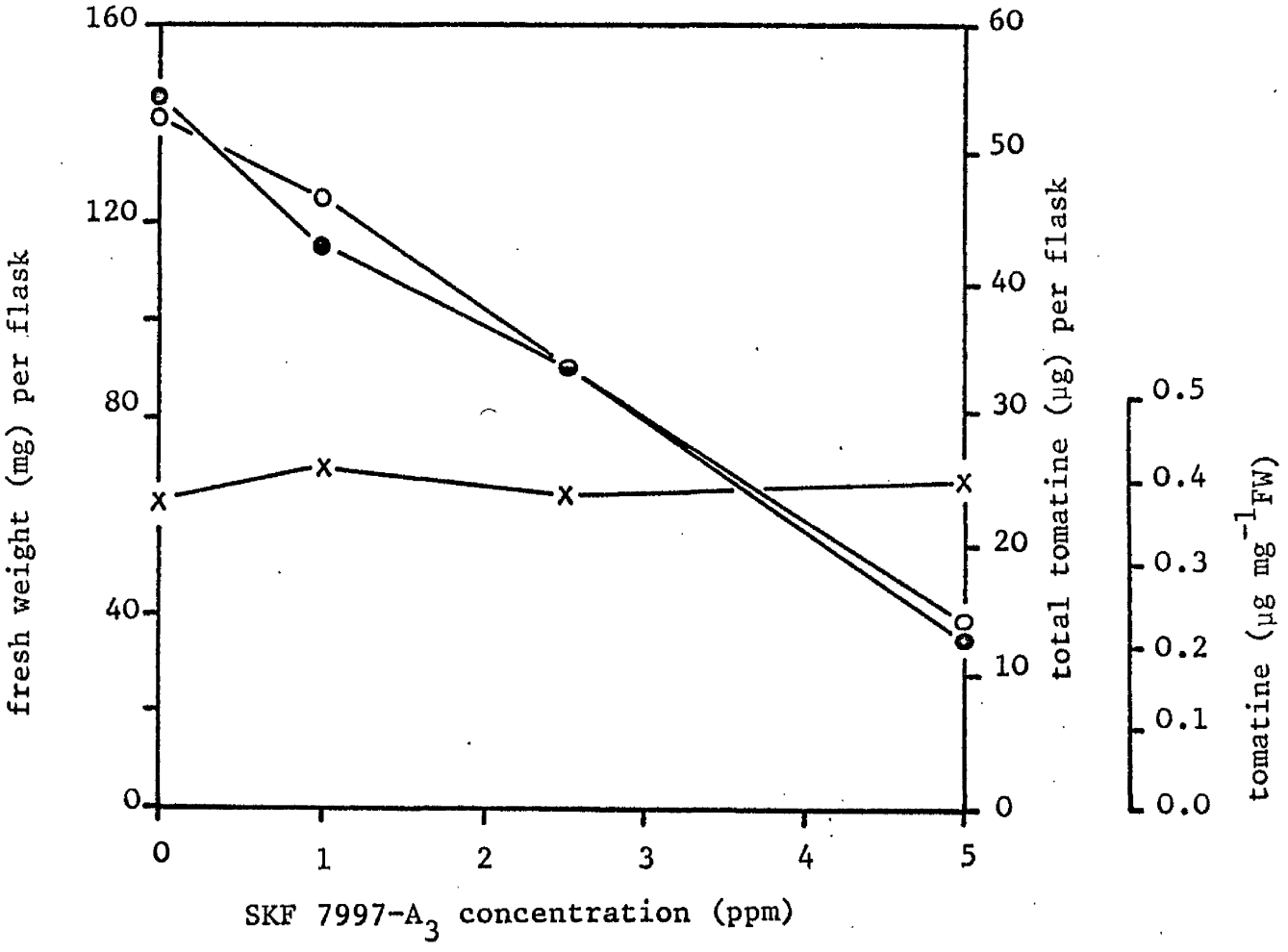

Fig.20b. Effect of SKF 7997-A_3 on tomatine production in cultured roots. (Data in appendix 5h.)

●——● fresh weight

o——o total tomatine

x——x tomatine concentration

PART IV

STUDIES WITH CALLUS CULTURES.

SECTION 1. CALLUS GROWTH AND TOMATINE PRODUCTION ON CHEMICALLY-DEFINED AND 'COCONUT-MILK' MEDIUM.

The cultures used in the following experiments were of 'spontaneous' root and hypocotyl callus (see p. 37). A chlorophyllous hypocotyl callus was obtained by transferring a slightly green sector of the 'spontaneous' hypocotyl callus to fresh medium and growing under constant illumination.

The decision to use, where possible, chemically-defined culture medium was prompted by such considerations as the uncertainty of supplies of fresh coconuts; the undefined nature of coconut-'milk', and the probable differences in its composition in coconuts of different ages; interest as to whether coconut-'milk' influenced tomatine synthesis in callus tissue.

Callus growth and tomatine production on 'coconut-milk' medium.

The above cultures were grown on a 'coconut-milk' medium containing 100 ppm meso-inositol (see table 2). This was referred to as culture medium A. After random selection of 12 flasks of each culture, fresh and dry weights of the callus were determined. Callus from the remaining flasks of each culture was bulked and analysed for tomatine. At the time of harvest, cultures appeared as shown in plate 1. (The calluses shown here, and in all other cases were 'average' cultures rather than the fastest-growing.)

Although the chlorophyllous hypocotyl callus had its origin in the non-chlorophyllous hypocotyl callus, dry weight production was only 18

PLATE 1. CALLUS CULTURES GROWN ON MEDIUM A.

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a) non-chlorophyllous hypocotyl callus

b) root callus

c) chlorophyllous hypocotyl callus

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a)

b)

c)

per cent of that in the latter (table 14a). Root callus produced almost three times as much dry matter as chlorophyllous hypocotyl callus but only about half as much as non-chlorophyllous hypocotyl callus.

Tomatine was detected only in non-chlorophyllous hypocotyl callus, but amounts present were very small, the alkaloid concentration being 0.07 per cent of that in seven-day old seedling hypocotyl (table 14b).

Callus growth and tomatine production on a chemically-defined medium (I).

Root callus and non-chlorophyllous hypocotyl callus were transferred from culture medium A to a chemically-defined medium (see table 2). Meso-inositol and 2,4-d were as for culture medium A and kinetin was present at 0.5 ppm. This was designated culture medium B.

Fresh and dry weights were recorded using 12 replicates of each culture and the bulked callus from the remaining flasks was analysed for tomatine.

Growth (as measured by dry-matter production) of both hypocotyl and root callus was less than that observed on medium A by about 30 per cent in each case (table 15). However, the 2:1 ratio of dry matter production in hypocotyl callus to that in root callus, observed in the previous experiment, was still maintained.

Tomatine could not be detected in either hypocotyl or root callus.

SECTION 2. ATTEMPTS TO INDUCE ORGANOGENESIS IN ESTABLISHED CALLUS CULTURES.

In order to decide if the capacity to synthesise tomatine was

TABLE 14a. Growth of callus cultures on medium A (containing coconut-'milk').

callus	fresh weight (g)	dry weight (mg)	$\frac{FW}{DW}$
hypocotyl	6.5 ±0.6	341.1 ±25.1	18.9
root	4.6 ±1.2	197.3 ±46.6	23.2
hypocotyl (chlorophyllous)	1.0 ±0.1	61.4 ±6.2	15.7

Results are expressed on a per flask basis.

TABLE 14b. Tomatine content of callus cultures grown on medium A.

callus	dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1}DW)
hypocotyl	4610.8	59.94	0.013
root	4310.3	0	-
hypocotyl (chlorophyllous)	4076.4	0	-

Results refer to bulked tissue.

TABLE 15. Growth of callus cultures on medium B (chemically-defined).

callus	fresh weight (g)	dry weight (mg)	$\frac{FW}{DW}$
hypocotyl	7.4 $\pm$1.0	238.5 $\pm$17.2	30.9
root	3.8 $\pm$0.8	139.3 $\pm$20.6	27.3

Results are expressed on a per flask basis.

associated with the degree of morphological organisation, attempts were made to induce formation of organised structures in callus tissue by altering the concentrations of certain components of the culture medium.

Alteration of 2,4-D and meso-inositol levels.

Root callus and hypocotyl callus, which had been maintained on medium B, were transferred to a similar medium but with 2,4-D concentration reduced to 0.15 ppm and meso-inositol increased to 400 ppm. With these modifications to their culture medium, Rao and Narayanaswami (1968) had induced root formation in potato callus within seven days. A number of the cultures were left to grow on this medium for an indefinite period while the remainder were subcultured regularly.

Hypocotyl and root tissues which were not subcultured, initially grew rapidly, but growth eventually declined and ceased altogether as the tissues became senescent. At no time during the prolonged growth period of 15 weeks were organised structures produced. In callus which was regularly subcultured, relatively high growth rates were maintained throughout, but no organogenesis had occurred after one year in culture. Nor was cellular differentiation observed in any of the above cultures.

Alteration of auxin and kinetin levels.

The approach adopted here was based on the finding by Skoog and Miller (1957) that organogenesis in tobacco callus could be controlled by altering the auxin/kinetin ratio in the culture medium. In the following experiments however, concentrations of one growth factor were

varied while that of the other was maintained constant.

Alteration of kinetin levels. The culture medium employed was identical to that used in the previous experiment, but kinetin concentrations were varied in different batches of culture medium. The concentrations tested were 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 ppm. Culture medium with different kinetin concentrations but lacking 2,4-D was also prepared, as was culture medium lacking both 2,4-D and kinetin. Each flask was inoculated with hypocotyl callus which had been maintained on the culture medium described in the previous experiment. Ten replicate flasks were used for each treatment.

After eight weeks growth, neither alteration of kinetin levels nor omission of 2,4-D had resulted in elaboration of organised structures. No evidence of cellular differentiation was obtained from microscopic examination of the calluses. However, as there were obvious differences in the growth of callus on different media, the cultures were harvested and fresh and dry weights determined.

From fig.21a it was deduced that, with 2,4-D at 0.15 ppm, the optimum concentration of kinetin for growth of hypocotyl callus was 0.15 ppm. When 2,4-D was omitted from the culture medium, no growth took place.

Alteration of 2,4-D levels. The culture medium was as used above, but kinetin was present at 0.15 ppm and a range of 2,4-D concentrations were tested viz. 0.01, 0.03, 0.1, 0.3, 1.0 and 3.0 ppm. Culture medium was also prepared with different auxin concentrations but lacking kinetin,

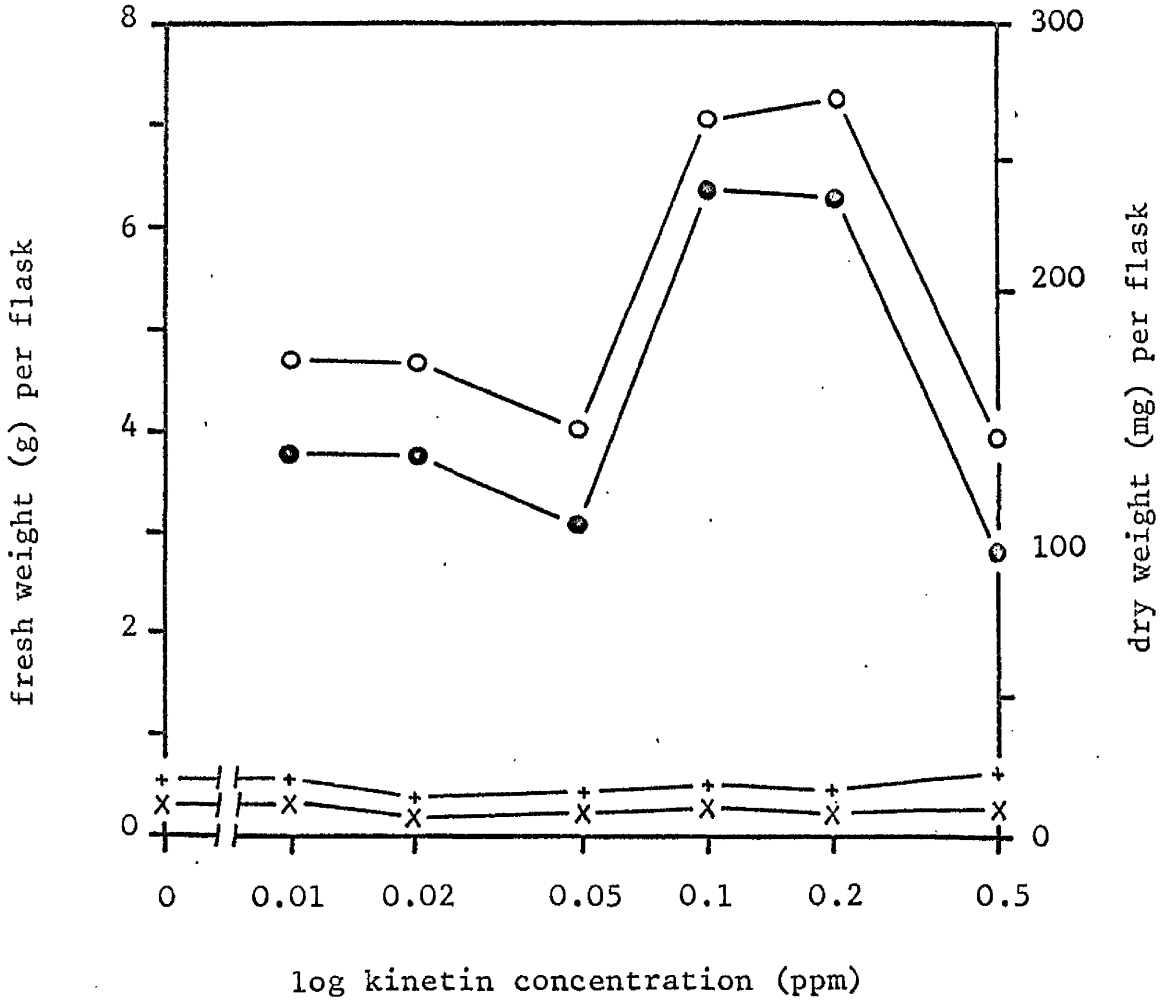

Fig.21a. Effect of kinetin on the growth of hypocotyl callus.

(Data in appendix 6a.)

●——● fresh weight + 2,4-D

○——○ dry weight + 2,4-D

X——X fresh weight - 2,4-D

+——+ dry weight - 2,4-D

as was culture medium lacking both growth substances. Each of the ten replicate flasks for each treatment was inoculated with hypocotyl callus of similar origin to that used above.

Again, no organogenesis or cellular differentiation was observed after eight weeks incubation but differences in callus growth were apparent. Fresh and dry weights of callus were therefore determined.

With kinetin at 0.15 ppm the optimum 2,4-D concentration for hypocotyl callus growth was 0.1 ppm (fig. 21b). The previously observed lack of growth on an auxin-free medium was confirmed, but omission of kinetin was less critical for callus growth and dry-matter accumulation was approximately 75 per cent of that on a culture medium containing kinetin.

Callus growth and tomatine production on a chemically-defined medium (II).

Despite the lack of success in inducing organ formation in callus tissue, the previous experiments indicated levels of 2,4-D and kinetin which allowed for rapid callus growth. Previously, it was found that hypocotyl callus and root callus grew less on chemically-defined medium B than on 'coconut-milk' medium A, and that hypocotyl callus did not elaborate tomatine on the former medium. It was decided therefore to investigate how callus growth and tomatine production on a culture medium based on the above data compared with growth and alkaloid production on medium A and B.

Hypocotyl callus and root callus were initiated on to the basic culture medium used in the previous two experiments but with kinetin and 2,4-D at 0.15 and 0.1 ppm respectively. This culture medium is hereafter

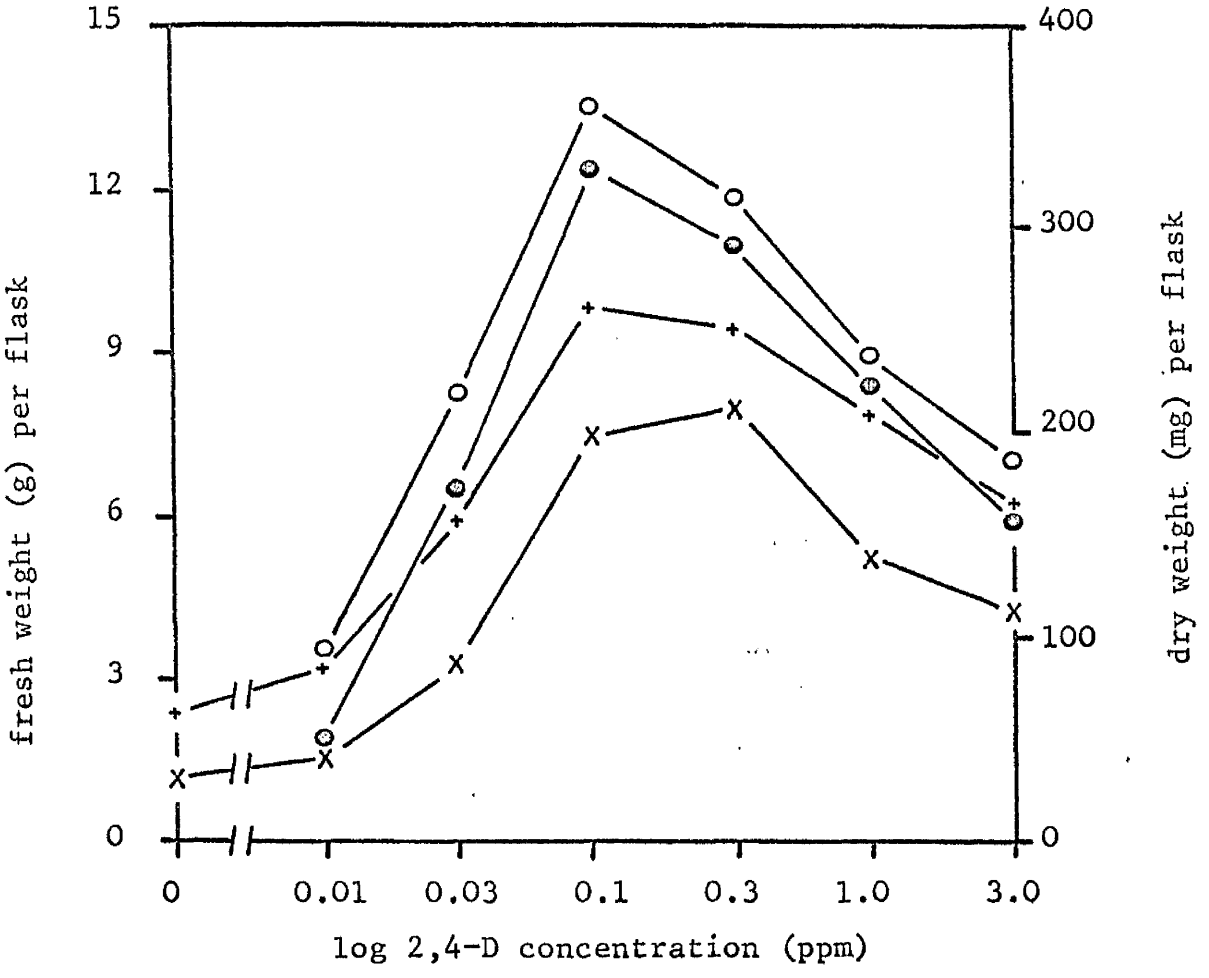

Fig.21b. Effect of 2,4-D on the growth of hypocotyl callus.

(Data in appendix 6b.)

●——●	fresh weight	+ kinetin
○——○	dry weight	
x——x	fresh weight	- kinetin
+——+	dry weight	

referred to as medium C. Fresh and dry weights were measured for each of 12 replicates and the remaining callus was bulked and analysed for tomatine.

Fresh and dry weights of both cultures were greater than those of the corresponding callus on medium B (table 16a). Fresh weights of both cultures were higher than those recorded on medium A but dry weights were of the same order.

Tomatine was present in hypocotyl callus and alkaloid concentration was comparable with that in hypocotyl callus grown on medium A (table 16b). As in all previous cases, tomatine was not detected in root callus.

SECTION 3. ATTEMPTS TO INDUCE ORGANOGENESIS IN NEWLY-INITIATED CALLUS CULTURES.

Various authors (Miller and Skoog, 1953; Street, 1965b) have reported that the regenerative capacity of callus tissue is often lost with increasing length of time in culture. In view of the lack of success in inducing organogenesis in established callus tissue, new cultures were initiated (see p. 38) in a further attempt to study the relationship between degree of organisation and synthesis of tomatine.

Tomatine content of organs used to initiate callus cultures.

The tomatine content of seedling hypocotyl, cotyledon and radicle were determined as standards against which to compare any capacity of resulting callus to elaborate tomatine.

Seven-day old tomato seedlings which had been germinated in the dark

TABLE 16a. Growth of callus cultures on medium C (chemically-defined).

callus	fresh weight (g)	dry weight (mg)	$\frac{FW}{DW}$
hypocotyl	14.5	389.1	37.2
	±0.8	±15.1	
root	6.8	223.5	24.8
	±1.8	±48.4	

Results are expressed on a per flask basis.

TABLE 16b. Tomatine content of callus cultures grown on medium C.

callus	dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1}DW)
hypocotyl	2889.8	24.60	0.009
root	2322.6	0	-

Results refer to bulked tissue.

at 25°C were divided up into hypocotyl, cotyledons and radicle, and tomatine analyses of these organs conducted. The extraction procedure was as used for cultured roots, the only modification being that cotyledon extracts were developed twice in diethyl ether to remove pigments before development using the standard two solvent-systems. Subsequent examination of the chromatograms revealed that neither the Rf of tomatine nor those of other extract components were altered by this modification. Each organ extraction was replicated four times. Because of a lower fresh weight/dry weight ratio in cotyledons than in the other two organs, results have been expressed on a dry-weight basis.

Tomatine was present in all three organs in almost identical concentrations (table 17).

Analysis of callus cultures for tomatine.

The culture medium employed only differed from medium C in that α-naphthalene-acetic acid (NAA) at 2.0 ppm was used as auxin source. After incubating organ explants for eight weeks, the callus tissue which developed, and which contained large numbers of roots, was transferred to fresh culture medium. Ages of cultures, stated hereafter, are dated from the time of this transfer.

Hypocotyl callus (plate 2) was harvested after 38 days and, after removing all visible roots, analysed for tomatine. Roots were also present in radicle and cotyledon callus at this time (plate 2), but insufficient tissue was available for alkaloid analyses.

TABLE 17. Tomatine content of seedling organs used to initiate callus cultures.

organ	No. per replicate	total dry weight (mg)	total tomatine (μg)	tomatine ($\mu g\ mg^{-1}$ DW)
hypocotyl	40	13.6 ±2.2	223.73 ±21.14	17.45 ±7.09
radicle	60	11.2 ±0.7	188.76 ±13.99	16.89 ±0.51
cotyledon	100 pairs	68.7 ±4.5	1111.49 ±83.67	16.14 ±0.39

PLATE 2. NEWLY-INITIATED CALLUS CULTURES SHOWING ROOTS

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a) hypocotyl callus

b) radicle callus

c) cotyledon callus

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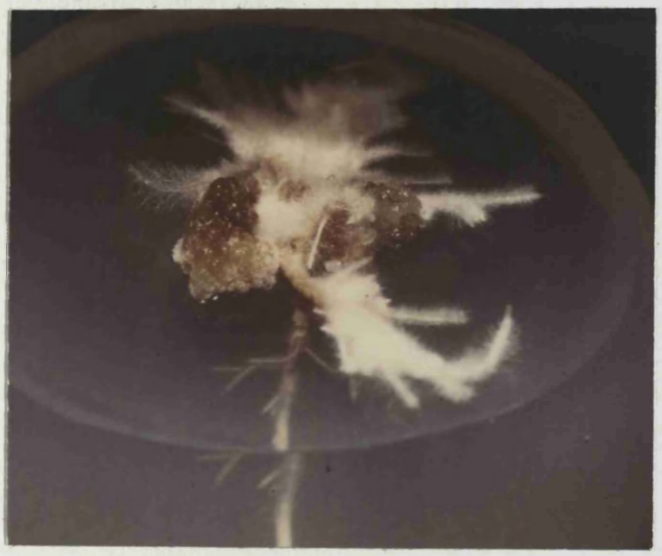
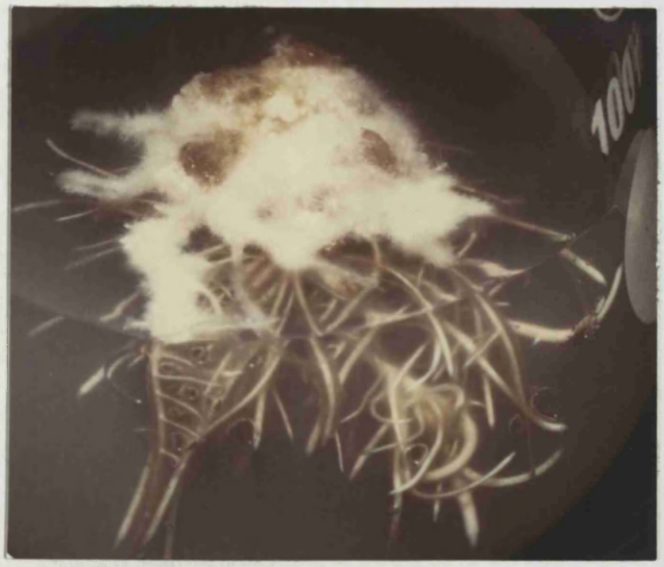
a)

b)



c)



Tomatine was present in hypocotyl callus. Alkaloid concentration, although only 2.5 per cent of that in the intact hypocotyl, was 3500 per cent of that in established hypocotyl callus grown on medium A (table 18).

It was possible that the relatively high tomatine level was due to the presence of root primordia in the callus which could not be removed.

Tomatine analyses of callus tissue at different stages of 'development'.*

Because organised structures were produced spontaneously by newly-initiated callus, further experiments to induce organogenesis were considered unnecessary. However, in view of the frequently observed loss of organogenetic capacity in callus cultures of increasing age, it was decided to study tomatine production in callus cultures over a period of time.

Cultures were harvested, not at regular intervals, but according to a subjective assessment of their degree of 'development'. The first analyses were carried out on 72-day old cultures in which roots were relatively abundant, but less so than after 38 days. At the second analyses, cultures were 176 days old. A few roots were present in some of the cotyledon and radicle calluses, but the hypocotyl callus was devoid both of roots and organised primordia. Cultures used for the third analyses were 447 days old and contained neither macroscopic nor

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*The term 'development' is used here to refer to the period of variable callus growth between initiation and establishment.

TABLE 18. Tomatine content of 38-day old hypocotyl callus.

No. of flasks	total dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} DW)
5	523.7	235.87	0.45

microscopic organised structures (plate 3). All visible roots were removed before the tissues were analysed.

As the number of roots declined with increasing age of callus, so also did the tomatine concentration in the tissues (fig. 22). Calculations showed that this was not a dilution effect. Tomatine was present in all cultures while roots were produced, but it was also detected in 175 days old hypocotyl callus which bore no organised structures. At the final harvest, when no organised structures were present in any of the cultures, no tomatine was detected. At all times (except at the final harvest), the highest tomatine concentration was in hypocotyl callus and the lowest in cotyledon callus.

Since passage lengths were not equal it was not possible to express callus growth graphically. Nevertheless observations were made and recorded. After 38 days growth the fastest and slowest growing cultures were the hypocotyl and radicle cultures respectively. Between 38 and 175 days, there was a decrease in the growth of all the cultures and at 175 days the cotyledon callus exhibited greatest growth with that of the hypocotyl and radicle cultures being almost equal. By the final harvest, after 447 days, growth of all the cultures had significantly increased, with the hypocotyl callus again showing fastest growth and the radicle callus slowest.

Tomatine analyses of roots produced by callus cultures.

Preliminary qualitative analysis of roots produced by 38-day old hypocotyl callus had shown tomatine to be present. Quantitative analyses

PLATE 3. CALLUS CULTURES, WHICH HAD INITIALLY PRODUCED ROOTS, AT A LATER STAGE OF 'DEVELOPMENT'.

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a) hypocotyl callus

b) radicle callus

c) cotyledon callus

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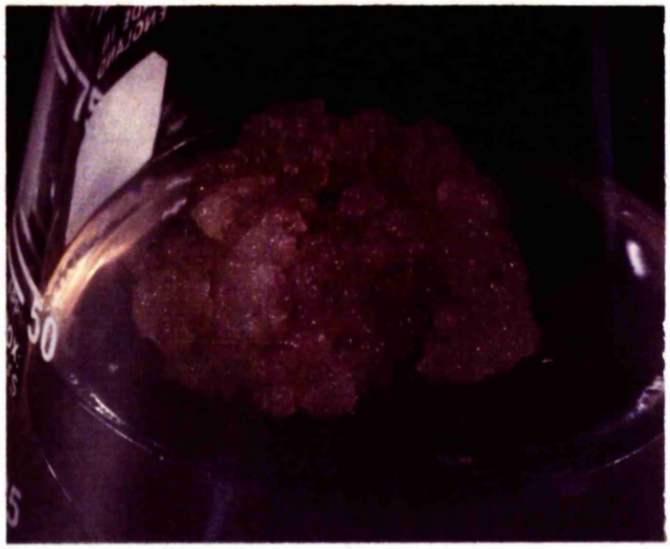
a)



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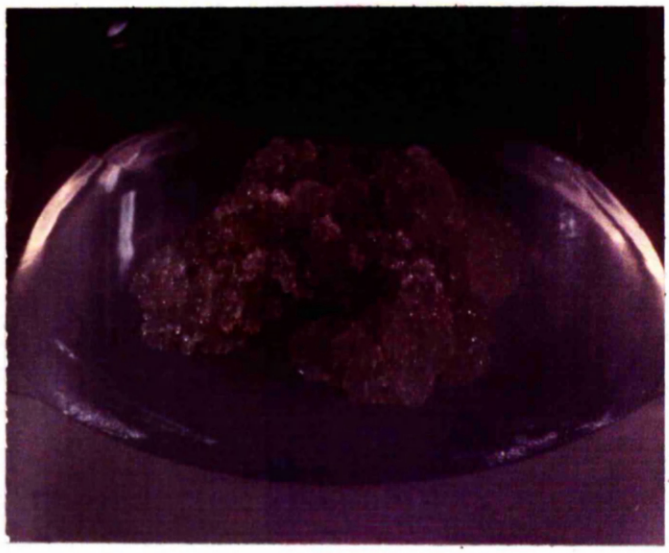

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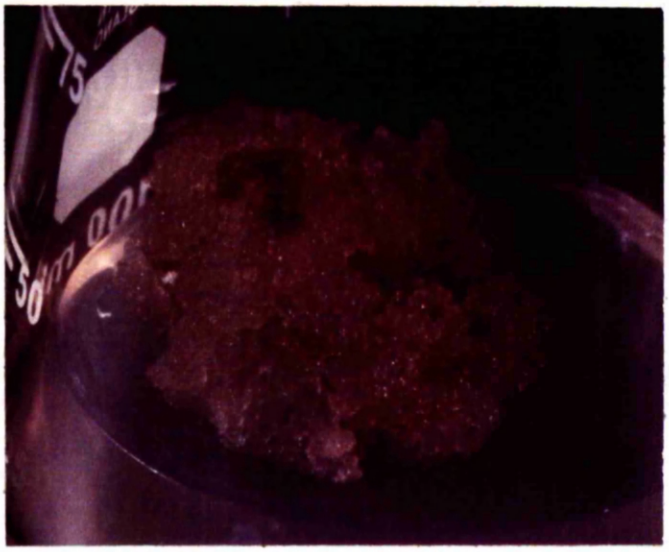

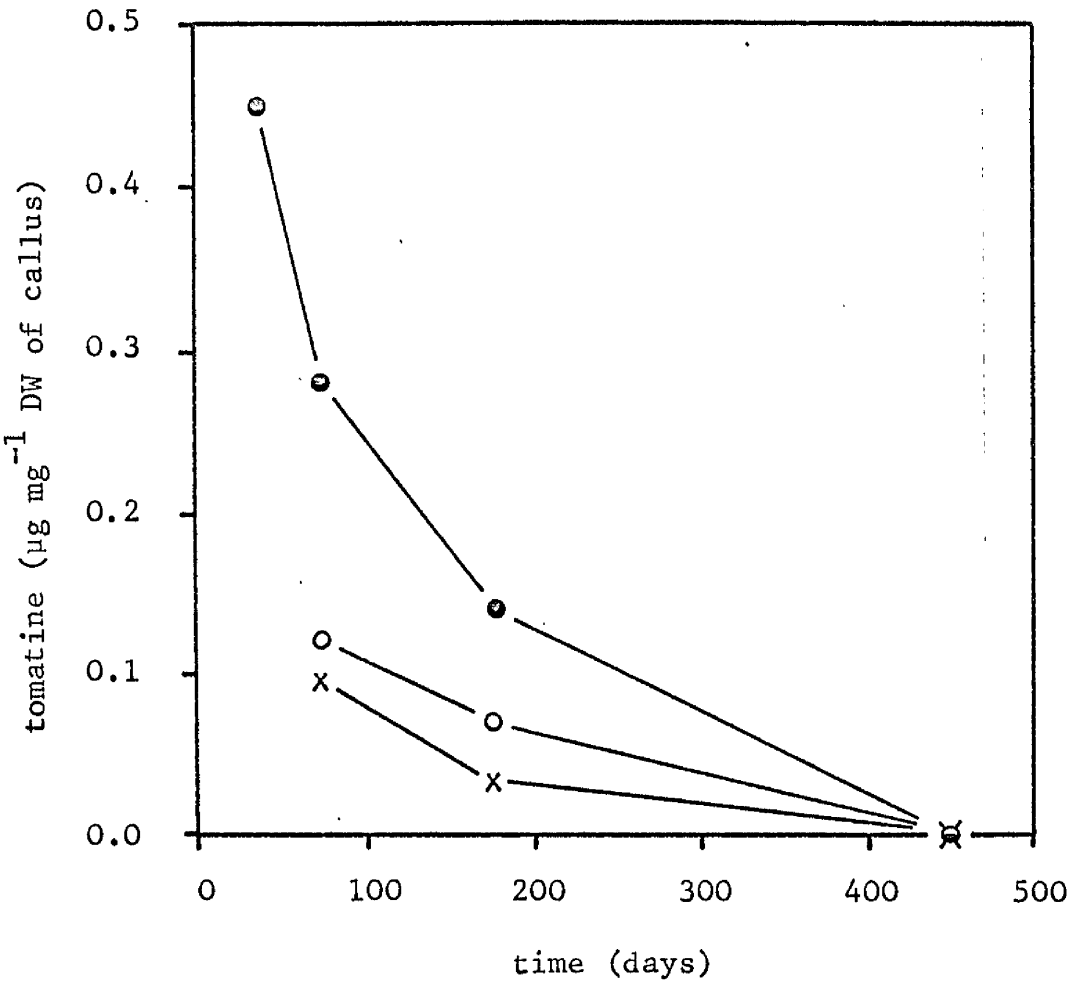

Fig.22. Tomatine content of callus cultures at different stages of 'development'. (Data for 38 days from table 18. Remainder of data in appendix 7.)

⊙——⊙ hypocotyl callus

o——o radicle callus

x——x cotyledon callus

were therefore conducted on roots from all three cultures.

Roots were removed from 72-day old cultures, and any remaining in the agar medium were separated out and thoroughly rinsed to remove adhering particles of solidified culture medium. Tomatine was extracted by the standard method used for cultured excised roots. Because of differences in the fresh weight/dry weight ratios, results have been expressed on a dry-weight basis.

Roots from hypocotyl and radicle callus had almost identical tomatine contents, but that of roots from cotyledon callus was lower (table 19). The ratio of tomatine concentrations in roots from hypocotyl, radicle and cotyledon callus (1.0 : 1.1 : 0.4) differed from that in the callus tissues from which they were derived (1.0 : 0.4 : 0.3).

Attempt to induce organogenesis in callus cultures.

The following experiment was carried out to investigate if the observed loss of capacity to synthesise tomatine in the above cultures could be reversed by re-establishing their capacity to produce roots.

Inocula from approximately 15-month old hypocotyl, radicle and cotyledon callus were transferred to a culture medium, similar to that used above but lacking NAA. In order to induce auxin limitation, cultures were left to grow without subculturing.

Up till the time of writing (c. 15 weeks) relatively little growth had taken place in any of the cultures and no organised structures had been produced.

TABLE 19. Tomatine content of roots produced by 72-day old callus cultures.

callus of origin	dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} DW)
hypocotyl	95.1	157.92	1.66
radicle	56.0	102.48	1.83
colyledon	87.6	60.48	0.69

Results refer to bulked tissue.

PART V

STUDIES WITH LIQUID SUSPENSION CULTURES.

SECTION 1. GROWTH AND TOMATINE PRODUCTION ON CHEMICALLY-DEFINED AND 'COCONUT-MILK' MEDIUM.

Having established that certain callus tissues were capable of synthesising tomatine, a study was made of the abilities of such cultures to elaborate the alkaloid when grown in an agitated, liquid medium. Two culture media were employed viz. medium A (containing coconut-'milk') and medium C (chemically-defined). Each was inoculated with hypocotyl callus which had been growing on the corresponding agar-solidified medium.

Growth and tomatine production in liquid medium A.

Eight weeks after initiation of the culture (i.e. at the end of the second passage), nine flasks were randomly selected and fresh and dry weights of the cells determined. The contents of a further nine flasks were bulked and, after filtering, the cells and 'spent' culture medium analysed for tomatine. Culture medium was analysed by the method described on p. 66.

When grown in liquid medium A, hypocotyl callus cells showed little or no growth (table 20). This was a marked change from the 24-fold weight increases which took place when grown on an identical, but agar-solidified, medium. The cultures did not exhibit the normal appearance of a suspension culture as the cells were aggregated into relatively large clumps.

No compounds resembling tomatine were detected in cell and culture medium extracts.

TABLE 20. Growth of suspension cultures of hypocotyl tissue in different liquid media.

culture medium	fresh weight (g)	dry weight (mg)	$\frac{FW}{DW}$
A	1.8 ±0.2	112.6 ±12.4	16.7
C ('yellow')	15.8 ±0.2	559.9 ±12.4	28.2
C ('green')	44.6 ±0.3	942.7 ±8.2	47.2

Results are expressed on a per flask basis.

Growth and tomatine production in liquid medium C.

Growth measurements and tomatine analyses were conducted using cultures which had been maintained through four passages (i.e. 16 weeks old). At time of harvest, two distinctive types of culture were apparent. The 'spent' culture medium was the normal yellow colour in some flasks, but in others it was green. Furthermore, growth appeared to have been more rapid in the 'green' culture medium than in the 'yellow'.

Two flasks of each type were used for measurements of fresh and dry weight. For tomatine analysis, cells from two flasks of each type were bulked. Filtered 'spent' medium from the two flasks of each type was also bulked for alkaloid analysis, but only after measurement of the volume obtained from each flask. Individual measurements of recoverable culture medium were made because the growth experiments had indicated that yields of 'spent' medium differed between the two types.

Despite the differences in culture medium colour, both types of culture grew as fine suspensions with few cell aggregates, none of which exceeded 2 mm diameter. The preliminary observation that growth was greater in the 'green' medium was confirmed. Fresh weight of tissue was almost three times as high, and dry weight almost twice as high in 'green' medium as in 'yellow' medium (table 20). The fresh weight/dry weight ratio of the cells was also higher in 'green' medium by a factor of about 2.

The weights of cells obtained for alkaloid analysis from the two flasks containing 'green' medium and from the two containing 'yellow' medium were 94.2g and 28.4g respectively. After filtering, the volumes

of 'green' medium obtained were 60 and 61 cm^3, and the volumes of 'yellow' medium 92 and 94 cm^3. These values are in keeping with the different fresh weight/dry weight ratios observed.

Tomatine was not detected in the cells or culture medium of either of the two types of culture.

DISCUSSION

Part of the work reported here has established that cultured excised roots of Lycopersicum esculentum are capable of synthesising tomatine. This confirms a similar finding made by Sander (1956). A statement in the same article that tomatine production is associated with the growth of the plant is of interest in view of the positive correlation found between alkaloid production and growth of cultured roots, as measured by weight. Similar correlations between these two factors have also been found by Solt (1957) and Hadwiger and Waller (1964) during the course of their studies of nicotine and ricinine biosynthesis respectively. Furthermore, the correlation between weight of roots and tomatine production was remarkably consistent and only on rare occasions were differential effects on these two factors observed. Dawson (1960) has also drawn attention to the consistency of the correlation between weight of cultured tobacco roots and nicotine production.

The changes in concentration of tomatine in cultured roots over a 24-day growth period cannot be explained on the basis of one mechanism due to accumulation of alkaloid during the first half of the growth period and lack of further accumulation in the second half.

Since tomatine concentration was calculated on a weight basis, the initial increase in alkaloid level suggests that during the first two days of culture tomatine synthesis occurs at a relatively greater rate than does accumulation of dry matter. The subsequent decline in tomatine concentration during the first half of the growth period emphasises the lack of direct proportionality between alkaloid production and weight of roots during active growth. This can probably be attributed to

accumulation of dry matter which is unrelated to growth (i.e. cell division and enlargement).

It is tempting to attribute the lack of further accumulation of tomatine between days 12 and 24 to limitations in precursors, but, as growth is still taking place during this period, it is unlikely that carbohydrate, steroid precursors or nitrogen are limiting. The possibility that tomatine is excreted into the culture medium to maintain a constant endogenous level can be ruled out by the inability to detect the alkaloid in 'spent' medium. It may be that the apparent cessation in tomatine biosynthesis is merely lack of net alkaloid production, and that tomatine is being synthesised in young lateral roots (which are mainly responsible for growth observed at this stage) and broken down at a corresponding rate in older senescent tissues. Tomatine degradation is known to occur in ripening tomato fruits (Sander and Grisebach, 1958) and a tomatine-degrading enzyme has been demonstrated in tomato leaves (Prokoshev et al., 1956). The possibility, therefore, that tomatine degradation also occurs in certain root tissues cannot be excluded.

The possibility that such a pattern of changes in alkaloid concentration in Lycopersicum roots might be a general phenomenon is strengthened by the work of Bruske (1966), who also observed an initial increase and subsequent decrease in tomatine concentration in cultured excised roots of L. pimpinellifolium.

The correlation between cultured root growth and tomatine production suggests that actively growing cells are the sites of alkaloid biosynthesis

Working with cultured tobacco roots, Solt et al. (1960) found that nicotine synthesis is confined to the root apex and is correlated with root growth, whereas anabasine synthesis takes place in the more mature root tissues and is not correlated with growth. The finding that mature regions of cultured tomato roots were incapable of elaborating tomatine infers that root apices are the sites of synthesis of this alkaloid. The disadvantages of such an indirect approach are appreciated, but it should be stressed that, because of its 'dynamic' nature, the root apex is not conducive to direct analysis. Probably the most suitable approach would be to expose the cultured root to a brief pulse of radioactive precursor (e.g. ^{14}C- mevalonic acid) and examine the distribution of labelled tomatine soon afterwards.

The fact that tomatine is found in equal concentrations in parts of the root which are of different physiological ages, and that different alkaloid concentrations are found in root systems of different ages, constitutes a paradox. However, this can be resolved by the fact that, as growth occurs, the root becomes progressively thicker towards the apex (Sinha, 1968). Thus, even with the same total alkaloid, a two-day old root and that part of a ten-day old root formed between 8-10 days (i.e. two days old) would exhibit differences in alkaloid concentration due to differences in the amount of dry matter present. Also, it is likely that the metabolic activity in the tip of an eight-day old root would be less than that in an inoculum tip. This could result in differences in rate of tomatine synthesis which would further contribute to resolving the above paradox.

The presence of tomatine in all parts of the root system further raises the question of its mode of distribution. The findings that tomatine is not present in exuded sap from decapitated tomato plants (Sander, 1956), or present in root culture medium seem to exclude distribution via the vascular tissues, as is the case with nicotine (Dawson, 1942). Since no evidence could be obtained of de novo synthesis in older parts of the root, a possible explanation of tomatine distribution is that the alkaloid in each cell was synthesised while that cell constituted part of the root apex and has been retained within the cell during its basipetal 'movement' in the root. Such a process could be tested by exposing inoculum tips to a brief pulse of ^{14}C- mevalonic acid and then returning to culture medium containing 'cold' mevalonic acid for a further 3-4 days. Detection of labelled tomatine as a 'band' near the middle of the main axis would be evidence in favour of the proposed mechanism.

The finding that growth of cultured tomato roots and tomato seedling radicles is unaffected by concentrations of exogenous tomatine which inhibit growth of lettuce seedling radicles raises the question of whether response to exogenous alkaloid is influenced by the presence of endogenous alkaloid. If toxicity of tomatine is principally due to its complexing with membrane sterols and so disrupting membrane permeability, then it would appear that in tomato cells formation of this complex is somehow prevented. There is mounting evidence that steroidal sapogenins are associated with membranes and membrane-bound organelles

(Stohs et al., 1969; Akahori et al., 1970), and so a similar intracellular location for tomatine cannot be ruled out. Such a location might also help explain the above findings. This is a problem worthy of further investigation.

That neither growth of cultured tomato roots nor their ability to synthesise tomatine is affected by exposure to light is not altogether unexpected considering the subterranean habit of plant roots. Nevertheless, various workers have reported light-alteration of growth of cultured roots of different species. In some cases exposure to light resulted in increases in growth (Gautheret, 1935; Street, 1953; Street et al., 1961), whereas in other cases growth was inhibited (Segelitz, 1938; Burström, 1959, 1960). Since growth of cultured tomato roots has been reported to be influenced by certain wavelengths emitted from a tungsten filament (Street, 1953), the lack of success here may be due to the particular light source employed (fluorescent - 'warm white'). It might be of interest to investigate the effect of ultra-violet light on tomatine synthesis in cultured roots, in view of the report by Conner (1937) that such wavelengths stimulate solanine synthesis in potato tubers.

The higher concentrations of tomatine in cultured roots incubated at sub- and supra-optimal temperatures cannot be satisfactorily explained by a single mechanism. The fresh weight, dry weight, main axis length and tomatine concentration of roots grown at the two temperature extremes (15°C and 35°C) were very similar to those recorded in two-day old roots grown at the normal temperature of 25°C, thus suggesting that only two days growth had taken place at these temperatures. Because of the time

required for temperature equilibration between incubator and culture medium and the changes in growth rate of roots which would take place during this time, it cannot be categorically stated that root growth took place during the first two days and then ceased. It is likely that growth continued beyond two days, but that total growth was equivalent to that which takes place during the first two days of culture at 25°C.

The elevated tomatine levels in roots cultured at 30°C is apparently due to differences in the temperature optimum for growth and tomatine synthesis. The fact that root growth is more adversely affected by a 20°C incubation temperature than is tomatine synthesis suggests that the former process is more sensitive to deviations from the optimum temperature than is the latter. These findings tentatively suggest that the enzymes involved in tomatine biosynthesis exhibit broader temperature optima than those involved in growth processes.

Both weight of cultured roots and total tomatine showed similar slight increases when urea or casamino acids were added to the culture medium (c.f. Hannay et al., 1959; Street et al., 1960). Neither of these compounds is an intermediate in tomatine biosynthesis, and the possibility that they directly influence alkaloid synthesis by increasing soluble nitrogen levels can be ruled out by the fact that the standard culture medium is rich in nitrate nitrogen. Because of the correspondence between changes in growth and tomatine content, the effects of these compounds on tomatine synthesis are likely to be indirect, exerted through cell processes involved in growth.

The reductions in all parameters of cultured root growth in the

presence of low concentrations of ammonium sulphate tend to be contrary to the findings of other workers (e.g. Skinner and Street, 1954; Sheat et al., 1959). Since low concentrations of ammonium ions in 'nitrate' medium are held to maintain or enhance (but not inhibit) root growth probably due to stabilisation of the pH of the culture medium (Street, 1965a), it is difficult to account for the inhibitory effects observed. However, the growth reductions at higher salt concentrations are in keeping with the toxic effects of high ammonium ion concentrations. Growth reduction implies a lower overall metabolic rate in growing cells and this could account for the decreases in the tomatine content of roots.

The failure of added precursors (i.e. acetate, mevalonate and cholesterol) to increase alkaloid concentration in cultured roots is comparable with the findings of certain other workers using cultured roots of different species. Solt et al. (1960) were unable to increase nicotine levels in cultured tobacco roots by adding nicotinic acid, ornithine or lysine, and Hadwiger and Waller (1964) showed that ricinine levels in cultured Ricinus roots were unaffected by additions of nicotinic or succinic acid. However, the above workers did find that these precursors were incorporated into the appropriate alkaloid, and, in fact, acetate (Sander and Grisebach, 1958) and cholesterol (Heftmann et al., 1967; Tschesche and Hulpke, 1966) have been reported to be incorporated into tomatine in intact plants. It is interesting that the cultured roots in which alkaloid levels could not be increased by addition of precursors also showed correlations between growth rate and rate of

alkaloid production. On the other hand, alkaloid levels in cultured roots of Atropa belladonna have been increased by addition of arginine or ornithine to the culture medium (Reinouts van Haga, 1957).

Acetyl-CoA can be regarded as the compound at which 'primary' metabolic processes, and 'secondary' biosynthetic pathways leading to steroid synthesis, diverge. Because of the importance of acetyl-CoA in energy-yielding processes, its rate of synthesis, conversion etc. is likely to be strictly regulated by other events in such pathways. This could explain the lack of effect of acetate itself on rate of tomatine biosynthesis.

Although mevalonic acid and cholesterol are often regarded as being associated with 'secondary' rather than 'primary' metabolism, both are involved in synthesis of sterols, the importance of which in the life of the cell is generally recognised, despite lack of precise knowledge of their function. It is probable therefore, that sterol biosynthesis is also subject to metabolic regulation.

The increases in weight of roots and total tomatine after addition of cholesterol to the culture medium suggest that endogenous sterols may be present in slightly limiting amounts in cultured tomato roots. The constancy of tomatine concentrations throughout this experiment indicates that increasing the pool size of sterols does not alter their relative distribution between the tomatine biosynthetic pathway and growth processes.

If sterols are, in fact, limiting in cultured tomato roots, then addition of sterol precursors such as mevalonic acid might be expected

to give similar results. This was not found to be the case. If, on the other hand, sterols (or mevalonic acid) are not limiting, then the constancy of the growth rate can be explained by metabolic regulation, but the reduction in alkaloid level is not compatible with such a mechanism. No adequate explanation can be offered for the observed effect of mevalonic acid on tomatine production.

The reductions in root growth brought about by SKF 7997-A_3, and the improbability of these reductions being caused by changes in the amount of tomatine present, draw attention to the importance of sterols in growth and development of cultured tomato roots. The reductions in root growth and tomatine production suggest that certain sterols can be utilised both in growth processes and tomatine biosynthesis. The finding that tomatine concentrations remained constant at all inhibitor levels indicates that there is no diversion of sterols destined to enter the tomatine biosynthetic pathway into other pathways more closely involved in growth, in order to compensate for reductions in this latter process.

Callus cultures derived from tomato have been initiated by other workers (Mehta, 1966; Fukami and Mackinney, 1967; Ulrich and Mackinney, 1969, 1970), but have not been examined for tomatine. The only other reports of the presence of tomatine in undifferentiated tissues come from Kovacs *et al.* (1964) and Callam and Callow (1964) who identified the alkaloid in crown-gall tumours of tomato. These tumours, however, were not grown aseptically *in vitro*.

The fact that the tomato callus tissues from which tomatine was isolated had been maintained in culture for at least two years prior to

analysis indicates that the tomatine biosynthetic pathway is operational in such tissues and that the alkaloid present is not that carried over from the original explant.

The presence of tomatine in 'spontaneous' hypocotyl callus and its absence in 'spontaneous' root callus does not necessarily imply that the tomatine-synthesising capacity of callus is determined by that of the organ from which it was derived. Raj Bhandary et al. (1969) found that callus derived from A. belladonna roots, which contain tropane alkaloids, was devoid of such compounds. Some workers (e.g. Speake et al., 1964; Furuya et al., 1966) have detected nicotine in tobacco callus whereas others (e.g. Benveniste et al., 1966; Krikorian and Steward, 1969) have not.

Evidence that certain biochemical pathways can be suppressed or blocked according to the metabolic status of the cell is supplied by the apparent absence of tomatine in chlorophyllous hypocotyl callus, which was derived from callus (non-chlorophyllous) which elaborated the alkaloid. In this case, loss of alkaloid-synthesising capacity was associated with a reduction in growth rate and development of chlorophyll. The reduced growth of the callus might result in a concomitant reduction in tomatine production, but against this must be considered the finding by Thomas and Stobart (1970) that exposure of greening callus of Kalanchöe crenata to light caused an increase in the activities of the enzymes catalysing conversion of mevalonic acid to mevalonate-5-pyrophosphate. It may be that, despite increases in their rates of synthesis, certain steroid precursors are preferentially utilised in chloroplast development. In fact, Conner (1937) reported that whereas

chloroplast development in potato tubers was induced by visible light, rate of solanine synthesis was enhanced by ultra-violet light.

The presence of tomatine in hypocotyl callus grown on media A and C, and its absence in hypocotyl callus grown on medium B suggests that conditions conducive to tomatine synthesis on medium A were absent in medium B, but restored in medium C. Because these culture media differed in more than one component, delimitation of the factors involved presents a difficult task. The fact that levels of meso-inositol in media A and B were identical, as were supplementary salt concentrations in media B and C, tends to eliminate these constituents as those influencing tomatine synthesis. The remaining possibilities are therefore coconut-'milk', 2,4-D and kinetin. These compounds are all known to influence growth rates and, in fact, callus growth on media A and C was very similar, while that on medium B was reduced. The differential capacity of hypocotyl callus to synthesise tomatine may therefore be the result of growth differences brought about by alterations in the levels and ratios of these growth substances. Coconut-'milk' and 2,4-D have been found to exert a synergistic action (Steward and Caplin, 1951) and the loss of this effect on medium B may have contributed to the loss of capacity to synthesise tomatine. Synergistic interaction also occurs between 2,4-D and kinetin (Das et al., 1956) and the degree of synergism in media B and C may have differed sufficiently (because of different auxin/kinetin ratios) to account for the observed differences in growth rates and tomatine levels. Other workers (e.g. Furuya et al., 1967; Furuya, 1968; Konosima et al., 1967) have reported that levels of

alkaloids in callus tissues are influenced by the nature, concentration and ratios of growth substances in the culture medium. However, it is obvious that, in this case, more critical studies are required to establish the causal factors.

Since modifications to the culture medium which induce organ formation in one tissue do not necessarily do so in tissues from different species (Street, 1965b), the inability to induce organogenesis in tomato callus does not come as altogether unexpected. The lack of regenerative capacity in hypocotyl callus cannot be attributed to loss of organised structures with time, as the culture was initiated from spontaneous cell proliferations in which no such structures were observed. Due to lack of experimentation it cannot be categorically stated that the cultures have entirely lost their capacity to elaborate organised structures. However, it should be borne in mind that certain tissues, despite being subjected to rigorous experimentation, cannot be made to initiate organs (Nobécourt and Hustache, 1954).

The decline in number of roots formed by newly-initiated callus with increasing length of time in culture, and the concomitant decreases in tomatine content of the tissues is, at first glance, indicative of a causal relationship between morphological organisation and tomatine biosynthesis. However, Dawson (1960) also observed a similar decrease in nicotone content of newly-initiated tobacco callus, but no organised structures were present. All visible roots produced by tomato callus were removed before analysis of callus, and since these were unlikely to secrete tomatine into the callus, any relationship between organisation

and alkaloid production must be based on the presence of organised root primordia within the callus. Visible roots, therefore, acted merely as indicators of the degree of organisation within the callus. In view of Dawson's findings, it may be that the contribution of the root primordia to the alkaloid content of the callus was negligible, but the large number of roots observed renders this unlikely. There is, in fact, some evidence that capacity to synthesise tomatine was lost in callus cells. No roots or root primordia were present in the 175-day old or 447-day old hypocotyl callus, although tomatine was still present after 175 days but absent after 447 days. This reduction in alkaloid content, could not be attributed to decrease in growth since, on the contrary, growth increased during this period. These findings imply that both callus cells and root primordia contributed to the extracted tomatine, and that the observed decreases in tomatine content of callus were due to both loss of tomatine-synthesising capacity in callus cells and loss of regenerative powers.

The relatively low tomatine content of roots derived from callus cannot be explained by postulating that rate of alkaloid biosynthesis in the 'parent' callus influences that in roots to which it give rise. Not only is there a lack of correspondence between the ratio of alkaloid concentration in roots of different origin and that in the 'parent' callus cultures, but it has been shown (Raj Bhandary et al., 1969) that roots derived from alkaloid-free callus of A. belladonna are capable of elaborating such compounds. The most likely explanation is based on the finding that the tomatine concentration in cultured excised roots decreased

with time. Since visible roots were removed from callus tissue used as inocula, the roots which were analysed were a maximum of 34 days old. Studies with cultured roots were only conducted up to 24 days, but the alkaloid content at this stage was also relatively low (2.4µg mg^{-1} dry weight) and the graph was still following a downward trend. It is possible therefore that the tomatine concentration in 34-day old cultured roots would be similar to that in roots produced by callus. This would then suggest that roots derived from callus tissue are similar to cultured excised roots in their capacity to synthesise tomatine.

Because of the lack of growth of hypocotyl callus cells grown in liquid medium A, it is difficult to draw conclusions about the absence of tomatine in this culture. However, tomatine was also absent from cultures grown in liquid medium C, where rapid growth was observed. Cells grown in liquid suspension often have different nutritional and hormonal requirements from those grown as callus on solidified nutrient medium (Street and Henshaw, 1965), and these differences may be sufficient to account for absence of tomatine (as has been previously discussed for callus cultures). In this respect it would be interesting to investigate if tomatine synthesis is restored in callus cultures initiated from cell aggregates of a suspension culture.

The small amounts or absence of tomatine in callus and suspension cultures, and the relatively high alkaloid levels in cultured roots is in keeping with the findings of other workers who have studied alkaloid biosynthesis in cultured tissues and organs (e.g. Speake et al., 1964; Raj Bhandary et al., 1969). It appears therefore, that tomatine bio-

synthesis (like synthesis of nicotine and tropane alkaloids) is related to the degree of morphological organisation pertaining within a system.

The findings of this work raise the question of why cultured tissues and organs derived from tomato are unable to elaborate tomatine to the same degree as found in the intact plant. This phenomenon is not unique to tomato cultures, but has been widely reported in callus cultures, and also in cultured roots of Hyoscyamus niger (Telle and Gautheret, 1947) and Datura stramonium (Stienstra, 1954).

The different tomatine levels in seedling roots and cultured excised roots can hardly be attributed to differences in the degree of organisation, but are probably associated with the presence or absence of attached shoot tissues. It is possible to attribute the lower alkaloid concentration in cultured roots to deficiences in compounds normally supplied by the shoot, but insufficient information is available to discuss this question in detail. Alternatively, the influence of the shoot may be exerted through its effect on root growth and development, and there is good evidence that such growth correlations are regulated by growth substances. Cultured roots are apparently able to synthesise auxin (Thurman and Street, 1960), gibberellic acid (Butcher, 1963) and possibly kinetin (Weiss and Vaadra, 1965) and, because they are isolated from the influences of the shoot, it is probable that their hormonal status differs from that of intact roots. This could account for the differences in tomatine content of cultured and intact roots, especially when considering that alkaloid levels in callus tissue are also reported to be influenced by growth substances. In fact, Solt (1957) has shown that rate

of nicotine synthesis in cultured tobacco roots is reduced by addition of IAA to the culture medium. Consequently, it would be interesting to study the effects of different growth substances on tomatine synthesis in cultured tomato roots.

Callus tissues have no analogue in the intact plant and hence caution must be exercised when drawing comparisons between the biosynthetic capacities of such tissues and intact plant tissues. Nevertheless, the fact that such cells are free from the constraints of the intact organ renders them convenient for study of factors controlling biosynthesis of certain metabolites.

Since knowledge is lacking of the actual cell types in the organ explant which gave rise to the callus cultures, it is obviously difficult to compare their tomatine-synthesising abilities. Consequently, the absence or low amounts of tomatine in certain cultures may genuinely reflect the capacity of the 'parent' cells in the intact plant to synthesise the alkaloid. However, the finding that tomatine levels decreased during the period of callus 'development' suggests that a certain degree of repression of tomatine synthesis occurs in callus tissue.

Bu'lock (1965) has suggested that in micro-organisms, changes in the state of development or organisation are associated with changes in 'primary' metabolism which result in accumulation of 'primary' metabolic intermediates. Release of this "primary metabolic pressure" is achieved by diversion of these intermediates into 'secondary' metabolism. That a balance exists between 'primary' and 'secondary' metabolism is unquestionabl

and hence there seems no reason why conditions might prevail in tissues such as callus where 'primary' metabolism makes such demands on available intermediates that little or no secondary products are formed.

It can be concluded that cultured tissues and organs derived from the tomato plant cannot, as yet, be induced to elaborate tomatine to the same degree as the corresponding structures in the intact plant. The lack of success from precursor studies emphasises how unnaturally large pools of metabolic intermediates can be counteracted by internal regulatory mechanisms. The problem therefore, in increasing secondary product levels in cultured plant systems appears to lie primarily in the biochemical modifications induced by in vitro conditions.

The fact that cultured excised roots are morphologically and anatomically similar to seedling roots indicates that in vitro conditions do not sufficiently simulate in vivo conditions to provide the necessary biochemical conditions for similar degrees of tomatine biosynthesis. In the case of callus cultures, such an argument does not necessarily apply. Even if in vivo conditions could be simulated in culture there is no guarantee that callus cells would possess the same secondary biosynthetic capacities as undifferentiated parenchyma cells. Krikorian and Steward (1969) have drawn attention to the artificial nature of standardised nutrient medium and constant environmental conditions. However, even if such modifications were possible, levels of secondary products might only attain those found in the intact plant. To achieve higher levels of secondary products in cultured systems than in the intact plant, without altering the degree of structural organisation, much greater

knowledge of the relationship between 'primary' and 'secondary' metabolism, metabolic regulatory mechanisms and factors governing growth and morphogenesis is obviously required.

The findings of this work are in agreement with the generally held opinion that use of cultured plant systems for large-scale production of important secondary products is not yet an economically feasible proposition. Nevertheless, plant tissue and organ culture techniques have much to offer in studies of secondary product biosynthesis and adoption of such an approach has been advocated by Bu'lock (1965). Such information might not only help to elucidate the biological significance of secondary products but, in conjunction with our growing knowledge of growth and metabolism of plant cells in vitro, might contribute to development of techniques for economic exploitation of cultured plant systems in the future.

SUMMARY

Excised root cultures, initiated from seedling radicles of tomato (Lycopersicum esculentum MILL. var. Suttons Best of All), have been found to synthesise the steroidal alkaloid tomatine, although alkaloid levels were lower than in intact radicles. There was no evidence of the aglycone, tomatidine, or modified forms of tomatine in cultured root extracts, and the alkaloid was not excreted into the culture medium.

A procedure has been devised for purifying extracted tomatine by thin-layer chromatography (TLC). The identity of extracted tomatine has been confirmed by TLC, colour reactions, visible/ultra-violet spectroscopy, mass-spectroscopy and sugar analyses.

A positive correlation has been found between growth of cultured roots, as measured by weight, and production of tomatine. This suggests that actively growing cells, and particularly root apices are the sites of tomatine synthesis. Indirect evidence which supports this hypothesis has been presented. However, tomatine does not appear to accumulate in apical regions, but is present in equal concentrations in all parts of the root. The implications of this finding in the mode of distribution of tomatine in the root are discussed.

Changes in the concentration of tomatine in cultured roots during growth have been discussed on the basis of differential rates of tomatine synthesis (and possibly degradation) and dry-matter accumulation.

Growth of cultured roots was unaffected by the presence of 'authentic' tomatine (up to 100 ppm) in the culture medium. Nor was such alkaloid

absorbed to any significant degree or chemically transformed. Radicle growth in tomato seedlings was likewise unaffected by 'authentic' tomatine, but that in lettuce seedlings was inhibited. The question has been raised of whether response of tissues to exogenous tomatine is influenced by the presence of endogenous tomatine.

The optimum temperature for tomatine synthesis appears to be higher than that for cultured root growth. There was also evidence that the former process is less adversely affected than the latter by deviations from the optimum temperature.

Addition of nitrogenous compounds or steroid precursors to the culture medium did not result in significant increases in tomatine levels in cultured roots. Nevertheless, these experiments, on the whole, confirmed the previously observed correlation between growth of cultured roots, as measured by weight, and tomatine production. No definite correlations could be established between tomatine production and other growth parameters.

Small amounts of tomatine have been found in 'spontaneous' hypocotyl callus grown on a medium supplemented with coconut-'milk', but when the tissue developed chlorophyll, no alkaloid could be detected. 'Spontaneous' root callus, grown on the same medium, was also devoid of tomatine. Hypocotyl callus was established on two different chemically-defined media. On one medium, tomatine was absent from the callus, but on the other it was present. Tomatine was not detected in root callus grown on either of these media. The changes in tomatine-

synthesising capacity of hypocotyl callus are thought to be due to differences in certain culture medium components, especially growth substances.

New callus cultures have been initated on a chemically-defined medium from hypocotyl, radicle and cotyledon of tomato seedlings. All cultures produced roots during the early stages of growth and tomatine was present in both callus tissue and roots. Alkaloid levels in roots were comparable with those in cultured excised roots of the same age. Both number of roots and tomatine levels in the callus tissues decreased with time, until eventually, neither roots nor alkaloid was produced by any of the cultures. There was evidence that the reduction in alkaloid levels was due to both decrease in numbers of roots and loss, by the callus cells, of the capacity to synthesise tomatine.

Liquid suspension cultures were initiated from 'spontaneous' hypocotyl callus using both 'coconut-milk' medium and chemically-defined medium but tomatine was not found in the cells or 'spent' medium of either culture.

It is concluded that tomatine biosynthesis is associated with the degree of morphological organisation pertaining within a cell system.

Possible reasons for, and implications of, the reduced capacities of cultured tissues and organs to synthesise tomatine, compared with those of the corresponding structures in the intact plant, are discussed.

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APPENDIX

APPENDIX 1a. Anthrone technique (McCready et al., 1950).

500 mg of anthrone (previously stored in a refrigerator at 4°C) was dissolved in 250 cm^3 of 95 per cent sulphuric acid, also at 4°C. The solution was prepared immediately before commencing the analysis and, if not bright yellow in colour, was discarded.

10 cm^3 of solution was dispensed into a boiling tube in an ice bath and 5 cm^3 of the sugar solution 'layered' into the tube. After sealing with aluminium foil and shaking vigorously to mix contents, the tube was placed in a boiling water bath for exactly ten minutes.

At the end of this time, the tube was transferred to an ice bath. When cool, 7 cm^3 of the mixture was transferred to a colorimeter tube and the colour intensity measured on an EEL colorimeter fitted with a red filter. The reference mixture used to zero the colorimeter contained 5 cm^3 of distilled water in place of sugar solution.

The amount of sugar was calculated by reference to a calibration graph.

APPENDIX 1b. Estimation of glucose. (Boehringer 'Biochemica Test Combination', TC-X-1).

The following were pipetted into a glass spectrophotometer cell (path length 10 mm):-

i) 2.60 cm^3 of 0.3M triethanolamine buffer, pH 7.5; 4mM MgSO4

ii) 0.20 cm^3 of sugar sample (no dilution required).

iii) 0.10 cm^3 of 12mM NADP.

iv) 0.10 cm^3 of 16mM ATP.

Contents were mixed with a plastic paddle and the absorbance (A_1) measured at 340 nm, against air, on a Pye 'Unicam' SP 500 spectrophotometer.

v) 0.02 cm^3 of suspension containing 1 mg cm^{-3} of hexokinase and 1 mg cm^{-3} of glucose-6-phosphate dehydrogenase.

Contents were mixed, as above, and after ten minutes the absorbance (A_2) noted.

$$\text{glucose concentration (mg 100 cm}^{-3}) = \Delta A_{340\ nm} \times 43.7$$

$$(\text{where } \Delta A = A_2 - A_1)$$

APPENDIX 1c. Estimation of galactose (Boehringer 'Biochemica Test Combination', TC-GA).

The following were pipetted into a glass spectrophotometer cell (path length 10 mm):-

i) 3.00 cm^3 of 0.2M phosphate buffer, pH 7.5.

ii) 0.10 cm^3 of 13mM NAD.

iii) 0.20 cm^3 of sugar sample (no dilution required).

Contents were mixed with a plastic paddle and the absorbance (A_1) measured at 340 nm, against air, on a Pye 'Unicam' SP 500 spectrophotometer.

iv) 0.02 cm^3 of suspension containing 5 mg cm^{-3} of galactose dehydrogenase.

Contents were mixed, as above, and after 40 minutes, the absorbance (A_2) noted.

galactose concentration (mg 100 cm^{-3}) = $\Delta A_{340\ nm} \times 48.0$

(where $\Delta A = A_2 - A_1$).

APPENDIX 2. Tomatine production in cultured roots over a 24 day growth period.

time (days)	No. per replicate	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	200 tips	0.55 ± 0.05	0.25 ± 0.03	0.45 ± 0.04
2	10 flasks	1.9 ± 0.1	1.36 ± 0.16	0.71 ± 0.08
4	3 flasks	5.1 ± 0.2	2.78 ± 0.25	0.55 ± 0.05
6	2 flasks	7.9 ± 0.5	3.22 ± 0.42	0.42 ± 0.07
8	1 flask	18.1 ± 1.0	10.21 ± 0.82	0.57 ± 0.05
10	1 flask	28.2 ± 3.4	11.93 ± 1.58	0.42 ± 0.01
17	1 flask	50.0 ± 7.7	15.40 ± 4.11	0.31 ± 0.13
24	1 flask	70.7 ± 4.2	15.99 ± 2.64	0.23 ± 0.03

Results are expressed on a per root basis.

APPENDIX 3. Effect of temperature on tomatine production in cultured roots.

temperature (°C)	No. per replicate	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
15	6 flasks	8.0 ± 0.5	6.87 ± 0.20	0.87 ± 0.06
20	2 flasks	70.3 ± 4.4	55.90 ± 2.84	0.80 ± 0.05
25	1 flask	204.8 ± 5.2	97.55 ± 3.46	0.48 ± 0.02
30	1 flask	134.9 ± 8.8	110.44 ± 11.02	0.82 ± 0.07
35	8 flasks	6.2 ± 0.2	5.12 ± 1.09	0.82 ± 0.15

Results are expressed on a per flask basis.

APPENDIX 4a. Effect of ammonium sulphate on the growth of cultured roots.

$(NH_4)_2SO_4$ concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	126.8 ± 5.0	39.0 ± 2.8	261.5 ± 25.9
5.3	104.3 ± 8.3	27.8 ± 3.0	137.1 ± 19.1
10.7	116.4 ± 3.6	34.8 ± 2.8	162.3 ± 21.2
53.4	132.3 ± 6.9	41.4 ± 3.2	192.5 ± 23.3
106.8	120.6 ± 5.7	38.0 ± 2.1	163.3 ± 10.0

APPENDIX 4b. Effect of ammonium sulphate on tomatine production in cultured roots.

$(NH_4)_2SO_4$ concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	162.6 ± 1.9	64.56 ± 5.52	0.40 ± 0.03
5.3	135.4 ±14.8	48.78 ± 4.37	0.37 ± 0.04
10.7	149.0 ±38.3	56.86 ±14.35	0.38 ± 0.02
53.4	88.2 ± 5.3	24.55 ± 7.46	0.28 ± 0.09
106.8	112.6 ±11.9	28.01 ±14.04	0.23 ± 0.10

Results are expressed on a per flask basis.

APPENDIX 4c. Effect of urea on the growth of cultured roots.

urea concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	100.1 ± 5.5	24.8 ± 1.6	145.8 ±11.3
25	97.5 ± 8.3	29.5 ± 3.5	172.4 ±28.5
50	105.5 ± 7.2	27.5 ± 3.3	172.3 ±28.9
100	105.5 ± 7.1	24.3 ± 2.4	183.8 ±21.6
200	120.9 ± 6.4	28.7 ± 3.0	208.3 ±28.2

APPENDIX 4d. Effect of urea on tomatine production in cultured roots.

urea concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	134.7 ±14.2	54.02 ± 8.31	0.40 ± 0.04
25	127.2 ±15.3	57.52 ± 3.81	0.46 ± 0.03
50	132.1 ± 5.2	54.70 ± 3.06	0.42 ± 0.01
100	155.4 ±21.0	64.97 ±10.33	0.42 ± 0.04
200	163.7 ± 4.2	65.97 ± 7.07	0.40 ± 0.03

Results are expressed on a per flask basis.

APPENDIX 4e. Effect of casamino acids on the growth of cultured roots.

CAA concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	83.8 ± 9.3	22.4 ± 4.2	109.4 ±19.6
50	101.4 ± 8.0	26.0 ± 2.1	152.1 ±18.0
100	115.1 ± 6.5	25.8 ± 2.1	181.8 ±24.2
250	94.7 ± 9.1	17.8 ± 3.6	95.9 ±17.5
500	70.0 ± 9.5	12.4 ± 2.6	71.9 ±14.6

APPENDIX 4f. Effect of casamino acids on tomatine production in cultured roots.

CAA concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	74.5 ± 3.8	33.46 ± 3.07	0.45 ± 0.03
50	92.7 ± 3.4	37.49 ± 5.13	0.40 ± 0.04
100	82.8 ± 5.2	28.39 ± 2.58	0.34 ± 0.02
250	99.0 ±12.1	38.79 ± 5.55	0.39 ± 0.15
500	93.1 ± 6.3	38.27 ± 4.27	0.41 ± 0.05

Results are expressed on a per flask basis.

APPENDIX 5a. Effect of sodium acetate on the growth of cultured roots.

acetate concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	142.5 ± 6.0	56.8 ± 5.0	276.0 ±35.4
1	140.9 ± 4.9	59.6 ± 3.4	298.1 ±33.6
5	135.3 ± 5.7	55.4 ± 4.9	237.0 ±34.3
10	119.0 ± 5.4	48.6 ± 3.9	201.9 ±33.2
20	107.3 ± 2.7	29.4 ± 2.4	115.3 ±15.4

APPENDIX 5b. Effect of sodium acetate on tomatine production in cultured roots.

acetate concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	129.0 ± 3.1	61.77 ± 3.16	0.48 ± 0.03
1	137.9 ±11.4	55.44 ± 8.58	0.40 ± 0.03
5	123.1 ±15.3	54.57 ± 6.42	0.45 ± 0.03
10	107.0 ±23.9	44.17 ±11.82	0.41 ± 0.03
20	104.2 ±14.0	42.49 ± 9.22	0.39 ± 0.05

Results are expressed on a per flask basis.

APPENDIX 5c. Effect of mevalonic acid lactone on the growth of cultured roots.

mevalonate concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	120.9 ± 4.9	36.1 ± 2.8	216.1 ±25.7
5	109.3 ± 7.9	30.4 ± 3.8	178.5 ±28.5
10	115.8 ± 6.9	36.3 ± 3.4	246.7 ±30.5
25	120.8 ± 2.9	34.6 ± 2.3	199.6 ±19.5
50	107.3 ± 9.8	29.0 ± 3.9	179.7 ±29.5

APPENDIX 5d. Effect of mevalonic acid lactone on tomatine production in cultured roots.

mevalonate concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	137.5 ± 6.8	69.07 ± 3.10	0.51 ± 0.05
5	140.2 ± 9.8	59.23 ± 6.55	0.42 ± 0.03
10	128.9 ±10.1	50.12 ± 4.93	0.39 ± 0.01
25	129.1 ±11.5	46.23 ± 8.60	0.36 ± 0.04
50	144.6 ± 6.0	50.90 ± 1.85	0.35 ± 0.01

Results are expressed on a per flask basis.

APPENDIX 5e. Effect of cholesterol on the growth of cultured roots.

cholesterol concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	129.2 ± 4.7	37.5 ± 3.6	121.7 ±13.6
5	116.4 ± 5.0	34.7 ± 2.3	109.3 ±12.9
10	99.5 ± 4.7	17.6 ± 1.1	60.3 ± 7.1
25	83.6 ± 7.0	11.8 ± 1.8	33.8 ± 7.0

APPENDIX 5f. Effect of cholesterol on tomatine production in cultured roots.

cholesterol concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	73.9 ± 5.0	23.27 ± 1.54	0.32 ± 0.01
5	77.5 ±10.9	28.77 ± 4.29	0.37 ± 0.02
10	104.6 ±10.5	31.22 ± 3.55	0.30 ± 0.01
25	88.0 ± 5.5	32.14 ± 3.41	0.36 ± 0.03

Results are expressed on a per flask basis.

APPENDIX 5g. Effect of SKF 7997-A_3 on the growth of cultured roots.

SKF concentration (ppm)	LMA (mm)	LN	TLL (mm)
0	126.9 ±10.8	31.9 ± 3.5	226.3 ±26.4
1.0	119.2 ±11.6	24.3 ± 2.4	181.3 ±23.1
2.5	110.6 ± 9.3	11.0 ± 2.9	80.8 ±19.0
5.0	69.1 ± 7.7	2.4 ± 0.6	16.7 ± 4.0
10.0	47.6 ± 5.9	0.1 ± 0.1	0.7 ± 0.7
25.0	33.5 ± 3.8	0	-

APPENDIX 5h. Effect of SKF 7997-A_3 on tomatine production in cultured roots.

SKF concentration (ppm)	fresh weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} FW)
0	144.7 ± 9.9	52.69 ± 3.12	0.37 ± 0.01
1.0	116.2 ±15.0	46.40 ± 4.18	0.41 ± 0.05
2.5	90.3 ± 3.2	33.66 ± 4.65	0.38 ± 0.06
5.0	35.3 ± 4.4	14.35 ± 2.25	0.40 ± 0.02

Results are expressed on a per flask basis.

APPENDIX 6a. Effect of kinetin on the growth of hypocotyl callus.

kinetin concentration (ppm)	2,4-D (0.15 ppm)	fresh weight (g)	dry weight (mg)	FW/DW
0	-	0.3 ± 0.03	19.5 ± 1.4	16.3
0.01	+	3.7 ± 0.6	171.0 ±21.6	21.9
	-	0.3 ± 0.03	22.9 ± 1.6	14.2
0.02	+	3.8 ± 0.9	171.3 ±27.2	22.0
	-	0.2 ± 0.03	14.3 ± 1.4	15.6
0.05	+	3.1 ± 0.9	145.2 ±27.3	21.3
	-	0.2 ± 0.03	16.3 ± 2.0	14.8
0.1	+	6.4 ± 1.3	264.4 ±42.0	24.0
	-	0.3 ± 0.03	20.3 ± 1.9	14.5
0.2	+	6.3 ± 1.3	273.6 ±44.9	23.0
	-	0.2 ± 0.03	17.7 ± 2.3	12.9
0.5	+	2.8 ± 0.6	147.2 ±33.7	18.9
	-	0.3 ± 0.08	24.3 ± 6.2	12.7

Results are expressed on a per flask basis.

APPENDIX 6b. Effect of 2,4-D on the growth of hypocotyl callus.

2,4-D concentration (ppm)	kinetin (0.15 ppm)	fresh weight (g)	dry weight (mg)	FW/DW
0	-	1.2 ± 0.3	61.6 ±13.3	18.7
0.01	+	1.8 ± 0.4	94.2 ±16.9	19.4
	-	1.7 ± 0.5	88.1 ±24.7	19.1
0.03	+	6.5 ± 2.2	221.6 ±54.6	29.3
	-	3.2 ± 0.9	150.9 ±33.3	21.5
0.1	+	12.3 ± 1.8	354.2 ±26.7	34.7
	-	7.5 ± 1.5	262.1 ±41.2	28.5
0.3	+	11.0 ± 2.0	313.4 ±41.0	35.1
	-	8.0 ± 2.0	251.4 ±46.6	31.6
1.0	+	8.3 ± 2.1	237.6 ±48.3	34.7
	-	5.2 ± 0.9	210.3 ±29.5	24.6
3.0	+	5.9 ± 2.0	187.3 ±44.1	31.6
	-	4.3 ± 3.3	165.8 ±30.8	25.8

Results are expressed on a per flask basis.

APPENDIX 7. Tomatine content of callus cultures at different stages of 'development' (including data from table 18).

callus	age (days)	length of last passage (days)	No. of flasks	total dry weight (mg)	total tomatine (µg)	tomatine (µg mg^{-1} DW)
hypocotyl	38	38	5	523.7	235.87	0.45
	72	34	6	485.7	136.08	0.28
	176	43	12	273.6	38.40	0.14
	447	64	10	1445.8	0	-
radicle	72	34	5	165.1	19.20	0.12
	176	43	11	240.7	17.70	0.07
	447	64	10	760.3	0	-
cotyledon	72	34	12	875.0	75.60	0.09
	176	43	12	490.9	15.25	0.03
	447	64	10	976.9	0	-