STEROIDAL OESTROGENS AND THE REGULATION OF GROWTH AND DEVELOPMENT IN *PHASEOLUS VULGARIS* L.

A thesis submitted to the University of Glasgow for the degree of Doctor of Philosophy in the Faculty of Science

by

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to

Marette,

Sarah and Edward

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Abbreviations

The S.I. units of measurement have been used throughout the thesis except for units of time where the concept of minutes, hours and days is more readily grasped than seconds.

K_A	association constant
K_D	dissociation constant
pK_D	$-\log_{10} K_D$
PPO	2,5-diphenyloxazole
Pi	phosphate
TMSi	trimethylsilyl
iPA	isopentyl adenosine
ABA	abscisic acid
GA_1	gibberellin A_1, and similarly for other gibberellins
FAP	6-furfurylaminopurine
IAA	indol-3yl acetic acid
IBA	indol-3yl butyric acid
IAAsp	indol-3-acetyl aspartic acid
IAN	indoleacetonitrile
NAA	naphthylacetic acid
NOA	naphthoxyacetic acid
NPA	N-1-naphthylphthalamic acid
PAA	phenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
FC	fusicoccin
MBOA	6-methoxy-2-benzoxazolinone
DMBOA	6,7-dimethoxy-2-benzoxazolinone
TIBA	triiodobenzoic acid
oestradiol	1,3,5(10)-estratrien-3,17β-diol*
oestrone	1,3,5(10)-estratrien-3-ol-17-one*
moxoestrol	11β-methoxyethynyl oestradiol
RU 16117	11α-methoxyethylnyl oestradiol
DES	diethylstilboestrol
RNA	ribonucleic acid

* for other steroids, trivial names are employed rather than the IUPAC-approved

Abbreviations continued

mRNA	messenger RNA
DNA	deoxyribonucleic acid
poly(A)	polyadenylate
ATPase	adenosine triphosphatase
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
EDTA	ethylenediaminetetra-acetic acid
UV	ultra violet
TLC	thin layer chromatography
GC	gas chromatography
IR	infra red
NMR	nuclear magnetic resonance
RIA	radio immuno assay
GC-MS	combined gas chromatography-mass spectrometry
HPLC	high pressure liquid chromatography
DNP	day-neutral plant
LDP	long-day plant
SDP	short-day plant
PP	petiolar pulvinus
DP	distal pulvinus
P	parental generation
F_3	third fillial generation
et al	*et alia*
%	per centum
pers. comm.	personal communication
i.e.	*id est*
e.g.	example given

in addition, the following trivial names were adopted in the older literature for oestrone and oestradiol

ova hormone	uncharacterised
progynon	oestradiol
α-follicle hormone	uncharacterised

Abbreviations continued (2)

dihydrofolliculin	oestradiol
folliculin	oestrone
ketohydroxyestrin	oestrone
theelol	oestriol
ovarian hormone	uncharacterised
oestrin	uncharacterised
ovarin	uncharacterised

Contents

SUMMARY

In this thesis the effects of steroidal oestrogens (a group of sex hormones in animals) on various aspects of the physiology of the dwarf French bean plant, *Phaseolus vulgaris*, are investigated in an attempt to clarify the roles, if any, of these substances in higher plants.

Chlorophyll levels of leaf discs senescing in darkness were not affected by solutions of oestrone and oestradiol (10^{-3}mol m^{-3} to 10^{-7}mol m^{-3}) or oestradiol-sulphate (10^{-3}mol m^{-3} to 10^{-7}mol m^{-3}). Experiments with radioactively-labelled oestrogens demonstrated that the ineffectiveness of the solutions was not due to lack of uptake. The metabolism of the applied oestrogens was investigated and it was shown that oestrone and oestradiol were probably interconverted by the senescing discs.

Germination of bean seeds was not influenced by the steroidal oestrogens tested, and the growth and development of seeds imbibed in oestrogen solutions did not differ from the controls. As with senescing leaf discs, ^{3}H and ^{14}C from radioactive oestrogens entered seeds. Indeed, the uptakes were similar: initially, a rapid phase of water uptake was paralled by an rapid increase in radioactivity content, but after this, while uptake of ^{3}H and ^{14}C fell slightly, the uptake of water was considerably reduced. This divergence is discussed with reference to the properties of the substances and the conditions of the tissues involved.

The effects of steroidal oestrogens on adventitious root formation on *P. vulgaris* explants were investigated. In these experiments, oestrone, oestrone-sulphate, oestrone-phosphate, oestradiol and oestradiol-sulphate were tested. With hypocotyl cuttings rooting in distilled water for 7d, applications of up to 100 µg oestrogen to primary leaves or 50 µg to apices did not affect the numbers of roots which emerged, and also when the oestrogens were supplied as 10^{-3}mol m^{-3} to 10^{-7}mol m^{-3} solutions in the rooting medium no effects were noted. However, the use of 0.1mol m^{-3} oestrogen-sulphates resulted in significant decreases in root numbers and caused the outgrowth of axillary buds at the cotyledonary and primary leaf nodes. In some plants the main shoot apex abscinded. 0.1mol m^{-3} oestrone-phosphate, on the other hand, did not invoke these responses.

In epicotyl and primary leaf explants a similar situation prevailed: oestrogen solutions of less than 0.1mol m^{-3} did not influence root production and only oestrogen-sulphates had any demonstrable effects. In both primary leaf and epicotyl cuttings the inhibition of root formation was almost complete and a hyperplastic tissue was formed. Also, in epicotyl cuttings

abscission layers were formed resulting in the loss of the main apex and some buds, and young trifoliate leaves became markedly incurled, the latter effect being obvious within 24h of cutting. These results are considered drawing on data from experiments on the uptake of ^{14}C and ^{3}H and anatomical investigations of the tissues involved.

In the course of these experiments the presence of the proximal petiolar pulvinus on primary leaf cuttings was found to inhibit root initiation. This effect is considered with reference to the water relations of such cuttings.

Growth and flowering were investigated in two types of experiment: firstly, experiments were carried out on whole plants, applying oestrogens to apices or primary leaves. In the second, the cuttings used in root initiation experiments were allowed to grow and develop until flowering had ceased and pod production commenced. Other than the changes in growth habit induced in 0.1mol m^{-3} oestrogen-sulphate-treated hypocotyl cuttings, no effects of oestrogens on growth or flowering were observed. Alterations in growth habit (axillary bud development) were found when radioactive oestrogens were applied to apices of cuttings, but this was an effect of the radiation rather than the oestrogens. The results of these experiments again are discussed with reference to the uptake and distribution of applied ^{3}H- and ^{14}C-oestrogens.

By correlating results from different experiments it was possible to gain a better knowledge of the plant materials used. This revealed that plant growth and development varied considerably, often showing distinct seasonal trends. The implications of this are considered.

The presence of oestrone and oestradiol in bean seeds was confirmed using combined gas chromatography-mass spectrometry techniques.

In the Discussion the results are viewed collectively with reference to the relevant literature and in the Conclusion the findings made in the thesis are considered in the light of the current views on hormone action outlined in the Introduction.

GENERAL INTRODUCTION

Growth and differentiation in complex multicellular organisms are widely believed to be under hormonal control. In higher plants there are five types of compound which are usually accepted as the basic plant hormones or plant growth substances, *viz*: auxins, gibberellins, cytokinins, abscisic acid and ethylene. There are, in addition, many other compounds which are naturally occurring in plants and influence a wide range of developmental processes therein. The precise chemistry of many of these substances is known, but their physiological roles have not been studied in detail. Such compounds include the phenolics, fatty acids, brassinolides, various terpenes, secondary growth substances and the steroidal oestrogens.

The physiology of steroidal oestrogens in mammals represents an active area of reaearch, not only in their role in the expression of sexuality, but also in their involvement in the abnormal development of cells. In plants, on the other hand, the study of steroidal oestrogens has been largely neglected since the 1930's when there was speculation concerning the possibly parallel roles of these substances in plant and animal developments.

This thesis is an account of certain aspects of steroidal oestrogen physiology in *Phaseolus vulgaris*. The Introduction is divided into four sections:-

1. A Synopsis of the Hormone Concept
2. Current Theories on the Mode of Hormone Action
3. Review of Oestrogens in Plants
4. Aims and Rationale of the Project

INTRODUCTION

1. A Synopsis of the Hormone Concept

The foundations of hormone physiology date back to 1690 when Overcamp suggested that the "ova" (follicles) secrete a substance into the blood-stream (in Newerla, 1944). The veracities of such endocrine secretions were confirmed in experiments involving the transplantation of fowl testes (Berthold, 1849). Control of organ development by substances circulating in the blood was demonstrated similarly: ovaries which were transplanted to other parts of the body and became vascularised, developed and functioned normally (Halban, 1900: Knauer, 1900). Later, Bayliss and

Starling (1902, 1904) reasoned that activity in the pancreas was stimulated by a blood-borne secretion from the intestinal wall and proposed that similar mechanisms involving transport of secreted substances by the bloodstream between sites of production and action would explain many physiological processes in animals. The word "hormone" was derived to describe these blood-carried chemical messengers (Starling, 1905). The continuing assimilation of information regarding the chemical control of biological processes necessitated that the original definition of a hormone be widened to include all cases where transport from tissue of production to tissue of action occurred (Huxley, 1935). Currently, a hormone is viewed as being a specific molecule which is produced in minute quantities in one part of an organism and is then transported to another part of that same organism where it has a specific effect.

The involvement of hormonal substances in plant physiology is not a modern concept. Sachs (1864, 1865, 1886) found that a stimulus for flowering (ultra-violet light) was perceived by the leaves although the effect was obvious at the apex, and he also referred to molecules other than nutrients affecting plant growth (1880, 1882). In the same period, Darwin and Darwin (1880) demonstrated in coleoptile phototropism and root geotropism that the regions of stimulus perception and adjustment growth were spatially separated, and concluded that an "influence" of some nature was transmitted between the sites of perception and action to elicit the response. The chemical nature of the influence was confirmed using decapitated coleoptiles: asymmetric placement of agar blocks containing the diffusate from excised apices resulted in coleoptile curvature (Went, 1926, 1927). However, that hormones control plant growth and development is still debated. After many years of extensive investigations factors controlling phenomena such as flowering, apical dominance and tuberisation have yet to be elucidated, and to these ends, studies on synthesis and translocation of the generally accepted plant growth substances largely have been inconclusive. Such failures to demonstrate the presence in plants of classically-defined hormones to control specific processes, and the difficulties encountered in fulfilling Jacobs' Rules for hormones (Jacobs, 1959) have led to the adoption of alternative nomenclatures e.g. plant growth substances etc. (see Weyers, 1978: Weyers *et al.*, 1980) and have allowed the arguments over the occurrence of hormones *per se* in plants to continue. Nevertheless, studies on compounds with plant growth regulating-activity continue, and investigations

into the mode of action of unequivocally identified endogenous substances may clarify their real significance in plants.

2. Current Theories on the Mode of Hormone Action

a) Steroid hormones in animals

The mode of action of hormones in animals has been a subject of much study and consequently is extensively documented. In synopsis, animal hormones i) act *via* receptors, either in the cytoplasm (mostly steroid hormones) or on the cell surface (generally polypeptide hormones and neuromediators), ii) are usually part of a feedback loop, iii) are seldom unique and iv) are balanced by counteracting effects of other hormones (Tomkins, 1975).

The mode of action of steroid hormones may be summarised as follows: steroid hormones circulating in the blood are taken up selectively by predetermined target cells (Jensen and Jacobson, 1962) in which they bind to specific receptor proteins (Baulieu *et al.*, 1967: Toft *et al.*, 1967). A major conformational change in the protein takes place on binding the steroid (Jungblut *et al.*, 1976) facilitating the dimerisation of hormone-receptor complexes (Little *et al.*, 1973: Notides, 1975) prior to translocation to the nucleus (Jensen *et al.*, 1968: Gorski *et al.*, 1968). There, by binding to effector sites (Baulieu, 1979), the hormone-receptor dimers somehow effect alterations in gene expression, resulting in specific changes in RNA and protein syntheses (Mueller *et al.*, 1958).

This summary, largely based on the oestradiol (2) receptor system, is believed to be generally true for steroid hormones (Jungblut *et al.*, 1976), but more detailed examinations of steroid hormone-receptor interactions have revealed that, in reality, various complicating factors exist and interact, giving lie to the simplicity of the system outlined above.

Firstly, transfer of steroid-receptor complexes to the nucleus did not guarantee effectiveness: only if the complexes bound to the high-affinity nuclear effector sites was transcription modified (Baulieu, 1979). It was likely that there were many other binding sites in the nucleus but these were thought to be weak-affinity acceptors (Williams and Gorski, 1972). Whether the synthesis of few or many proteins was involved in the final response, the high-affinity sites were probably few in number and easily

concealed by the bulk presence of low-affinity acceptors (Yamamoto and Alberts, 1976). Therefore, although the concentration of hormone-receptor complexes in the nucleus as well as their rate of turnover therein was related to the extent of the effect obtained (Anderson *et al.*, 1974) the relationship between specific receptor binding in the nucleus and the magnitude of response to the hormone has not been quantified (Baulieu, 1979).

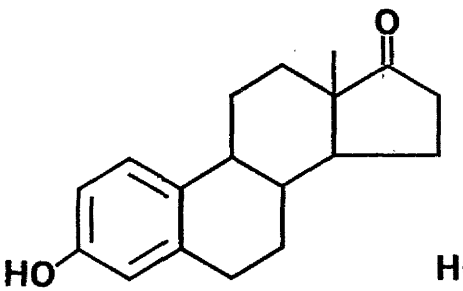
Neither was the translocation of receptor to the nucleus always a prerequisite for hormone action; cytoplasmic receptors were found in nuclei in the absence of hormones (Mester and Baulieu, 1972: Zava and McGuire, 1977) and receptors were translocated when bound to anti-hormones (Bouton and Raynaud, 1975).

Since receptors were first isolated (Toft and Gorski, 1966) the view of their role in hormone-mediated processes has changed dramatically and the relative importance of receptors and hormones has been questioned (Edelman, 1970: Hughes *et al.*, 1976: Jungblut *et al.*, 1976: Clark *et al.*, 1977). It was demonstrated that the presence of receptor was necessary for steroid hormone responses (Anderson *et al.*, 1974), oestradiol receptor proteins slowly dimerised in the absence of oestradiol (Hughes *et al.*, 1976), receptors could elicit effects in the absence of hormones, and in some cancers receptors maintained transcription autonomously (Jungblut *et al.*, 1976). Thus the binding of steroid hormones by receptors may be regarded as the activation of a transcription-regulating protein (Higgins *et al.*, 1973: Milgrom *et al.*, 1973a) and the role of hormones may lie in the control of the rates of biological processes, not in their initiation (Jungblut *et al.*, 1976).

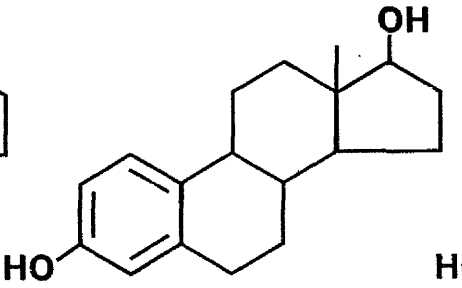
Many proteins are capable of binding steroids, but hormone-receptors were shown to be distinct in that the association constants* of receptor-ligand complexes were high, at least pK_D 9 (Barbanel *et al.*, 1977) and the receptor-ligand binding properties corresponded to the biological activity of the ligands (Baulieu, 1973). Similarly, there was good correlation between competition for oestradiol receptors and biological activity of analogues (Müller and Wotiz, 1977) and

* The association constant (K_A) is a measure of the affinity of a receptor for its ligand. The dissociation constant (K_D) is the reciprocal of K_A (Kende and Gardner, 1976). The term pK_D ($=-\log_{10}K_D$) will be used throughout.

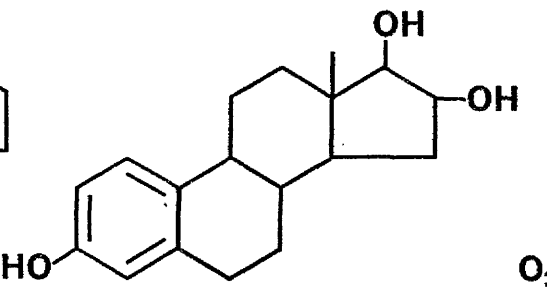
Fig. 1. Structural formulae of steroidal oestrogens and other animal sex hormones



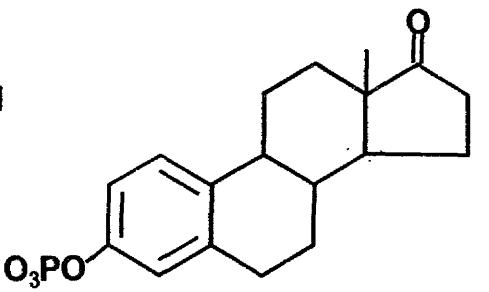
(1) oestrone



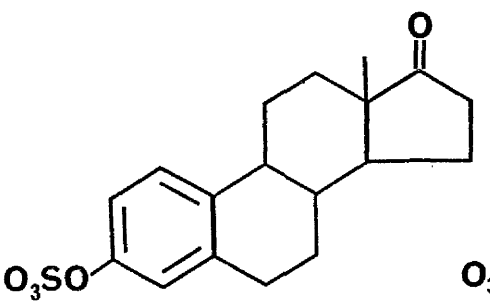
(2) oestradiol



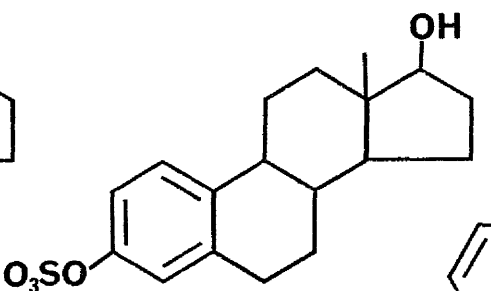
(3) oestriol



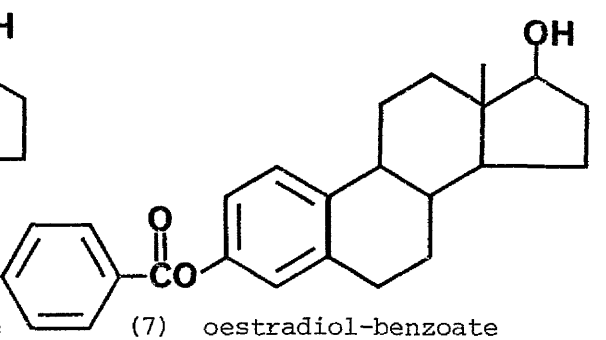
(4) oestrone-phosphate



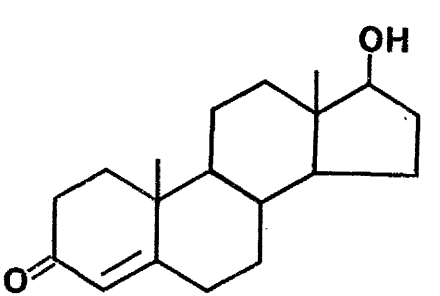
(5) oestrone-sulphate



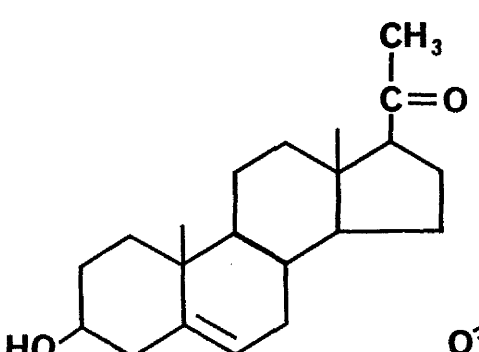
(6) oestradiol-sulphate



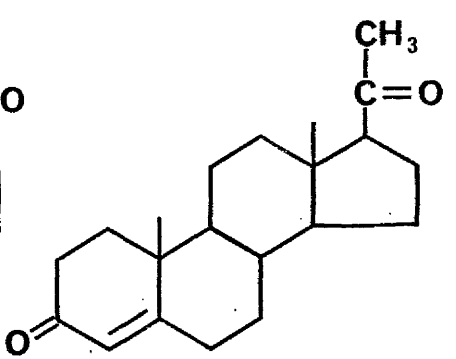
(7) oestradiol-benzoate



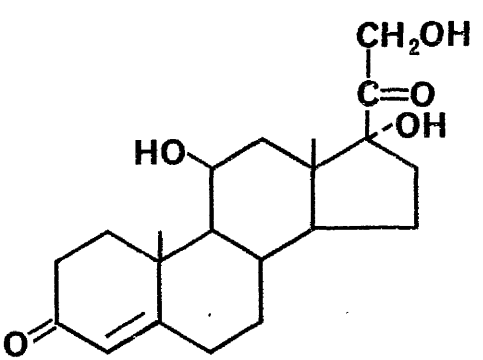
(8) testosterone



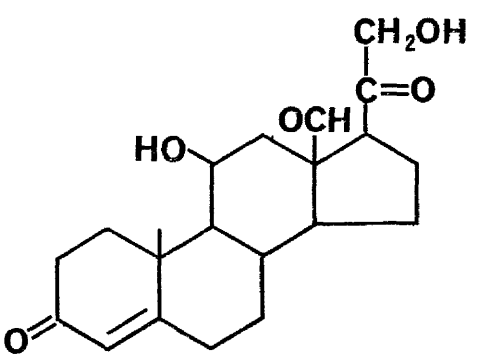
(9) pregnenolone



(10) progesterone



(11) glucocorticosteroid e.g. cortisol



(12) mineralo corticosteroid e.g. aldosterone

oestradiol-receptors of different organs from different animals had common antigenic determinants (Greene *et al.*, 1977). The binding of oestradiol by receptors was non-covalent: bound labelled oestradiol was displaced by excess unlabelled oestradiol (Katzenellenbogen *et al.*, 1973). The hydroxyl groups at the 3 and 17 positions on the oestradiol molecule were essential steric requirements for binding, with the distance between the groups and the conformation of any sub-units being of great importance when binding affinity was considered (Ananchenko *et al.*, 1975). To be a specific receptor, then, a molecule must exhibit binding specificity, saturability and high affinity (Barbanel *et al.*, 1977).

Each target cell contains 1,000 to 50,000 receptors, the concentration varying under hormonal, developmental, genetic, pathological and pharmacological circumstances (Baulieu, 1979). Oestradiol receptors in uteri showed seasonal and circadian fluctuations, and a third cycle with an irregular period (9 to 15 days) also interacted. Thus, it was unlikely that true base-line levels of receptors could exist (Hughes *et al.*, 1976). The concentration of oestradiol receptors could be controlled by a depletion-replenishment sequence (Little *et al.*, 1975) and as receptor levels doubled within 24h of oestradiol administration (Hsueh *et al.*, 1976), *de novo* synthesis of receptors must have occurred whether it was thought that recycling had taken place (Hsueh *et al.*, 1976) or not (Hughes *et al.*, 1975 : Little *et al.*, 1975). Oestradiol also caused an increase in the responsiveness of cells to progesterone (10) via an increase in receptor levels (Milgrom *et al.*, 1973b) and progesterone interfered with the synthesis of oestradiol receptors (Hsueh *et al.*, 1975, 1976) at the nuclear level (Clark *et al.*, 1977).

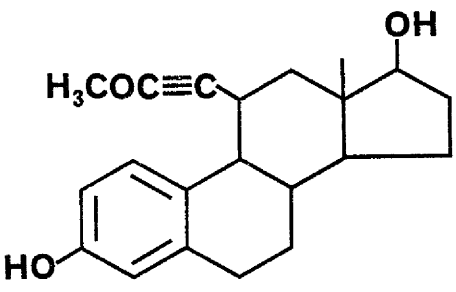
There are five physiologically well-defined classes of steroid hormones, namely oestrogens, androgens, progesterone, glucocorticosteroids (11) and mineralocorticosteroids (12), each with its own class receptors (Baulieu, 1979). However, oestradiol receptors in the mouse uterus and hypothalamus differed with respect to nuclear binding (Fox, 1977) and the oestradiol-binding component in the neonatal rat brain dwarfed the adult oestradiol receptor in magnitude and did not share its ligand-binding properties (McEwen *et al.*, 1975). Thus, the receptors for one hormone probably differ between tissues and those present at different stages of development in a tissue need not be identical. Furthermore, different receptors for the same class of

hormone can be present in a tissue at the same time (Tchernitchin *et al.*, 1975) and a given hormone may interact with different classes of receptor, e.g. oestradiol bound to androgen receptors and testosterone (8) may have bound to oestrogen receptors (Jung-Testas and Baulieu, 1974) although the latter may have been complicated by local metabolism of testosterone to oestradiol (Natfolin *et al.*, 1975). Progesterone has been found to bind to all classes of receptor excepting oestrogens and, therefore, a hormone can be active in the absence of its own receptor (McGuire *et al.*, 1977). However, the binding of unnatural ligands may cause abnormal conformational changes in receptors and any effects elicited need not correspond to those usually associated with the hormone or receptor (Baulieu, 1979). Members of the one class of hormone also competed for receptor sites, e.g. oestriol (3) inhibited oestradiol activity by binding to the same receptor but not as strongly. This may have reflected a regulatory role for oestriol (Brecher and Wotiz, 1967).

It was demonstrated that binding characteristics of hormones and analogues to receptors could vary (e.g. McEwen *et al.*, 1975) and this affected their relative performances, usually via the lengths of time spent by active hormone-receptor complexes in the nucleus (Baulieu, 1979). Similarly, the binding of hormones by plasma proteins, although mostly loose and non-specific, could influence the availability of steroids, and different binding properties of hormones and analogues could affect their relative availabilities and potencies (Milgrom *et al.*, 1970). Additionally, the plasma protein concentration varied in different tissues (McEwen, 1976).

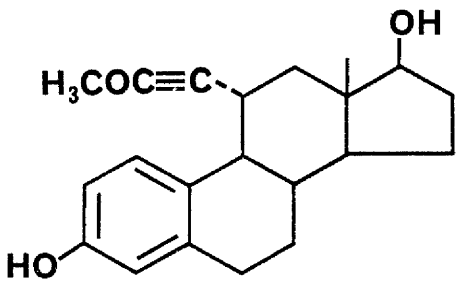
Hormone effects have also been reduced by antihormones, compounds which compete with a hormone for its receptor and so lower the effective receptor concentration (Baulieu, 1979). The binding properties of antihormones usually are different to those of their active counterparts. Moxoestrol (11β-methoxyethynyl oestradiol) (13) is a highly potent oestrogen, but RU 16117 (11α-methoxyethynyl oestradiol) (14) is very weak on its own, and may be antagonistic if combined with active oestrogens.

Fig. 2. Structural formulae of :



(13) moxoestrol

and



(14) RU 16117

Moxoestrol and RU 16117 bound to the mouse uterine oestradiol receptors with similar affinities, but RU 16117 dissociated much more readily. Both complexes translocated to the nucleus, although RU 16117 translocation was slower and quantitatively less (Bouton and Raynaud, 1975). Also, the replenishment of oestradiol receptors was reduced greatly in the presence of antioestrogens (Clark *et al.*, 1973). This may have been due to abnormal binding of antihormone-receptor complexes in the nucleus (Ruh and Baudendistel, 1977) and this could also have caused their long nuclear retention times when compared to oestrogen-receptor complexes (Baudendistel and Ruh, 1976: Ruh and Baudendistel, 1977). As well as preventing receptor recycling, the modified binding probably blocked further synthesis of receptors (Ruh and Baudendistel, 1977). Receptor replenishment, however, was paralleled by an increased rate of breakdown in the cytoplasm, and continuation of this in conjunction with the lacks of recycling and synthesis would have deplenished receptor levels, and rendered the tissue unresponsive to the hormone (Katzenellenbogen *et al.*, 1979).

The mode of hormone action with regard to the regulation of gene expression, therefore, is absolutely dependent on receptors. However, the possibility of receptor-independent effects of steroid hormones has never been discounted (Jungblut *et al.*, 1976) and recent developments may have justified this belief. Within the cell, oestradiol inhibited the effect of pregnenolone (9) on meiotic maturation in *Rana pipiens* ovarian follicles by binding to 3β-hydroxysteroid dehydrogenase, preventing conversion of pregnenolone to progesterone, the steroid which initiated meiosis (Spiegel *et al.*, 1978). The co-enzyme-like action of steroid hormones on isolated enzyme systems and the role of steroids as allosteric effectors are well known (Williams-Ashman, 1965). The initial cell divisions in *Psammechinus miliaris* and other animal embryos were susceptible to oestradiol: 2×10^{-3} mol m^{-3} oestradiol caused atypical cleavages and 10^{-2} mol m^{-3} oestradiol completely inhibited cell division, apparently acting in a similar manner to colchicine, which affects the mitotic spindle (Agrell, 1954, 1955).

Steroid hormones have also been found to elicit effects without entering cells. In *Xenopus laevis* oocytes progesterone promoted meiosis. An analogue of progesterone, androsta-4-ene-3-one-17β-carboxy acid, when linked to a high molecular weight water-soluble polymer, could not enter cells but still promoted meiosis. Moreover, progesterone was found to

bind to the cell membrane surface (Pietras and Szego, 1977). Drugs which displaced membrane-bound Ca^{++} or altered Ca^{++} distribution in cells also mimicked progesterone action, and ionophore 23187 in the presence of Ca^{++} or Mg^{++}, specific Ca^{++} ionophores and lanthanum stimulated meiotic maturation. Injection of oocytes with the chelating agent EDTA, however, suppressed the progesterone effect, indicating that Ca^{++} may have acted as a second messenger for the steroid signal at the membrane level (Baulieu *et al.*, 1978). In addition, mitotic activity in rat bone marrow and thymus was stimulated by Ca^{++} and Mg^{++}. The effects of these were blocked by oestradiol and testosterone respectively through the inhibition of uptake of the divalent cations (Perris and Morgan, 1976), and oestradiol also interacted with the surface of uterine cells (Pietras and Szego, 1977).

The question as to whether such cell surface interactions related to the entry of steroid hormones into cells remains open (Milgrom *et al.*, 1973c)and the same may also be said of other steroid hormone effects on cell membrane properties such as permeability and polarization (Baulieu, 1979). The other classes of animal hormones, the neuromediators and polypeptide hormones are known to act at the plasma membrane level (Hechter and Lester, 1960), influencing membrane enzymes and cation transport systems (Baulieu *et al.*, 1978), and there is also evidence for some involvement in gene activation (see Davidson, 1965).

Therefore, although much information regarding animal hormones has accumulated, the precise molecular mechanisms by which they act are not yet fully characterised, whether concerning receptor-mediated interactions with the nucleus, direct interactions with enzymes, or influences on membrane-associated events.

b) Hormone action in plants

The stimulation of cell extension in *Avena* coleoptiles by auxins and gibberellin-induced α-amylase production in barley seeds are two of the best-characterised responses to plant hormones, yet, on a molecular basis, the modes of action are not well understood. However, there is reason to suspect that hormones in plants act in the same ways as their animal counterparts: target cells unquestionably exist for plant hormones, e.g. gibberellins produced in the germinating barley embryo induced synthesis of specific enzymes in aleurone layer cells (Yomo and Varner, 1971), in etiolated pea plants epicotyl cells

elongated in response to auxin produced by the apex (Osborne, 1978) and the guard cells of spinach stomata were affected by abscisic acid synthesised in the chloroplasts of mesophyll cells (Loveys, 1977).

Although in each example the active substance met the requirement (in the definition of hormone) that the sites of synthesis and action should by spatially separated, it is clear that the transport distances involved in plants can be much shorter than those in animals. The respective transport systems involved are not truly comparable, but this should not be allowed to over-ride the fundamental consideration concerning the level at which spatial separation should hold when subcellular compartmentation is taken into account, especially in plants. It has even been suggested that the space between adjacent molecules (synthesis and action) would be sufficient (van Overbeek, 1950).

There is no direct evidence to confirm the theory that hormones in plants, *via* binding to cytoplasmic receptor proteins, interact with the nucleus *cf.* steroid hormones in animals (e.g. Ginzburg and Kende, 1968), but, for sustained responses to hormones, plants require *de novo* syntheses of RNA and protein. It was established that treatment with GA_3 (17) induced α-amylase and protease syntheses in barley aleurone cells (Varner and Chandra, 1964 : Filner and Varner, 1967 : Jacobsen and Varner, 1967) and there was good evidence that a transcriptional control mechanism was involved in gibberellin action. Most eukaryotic mRNA molecules have a polyadenylate (poly(A)) sequence. GA_3 enhanced poly(A)-RNA synthesis in aleurone cells (Ho and Varner, 1974 : Jacobsen and Zwar, 1974) and, in a cell-free protein synthesis system from wheat embryos, poly(A)-containing RNA from GA_3-treated aleurone layers caused production of α-amylase. Moreoever, the time course for production of mRNA for α-amylase was parallel with that for the rate of α-amylase synthesis following gibberellin treatment of barley seeds (Higgins *et al.*, 1976). Studies with potato tubers have shown qualitative changes to occur in non-histone chromosomal proteins after GA_3 treatment. Such changes may affect the quality of transcription (Kahl *et al.*, 1979).

Gene derepression by IAA (31) may be exhibited in pea. Cellulase activity in decapitated epicotyls was induced by IAA treatment whereas other hydroxylases were unaffected (Datko and Maclachlan, 1968) and this induction was blocked by inhibitors of RNA and protein syntheses (Fan and Maclachlan, 1966). *In vitro*, polysomes from both IAA-treated and control tissues were capable of protein synthesis, but only in the

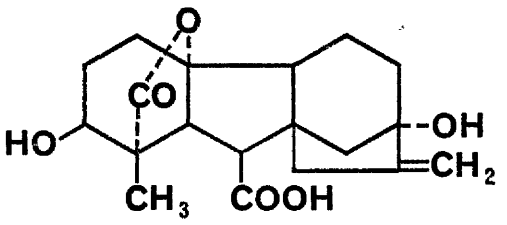
former was cellulase produced (Davies and Maclachlan, 1969). Together, these data indicate that IAA treatment led to derepression of the gene for cellulase mRNA.

Hormone-receptor studies have proved difficult in plants, as witnessed by the large proportion of investigation systems which have not been pursued after preliminary experiments (Venis, 1977). Misinterpretation of data also has been an inhibitory influence: the first reported isolation of cytoplasmic IAA-protein complexes was from whole plants and explants of pea (Siegel and Galston, 1953) but this was later found to be an artefact resulting from the protein precipitation procedure used (Andreae and van Ysselstein, 1960). Similarly, the binding of an IAA-oxidation product to pea RNA (Kefford *et al.*, 1963) was a function of the assay system (Galston *et al.*, 1964) and incorporation of radiocarbon from ^{14}C-IAA into 4S-RNA (Bendana *et al.*, 1965) was not due to IAA-binding but to recycling of the radioactively-labelled side chain into RNA-associated polysaccharides (Davies and Galston, 1971).

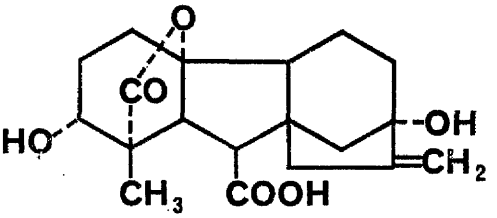
However, binding of gibberellins, cytokinins, abscisic acid and auxins to subcellular fractions has now been confirmed. With isolated pea nuclei, gibberellin-enhanced RNA synthesis required a factor from the cytoplasm (Johri and Varner, 1968), and tritium-labelled proteins were extracted from pea 12 hours after application of ^{3}H-GA$_1$. Two peaks of radioactivity were found in the 20,000 g supernatant, both possibly due to bound, but otherwise unchanged ^{3}H-GA$_1$. Parallel protein fractions isolated from untreated plants bound ^{3}H-GA$_1$ *in vitro*, and GA$_8$ (20) and pseudo-GA$_1$ (16) did not compete. Binding was non-covalent and ^{3}H-GA$_1$ could be displaced by excess unlabelled GA$_1$ (15) (Stoddart *et al.*, 1974). ^{3}H-GA$_3$ also bound non-covalently to cytoplasmic proteins in pea stems and four ^{3}H-GA$_3$-protein complexes were separable. GA$_{4+7}$ (18, 19) and GA$_{13}$ (22) were competitors of GA$_3$ for binding sites, but no binding affinities were presented. On the other hand, GA$_{13}$ is not an active gibberellin and it is GA$_1$ not GA$_3$ which is active in pea (Konjević *et al.*, 1970). Therefore, the biological significance of this binding is questionable.

Specific and reversible non-covalent binding of ^{3}H-GA$_1$ (pK_D 5.82) has been found in wheat aleurone layer subcellular fractions enriched with aleurone grains, and these accounted for 85% of the gibberellin-binding-activity. Binding required the presence of Ca^{++}, and was prevented by

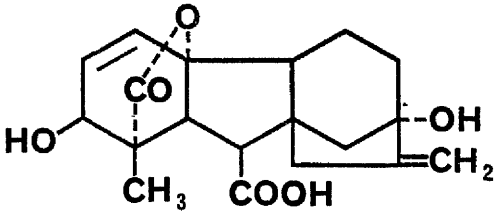
Fig. 3. Structural formulae of gibberellins



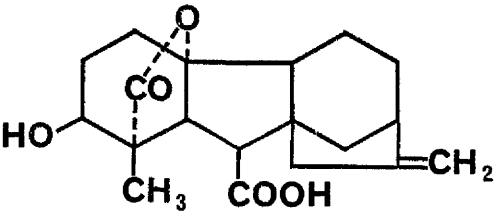
(15) GA_1



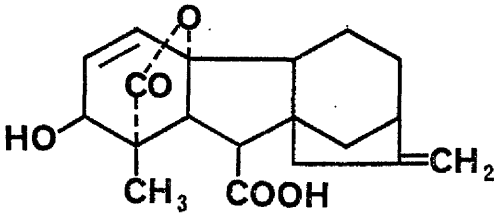
(16) pseudo-GA_1



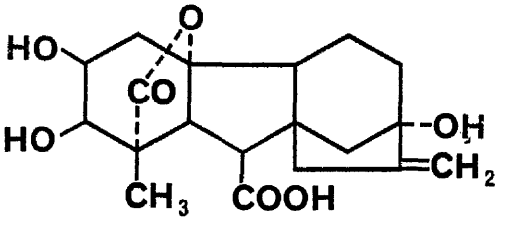
(17) GA_3



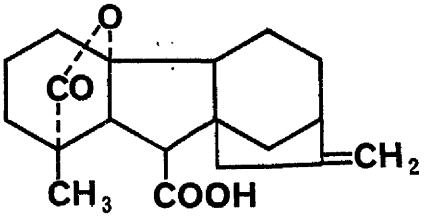
(18) GA_4



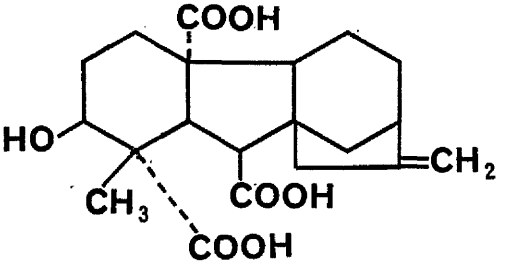
(19) GA_7



(20) GA_8



(21) GA_9



(22) GA_{13}

abscisic acid, a known gibberellin-antagonist in germination (Jelsema *et al.*, 1977). On the other hand, when lettuce hypocotyl sections were incubated in $^{3}H\text{-}GA_1$, differential centrifugation gave only one subcellular fraction (2000g) which interacted significantly with tritium. Unlabelled GA_1 and GA_9 (21), but not GA_8, competed with $^{3}H\text{-}GA_1$ for binding sites. The 2000g pellet was composed mostly of cell wall material and GA_1 probably was bound covalently to a polysaccharide, not electrostatically to a polypeptide (Stoddart, 1979a,b). This does not preclude the binding from a biological role: segment growth and tritium incorporation by the cell wall fraction were highly correlated (Stoddart and Williams, 1979: Stoddart, 1979a), binding occurred before elongation growth (Stoddart and Williams, 1980), gibberellin-induced cell elongation in lettuce was mediated *via* an increase in cell wall plasticity indicating that the primary site of action was within the cell wall (Silk and Jones, 1975: Stuart and Jones, 1977) and, in any case, gibberellin responses in plants differ in their kinetics from receptor-mediated responses to steroid hormones in animals (Kende and Gardner, 1976).

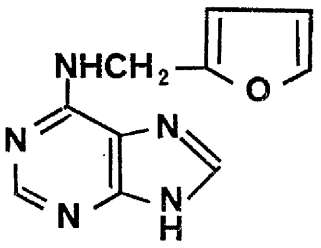
Matthyse and Abrams (1970) found that kinetin (23) could induce RNA transcription *in vitro* only if a mediator protein isolated from pea bud chromatin was present. Although described as a receptor, binding of kinetin to the protein was not demonstrated (Matthyse and Abrams, 1970). Ribosomes (83S) from chinese cabbage leaves bound kinetin and benzylaminopurine (24) with low affinity, and adenine derivatives (inactive as cytokinins) less tightly, but this and biological activity did not always correlate (Berridge *et al.*, 1970). Low-affinity and high-affinity sites were found for benzyladenine (25) in ribosomes from tobacco callus and wheat germ (Fox and Erion, 1975). The "high affinity" (pK_D 6.22) binding agents were identified as 65,000 dalton proteins, present at a concentration of one per ribosome and removable in the active form with 0.5M KCl. The ability to compete with benzyladenine for high-affinity sites correlated well with the cytokinin activity of test compounds (Fox and Erion, 1975). A single polypeptide (51,000 daltons) which bound cytokinins was partially purified from tobacco leaves (Yoshida and Takegami, 1977). The protein bound specifically to the 40S ribosomal subunit and the binding was increased 300 per cent in the presence of benzyladenine (Takegami and Yoshida, 1977). However, although RNA polymerase activity also was stimulated by the cytokinin-binding protein (Yoshida and Takegami, 1977) the very low pK_D for

benzyladenine (4.40) (Takegami and Yoshida, 1975) made it unlikely that the protein could act as a cytokinin-receptor (Polya and Bowman, 1979). In addition, the high levels at which this protein was present would not be compatible with a hormone-receptor function (Polya and Davies, 1978). The other possibility, that the polypeptide was an initiation factor in protein synthesis, remained, the molecular size being of the correct order for such a function (Takegami and Yoshida, 1977). A cytokinin-binding glycoprotein of similar dimensions has been isolated from the aquatic fungi, *Achyla* and *Blastocladiella*, cytokinins acting as allosteric regulators of Ca^{++} binding.

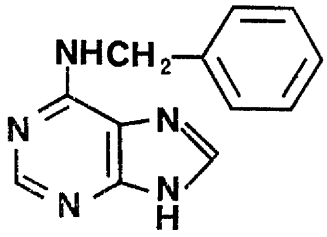
Tobacco cells in culture contained two membrane-associated cytokinin-binding proteins, neither of which was associated with ribosomal fractions. *In vitro* binding to the major protein was low-affinity (pK_D 5.11), heat stable at 378 K and not specific for biologically active molecules. The second protein, however, demonstrated higher affinity (pK_D 6.85), heat-labile binding which, using halogenated benzyladenine derivatives with a wide range of cytokinin-activity, was shown to be specific for active cytokinins (Sussman *et al.*, 1975 : Sussman and Kende, 1978).

The high-affinity cytokinin binding protein (*ca.* 180,000 daltons) from wheat germ had pK_D 6.70, and kinetin was specifically displaced by low concentrations of synthetic and naturally occurring biologically active cytokinins, including some ribosides, but not by adenine derivatives which lacked cytokinin-activity. However, zeatin (26), a highly potent endogenous cytokinin, was an exception (Polya and Davies, 1978) and a wide variety of structurally distinct molecules which can affect cytokinin-modified processes also interacted with the 180,000 dalton protein. This qualitatively wide ligand specificity and the high incidence of the protein indicated that it was unlikely to be a cytokinin-receptor (Polya and Bowman, 1979). Also working with wheat germ, Fox and Erion (1977) isolated one high-affinity, cytokinin-specific binding protein (100,000 daltons) from the ribosomal faction, and two from the post-ribosomal supernatent (pK_D 6.00 - 6.30). One of these may have been identical to that in the ribosomal pellet, and the other, at 30,000 daltons, was smaller (Fox and Erion, 1977). Although substantial differences existed between these two high-affinity binding proteins and that found by Polya and Davies (1978), it was possible that the different macromolecules

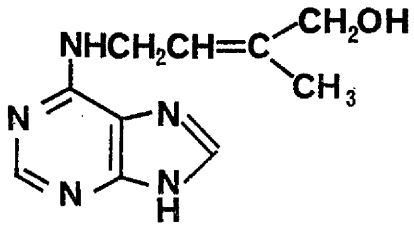
Fig.4. Structural formulae of kinins, abscisic acid and ethylene



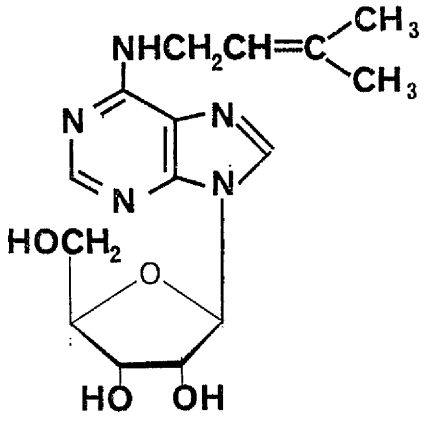
(23) Kinetin =
(28) 6-furfurylaminopurine



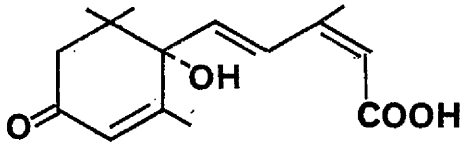
(24) benzylaminopurine =
(25) benzyladenine



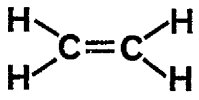
(26) zeatin



(27) iPA (isopentyl adenosine)



(29) abscisic acid



(30) ethylene

contained the same cytokinin-binding subunit (Polya and Davies, 1978). Again, however, the binding proteins were present at concentrations in excess of that expected for a hormone receptor (Polya and Davies, 1978).

In the preceding reports on cytokinin binding, no attempts were made to correlate the presence of binding proteins with specific cytokinin effects. However, the occurrence of binding proteins and cytokinin-sensitivity have been linked in a moss. Certain cells of *Funaria hygrometrica* can respond to cytokinins: bud initiation in caulonema cells of protonemata had commenced within 10 hours of cytokinin treatment (Brandes, 1967) and those cells accummulated radiocarbon from ^{14}C-benzyladenine. Radioactivity could be cleared by washing with distilled water, indicating a non-covalent interaction (Brandes and Kende, 1968). Isolation of caulonema (budding) cells resulted in their reversion to cytokinin-insensitive chloronema (non-budding) cells, and protein extraction followed by microgelelectrophoresis demonstrated that three bands of protein present in caulonema cells were absent from chloronema cells. These proteins bound benzylaminopurine ten times more strongly than the other proteins extracted (Bopp *et al.*, 1976: Erichsen, *et al.*, 1977). Also, benzyladenine bound to particulate factions from protonemata. This was not associated with RNA (Gardner *et al.*, 1976, 1978) and zeatin, kinetin and iPA (27) were competitors of benzyladenine for binding sites. On the other hand, neither ribosides of benzyladenine, zeatin and iPA nor any of the biologically inactive compounds tested were effective competitors of benzyladenine (Gardner *et al.*, 1978). Uptake and specific binding of ^{3}H-benzyladenine also has been demonstrated *in vivo* (Gardner *et al.*, 1975a).

Cytokinins influence sex expression in the female direction in *Mercurialis annua* and benzylaminopurine was found to bind specifically *in vitro* to several subcellular fractions from flower buds, with zeatin, FAP (28) and iPA, but not their ribosides, effective competitors of benzylaminopurine for binding sites. Binding was greatest, however, in the ribosomal fraction from male buds, almost twice the level found in female buds. Paradoxically, female buds had a greater endogenous level of cytokinins (Chung *et al.*, 1977 : Durand *et al.*, 1978). Whether such binding had any biological significance regarding the mode of action of cytokinins was not ascertained, but it must be borne in mind that the significance of any cytokinin-protein interaction could be questioned.

Cytokinins have been shown to bind specifically to materials such as glass (Gardner *et al.*, 1975b, 1978) and talcum powder (Gardner *et al.*, 1975b, 1976, 1978 : Sussman and Kende, 1978). The binding characteristics differed between physical systems and high-affinity biological materials in certain details, but still the difficulty of separating biologically significant interactions from physical adsorption by biological materials remained (Gardner *et al.*, 1975b, 1978). Further doubts on the possibility of cytokinin-receptors could result indirectly from studies on cytokinin-antibodies induced in rabbits. The pK_D's for benzylaminopurine (8.89) and kinetin (8.82) (Constantinidou *et al.*, 1978) were of the order expected for receptor proteins, but were much higher than cytokinin-specific-binding protein so far located in plants.

It has also been suggested that cytokinin action in enzyme induction could be a membrane-related phenomenon. Benzyladenine treatment induced betacyanin synthesis in *Amaranthus*, and this was thought to have been achieved *via* a selective increase in K^+ transport (Elliott, 1976). Similarly, the increase in fresh weight of soybean cotyledons and leaf discs induced by cytokinin treatments probably involved specific effects on ion transport (Ilan *et al.*, 1971 : Ilan, 1971).

Subcellular fractions from *Vicia faba* leaves contained a high-affinity and a low-affinity binding site for abscisic acid (29). The high affinity site may have been associated with the plasma membrane fraction and its pK_D (7.30 - 7.52) was in agreement with the range of abscisic acid concentrations effective in stomatal closure. Also, maximal binding activity coincided with maximum specific activities of glucan synthetase and an Mg^{++}-dependant, K^+-stimulated ATPase, enzymes which probably are plasmalemma-associated (Hocking *et al.*, 1978).

Binding sites for ethylene have been found in tobacco leaves (Sisler, 1979), but the binding was not obviously associated with any physiological process and the affinity was not high (pK_D 5.17). A cell-free system from developing cotyledons of *Phaseolus vulgaris* recently has been found to bind ethylene (30) with very high affinity (pK_D 9.19) (Bengochea *et al.*, 1980a), and only biologically-active ethylene analogues were competitors (Bengochea *et al.*, 1980b). The binding proteins were associated with membranes, probably endoplasmic reticulum or dictyosomes (Evans *et al.*, pers. comm.). The relevance of this binding must be questioned, however, as ethylene has no known role in bean cotyledon development, and the possibility that these sites are involved in ethylene

metabolism has been raised. On the other hand, a similar binding site seemed to be present in abscission zones in *Phaseolus* (see Bengochea *et al.*, 1980a).

From the evidence presented it is clear that receptor physiology for gibberellins, cytokinins, abscisic acid and ethylene is yet embryonic. However, as with the majority of hormone studies in plants, systems involving auxins have been more extensively investigated.

There are indications that auxin-induced increases in RNA synthesis may require the presence of a protein factor. Such a mediator protein was required in tobacco and soybean auxin-target cells for the promotion of RNA production by 2,4-D (39) treatment (Matthyse and Phillips, 1969). Only the nucleolar enzyme RNA polymerase I was stimulated by 2,4-D applications in pea, maize (Venis, 1971) and soybean (Hardin and Cherry, 1972 : Rizzo *et al.*, 1977), RNA polymerases II and III being unaffected (Rizzo *et al.*, 1977). Also, a protein fraction from soybean cotyledons stimulated RNA polymerase I from hypocotyls, but the enzyme from 2,4-D-pretreated plants was not affected (Hardin *et al.*, 1970). However, application of 2,4-D doubled the endogenous RNA polymerase I activity (O'Brien *et al.*, 1968 : Hardin and Cherry, 1972) and it was unlikely that further stimulation of the enzyme *in vitro* could occur (Hardin *et al.*, 1970). A two-fold increase in RNA polymerase-activity was induced in lentil roots by IAA treatment (Teissere *et al.*, 1973) and four fractions, α, β, γ and δ, of the non-histone chromosomal proteins stimulated RNA polymerase-activity. Two of these fractions, γ and δ, studied in more detail, seemed to be RNA synthesis initiation factors, and the level of γ, which was specific for RNA polymerase I, seemed to be doubled in the presence of auxin (Teissere *et al.*, 1975). Re-examination of the data, however, revealed inaccurate interpretations, and the level of stimulation by auxin treatment was no more than 25 per cent (Venis, 1977). These and other similar observations concerning protein mediators and auxin-stimulated RNA synthesis cannot be considered as evidence for auxin receptors until interactions between auxins and these proteins have been demonstrated (Kende and Gardner, 1976).

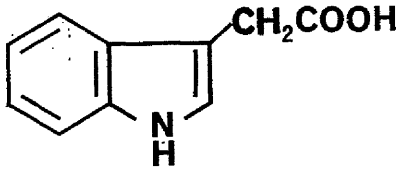
An auxin-receptor protein has been isolated from coconut endosperm nuclei (Mondal *et al.*, 1972 : Biswas *et al.*, 1975). In the presence of nucleoplasmic RNA polymerase, 10^{-3} mol m^{-3} IAA, DNA and an initiation factor, RNA synthesis was doubled by auxin-receptor protein, mostly

through chain initiation; electrophoresis demonstrated that new types of RNA had been produced. *In vitro* binding of auxin-receptor protein to ^{3}H-DNA was enhanced by 10^{-4}mol m^{-3} IAA (Biswas *et al.*, 1975) supporting the theory that auxin acts *in vivo* by transporting an inherently active regulatory protein from the cytoplasm to the nucleus (Hardin *et al.*, 1972). The receptor was believed to be a single polypeptide unit of 100,000 daltons, with pK_D 5.12 for IAA. Each protein was found to have 0.5 binding sites for auxin, indicating either that two receptors bound one auxin molecule or that partial inactivation of receptor had occurred during purification. The latter was thought the more likely (Biswas *et al.*, 1975) but, parenthetically, dimerisation of receptors as an activation requirement should not be discounted. However, the specificity of this system for auxin and the optimal concentration of IAA were not determined, and the concentration of IAA used (10^{-3}mol m^{-3}) in RNA synthesis experiments would have left the majority of receptor sites unoccupied if the estimated pK_D (5.12) was accurate (Venis, 1977). In any case, such a low pK_D may not be compatible with a receptor role for this protein.

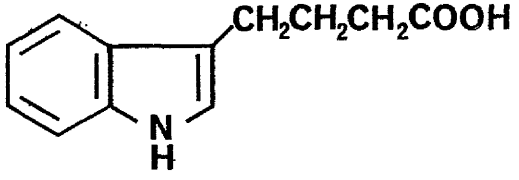
Cytoplasmic proteins in soybean cotyledons bound auxins, and the abilities of NAA (35), IBA (32) and PAA (36) to displace bound ^{14}C-IAA correlated well with their biological activities (Ihl, 1976). High affinity auxin-specific binding was demonstrated in *Phaseolus vulgaris* seedlings. The 315,000 dalton protein bound IAA more tightly (pK_D 7.05) than NAA (pK_D 5.77), but the affinities for 2,4-D and 2,4,5-T (40) which also bound, were not given (Wardrop and Polya, 1977).

A high-affinity, low capacity receptor for IAA has been located (Oostrom *et al.*, 1975) and partially purified from tobacco-pith explants (Oostrom and van Loopik-Detmers, 1976). Possibly due to the low levels of receptor present in crude extracts, the calculated pK_D values for IAA from separate experiments ranged from 7.85 to 8.04 (Oostrom *et al.*, 1975). The receptor was present both in IAA-treated and control tobacco-pith explants (Bogers *et al.*, 1978 : Mennes *et al.*, 1978). When IAA was present in the culture medium, RNA synthesis was increased during the second day of incubation and DNA synthesis and cell division were induced within four days. Of the IAA initially in the culture medium (10^{-2}mol m^{-3}) less than 20 per cent was taken up by the cells, wherein rapid metabolism to IAAsp (33), followed by slow production of an unidentified compound, occurred. At equilibrium, less than 2 per cent

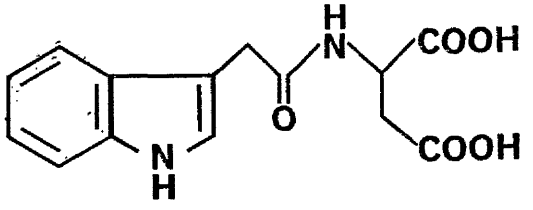
Fig. 5. Structural formulae of auxins



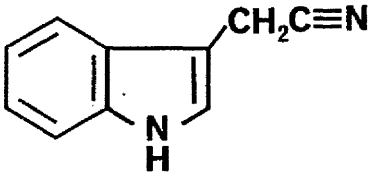
(31) IAA



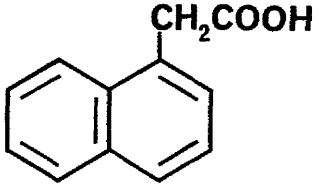
(32) IBA



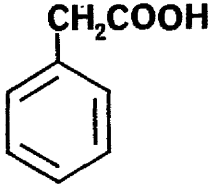
(33) IAAsp



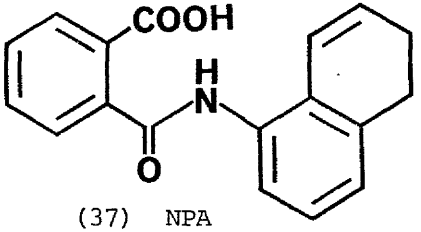
(34) IAN



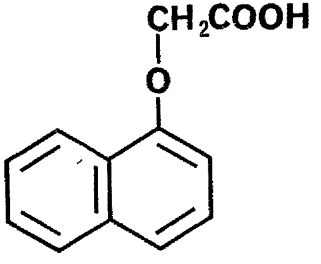
(35) NAA



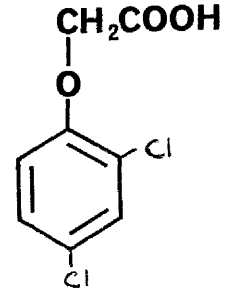
(36) PAA



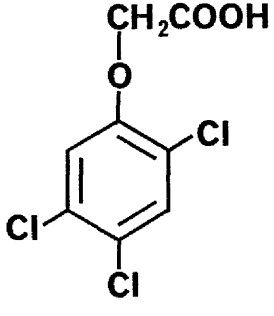
(37) NPA



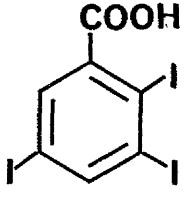
(38) NOA



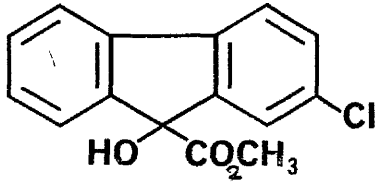
(39) 2,4-D

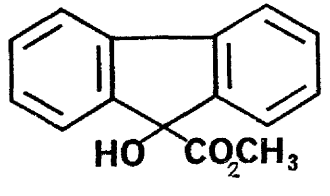


(40) 2,4,5-T



(41) TIBA





(42) Morphactins

e.g. Methyl-2-chloro-9-hydroxy-fluorene-9-carboxylate and Methyl-9-hydroxyfluorene-9-carboxylate

(used in van den Driessche and Cerf, 1977)

of the IAA taken up remained unmetabolised, resulting in an IAA concentration of 10^{-4} mol m^{-3} in cells, still sufficient to saturate the high-affinity binding sites (pK_D 8.00) (Bogers *et al.*, 1978)

The most frequently found auxin-binding sites however, have been membrane-associated. An unidentified particulate IAA-binding system from pea seedling buds lost much of its activity when apical dominance was released, and binding activity correlated well with the decreased ability of auxin to inhibit bud growth. NAA and IBA were competitors of IAA for binding sites (Jablanović and Noodén, 1974), but the competition by benzyladenine must cast doubts over the relevance of this binding. Developmental links also were noted in tomato: specific binding of NAA occurred in stems and young fruit. On the other hand, leaves and roots displayed little auxin-binding and the specific binding by fruit decreased with age (Mudge and Poovaiah, 1978). Auxins are believed to be a controlling factor in chloroplast development, possibly acting via membranes. Application of morphactins (42), a group of anti-auxins (transport inhibitors), caused ultrastructural alterations in chloroplasts and, moreover, inhibited the binding of IAA to chloroplast membranes (van den Driessche and Cerf, 1977).

Parallel with the location of cytoplasmic IAA-binding proteins in tobacco-pith explants, NAA-binding to membrane fractions (pK_D 6.48) also was demonstrated (Mennes *et al.*, 1978: Vreugdenhil *et al.*, 1979). The affinity for IAA was less (pK_D 4.78), but, in general, the affinities of auxin analogues correlated with their activities in bio-assays (Vreugdenhil *et al.*, 1979) although such low affinities would not indicate high specificity for auxins.

Zea mays coleoptiles have been used commonly in auxin-binding studies: membrane preparations exhibited NAA and IAA binding, and ^{14}C-NAA could be displaced by IAA and 2,4-D, but not NPA (37), GA_3 or abscisic acid. Estimated pK_D values were 5.40 - 5.52 for IAA and 5.70 - 6.00 for NAA (Hertel *et al.*, 1972). Methodological improvements allowed a more accurate assessment and the revised pK_D for NAA was 4.30 (Hertel, 1974). More detailed investigations demonstrated that two high-affinity auxin-binding sites were present : site 1 had pK_D's of 4.82 for NAA and 5.77 for IAA, whereas 5.80 and 5.24 were the respective values at site 2. IAA and NAA competed for both sites, and GA_3 and auxin analogues, including compounds without auxin-activity, were competitive

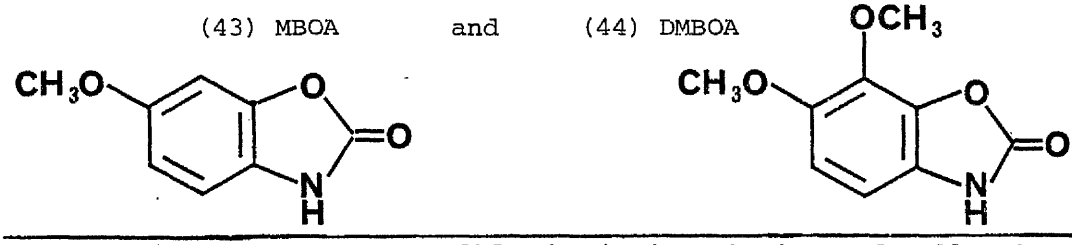
with NAA for site 1. On the other hand, site 2 was auxin-specific in that only active auxins, anti-auxins and auxin-transport inhibitors were competitors of NAA (Batt *et al.*, 1976). As well as having different binding properties, the two types of receptor were found in different membrane fractions, tentatively identified as the plasmalemma for site 2 and dictyosomes or endoplasmic reticulum (ER) for site 1 (Batt and Venis, 1976). However, it was shown later that site 1 was located only in the ER fraction (Hertel *et al.*, 1976) with site 2 on the tonoplast (Dohrmann *et al.*, 1978), and a third site, saturable for 2,4-D, present on the plasma membrane (Hertel *et al.*, 1976 : Dohrmann *et al.*, 1978). Binding at site 1 was not associated with RNA: rough ER, stripped of ribosomes, still had NAA attached (Ray, 1977). With purified membrane fractions, NAA was found to bind with pK_D 6.24 (Ray *et al.*, 1977a), 7.34 (Cross and Briggs, 1978a) and 7.17 (Cross and Briggs, 1979) much higher values than previous estimates. The rapid reversibility of binding suggested that covalent interactions had not occurred, yet the insensitivity of binding to high monovalent salt concentrations (Poovaiah and Leopold, 1976 a,b : Ray *et al.*, 1977a) indicated that binding was not primarily ionic (Ray *et al.*, 1977a). NAA-binding to particular fractions was increased by 10mol m^{-3} La^{+++}, Ca^{++} and Mg^{++}, and the $CaCl_2$-induced increase correlated well with the inhibition of auxin-induced elongation (Poovaiah and Leopold, 1976a,b). In contradiction, Ray *et al.*, (1977a) found that Ca^{++} and Mg^{++} concentrations greater than 5 mol m^{-3} inhibited NAA-binding.

The possibility that site 1 could be a precursor of site 2 has been raised (Dohrmann *et al.*, 1978). Site 1 binding was not auxin-specific (Batt *et al.*, 1976), but a factor (SF) from supernatant fractions of corn coleoptiles modified receptor-hormone interactions, narrowing the range of compounds bound by site 1 (Ray *et al.*, 1977b). SF inhibited NAA-binding to microsomal membranes (Cross and Briggs, 1978a,b), intact membranes and solubilized site 1 protein (Cross and Briggs, 1979) by altering the K_D (Cross and Briggs, 1978b). If sufficient SF was added, the inhibition of NAA-binding was complete, whereas binding of IAA, 2,4-D and NOA (38) were unaffected (Cross and Briggs, 1979). Previously it had been found that in SF-inhibited NAA-binding no reduction in the number of sites had occurred and that the SF-effect could not be pursued to completion (Ray *et al.*, 1977a). The large reductions in site 1

affinities for NAA and inactive auxin analogues (Ray *et al.*, 1977b) effectively resulted in an increased specificity of binding towards active auxins and this may represent receptor transformation (Venis, 1977) with site 1 as the precursor of site 2 (Dohrmann *et al.*, 1978). SF was identified as a mixture of 6-methoxy-2-benzoxazolinone (MBOA) (43) and 6,7-dimethoxy-2-benzoxazolinone (DMBOA) (44), with the latter about 50 times more active in NAA-binding inhibition (Venis and Watson, 1978).

Fig. 6. Structural formulae of:

(43) MBOA and (44) DMBOA



Auxins are known to modify the ionic relations of cells via ATPase-activation, e.g. for K^+ uptake and H^+ efflux (see Marrè 1977a,b). The auxin-receptors of *Zea mays* coleoptiles did not have ATPase activity (Cross *et al.*, 1978) but, with mung bean hypocotyl membrane fractions, there was highly specific IAA-binding to a Mg^{++}-activated ATPase-containing plasmalemma-rich isolate *in vitro* (Kasamo and Yamaki, 1973). 1PA, IBA and IAN (34) did not compete with IAA and direct binding of IAA to ATPase was suggested (Kasamo and Yamaki, 1976).

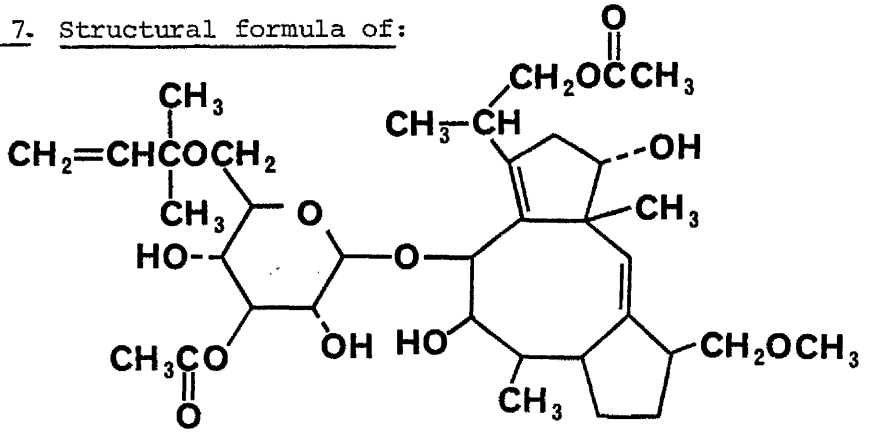
An auxin-binding site was located in the plasma membrane fraction from *Cucurbita pepo* hypocotyls (Jacobs and Hertel, 1978). This site (pK_D 5.82 for IAA) may be equivalent to site 3 in maize (Hertel *et al.*, 1976) : Dohrmann *et al.*, 1978) and could be involved in auxin transport (Jacobs and Hertel, 1978). That auxin transport inhibitors could affect auxin-mediated processes e.g. root geotropism in *Pisum sativum* (Gaither and Abeles, 1975) was indicative of the potential economic importance of these compounds as growth regulators. However, their mode of action is not fully understood. Sites on *Zea mays* plasma membrane bound on auxin-transport inhibitor NPA specifically, strongly and non-covalently (Lembi *et al.*, 1971) but NPA did not compete for specific auxin-receptors on membranes (Hertel *et al.*, 1972). IAA, 2,4-D and NAA were not bound by the NPA-site and TIBA (41), GA_3 and ABA did not compete with NPA. TIBA moved basipetally in coleoptile segments and, therefore, may have

utilized auxin-binding sites on the plasma membrane. The saturation kinetics of NPA effects on auxin transport correlated well with the affinity of the NPA-binding site (pK_D 6.66) (Thomson *et al.*, 1973), recently re-estimated to be 7.47 (Trillmich and Michalke, 1979). Also, the displacement of ^{3}H-NPA from membrane sites by NPA-derivatives and morphactins was in agreement with their relative activities as auxin transport inhibitors (Thomson and Leopold, 1974). NPA and TIBA were found to increase the affinity of the plasmamembrane site for auxin in fractions from *Cucurbita pepo* hypocotyls i.e. inhibit auxin transport by preventing the release of bound auxin. Only at concentrations exceeding 10^{-2}mol m^{-3} did NPA and TIBA compete for the auxin-specific sites and so reduce auxin binding (Jacobs and Hertel, 1978). It was suggested that the presence of specific binding sites for auxin transport inhibitors may reflect the presence of an unidentified plant hormone (Thomson, 1972) and a preliminary report of the isolation of a substance (Kartoffelfaktor) from potatoes and maize which bound to the NPA-receptor with high-affinity (pK_D 7.70) and specifically inhibited auxin transport, has been made (Reichert *et al.*, 1978).

Thus, auxins probably have two sites of action, cytosolic receptors and intracellular membrane-bound receptors (Venis, 1977) with the possibility that a third receptor, associated with the plasma membrane and primarily involved in auxin transport, may also mediate some responses to auxin (Jacobs and Hertel 1978). However, just as with steroid receptors in animals, the auxin-receptors in different organs of the same plant need not be identical e.g. pea buds and roots (Matthyse, 1970).

Specific binding of the fungal toxin, fusicoccin (FC) (45), has been found in plasma membrane fractions from oat roots (Stout and Cleland, 1978, 1979), maize coleoptiles (Hertel *et al.*, 1976 : Beffagna *et al.*, 1977 : Dohrmann *et al.*, 1977 : Tognoli *et al.*, 1979), spinach leaves (Beffagna *et al.*, 1977) and pea internodes (Lado *et al.*, 1976), but chloroplast and mitochondrial membrane-fractions from *Zea* coleoptiles

Fig. 7. Structural formula of:



(45) fusicoccin A

and spinach leaves had no FC-binding activity (Beffagna *et al*, 1977). Furthermore, specific FC-binding by maize coleoptile plasma membranes occurred *in vivo* (Pesci *et al*., 1978) and the binding sites in *Zea* coleoptiles (Hertel *et al*., 1976) and roots (Pilet, 1976) and pea internodes (Lado *et al*., 1976) were distinct from auxin-binding sites. Bound ^{3}H-FC in *Zea* coleoptile plasmalemma fractions was not readily displaced by unlabelled FC, the affinity for ^{3}H-FC being very high (pK_D 8.70). Yet 0.1mol m^{-3} FC was required to saturate the binding sites (Dohrmann *et al*., 1977) suggesting a high concentration of high affinity binding sites. The degree of FC-binding, however, was found to parallel the K^+-stimulated ATPase-activity present in oat root plasma membrane fractions (Stout and Cleland, 1978) and it was suggested that FC might bind to the enzyme (Stout and Cleland, 1979). Using *Zea* coleoptile plasmalemma fractions, Pesci *et al*., (1978) and Tognoli *et al*., (1979) demonstrated the catalytic subunit (for H^+/K^+ exchange) and the FC-binding protein were discrete, but both probably components of a multi-unit ATPase system. Also, FC-binding was inhibited by DES (69) (Tognoli *et al*., 1979), a synthetic oestrogen which is known to inhibit non-competitively ATPase-activity from plasma membrane fractions of oat roots (Balke and Hodges, 1979b) and also inhibit FC-effects (Marrè, 1978). Therefore, it is conceivable that FC-binding may indicate the presence of a receptor site for an unidentified plant hormone or enzyme co-factor, *c.f.* specific NPA binding.

Many enzyme systems and biosynthetic pathways are very similar in plants and animals, and, purely on theoretical grounds, there would seem no reason to doubt that modes of hormone action should be parallel in the two life-forms. In the first instance, animals (Jensen and Jacobson, 1962) and plants (Osborne, 1978) both have target cells for hormones. Secondly, many hormone effects in animals are brought about by direct interaction with enzymes, some of which are associated with the cell membrane (see Baulieu *et al*., 1978) and, in all probability, the same is true in plants. For the third part, hormones in plants and animals can effect alterations in the syntheses of specific RNAs and proteins. In animals, this is probably achieved *via* receptor proteins (e.g. O'Malley and Means, 1974), but there is no clear evidence for this in plants: although receptors have been found, translocation of hormone-receptor complexes to the nucleus and accumulation of radioactivity from labelled hormones by nuclei have yet to be demonstrated (Ginzburg and Kende,1968).

However, it must be borne in mind that the precise mode of action of hormone-receptor complexes in modifying gene expression in animal systems remains unresolved (Baulieu, 1979).

Initially, there seemed to be clear differences between the two major classes of animal hormones in terms of mode of action, but now the issue is less simple: polypeptide hormones and neuromediators generally act on enzymes at the cell membrane (Baulieu *et al.*, 1978) but may also be involved in gene activation (Davidson, 1965), and steroidal hormones, although mostly concerned with modifications of gene transcription, elicit effects also at the cell surface (e.g. Pietras and Szego, 1977) and through interactions with enzyme systems (e.g. Spiegel *et al.*, 1978). Of the plant hormones, auxins, gibberellins, cytokinins and abscisic acid all appear to act at both membrane and nuclear levels by virtue of effects on ion fluxes and RNA synthesis respectively.

The biological significances of many of the binding sites located for plant hormones are open to question on several counts: in very few plant systems do the values obtained for binding affinities or dissociation constants come within two orders of magnitude of those commonly found in animal hormone-receptor interactions. As yet, no unequivocal localizations of plant hormone receptors have been made (Rosenstock and Kahl, 1978). Many seem to be associated with intracellular membranes, but cell fractionation techniques can give rise to anomalous findings and the true subcellular location of a macromolecule, whether membrane-bound or free in the cytosol, need not coincide with its position after differential centrifugation. Although the same may be said of studies with animal systems, the lack of plastids and cell walls tends to simplify the situation. Receptors, however, are not the sole class of proteins which would be expected to bind hormones with high affinity and specifity: by virtue of the minute quantities in which hormones occur, and their rapid metabolism (in plants and animals), catabolic enzymes must have a high affinity and specifity for their hormone substrates (Kende and Gardner, 1976). A further complication is that both plant (e.g. Gardner *et al.*, 1978) and animal hormones (Cuatrecasas and Hollenberg, 1975) can bind to non-biological materials, often with high affinity and specificity.

Whereas the main mode of action of animal steroid hormones almost certainly involves interactions at the nuclear level, it is quite conceivable that the situation in plants is different: the rapid growth responses in coleptiles to applied auxins imply that the mode of action does not concern the nucleus, but rather that the site is located within the cell wall or is at the cell membrane surface. Even where protein and/or RNA synthesis is required for the effects of a hormone to become apparent, the challenges involved in proving interactions between hormone-receptor complexes and nuclei in plants are immense. The large amounts of protein and RNA in plant nuclei are, in themselves, difficult problems to surmount and there is also the possibility that in plants such hormone-receptor-nucleus interactions may not be required to initiate at least some responses *via* protein synthesis: plant mRNA, especially that in seeds, may be long-lived and it has been suggested that, in senescence, the genes for chlorophyll degradation are transcribed before the cell is mature and the mRNA is stored until the appropriate stimulus, be it hormonal or environmental, is perceived (Thomas, 1973). Although this may represent a special case, the implications are clear, especially when phytochrome-mediated responses are considered, and, while not denying the possibility of earlier hormone-nucleus interactions, such a system might explain some of the rapid responses of plants to their environment and to applied hormones.

Thus the picture of hormone action remains cloudy, particularly in plants. Significant advances have been made concerning steroid hormones in animals, although the mechanism by which genes are derepressed has not yet been elucidated. In contrast, plant physiologists have made little progress: more is known about the modes of action of fungal toxins such as FC and the oestrogen-analogue DES than the plant hormones themselves. Clearly though, with time and improved techniques, a greater understanding will be achieved.

A greater insight into the mode of hormone action might yield some information on the mechanism by which cells become hormone-responsive. That a cell should respond to a hormone, the response often manifested many reactions removed from the initial hormone-receptor interaction, is a reflection of the complex genetic determination of cell function laid down during differentiation (Tepperman and Tepperman, 1960).

3. Review of Oestrogens in Plants

The theme of parallel development in plant and animal hormone systems extends beyond the proposed modes of hormone action, e.g. animals produce significant amounts of IAA (Bertilsson and Palmér, 1972), the main endogenous auxin in plants (Greenwood *et al.*, 1972), and gibberellins, which are masculinizing agents in some plants (Krishnamoorthy, 1975), can substitute for oestrogens in the maintenance of oestrus in castrated female rats (Maillet and Bouton, 1969: Maillet *et al.*, 1969). However, the substances most likely to have a significant role both in plants and animals are the steroidal oestrogens.

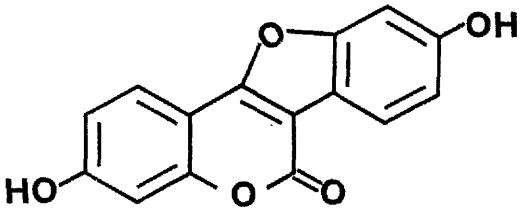
The foundations for this belief can be traced far into history: in ancient Egypt, stimulation of oat seedling growth by women's urine was used as an early determination of pregnancy (in Gad-Andresen and Jarlov, 1934), an assay which was also successful more recently using barley as the test plant (Küstner, 1932). The effects of certain fodder plants in hastening the appearance of sexual functions in animals were known long before oestrogens were identified, and in almost every culture, mankind has utilized an enormous number of plants as antifertility agents (see Farnsworth *et al.*, 1975a). The realisation that oestrogens may have parallel roles in plants and animals, though, came somewhat later. It was the demonstration of oestrogen-control of sexual development in animals and the knowledge that sexual function could be induced in immature or sexually quiescent animals by feeding with particular plants (Allen and Doisy, 1927) which prompted the search for oestrogens in the plant kingdom.

a) The occurrence of steroidal oestrogens in plants

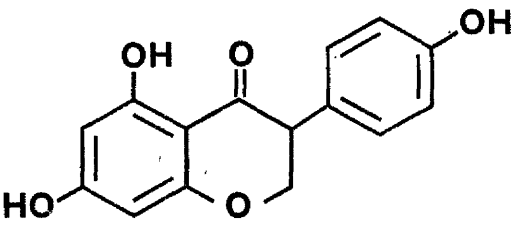
The first extraction of oestrogenic activity from plants was probably by Kountz (unpublished, in Allen and Doisy, 1927): flax and hemp seed extracts were partially active in the vaginal smear reaction but, at the time, Kountz did not regard the pro-oestrus response as sufficient to demonstrate the presence of oestrogens. However, the partial response was later taken to be acceptable, and many identifications of oestrogen-activity in plant extracts followed utilizing the ovariectomised mouse bioassay (Allen and Doisy, 1923). A preliminary report of oestrogenic activity in female flowers (Loewe, 1925) was later expanded, and female willow flowers (*Salix caprea*) were shown to contain up to 200 mouse units per kg (M kg^{-1}), with much

lower activities (3M kg^{-1}) extractable from *Althea rosea* leaves and stems, *Impatiens parviflora* stems and *Nuphar lureum* ovaries (Loewe and Spohr, 1926: Loewe *et al.*, 1927). *Beta vulgaris* seeds, parsley roots, potato tubers, cherry and plum fruit flesh (Dohrn *et al.*, 1926) and rice and oat seed embryos (Fellner, 1926) contained various degrees of oestrogen-activity. In the same period, yeast cells (Dohrn *et al.*, 1926: Glimm and Wadehn, 1928) and bacteria (Silberstein *et al.*, 1932: Pedersen-Bjergaard, 1933) also were found to contain oestrogenic substances. Even at this time, however, the difficulties in this field were becoming obvious. Walker and Janney (1930), in a wide-ranging search for oestrogens in plants, found that high concentrations of oestrogenic materials were associated only with rapidly growing green parts of plants; green parts beyond the rapid-growth phase, and roots, tubers and fruit, regardless of the growth phase, all had no oestrogen-activity. This was strictly in contrast to the findings of Dohrn *et al.*, (1926) mentioned above. Over the years though, a very wide variety of plants were shown by bioassay to contain oestrogenic substances (reviewed by Löve and Löve, 1945: Bradbury and White, 1954), but the applicability of this method to plant extracts is debatable. Bioassays are relatively unspecific and therefore cannot be used as a means of identification despite sometimes having great sensitivity. Additionally, even if a response is elicited it is by no means certain that the applied substance itself is active as it is liable to metabolism by the assay system. A specific complication to oestrogen bioassays has been that plants contain a number of active substances other than oestrone (1), oestradiol and oestriol (Cheng *et al.*, 1953). The oestrogenic substance extracted from *Butea superba* tubers (Schoeller *et al.*, 1940) was shown by chemical analysis not to be a steroidal oestrogen, and it was suggested that oestrone might not be as widely distributed in the plant kingdon as had been imagined (Butenandt, 1940). The phyto-oestrogens, mostly isoflavonoids e.g. coumestrol (46), genistein (47), biochanin A (48) and formononetin (49) (reviewed extensively by Farnsworth *et al.*, 1975b), are competitive inhibitors

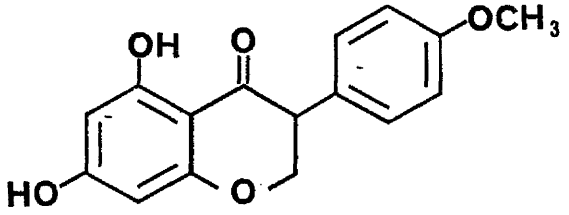
Fig.8. Structural formulae of some phyto-oestrogens



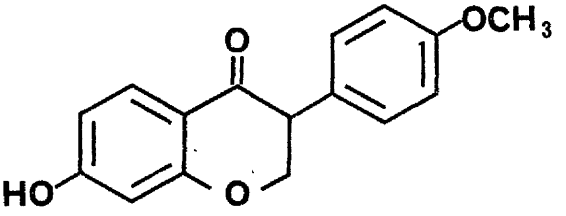
(46) coumestrol



(47) genistein



(48) biochanin-A



(49) formononetin

of oestradiol in animals, but bind to oestradiol receptors much more weakly (Shutt and Cox, 1972: Newsome and Kitts, 1975). As competitive inhibitors the phyto-oestrogens inhibit oestradiol-effects in the presence of oestradiol, but in its absence they are oestrogenic to some extent (Shutt, 1967). The presence of these compounds at high levels in certain forage plants is potentially serious, and has economic implications: sheep grazing on these plants have become infertile (Shutt, 1976). The degree of purification of a plant extract, therefore, to a great extent determines the activity shown in oestrogen bioassays.

The use of physico-chemical methods (Table 1c) has allowed more specific identifications to be made, and, by using a variety of techniques, oestrogens have been found in a number of plants (Table 1a). The main steroidal oestrogens are oestrone, oestradiol and oestriol (Fieser and Fieser, 1956).

The earliest attempts to identify definitively steroidal oestrogens in plants were made using physical properties such as melting point and chemical derivatizations. While, in general, no physical or chemical property is unique to a substance, analysis of a series of such properties reduces the range of possible identities, and thus α-follicle hormone and oestriol were identified from palm kernel (Butenandt and Jacobi, 1933) and willow flowers (Skarzynski, 1933a,b).

Table 1a. Cited plant sources of steroidal oestrogens

plant	plant parts	oestrogen	modes of identification	references
Angiosperms:				
Class Magnoliatae				
Subclass Hamamelidae				
Order Urticales				
Family Urticaceae				
Humulus lupulus	flowers	oestradiol	unknown	Anguelakova *et al.* (1972)
Order Fagales				
Family Betulaceae				
**Alnus glutinosa*	pollen	oestrone	9,11,12,16	Kvanta (1968)
Subclass Caryophyllidae				
Order Caryophyllales				
Family Chenopodiaceae				
Chenopodium rubrum	aerial plant parts	unidentified	9,12	Kopcewicz (1972c)
Subclass Dilleniidae				
Order Malvales				
Family Sterculiaceae				
Glossostemon bruguieri	roots	oestrone	2,3,9,11,16	Amin *et al.* (1969)
Order Violales				
Family Cucurbitaceae				
Cucurbita pepo	distribution during development in stems and leaves	oestrone, unidentified	9,12	Kopcewicz and Chrominski (1972)

Table 1a continued.....(1)

Order	Salicales				
Family	Salicaceae				
	Salix caprea	female flowers	oestriol	1,2,6,7, 1,2,3,7,11	Skarzynski (1933a) Skarzynski (1933b)
Order	Capparales				
Family	Cruciferae				
	Sinapis alba	distribution during development; leaves and seed	unidentified	12,18	Brooks *et al.* (unpublished)
Subclass	Rosidae				
Order	Rosales				
Family	Rosaceae				
	Malus silvestris	seeds	oestrone	7,12,14	Gawienowski and Gibbs (1969)
	Prunus armeniaca	seeds	oestrone, oestradiol	1,12	Awad (1974)
Family	Leguminosae				
	Phaseolus vulgaris	distribution during development;			
		1) aerial plant parts	unidentified	9,12	Kopcewicz (1977a)
		2) whole plants (pods removed), pods	oestrone	9,12	Kopcewicz (1971b)
		3) whole plants (pods removed)	unidentified	9,12	Kopcewicz (1972a)
		4) seeds, flowers, leaves	oestradiol	12,13,14,15, 18,19,20	Young (1977)

Table 1a continued.....(2)

	Phaseolus vulgaris (continued)	rooted cuttings, seeds, leaves	oestradiol	12,13,14,15, 18,19,20	Young *et al.* (1977)
		seeds, flowers, leaves	oestradiol	12,13,14,18,20	Young *et al.* (1978)
Order	Sapindaceae				
Family	Rutaceae				
	Belva chal	bark	oestradiol	12	Mannan and Ahmad (1976)
Order	Euphorbiales				
Family	Euphorbiaceae				
	Phyllanthus neruri	roots	oestradiol	12	Mannan and Ahmad (1976)
Order	Myrtales				
Family	Lythraceae				
	Punica gratanum	seeds	oestrone	1,9,12	Bennett *et al.* (1966b)
				12,18	Dean *et al.* (1971)
Order	Umbellales				
Family	Araliaceae				
	Panax ginseng	roots	oestrone, oestradiol, oestriol	unknown	Anguelakova *et al.* (1972)
Subclass	Asteridae				
Order	Polemoniales				
Family	Solanaceae				
	Hyoscyamus niger	leaves and stems	oestrone, unidentified	9,12	Kopcewicz (1972b)
Family	Boraginaceae				
	Heliotropium indicum	roots	oestradiol	12	Mannan and Ahmad (1976)

Table 1a continued.....(3)

Order	Lamiales				
Family	Labiatae				
	Perilla ocimoides	aerial plant parts	oestrone, unidentified	9,12	Kopcewicz (1972c)
	Salvia splendens	distribution during development; whole plants	unidentified	9,12	Kopcewicz (1972b)
Order	Scrophulariales				
Family	Oleaceae				
	Olea europaea	kernel olive oil	oestrone oestrone ester	9,11,12,16,17 7,11,12	Amin and Bassiouny (1979)
Order	Asterales				
Family	Compositae				
	Synedrella nudiflora	roots	oestradiol	12	Mannan and Ahmad (1976)
Class	Liliatae				
Subclass	Commelinidae				
Order	Cyperales				
Family	Graminae				
	**Dactylis glomerata* **Phleum pratense* **Secale cereale* **Zea mays*	pollen	oestrone	9,11,12,16	Kvanta (1968)
	Zea mays	corn oil	oestrone	7,11,12	Amin and Bassiouny (1979)
Subclass	Arecidae				
Order	Arecales				
Family	Palmae				
	Eleais guineensis	kernel	oestrone	1,2,4,5,7,11	Butenandt and Jacobi (1933)
	Hyphaene thebaica	kernel, pollen	oestrone	2,3,4,11,16	Amin and Paleologom (1973)

Table 1a continued.....(4)

	Phoenix dactylifera	pollen	oestrone	1,4,9,11	el Ridi and el Wafa (1947)
				9,11	Hassan and el Wafa (1947)
				8,12,16	Bennett *et al.* (1966a)
				2,3,9,11	Amin *et al.* (1969)
				12	Mahran *et al.* (1976)
		seed	oestrone	6,8,9,10,12,13	Heftmann *et al.* (1965)
				8,9,10,12	Bennett *et al.* (1966a)
Subclass	Liliidae				
Order	Liliales				
Family	Liliaceae				
	Hyacinthus orientalis	distribution during development; bulb heel, bulb scales, leaves, inflorescence, roots	unidentified	9,12	Kopcewicz *et al.* (1973)
Gymnosperms:					
Subclass	Coniferopsida				
Order	Coniferales				
Family	Pinaceae				
	**Pinus montana*	pollen	oestrone	9,11,12,16	Kvanta (1968)

* mixed pollen sample (Kvanta, 1968)

Table 1b. Techniques used in identification of steroidal oestrogens in plant extracts

Procedure

1. bioassay
2. melting point
3. mixed melting point
4. chemical formula
5. optical rotation
6. solubility
7. derivatization
8. reduction to oestradiol
9. colour tests
10. fluorescence
11. ultraviolet (UV) absorption spectroscopy
12. thin-layer chromatography (TLC)
13. column chromatography
14. gas chromatography (GC)
15. radio-gas chromatography
16. infra-red (IR) absorption spectroscopy
17. nuclear magnetic resonance (NMR) spectroscopy
18. radioimmunoassay (RIA)
19. recrystallisation to constant specific activity
20. combined gas chromatography-mass spectrometry (GC-MS)

Table 1c. Evaluation of techniques

Procedure	Speed	Specificity	Sensitivity
bioassay	slow	non-specific	variable
RIA	rapid	non-specific	sensitive
colour reactions	rapid	non-specific	variable
chromatographic mobility	rapid	non-specific	variable
gas chromatography	rapid	semi-specific	sensitive
optical rotation	rapid	variable	sensitive
UV spectroscopy	rapid	non-specific	insensitive
IR and NMR spectroscopy	rapid	can be uniquely specific	sensitive
GC-MS	rapid	highly specific, aided by various types of ionisation and computational facilities	sensitive

The use of chemical conversions, usually reduction of presumed oestrone to oestradiol, or derivatizations have also suffered from the fact that identification of the reaction product has been by semi-specific methods.

The published studies on oestrogen distribution during growth and development mostly have been made by Kopcewicz: in *Phaseolus vulgaris* (DNP), four unidentified oestrogen-like substances first appeared at flower bud formation, reaching a maximum during flowering and pod formation (Kopcewicz, 1971b) The LDPs *Hyoscyamus niger* and *Salvia splendens* displayed synthesis of oestrogens, beginning at bud formation and peaking at flowering. Three oestrogen-like substances were isolated from each species, and the six oestrogens were discrete, with oestrone possibly present in *Hyoscyamus* (Kopcewicz, 1972b). Similarly, high oestrogen levels were associated with formation of generative organs in the SDPs *Perilla ocimoides* and *Chenopodium rubrum*. Two oestrogens were isolated from each plant, *Perilla* may have contained oestrone, and it was suggested that each plant species produced its own oestrogen-like substances which controlled flowering (Kopcewicz, 1972c). Also it was proposed that ethylene may promote femaleness in *Cucubita pepo* through oestrogens (Chrominski and Kopcewicz, 1972), and subsequently ethylene-induced female sex expression was correlated with oestrogen content, again with oestrone possibly present (Kopcewicz and Chrominski, 1972). In *Hyacinthus orientalis*, a cold-requiring plant, the lowest oestrogen levels were found in unrooted dormant bulbs, only the leaves and inflorescences containing oestrogens. With bulbs not cold-treated, but potted and grown in greenhouses, the oestrogen content of leaves and inflorescences had doubled at flowering, but no oestrogens were present in the roots or fleshy scales. Cold-treated bulbs also had increased oestrogen levels, but throughout the plant (Kopcewicz *et al.*, 1973). Conversely, Brooks *et al.* (unpublished) found that leaves of *Sinapis alba* plants which had flowered contained less oestrogen than did leaves from vegetative plants of the same batch and age, and, in general, the oestrogen content fell after floral bud initiation. The implied connection between oestrogens and flowering is clear and the work of Brooks *et al.* was not necessarily contradictory to that of Kopcewicz. Young, however, found similar levels of oestrogens in seeds and vegetative and flowering plants of *Phaseolus vulgaris* (Young, 1977: Young *et al.*, 1978).

Kopcewicz has also tested the effects of plant hormones on oestrogen levels: in *Phaseolus* IAA and GA_3 had no effect but kinetin treatment induced an increase and ABA a decrease (Kopcewicz, 1971a, 1972a). Ethylene treatment resulted in increased levels of oestrogens in *Cucubita pepo* (Kopcewicz and Chrominski, 1972).

However, several criticisms can be levelled at Kopcewicz's work, as he is aware (see van Rompuy and Zeevaart, 1979). The determinations of oestrogens (see Table 1a) were by equivocal means (Table 1b). Colour tests in general may not be suitable for plant extracts, and the Kober reaction used by Kopcewicz can be influenced by artefacts from acid hydrolysis (Adlercreutz *et al.*, 1967). Furthermore, no attempt was made to identify the oestrogen-like substances extracted and Kopcewicz has not been able to confirm the presence of Kober-positive materials in flowers and plants. Previously, the positive response may have been due to phenolics (see van Rompuy and Zeevaart, 1979). In most experiments several plant parts were excluded from extraction (see Table 1a), but the plant organs removed were not consistent, and no rationale was given for these exclusions. Only in *Hyacinthus* (Kopcewicz *et al.*, 1973) was a complete investigation of plant parts attempted. Also, in the studies of the effects of plant growth regulators on oestrogen levels, it was not clear if the alterations in presumed oestrogen contents were related directly to the hormone in question or the modifications of development induced by treatment.

Until recently, the methods used in all identifications (Tables 1b,c) have been equivocal. The results obtained, therefore, have depended greatly on the extraction and purification procedures employed. Also, as oestrogens are continually produced by man, the possibility of contamination has been ever-present. These constraints, coupled with reported failures to isolate oestrogens from plants (Jacobsohn *et al.*, 1965: Bennett *et al.*, 1967: van Rompuy and Zeevaart, 1975, 1976, 1979: Bledsoe and Ross, 1978), have caused doubts as to their endogeneity and hindered their acceptance as possible natural growth regulators in plants. However, the accumulated evidence over the years had suggested that steroidal oestrogens were present, and this was confirmed by the incorporation of biosynthetic label from ^{14}C-mevalonic acid, ^{14}C-oestrone and ^{3}H-oestrone-sulphate into oestradiol by *Phaseolus vulgaris* hypocotyl cuttings and leaf homogenates (Young

et al., 1977, 1979) and the identification of oestradiol in *P. vulgaris* seeds and leaves by GC-MS (Young *et al.*, 1977, 1978), the only methods to give a reasonably definitive identification for small quantities of an endogenous compound in a complex extract. This has added considerably to the credibility of oestrogens as plant growth regulators. The levels of oestrogens estimated by RIA, 2-3.4 $\mu g\ Kg^{-1}$ in *Phaseolus* seeds (Young *et al.*, 1978) and 3-10 $\mu g\ Kg^{-1}$ in *Punica* seeds (Dean *et al.*, 1971) were very much lower than previous estimates, e.g. 17 $mg\ Kg^{-1}$ in *Punica* seeds (Bennett *et al.*, 1966b), made using less sensitive techniques. Indeed, when questioning the occurrence of steroidal oestrogens in plants, Jacobsohn *et al.* (1965) indicated that the level of oestrone in palm kernels could not have exceeded 10 $\mu g\ Kg^{-1}$, their limit of resolution, and such endogenous levels would be more in line with those expected for a hormone.

The failures of van Rompuy and Zeevaart (1975, 1976, 1979) to confirm the presence of oestrogens in several previously examined species using GC-MS may have been due to inadequate extraction procedures and inefficient purification and separation techniques. In addition, the high level of radioactivity added as a tracer (1 μCi = 5.5 μg oestrone and oestradiol) especially when viewed in conjunction with the use of a low efficiency (16 per cent) purification system, could have concealed the presence of small amounts of oestrogens, particularly in small samples. By inference (in van Rompuy and Zeevaart, 1979) 1 Kg apple seeds were extracted but no mention was made of sample size in the other species examined.

b) Uptake and distribution of applied oestrogens

If a substance is to be considered as a hormone, information regarding its endogenous levels, distribution and metabolism must be at hand. Equally, details of uptake, distribution and metabolism are prerequisites for interpretation of the effects of exogenous compounds. Unfortunately, these aspects have been generally ignored in studies of effects of oestrogens in plants, even where specific responses have been elicited. In the early literature, uptake of applied substances was rarely mentioned: barley seedlings took up folliculin (von Euler *et al.*, 1934) and, with hyacinths, the alkali salt of folliculin (formed

by treatment with NaOH) was more readily absorbed than the native hormone (Schoeller and Goebel, 1934). Of course, the metabolism of folliculin by hyacinths (von Euler and Zondenk, 1934) and maize (Marzetti, 1935) implied that uptake had occurred, and the same may be said of the many experiments in which plants responded to oestrogen treatments. Indeed, it seemed only when applications were without effect that the question of uptake was considered. Many of these experiments involved plants grown in soil watered with oestrogenic solutions and it was felt that absorption by materials (such as humus) in the soil (Rose and Hamon, 1939: Botjes, 1941) or destruction by microorganisms (Botjes, 1941) would have prevented uptake of oestrogens by roots.

The lack of suitable technologies to follow uptake and distribution no doubt greatly hampered the early workers: Löve and Löve (1945) had postulated that oestrogens applied to flowers (*Melandrium dioicum*) could not have been translocated away to other flowers on the same plant as only those treated responded to oestrogens. The advent of radioisotope usage has made such studies possible, but the major drawback has been that unless identities of label-containing compounds are established, merely uptake and translocation of radioactivity are observed. In this context it is important to have the label at a site on the molecule where exchange is unlikely.

Radiochemicals have been used to monitor the distribution of sterols (Atallah *et al.*, 1975) and oestrogens (Young, 1977: Young *et al.*, 1977, 1979: Hewitt *et al.*, unpublished) taken up from solutions by cuttings, but uptake itself was not studied in detail. Theoretically in this method, known as "wick-feeding", cuttings stood in solution take up the applied chemical in the transpiration stream (Atallah *et al.*, 1975). In the study of sterol translocation in *Pelargonium hortorum* cuttings, autoradiography was used to demonstrate that radioactivity was distributed throughout the explant after 48 hours (Atallah *et al.*, 1975). The theory behind wick-feeding, however, did not really hold for steroidal oestrogens: with ^{14}C-oestrone and ^{14}C- oestradiol applied to hypocotyl cuttings taken from plants at various growth stages, usually at least 90 per cent of the label taken up was retained at the cutting base (Young, 1977: Young *et al.*, 1977, 1979: Hewitt *et al.*, unpublished). The hydrophobic nature of the steroidal substances may have

caused them to leave the aqueous medium in favour of the more suitable lipoid environments provided by membranes. Using ^{3}H-oestrone-sulphate, however, a much more even distribution of label was noted (Young, 1977: Young *et al.*, 1977, 1979). Oestrone-sulphate is water soluble, and its negative charge should prevent non-specific binding to xylem vessels, which consist of negatively-charged cell wall materials.

The degree of translocation of radiolabel from sterols applied to sunflower and geranium leaves varied considerably but much of the label remained at the site of application (Atallah *et al.*, 1975). The translocation of ^{14}C-metabolites from leaves supplied with $^{14}CO_2$ has been studied in *Phaseolus vulgaris*: the metabolites from young leaves which had just begun exporting assimilates were rich in putative steroids and these were translocated upwards in the phloem towards the apex. It was only when the leaf had expanded further and its metabolism switched to predominantly sucrose production that downward translocation of ^{14}C-metabolites occurred (Biddulph and Cory, 1965).

Preliminary experiments with *P. vulgaris* seeds have suggested that ^{14}C and ^{3}H from labelled oestrogens entered with water during imbibition, and that uptake of label continued after water uptake had been reduced. During the growth of seeds imbibed in radioactive oestrogen solutions, most of the label remained associated with cotyledons (Hewitt *et al.*, unpublished).

Curiously, these have been the only utilizations of such techniques to follow oestrogen uptake and distribution despite the absolute requirement for this information if any sensible comments are to be made concerning results from application experiments. This disturbing situation has done nothing to enhance the case for oestrogens as plant hormones.

c) Metabolism of applied oestrogens

The establishment of oestradiol synthesis in *Phaseolus* (Young *et al.*, 1977) was at least as important as the absolute confirmation of its presence. However, the anabolic and catabolic pathways of steroidal oestrogens have not yet been investigated fully in higher plants (Grunwald, 1978). Microbial organisms, on the other hand, due to their economic importance in the preparative organic chemistry industry, have been studied in more detail with regard to oestrogen

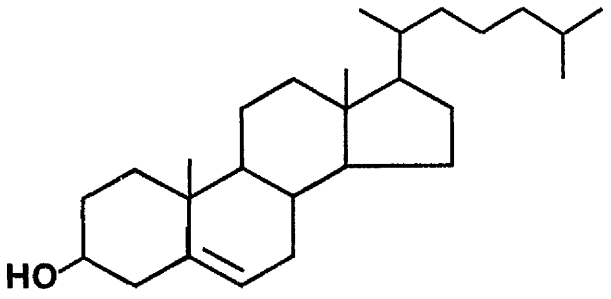
metabolism. An enzymic preparation from mushrooms, tyrosinase, which contained phenol oxidase, inactivated oestrone and oestradiol (Westerfield, 1940) probably by production of 2-hydroxy-oestradiol (53) (Jellink and Brown, 1971).

Fusarium spp. produce oestrogens if given suitable substrates, and are also capable of oestrogen catabolism. *F. solani* produced oestrone and oestradiol from 19-nor-testosterone (52) (Kondo, 1964) and oestrone from progesterone, where the rapid side-chain degradation in 20-keto pregnanes to corresponding 17-ketones was accompanied by simultaneous aromatization of ring A (Vischer and Wettstein, 1953). In *F. moniliforme* (*Gibberella fujikaroi*), oestrone and oestradiol were converted to the respective 15α-hydroxy derivatives (58,59) and the 3-methyl ether of oestradiol (62) was hydroxylated at the 6β-position (63) (Crabbe and Casas-Campillo, 1964: Casas-Campillo and Bautista, 1965). Hydroxylations at other positions on the steroid nucleus could be achieved using different fungi: *Glomerella fusarioides*, *G. glycines* and *Aspergillus corneus* produced the 7α-hydroxy derivatives (55, 56) of oestrone and oestradiol, *Martierella alpina*, reduced oestrone to 6β-hydroxy-oestradiol (54) (Laskin, *et al.*, 1964) and 11β-hydroxy-oestradiol (57) was formed from oestradiol by *Neurospora crassa* with interconversion of oestrone and oestradiol also noted here (Maugra *et al.*, 1973) and with *Candida albicans* (Gharf *et al.*, 1975).

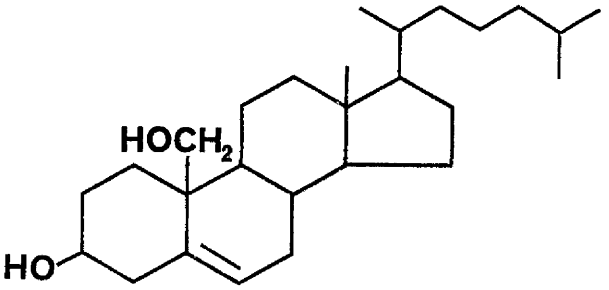
Aspergillus flavus has been found to produce oestrogen conjugates: of the oestradiol added, 60 per cent was recovered as oestrone-3-sulphate (5) and 10 per cent as oestradiol-3-sulphate (6) (Schubert and Groh, 1971). Also, an enzyme preparation, mylase-P, from *A. oryzae* hydrolysed most of the oestrogen conjugates in the urine of pregnant mares, and this was due to a phenol sulphatase, not a general sulphatase or phenol oxidase (Cohen and Bates, 1949).

The acetate, butyrate and propionate of oestrone (64,65,66) were converted mostly to oestradiol by fermenting yeasts. Little oestrone was produced and oestrone could not be reduced to oestradiol in the same systems (Mamoli, 1938). On the other hand, Wettstein (1939) found the latter reaction possible, and the production of oestradiol by *Saccharomyces* from chemically synthesised oestrone can be used to split racemic mixtures of the steroid, only the biologically active enantiomer being accepted as a substrate (Vischer *et al.*, 1956).

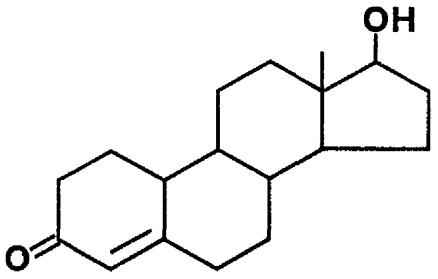
Fig. 9. Structural formulae of some sterols and oestrogen derivatives



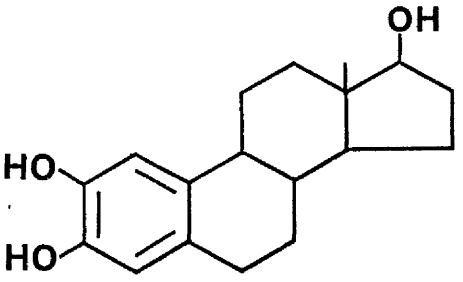
(50) cholesterol



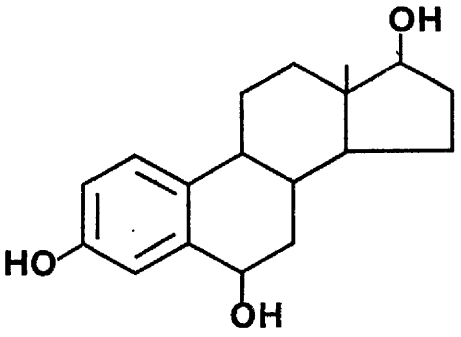
(51) 19-hydroxy-cholesterol



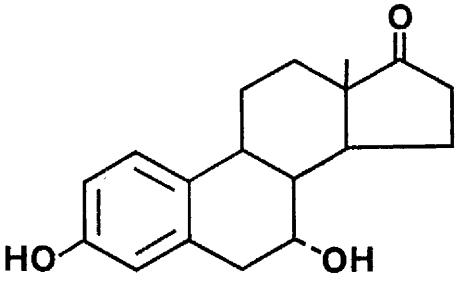
(52) 19-nor-testosterone



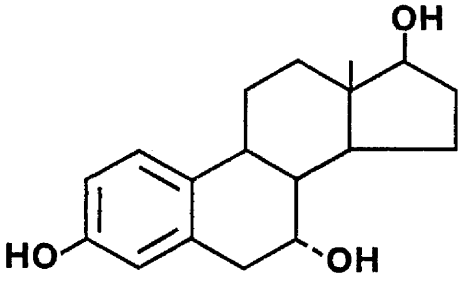
(53) 2-hydroxy-oestradiol



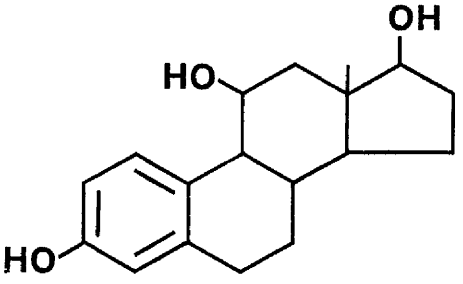
(54) 6β-hydroxy-oestradiol



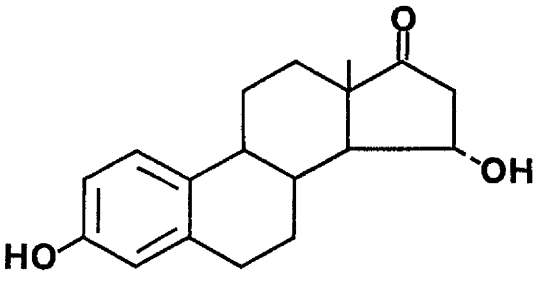
(55) 7α-hydroxy-oestrone



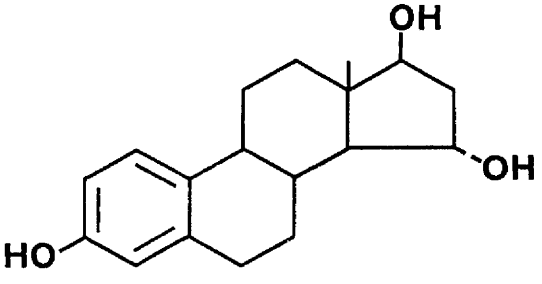
(56) 7α-hydroxy-oestradiol



(57) 11β-hydroxy-oestradiol

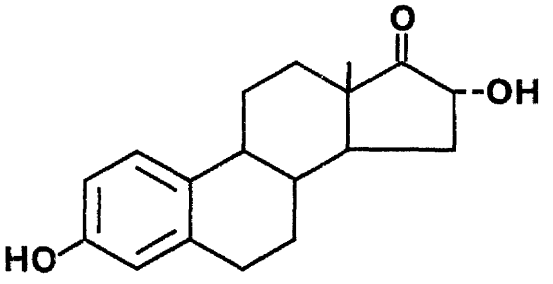


(58) 15α-hydroxy-oestrone

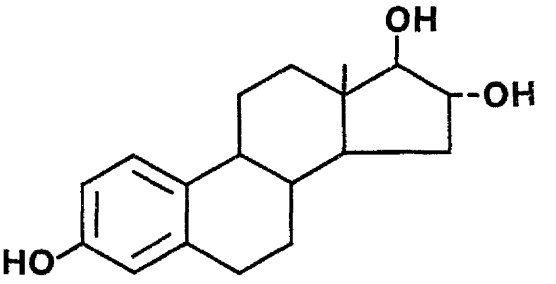


(59) 15α-hydroxy-oestradiol

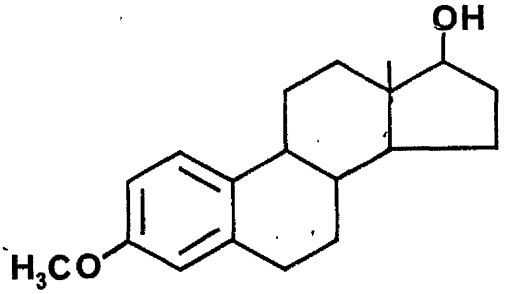
Fig.9 continued



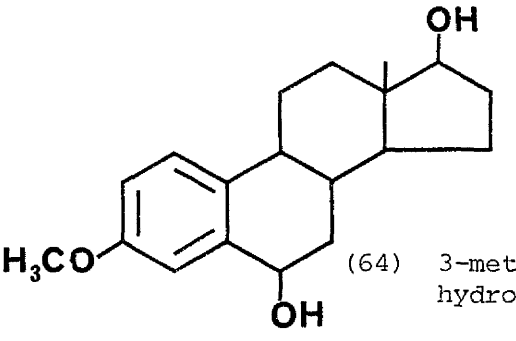
(60) 16α-hydroxy-oestrone



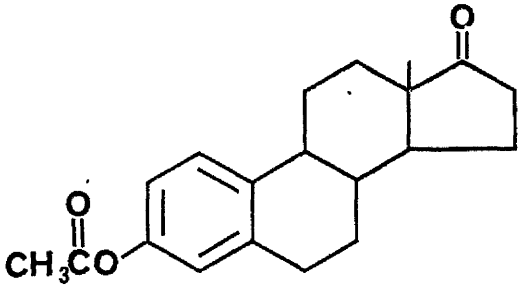
(61) 16α-hydroxy-oestradiol



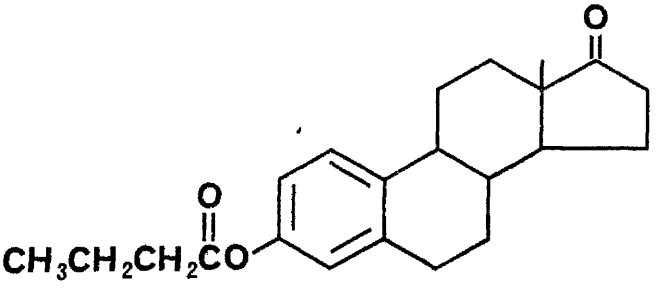
(62) 3-methoxy-oestradiol



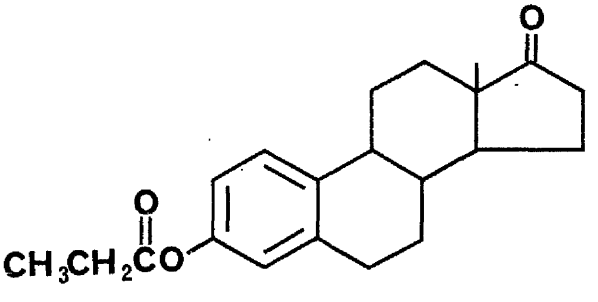
(64) 3-methoxy-6β-hydroxy-oestradiol



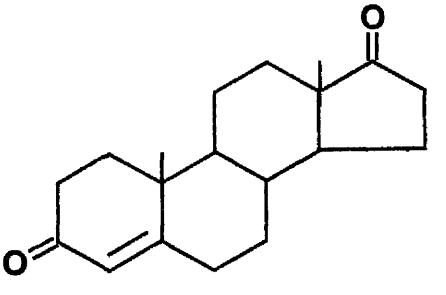
(64) oestrone-acetate



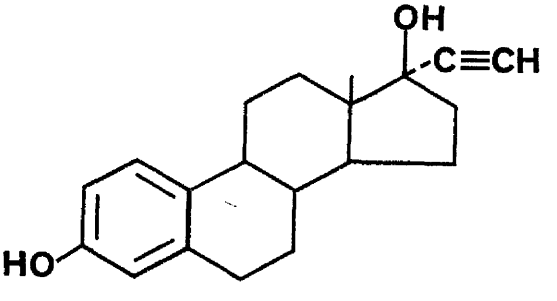
(65) oestrone-butyrate



(66) oestrone-propionate



(67) 4-androsten-3,17-dione



(68) ethynyl-oestradiol

Oestrone was produced from cholesterol (50) and 19-hydroxy-cholesterol (51) via C_{22}-acids by *Nocardia restrictus* (Sih *et al.*, 1967) and the build-up of oestrone following side chain removal and ring A aromatization varied from 8 to 30 per cent, depending on the strain used (Sih and Wang, 1965). In microorganisms however, the enzymic aromatization of ring A was not identical to that in the human placenta: with *N. restrictus*, 1,2-dehydrogenation yielded β-hydroxy ketones which underwent spontaneous rearrangement (non-enzymic reverse aldolation) to produce phenols. A flavoprotein electron-acceptor was required for activity and aromatization could proceed without $NADPH_2$ and O_2 (Sih and Rahim, 1963). Oxidation of oestrone by *N. restrictus* was found to involve cleavage of ring A prior to ring B (Coombe *et al.*, 1966) whereas most microorganisms oxidize steroids by splitting the B ring first (Sih and Wang, 1963). Bacteria also can produce and metabolise oestrogens: oestradiol was derived from 4-androsten-3,17-dione (67) by *Escherichia coli* (Goddard and Hill, 1972) and oestrone and oestradiol from 19-nortestosterone by *Cornybacterium simplex* (Kushinsky, 1958). Oxidation of oestradiol to oestrone has been reported in *Streptomycetes* (Heuesghen and Welsch, 1949) and in 48 *Actinomycetes spp.*, with four also producing oestriol and nine yielding unidentified hydroxy-derivatives (Kogan *et al.*, 1965).

The use of microorganisms in the manufacture of steroidal oestrogens and derivatives is somewhat paradoxical as the occurrence of the substances in microorganisms has yet to be confirmed. The production of a substance from specific substrates in artificial cultures cannot be taken as definitive proof of endogeneity, especially when the reactions involved often are strain specific. In higher plants, on the other hand, where a much wider range of species have been cited as oestrogen sources, very little work has been completed on oestrogen metabolism, despite the realisation, dating back to the early experiments, that plants metabolise applied oestrogens: of the folliculin added to hyacinth culture water, none was recovered from the solution or from the plant. It was shown that living roots or a pulp of root material inactivated folliculin enzymically (von Euler and Zondenk, 1934), confirmed in *Zea* by Marzetti (1935). Extracts of many plants, but especially potatoes, inactivated oestrogens and the presence of a specific "estrinase" was proposed (Zondenk and Sklow, 1942). However,

this was refuted when potato phenol oxidase was found to have a high capacity for oestradiol oxidation (Bergström *et al.*, 1945). Oestradiol breakdown did not involve oestrone production and the phenol oxidase content of the 20 strains tested varied by a factor of 10 and also increased rapidly during growth.

As far as oestrogen synthesis is concerned, it is possible that the pathways in plants and animals are very similar as the intermediates in animals are also found in plants (Grunwald, 1978). The production of steroids by whole plants has been monitored using $^{14}CO_2$ (Biddulph and Cory, 1965) and ^{14}C-MVA (Bennett *et al.*, 1967: Bledsoe and Ross, 1978), but the components of the steroid fractions were not positively identified. However, the biosynthesis of ^{14}C-oestradiol from ^{14}C-MVA applied by wick-feeding *Phaseolus vulgaris* hypocotyl cuttings has been demonstrated along with the production of ^{14}C-oestradiol from ^{14}C-oestrone and ^{3}H-oestradiol from ^{3}H-oestrone-sulphate (Young *et al.*, 1977, 1979). The conversion of ^{14}C-oestrone to ^{14}C-oestradiol was slow, as much of the label recovered up to 35 days after application was contained in oestrone. However, although ^{14}C-oestradiol was synthesised from ^{14}C-MVA, ^{14}C-oestrone was not present in sufficient quantities to permit formal identification, and a large proportion of the label applied in ^{14}C-oestrone could not be extracted and therefore remained uncharacterized (Young *et al.*, 1977, 1979).

However, distribution studies revealed 90 per cent of the applied ^{14}C to have remained in the hypocotyl and roots (Young *et al.*, 1979) and such high levels concentrated at particular sites could have resulted in anomalous metabolism patterns, particularly as long incubation periods were employed. Additionally, the low levels of oestrogens transported beyond the hypocotyl necessitated the use of large numbers of plants to obviate overloading effects even although only leaf material was extracted. The greater transport of ^{3}H-oestrone-sulphate has alleviated this to some extent, but until oestrogen-sulphates have been unambiguously characterized from plants, results from experiments involving their usage must be interpreted carefully.

A further difficulty with the use of wick-feeding is that of sterility and micro-organisms contaminating feeding solutions not only metabolise applied solutions, but also may enter the vascular system of cuttings (Nowak and Rudnicki, 1975).

Cell-free preparations have been used frequently to establish metabolic pathways, notably with leaf homogenates in the study of sterol, pregnane and androstane biosyntheses (see Stohs and Rosenburg, 1975: Stohs, 1977). Using such a system from bean leaves, Young confirmed the transformation of ^{14}C-oestrone to ^{14}C-oestradiol (Young *et al.*, 1979). Here, 43 per cent incorporation of ^{14}C into oestradiol occurred within 2 hours, with NAD a more suitable cofactor than NADP, and this rapid metabolism of ^{14}C-oestrone has raised the question of penetration of oestrogens applied to plants: the slow interconversion of oestrone and oestradiol in cuttings may have been due to compartmentation of the required enzyme or the applied oestrogens. In gibberellin synthesis a cytochrome P_{450}-like NADPH reductase system is involved in the production of kaurenoic acid from kaurene (Murphy and West, 1969). A similar system operates in the oxidation of steroids, barbiturates and alcohols in animals (Estabrook *et al.*, 1973), and it is possible that an enzyme system of this type may be involved in oestrogen metabolism in plants. As this enzyme is cytosolic (Murphy and West, 1969), the slow rates of reaction in cuttings may have been due to compartmentation of oestrogens by entry into lipoid environments such as membranes.

d) Effects of steroidal oestrogens in plants

Ever since oestrogens were found in plants, their possible functions have been investigated. The concept of parallel regulatory mechanisms for growth and development in animals and plants was the driving force behind studies into the effects of oestrogens on growth, flowering (sexual maturity) and sex expression.

There have been numerous reports on the involvement of oestrogens in a variety of developmental phenomena in a wide range of plants. Some of these findings have been substantiated in further work, but also many have been contradicted. Over the years, several reviews of oestrogen effects have been made, but, generally, only selective aspects have been discussed (e.g. Thimann, 1935: Bonner, 1937: Loehwing, 1938: Thimann and Bonner, 1938: Löve and Löve, 1945: Heslop-Harrison, 1957: Heftmann, 1963, 1971, 1974a,b, 1975a,b, 1977: Grunwald, 1975, 1978, 1979: Geuns, 1978: Zeevaart, 1978: Hewitt and Hillman, 1980: Hewitt *et al.*, 1980); a comprehensive account of the effects of oestrogens in

plants has never been compiled. The available reports are copious, therefore, rather than deal with each individually, the wide-ranging effects of oestrogens are presented *in tabulae* and only the more important aspects discussed in detail.

From Tables 2a,b,c, it is obvious that the effects of oestrogens in plants are not clear-cut, and the relevance of results has always been debated (e.g. Thorsrud and Ödelien, 1934: von Euler and Larsson, 1934: Bonner, 1937: David and Gouzon, 1939). The frequent failures met with when experiments were repeated by others could be attributed to a number of factors. In many experiments which yielded negative results, plants were grown in soil watered with oestrogen solutions (e.g. Lemmermann and Behrens, 1934) and it was questionable if any of the applied substance was available to the plant, due to absorption by soil components (Rose and Hamon, 1939: Botjes, 1941) or metabolism by micro-organisms (Botjes, 1941). On the other hand, some attempts to repeat experiments using water-culture techniques also failed; Gad-Andresen and Jarlov (1934) confirmed the results of Schoeller and Goebel (1931a) with hyacinth, but Virtanen *et al.* (1934) could not, despite using Schoeller's hormone preparation. Possible explanations for such difficulties relating to the use of different cultivars of a species, different developmental stages of plants and different growth conditions were put forward, and also it was not common practice to use distilled water, but tap-water in growth media (Harder and Störmer, 1935a).

The different degrees of purification of oestrogens for use in these early experiments probably was a major cause of discrepancies between results. A commercial preparation, Progynon, was widely used, and this was known to contain the plant hormone, auxin, also produced by animals (Schoeller, 1933) and lipids which were detrimental to plant growth (Schoeller and Goebel, 1931a). The auxin present was estimated at $C_{18}H_{32}O_5$ (Kögl and Haagen-Smit, 1931), and although it would seem more similar to the gibberellins than to IAA it was more likely to be an IAA-conjugate. Purification of progynon to remove the auxin resulted in loss of growth promotion in pea (Behrens, 1935), and Gad-Andresen and Jarlov (1934) proposed that the positive effects achieved with progynon treatments were due to the auxin content. However, specially prepared crystalline follicular hormones still had effects on plant growth and development (e.g. Schoeller and Goebel, 1931a, 1932: Scharrer and Schropp, 1935: Zollikofer, 1938).

Table 2a. Effects of Steroidal Oestrogens 1. Growth (Higher plant classifications after Cronquist, 1968)

Plant	Oestrogen	Influence	Reference
Class Magnoliatae			
Subclass Hamamelidae			
Order Urticales			
Family Urticaceae			
Cannabis sativa	progynon, crystalline follicular hormone	stimulation	Orth (1934)
	oestradiol	inhibition	David and Gouzon (1939)
Subclass Caryophyllidae			
Order Caryophyllales			
Family Caryophyllaceae			
Agrostemma githago	progynon, α-follicle hormone and Na salt*	no effect	Harder and Störmer (1935b)
Dianthus caryophylla	oestrin	stimulation	Schoeller and Goebel (1935)
Stellaria media	progynon, α-follicle hormone and Na salt	no effect	Harder and Störmer (1935b)
	progynon	stimulation	Hitzer (1935)
Family Chenopodaceae			
Beta vulgaris	crystalline follicular hormone	yield increase	Scharrer and Schropp (1935)
	ovarin	yield increase	Marinchik (1939)
Spinacia sp.	folliculin	increase	Gentcheff and Gustafsson (1940)
Family Amaranthaceae			
Amaranthus hybridii	progynon, α-follicle hormone and Na salt	no effect	Harder and Störmer (1935b)

* the salt formed on treatment of α-follicle hormone with NaOH

Table 2a continued.....(1)

Order	Polygonales			
Family	Polygonaceae			
	Rumex tenuifolius	oestrone, oestradiol, oestradiol-benzoate (17)	stimulation (pollen tubes)	Löve and Löve (1945)
Subclass	Dilleniidae			
Order	Violales			
Family	Begoniaceae			
	Begonia sp.	progynon, α-follicle hormone	no effect	Harder and Störmer (1935b)
Family	Cucurbitaceae			
	Cucumis melo	dihydrofolliclin	no effect	Castan (1940)
Order	Capparales			
Family	Cruciferae			
	Brassica oleracea capita	ova hormone	stimulation	Mabuti (1939)
	Capsella bursa pastoris	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	Lepidium sativum	progynon	no effect	Rodecurt (1932)
		dihydrofolliculin	inhibition	Castan (1940)
		oestrone	stimulation	Zollikofer (1942)
		oestrone	inhibition	von Euler (1946)
	Raphanus sativus	follicular hormone	stimulation	Liebe (1936)
		oestrone	no effect	Bonner and Haagen-Smit (1939): Bonner and Devirian (1939)
		oestrone	stimulation	Zollikofer (1942)
	Sinapis alba	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
		oestradiol	inhibition	David and Gouzon (1939)
		oestrone	stimulation	Bonner and Bonner (1940)
		dihydrofolliculin	no effect	Castan (1940)
	Iberis amara	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)

Table 2a continued.....(2)

Order	Primulales			
Family	Primulaceae			
	Primula vulgaris	oestrin	stimulation	Schoeller and Goebel (1935)
Subclass	Rosidae			
Order	Rosales			
Family	Crassulaceae			
	Bryophyllum calcinium	ketohydroxyestrin, theelol	no effect	Tincker (1935)
Family	Grossulariaceae			
	Ribes odoratum (leaves)	oestrone (low, high)	increase fresh weight	Grace (1939a)
		oestrone (intermediate)	decrease fresh weight	
Family	Leguminosae			
	Glycine max	ovarian hormone (low)	stimulation	Yun and Hong (1934)
		ovarian hormone (high)	inhibition	
		crystalline follicular hormone	no effect	Sharrer and Schropp (1935)
		crystalline follicular hormone	increase yield	Sharrer and Schropp (1937)
	Lathyrus odoratus	crystalline follicular hormone	no effect	Scharrer and Schropp (1937)
	Medicago sativa	crystalline follicular hormone	increase yield	Scharrer and Schropp (1937)
	Phaseolus vulgaris	progynon	no effect	Küstner (1933)
		oestradiol	inhibition (radicle)	Faller (1942)
		oestrone, oestradiol	no effect	Hewitt *et al.* (unpublished)
	Pisum sativum	progynon	no effect	Küstner (1933): Harder and Störmer (1935b)
		progynon	stimulation	Behrens (1935): Fults and Payne (1947)
		crystalline follicular hormone & Na salt*	no effect	Behrens (1935): Harder and Störmer (1935b)*

Table 2a continued.....(3)

	Pisum sativum (continued)	oestrone	stimulation	Kögl and Haagen-Smit (1936): van Santeen and Koningsberger (1939): Bonner and Bonner (1940): Helmkamp and Bonner (1953): Kopcewicz (1969a,b, 1970d)
		folliculin	stimulation	Bonner and Axtman (1937): Gentcheff and Gustafsson (1940)
		oestrone	no effect	Bonner and Devirian (1939): Jones and Roddick (1977): Hewitt *et al.* (1980)
		oestradiol-benzoate	no effect	Rose and Hamon (1940)
		oestradiol (low)	stimulation	Kopcewicz (1969a, 1970d)
		oestradiol (high)	no effect	Kopcewicz (1969a)
		oestrol	stimulation	Kopcewicz (1970d)
		oestrone	no effect	Kopcewicz (1969a)
		oestradiol, oestrone-phosphate, oestradiol-sulphate	no effect	Hewitt *et al.* (1980)
	Trifolium rubens	crystalline follicular hormone	increase yield	Scharrer and Schropp (1937)
	Vicia faba	crystalline follicular hormone	increase yield	Scharrer and Schropp (1935)
		follicular hormone	stimulation	Neurath (1936)
		oestradiol-benzoate	no effect	Rose and Hamon (1940)
Order	Euphorbiales			
Family	Euphorbiaceae			
	Mercurialis annua	progynon, crystalline follicular hormone	stimulation	Orth (1934)

Table 2a continued.....(4)

Order	Umbellales			
Family	Umbelliferae			
	Daucus carota	oestradiol	stimulation culture growth	Gioelli (1942)
		oestrone	increase yield* (pots): no effect* (in field)	Soding *et al.* (1949)
Subclass	Asteridae			
Order	Polemoniales			
Family	Solanaceae			
	Lycopersicum esculentum	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
		follicular hormone	stimulation (after toxicity)	Havas and Caldwell (1935)
		oestrin	stimulation, increase yield	Schoeller and Goebel (1935)
		oestrone	increase yield	Georgieff (1937)
		dihydrofolliculin	no effect	Botjes (1941): Frunzeti *et al.* (1961)
		progynon	stimulation	Emmerich (1942)
		oestradiol	increase yield	Milcu *et al.* (1959)
Family	Convolvulaceae			
	Pharbitis hispida	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
Order	Lamiales			
Family	Boraginaceae			
	Myosotis palustris	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
Order	Scrophulariales			
Family	Scrophulariaceae			
	Antirrhinum majus	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
		dihydrofolliculin	no effect	Frunzeti *et al.* (1961)

* *in hormone mixture*

Table 2a continued.....(5)

	Veronica tourne-fortii	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
Order	Dipsacales			
Family	Valerianaceae			
	Valerianella olitoria	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
Order	Asterales			
Family	Compositae			
	Bidens tripartitus	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	Callistephus sinensis	folliculin	inhibition	Chouard (1934)
		progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	Chrysanthemum sp.	oestrin	stimulation	Schoeller and Goebel (1935)
		progynon, crystals Na salt	no effect	Störmer (1936)
	Coreopsis sp.	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	Lactuca sativa	ketohydroxyestrin, theelol	no effect	Tincker (1935)
	Senecio vulgaris	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	Xanthium strumarium	oestrone	stimulation	Bonner and Bonner (1940)
Class	Liliatae			
Subclass	Commelinidae			
Order	Cyperales			
Family	Graminae			
	Avena sativa	ovarin	stimulation	Krasnosel'skaia-Maksimova (193

Table 2a continued.....(6)

Avena sativa continued	progynon	no effect	Lemmermann and Behrens (1934): Scharrer and Shropp (1934): Harder and Störmer (1935b)
	α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	crystalline follicular hormone	no effect	Scharrer and Schropp (1935)
	progynon	*inhibition*	*Emmerich* (1942)
	oestrone	stimulation	Zollikofer (1942)
	oestradiol	stimulation	Masuda *et al.* (1970)
Hordeum vulgare	progynon	no effect	Küstner (1933): Gad-Andresen and Jarlov (1934): Harder and Störmer (1935b)
	progynon	stimulation	Scharrer and Schropp (1934)
	folliculin	stimulation	von Euler *et al.* (1934a,b)
	α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	crystalline follicular hormone	stimulation	Scharrer and Schropp (1935)
	oestradiol	*inhibition*	von Euler and Perje (1945)
Oryza sativa	progynon	stimulation, increase yield	Emmerich (1942)
Panicum miliaceum	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
Poa annua	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
	oestrone	stimulation	Zollikofer (1936a,b,1937,1948)
	crystallised follicular hormone	stimulation	Zollikofer (1936a,b,1937)
Secale cereale	progynon	no effect	Scharrer and Schropp (1934)
	crystalline follicular hormone	stimulation, increase yield	Scharrer and Schropp (1935,1937
Setaria italica	progynon, α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)

Table 2a continued.....(7)

	Triticum aestevum			
		progynon	stimulation	Scharrer and Schropp (1934)
		progynon	no effect	Harder and Störmer (1935b)
		α-follicle hormone & Na salt*	no effect	Harder and Störmer* (1935b): Voss (1938)
		crystalline follicular hormone	increase yield	Scharrer and Schropp (1935)
		oestriol	stimulation over F control	Grace (1939b)
		progynon	inhibition	Ludwig and von Ries (1940)
		oestrone, oestradiol, oestradiol-benzoate	no effect	Hinton and Moran (1960)
	Zea mays	progynon	stimulation	Orth (1934) : Scharrer and Schropp (1934) : Harder and Störmer (1935b) : Fiedler (1936)
		α-follicle hormone & Na salt	no effect	Harder and Störmer (1935b)
		folliculin	inhibition	Marzetti (1935)
		crystalline follicular hormone	increase yield	Scharrer and Schropp (1937)
		crystalline follicular hormone	stimulation	Orth (1934)
Subclass	Arecidae			
Order	Arales			
Family	Araceae			
	Zantedeschia aethiopica	progynon	stimulation	Schoeller (1933)
Subclass	Lilidae			
Order	Liliales			
Family	Liliaceae			
	Convallaria majalis	progynon	stimulation	Schoeller (1933)
		folliculin, dihydrofolliculin	stimulation	Janot (1934)

Table 2a continued.....(8)

Convallaria majalis continued	ketohydroxyestrin, theelol	no effect	Tincker (1935)
Hyacinthus orientalis	follicular hormone	stimulation	Madaus (1933): Wasicky *et al.*, (1933): Harder and Störmer (1935a)
	progynon	stimulation (shoot)	Schoeller and Goebel (1931a): Schoeller (1933): Gad-Andresen and Jarlov (1934): Harder and Störmer (1935a)
	progynon	inhibition (root)	Gad-Andresen and Jarlov (1934)
	folliculin, dihydro-folliculin	stimulation	Janot (1934)
	Na-salt follicular hormone	stimulation	Schoeller and Goebel (1934)
	folliculin	no effect	von Euler and Zonderk (1934): Janot (1935)
	follicular hormone	no effect	Havas and Caldwell (1935)
	Na-salt folliculin	no effect	Janot (1935)
	ketohydroxyestrin, theelol	no effect	Tincker (1935)
	oestrone	stimulation	Glaser and Ranftl (1942)

Stimulation of vegetative growth by oestrogens had the characteristics of an auxin-type growth response curve, i.e. low concentrations stimulated growth whereas high concentrations had no effect or were inhibitory (Yun and Hong, 1934 for soybean: Kopcewicz, 1969a for *Pisum sativum*). Recent attempts to confirm Kopcewicz's findings, however, have been unsuccessful (Jones and Roddick, 1977: Hewitt *et al.*, 1980). Maximal growth stimulation in 6-day old, red-light-grown Cud Kelwedonu pea seedlings was achieved with 0.1 µg oestrone or oestradiol, with 10 µg ineffective (Kopcewicz, 1969a). Subsequently 0.1 µg oestrone and 0.001 µg GA_3 were found to stimulate growth to the same extent (Kopcewicz, 1969b). Using Alaska, Meteor, Feltham's First and Little Marvel pea varieties, Jones and Roddick (1977) found no stimulatory effect on growth by oestrone or oestradiol (0.1 µg to 40 µg) applied to the third internode or to the axil or hollow petiole of the first true leaf. Furthermore, at least 0.1 µg GA_3 was required to stimulate significantly growth in the dwarf varieties (Meteor and Little Marvel) grown in red-light (1 µg if grown in natural light) and in Alaska and Feltham's First, tall varieties where only seedlings grown in red light responded, at least 0.4 µg GA_3 applied to the third internode or injected into the petiole of the first leaf was required to give significant growth stimulation (Jones and Roddick, 1977). Although Kopcewicz always made applications to apices in later work, the site(s) of application here (Kopcewicz 1969a,b) was not specified. Therefore, whether the differences between the sets of results were due to the different sites of applications or were expressions of varietal variations in responsiveness within *P. sativum* was unclear. Application of 0.1 µg to 50 µg oestrogens to apices of Little Marvel seedlings (6-day old, red-light-grown) however, did not induce growth stimulation, adding to the doubts already expressed over the generality of oestrogen-stimulated growth (Hewitt *et al.*, 1980).

Administration of high oestrogen concentrations to hyacinths (Madaus, 1933), *Primula*, *Chrysanthemum* and *Fuchsia* (Schoeller and Goebel, 1931b) favoured leaf development, whereas low concentrations induced or increased flower production. This was in contradiction to the postulate of Kopcewicz discussed earlier, which associated increased oestrogen levels with flowering. Endogenous production was also thought to account for the lack of effect of dihydrofolliculin in *Callistephus sinensis*, an LDP, under LDs, whereas flowering was induced under SDs (Chouard, 1937, 1938).

Table 2b. Effects of steroidal oestrogens 2. Flowering

plant		oestrogen	influence	reference
Class	Magnoliatae			
Subclass	Magnoliidae			
Order	Ranunculales			
Family	Berberidaceae			
	Berberis Neubertii	ketohydroxyestrin, theelol	no effect	Tickner (1935)
Subclass	Hamamelidae			
Order	Fagales			
Family	Corylaceae			
	Corylus avellana	oestrone	increase	Zollikofer (1939)
Subclass	Caryophyllidae			
Order	Caryophyllales			
Family	Caryophyllaceae			
	Dianthus caryophyllus	oestrin	increase	Schoeller and Goebel (1935)
		folliculin	increase	Liebe (1936)
Subclass	Dilleniidae			
Order	Violales			
Family	Cucurbitaceae			
	Ecballium elaterum	oestrone, oestradiol, oestriol	increase	Kopcewicz (1971c)
Order	Capparales			
Family	Cruciferae			
	Brassica oleracea botrytis	oestradiol	increase	Leshem (1967)
	Brassica oleracea capita	folliculin	induction	Ionescu (1962)
	Iberis amara	folliculin	increase	Chouard (1936)
	Malcolmia maritima	folliculin	increase	Chouard (1936)
	Sinapis alba	oestradiol	increase	David and Gouzon (1939)
Order	Primulales			
Family	Primulacaea			
	Anagallis arvensis	folliculin	increase	Chouard (1936)
	Primula vulgaris	progynon	increase	Schoeller and Goebel (1931b)
		oestrin	increase	Schoeller and Goebel (1935)

Table 2b continued.....(1)

Subclass	Rosidae			
Order	Rosales			
Family	Rosaceae			
	Prunus sp.	progynon, α-follicle hormone	no effect	Harder and Störmer (1934a,b)
Family	Leguminosae			
	Pisum sativum	folliculin	no effect	Virtanen and von Hausen (1933)
		progynon	no effect	Virtanen *et al.* (1934)
		folliculin	decrease	Borgström (1939)
		oestradiol-benzoate	no effect	Rose and Hamon (1940)
	Vicia faba	oestradiol-benzoate	no effect	Rose and Hamon (1940)
Order	Myrtales			
Family	Onagraceae			
	Fuchsia sp.	progynon	increase	Schoeller and Goebel (1931b)
		oestrone	induction	Burkhardt (1941)
Order	Cornales			
Family	Cornaceae			
	Cornus sp.	progynon, α-follicle hormone	no effect	Harder and Störmer (1934a,b)
Order	Euphorbiales			
Family	Euphorbiaceae			
	Euphorbia peplus	folliculin	increase	Chouard (1936)
	Mercurialis perennis	folliculin	increase	Chouard (1936)
Order	Sapindales			
Family	Sapindaceae			
	Aesculus hippocastanum	oestrone	increase	Zollikofer (1939)
Order	Linales			
Family	Linaceae			
	Linum grandiflorum	folliculin	increase	Chouard (1936)

Table 2b continued.....(2)

Subclass	Asteridae			
Order	Polemoniales			
Family	Solanaceae			
	Lycopersicum esculentum	follicular hormone	no effect	Havas and Caldwell (1935)
		oestrone	induction	Georgieff (1937)
		dihydrofolliculin	no effect	Botjes (1941)
		folliculin	induction	Milcu *et al.* (1959)
Order	Lamiales			
Family	Boraginaceae			
	Omphalodes linifolia	folliculin	increase	Chouard (1936)
Family	Labiatae			
	Salvia splendens	oestradiol	induction	Kopcewicz and Porazinski (1974)
Order	Scrophulariales			
Family	Oleaceae			
	Forsythia viridis	progynon, α-follicle hormone	no effect	Harder and Störmer (1934a,b)
		oestrone	increase	Zollikofer (1939)
Order	Asterales			
Family	Compositae			
	Callistephus sinensis	folliculin	increase	Chouard (1934)
		folliculin	induction	Chouard (1934,1936)
		folliculin	no effect	Chouard (1937)
		dihydrofolliculin	induction(SDs)	Chouard (1937,1938)
			no effect(LDs)	
		dihydrofolliculin	no effect(SDs)	Botjes (1941)
		progynon	no effect	Emmerich (1942)
	Chrysanthemum sp	progynon	increase	Schoeller and Goebel (1931b)
		oestrin	increase	Schoeller and Goebel (1935)
		progynon, pure crystals Na salt	no effect	Störmer (1936)
	Cichorium intybus	oestradiol	induction	Kopcewicz (1970a)
	Dimorphotheca pluvialis	folliculin	increase	Chouard (1936)

Table 2b continued.....(3)

Class	Liliatae			
Subclass	Commelinidae			
Order	Cyperales			
Family	Graminae			
	Avena sativa	ovarin	decrease	Krasnosel'skaia-Maksimova (1933)
	Poa alpina	oestrone, crystallised follicular hormone	increase	Zollikofer (1936a)
		oestrone	induction	Burkhardt (1941)
		oestrone	increase	Zollikofer (1948)
	P. annua	folliculin	increase	Chouard (1936)
	Zea mays	progynon	induction	Schoeller and Goebel (1931a)
	Festuca pratensis x Lolium perenne	ketohydroxyestrin, theelol	no effect	Tickner (1935)
Subclass	Arecidae			
Order	Arales			
Family	Araceae			
	Calla oethospica	crystalline follicular hormone	induction	Schoeller and Goebel (1932)
	Zantedeschia aethiopica	progynon	induction	Schoeller (1933)
		progynon, α-follicular hormone	no effect	Harder and Störmer (1934a,b)
Family	Lemnaceae			
	Lemna minor	oestradiol	induction	Czygan (1962)
Subclass	Liliidae			
Order	Liliales			
Family	Liliaceae			
	Allium cepa	progynon, crystalline estrin	induction	Schoeller and Goebel (1931a)
	Convallaria majalis	progynon	induction	Schoeller (1933)
		progynon, α-follicular hormone	no effect	Harder and Störmer (1934a,b)
		folliculin, dihydrofolliculin	induction	Janot (1934)
	Hyacinthus orientalis	progynon	induction	Schoeller and Goebel (1931a), Schoeller (1933), Gad-Andresen and Jarlov (1934)

Table 2b continued.....(4)

	Hyacinthus orientalis cont.	low follicular hormone	increase	Madaus (1933)
		high follicular hormone	decrease	
		follicular hormone	induction	Wasicky *et al.* (1933)
		progynon, α-follicular hormone	no effect	Harder and Störmer (1934a,b, 1935a)
		folliculin, dihydrofolliculin	induction	Janot (1934)
		Na-salt follicular hormone	induction	Schoeller and Goebel (1934)
		folliculin	no effect	von Euler and Zondenk (1934)
		follicular hormone	no effect	Havas and Caldwell (1935)
		folliculin, Na-salt folliculin	induction	Janot (1935)
		progynon, pure crystals Na-salt	no effect	Störmer (1936)
		oestrone	induction	Burkhardt (1941)
	Narcissus sp.	progynon, α-follicular hormone	no effect	Harder and Stormer (1934a,b)
Family	Iridaceae			
	Crocus sp.	progynon, α-follicular hormone	no effect	Harder and Störmer (1934a,b)

Other, more circumstantial, evidence has linked steroids (not necessarily oestrogens) with flowering. SK&F 7997A_3, which inhibits conversion of lanosterol to cholesterol in animals, has been found to inhibit floral induction in *Lolium temulentum*, LDP, (Evans, 1964) and the SDPs *Pharbitis nil* (Bonner *et al.*, 1963: Sachs, 1966: Ogawa and King, 1979) and *Xanthium strumarium* (Bonner *et al.*, 1963: Bledsoe and Ross, 1978). In the latter plant, application in LDs of a crude, steroid-containing extract from induced plants was found to induce flowering (Lincoln *et al.*, 1961). Although interference with steroid synthesis by SK&F 7997A_3 was implied, induced and non-induced untreated plants displayed no obvious difference in their ^{14}C-MVA-metabolism patterns (Bonner *et al.*, 1963: Bledsoe and Ross, 1978). However, metabolism of ^{14}C-MVA was altered slightly in treated plants (Bledsoe and Ross, 1978). The gross similarities between components of the sterol fractions in induced and non-induced plants did not contradict the possibility of involvement of steroids in floral induction: the substance involved may have been present in amounts not detectable by the procedures used (Bonner *et al.*, 1963). SK&F 7997A_3 may also reduce growth (Douglas and Paleg, 1972), and any effect of this inhibitor on floral induction was not necessarily direct or specific as total photosynthate transport to the apex was reduced (Ogawa and King, 1979) and also sterols are now known to be required in plants as membrane components (Grunwald, 1978).

Unquestionably the most important series of experiments involving oestrogens and plants were those of Löve and Löve (1945) concerning regulation of sex expression in dioecious plants: not only were clear-cut feminising effects of oestrogens observed, but also the repetition and number of plants involved made the results indisputable. Surprisingly, this line of study has not been pursued despite the consistently feminising effect of oestrogens in the few other published reports.

Very little work has been done regarding the effects of oestrogens on germination, RNA synthesis, root initiation, senescence, apical dominance and cellular metabolism, and the results, as with those for growth and flowering, have been inconsistent or even contradictory. There was, however, a general promotion of mitotic activity by oestrogens (Orth, 1934: Löve and Löve, 1945: Weyland, 1948) with chromosome aberations (Faller, 1942), an effect also found in animal embryos (Cagianut, 1956), and pronounced disturbances of mitosis (von Euler

Table 2c. Effects of steroidal oestrogens 3. Other effects

Plant	Oestrogen	Effect	Reference
Class Magnoliatae			
Subclass Magnoliidae			
Order Ranunculales			
Family Berberidaceae			
Berberis neubertii	ketohydroxyestrin,	root initiation,	Tincker (1933)
	theelol	no effect	
Subclass Hamamelidae			
Order Urticales			
Family Urticaeae			
Cannabis sativa	progynon, crystalline follicular hormone	sex expression, no effect	Orth (1934)
	oestradiol	sex expression, feminise	David and Gouzon (1939)
Subclass Caryophyllidae			
Order Caryophyllales			
Family Caryophyllaceae			
Gypsophila sp.	folliculin, dihydrofolliculin	cell oxidation potential, reduction	Joyet-Lavergne (1939)
Melandrium dioicum	oestrone	sex expression, feminise	Löve and Löve (1940, 1945)
	oestradiol, oestradiol-benzoate	sex expression, feminise	Löve and Löve (1945)
	oestrone, oestradiol, oestradiol-benzoate	germination, increase:cell division at root tips, increase	
Family Chenopodiaceae			
Beta vulgaris	ovarin	sugar content, increase	Marinchik (1939)
		protein content, increase	

Table 2c continued.....(1)

	Spinacia sp.	folliculin	sex expression, feminise apical dominance, release	Hylmö (1940)
Family	Polygonaceae			
	Rumex acetosa	oestrone	sex expression, feminise (weakly)	Löve and Löve (1940, 1945)
		oestradiol, oestradiol-benzoate	sex expression, feminise (weakly)	Löve and Löve (1945)
		oestrone	cell division at root tips, increase	
	Rumex tenuifolius	oestrone	sex expression, no effect	
Subclass	Dilleniidae			
Order	Malvales			
Family	Malvaceae			
	Gossypium hirsutum	oestradiol	respiration, no effect	Milcu and Ionescu (1959)
Order	Violales			
Family	Cucurbitaceae			
	Cucumis sativus	oestradiol	sex expression, feminise	Gawienowski *et al.* (1971)
	Ecballium elaterium	oestrone, oestradiol, oestriol	sex expression, feminise	Kopcewicz (1971c)
Order	Caparales			
Family	Cruciferae			
	Brassica oleracea (*botrytis*)	oestradiol (high, low)	root formation, increase	Leshem (1967)
		oestradiol (intermediate)	root formation, no effect	
	Brassica oleraceae (*capita*)	folliculin	chlorosis, induction ascorbic acid content, increase respiration, increase	Ionescu (1962)
	Lepidium sativum	folliculin	germination, inhibition	Meyer (1936)
		oestrone	germination, increase	Zollikofer (1942)
	Raphanus sativus	oestrone	germination, increase	Zollikofer (1942): Martinez-Honduvilla *et al.* (1975)
		oestradiol	germination, increase	Martinez-Honduvilla *et al.* (1975)

Table 2c continued.....(2)

Subclass	Rosidae			
Order	Rosales			
Family	Saxifragaceae			
	Ribes odoratum	oestrone	root initiation, no effect	Grace (1939a)
Family	Rosaceae			
	Spiraea vanhouttei	oestrone	root initiation, no effect	Grace (1939a)
Family	Leguminosae			
	Ervum lens	folliculin	germination, inhibition	Meyer (1936)
	Glycine max	crystalline follicular hormone	protein content, increase	Scharrer and Schropp (1937)
	Lathyrus odoratus	crystalline follicular hormone	protein content, decrease	Scharrer and Schropp (1937)
	Lupinus albus	oestrone, oestradiol	germination, inhibition	Martinez-Honduvilla *et al.* (1975)
	Phaseolus vulgaris	oestradiol	mitotic frequency, increase : mitotic disturbance, increase	Faller (1942)
	Pisum sativum	oestrone	root initiation, enhance IBA effect	Went and Thimann (1937)
		oestradiol-benzoate	tumor development, no effect	Rose and Hamon (1939)
		folliculin	sugar content, increase : vitamin C content, increase: protein content, increase	Ionescu (1960)
		oestrone, oestradiol	germination, increase	Martinez-Honduvilla *et al.* (1975)
		oestradiol	RNA synthesis, increase	Mitra and Sen (1976)
		progynon	cell division, increase	Orth (1934)
	Vicia faba	oestradiol	RNA synthesis, increase	Mitra and Sen (1976)
Order	Cornales			
Family	Cornaceae			
	Cornus alba	oestrone	root initiation, no effect	Grace (1939a)

Table 2c continued.....(3)

Order	Euphorbiales			
Family	Euphorbiaceae			
	Mercurialis annua	progynon, crystalline follicular hormone	sex expression, no effect	Orth (1934)
Order	Linales			
Family	Linaceae			
	Linum usitatissimum	crystalline follicular hormone	protein content, decrease: oil content, increase	Scharrer and Schropp (1937)
Order	Umbellales			
Family	Umbelliferae			
	Daucus carota (*cultures*)	oestradiol	chlorophyll content, increase	Gioelli (1942)
Subclass	Asteridae			
Order	Polemoniales			
Family	Solanaceae			
	Capsicum mexicanum	folliculin	respiration, increase	Ionescu (1965)
	Lycopersicum esculentum	follicular hormone	tumor growth, increase	Havas (1935)
		follicular hormone	chlorosis, induction	Havas and Caldwell (1935)
		follicular hormone	tumor growth, inhibition	Stapp (1937)
		progynon	germination, no effect	Emmerich (1942)
		oestradiol	fruit acidity, reduction: protein content, decrease: vitamin C content, decrease: sugar content, no effect	Milcu *et al.* (1959)
		oestradiol	respiration, no effect	Milcu and Ionescu (1959)
		folliculin	sugar content, increase	Ionescu (1960)
		dihydrofolliculin	catalase activity, increase	Frunzeti *et al.* (1961)
		dihydrofolliculin	respiration, increase	Ionescu (1965)

Table 2c continued.....(4)

Solanaeceae continued				
	Solanum melongena	dihydrofolliculin	respiration, increase	Ionescu (1965)
	S. tuberosum	oestradiol	respiration, no effect	Milcu and Ionescu (1959)
		folliculin	sugar content, increase	Ionescu (1960)
Order	Scrophulariales			
Family	Schrophulariaceae			
	Antirrhinum majus	dihydrofolliculin	catalase activity, decrease	Frunzeti *et al.* (1961)
Order	Dipsacales			
Family	Caprifoliaceae			
	Lonicera tartarica	oestrone	root initiation, no effect	Grace (1939a, 1940)
		oestrone	root initiation, reduce IBA effect	Grace (1940)
Order	Asterales			
Family	Compositae			
	Callistephus sinensis	folliculin	chlorosis, induction	Chouard (1934)
	Lactuca sativa	oestrone, oestradiol	germination, increase	Martinez-Honduvilla *et al.* (1975)
Class Liliatae				
Subclass	Commelinidae			
Order	Cyperales			
Family	Graminae			
	Anthoxanthum cristatum	oestrone	cell division at root tips, increase	Löve and Löve (1945)
	Avena sativa	oestrone	germination, increase	Zollikofer (1942)
	Hordeum vulgare	oestradiol	pronounced mitotic disturbance	von Euler and Perje (1945)
	Oryza sativa	progynon	germination, no effect	Emmerich (1942)
	Poa annua	oestrone*	germination, no effect	Soding and Wagner (1955)

* given in hormone mixture

Table 2c continued.....(5)

	Triticum aestevum	oestriol	injury-effects F, reduction: germination, increase over F controls	Grace (1939b)
		oestradiol	respiration, no effect	Milcu and Ionescu (1959)
	Zea mays	progynon	sex expression, feminise	Schoeller ane Goebel (1931b)
		crystalline follicular hormone	protein content, decrease	Scharrer and Schropp (1937)
Subclass	Arecidae			
Order	Arecales			
Family	Palmae			
	Cocos sp.	oestradiol	RNA synthesis, increase	Mitra and Sen (1976)
Order	Arales			
Family	Lemnaceae			
	Lemna minor	oestradiol	germination, inhibition	David and Gouzon (1939)
Subclass	Liliidae			
Order	Liliales			
Family	Liliaceae			
	Allium cepa	oestrone	mitosis, frequency increased	Weyland (1948)
	Hyacinthus orientalis	follicular hormone	chlorophyll content, no effect	Havas and Caldwell (1935)
		oestrone	wilting, inhibition : chlorophyll content, increase	Glaser and Ranftl (1942)

and Perje, 1945) also reported. Those cells and tissues not yet irreversibly determined were most susceptible to oestrogens (Weyland *et al.*, 1949) and, indeed, the anatomical and morphological changes induced by oestrogen treatments were just as obvious in the F_3 as in the P-generation. The effects of carcinogens similarly were inheritable (Weyland, 1948: Weyland *et al.*, 1949) and, although the effects of oestrogens on plant tumor growth were not consistent, it was interesting that implantation of crown gall from *Pelargonium* or injection with an *Agrobacterium tumefaciens* suspension resulted in young female mice and rats reaching sexual maturity before controls (Havas, 1935). Applications of brassins (lipidic extracts of *Brassica napus* pollen) were found to give an overall enhancement of growth in *Phaseolus vulgaris* (Mitchell *et al.*, 1970: Mitchell and Gregory, 1972) with anatomical changes in treated stems also apparent (Worley and Mitchell, 1971). The active component of the extract, brassinolide (74), was isolated and characterised and found to be a steroid (Grove *et al.*, 1979).

Just as in Angiosperms, oestrogens also have effects in Gymnosperms, Pteridophytes, Algae, Fungi and Bacteria (Tables 3a,b).

The increased germination of pine seeds cannot be doubted, but little is known about oestrogen effects in algae and some of the events occurring in ferns require more detailed discussion. In *Equisetum arvense* grown on a mineral medium, male prothalli developed before female and were more numerous: however, addition of testosterone to the culture medium caused female prothalli to develop more quickly and to be more numerous than male (Laroche *et al.*, 1972: Olivry and Laroche, 1972). The added testosterone may have been metabolised to oestradiol (Geuns, 1978) and progynon had previously been found to favour development of female prothalli (Orth, 1934). Here, on the other hand, oestradiol (10^{-3}mol m^{-3}) did not affect male sexualisation but inhibited archegonium differentiation (Olivry and Laroche, 1972) and the latter was also evident in *Gymnogramme sulphurea* (Montardy-Pausader, 1973).

Effects on cell division were similar to those in higher plants: cells and tissues capable of mitotic activity were affected most readily in *Aspidium fliximas* (Weyland, 1948) and a general stimulation of cell division was found. High levels of oestrone, however, inhibited growth (i.e. cell division) in *Euglena* (Buetow and Levedahl, 1958) and oestradiol caused unequal embryo cleavage in *Fucus* (Pollock, 1969) *c.f.* animal embryos (Agrell 1954, 1955).

Table 3a. Effects of Steroidal Oestrogens 4. Gymnosperms, Pteridophytes and Algae

Plant	Oestrogen	Effect	Reference
Gymnosperms:			
Class Coniferopsida			
Order Coniferales			
Family Pinaceae			
Pinus pinea	oestrone, oestradiol	germination, increase	Martinez-Honduvilla (1974): Martinez-Honduvilla *et al.* (1976)
	oestrone, oestradiol	RNA synthesis, increase	Martinez-Honduvilla *et al.* (1976)
P. sylvestris	oestrone, oestradiol	germination, increase	Kopcewicz (1970b)
	oestrone, oestradiol, oestriol	growth, no effect	Kopcewicz (1970d)
Pteridophytes			
Class Sphenophyta			
Order Equisitales			
Family Equisitaceae			
Equisetum arvense	progynon	growth, stimulation: sex-expression, feminise	Orth (1934)
	oestradiol	growth, inhibition: archegonium differentiation, inhibition	Olivry and Laroche (1972)
Class Filicophyta			
Subclass Leptosporangidae			
Order Filicales			
Family Schizaceae			
Lygodium japonicum	oestradiol	sex expression, feminise	Colonval-Elenkova (1960)
Family Polypodaceae			
Aspidium fiximas	oestrone	mitosis, stimulation	Weyland (1948)
Gymnogramme sulphurea	oestradiol	spore germination, increase: cell division, increase: sex expression, feminise	Montardy-Pausader (1973)

Table 3a continued.....(2)

Algae				
Class	Euglenophyta			
Order	Euglenales			
Family	Euglenaceae			
	Euglena gracilis	oestrone (0.1mg/l)	growth, stimulation	Buetow and Levedahl (1958)
		oestrone (1.0mg/l)	growth, inhibition	
Class	Phaeophyta			
Order	Fucales			
Family	Fucaceae			
	Fucus distichus	oestradiol	growth, inhibition	Pollock (1969)
			inequal embryo cleavage	

Table 3b. Effects of steroidal oestrogens 5. Fungi

Class	Phycomycetes			
Order	Mucorales			
Family	Mucoraceae			
	Absidia coerula	progynon	growth, increase : sexual reproduction, no effect	Orth (1934)
	Rhizopus nigricans	oestrone	sexual reproduction, inhibit	Plumb and Durrell (1933)
Order	Peronosporales			
Family	Pythiaceae			
	Phytophthora sp.	oestradiol	oospore formation, no effect	Hendrix and Guttman (1969)
	Pythium artotrogus	oestrone	oospore formation, no effect : growth, inhibition	Al Hassan and Fergus (1969)
	P. periplocum	oestradiol	sexual reproduction, inhibition: growth, inhibition	Hendrix and Guttman (1968)
	P. spp.(8 isolates)	oestradiol	oospore formation, inhibition (3 isolates): oospore formation, reduction (3): oospore formation, no effect (2): growth, inhibition (6) : growth increase (2)	Hendrix and Guttman (1969)
Class	Ascomycetes			
Order	Aspergillales			
Family	Aspergillaceae			
	Aspergillus clavatus	oestrone (low), oestradiol (low)	growth, increase	El-Sherif and Refai (1975)
		oestrone (high), oestradiol (high)	growth, inhibition	
	A. fumigatus	oestrone (high), oestradiol (high)	growth, inhibition	El-Sherif and Refai (1975)
	A. niger	folliculin	growth, stimulation	Wasicky *et al.* (1933): Bertrand and Weber (1936)

Table 3b continued.....(1)

	A. niger cont.	progynon, crystalline follicular hormone	growth, no effect	Lund (1936)
		oestradiol	growth, no effect	Jefferson and Sisco (1967)
		oestrone, oestradiol	asperonone content, increase	Jefferson (1967)
		oestrone	growth, no effect	El Sherif and Refai (1975)
	A. oryzae	oestradiol	amylase activity, increase	Karosawa (1957)
	A. sojae	oestradiol	amylase activity, no effect	Karosawa (1957)
	A. tamarii	oestradiol	amylase activity, no effect	Karosawa (1957)
	Penecillium puberulum	oestradiol	deoxycorticosterone-induced growth inhibition, reversed: cholesterol-and DES-induced growth inhibition, no effect	Lester and Hechter (1961)
Order	Saccharomycetales			
Family	Saccharomycetaceae			
	Hansenula anomala	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	H. saturnus	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	Saccharomyces cerevisiae	progynon, crystalline follicular hormone	growth, no effect	Lund (1936)
		oestradiol	aerobic fermentation glucose, increase: exogenous respiration, no effect	Meier and Schuler (1956)
		oestrone, oestradiol	O_2 uptake, no effect	Salmony (1956)
		oestradiol	endogenous respiration, increase	Dirscherl and Geissler (1960): Romo *et al.* (1967)
		oestrone	endogenous respiration, increase	Dirscherl and Geissler (1960)
		oestrone, oestradiol	exogenous respiration, increase	Dirscherl and Geissler (1960)
		oestradiol	exogenous respiration, inhibition: glucose uptake, decrease	Romo *et al.* (1967)
		oestradiol	mating-type *a* growth, increase: mating-type α growth, no effect	Takao *et al.* (1970): Yanagishima and Shimoda (1970): Yanagishima *et al.* (1970)
		oestradiol	*a* hormone-induction, no effect	Yanagishima (1971)

Table 3b continued.....(2)

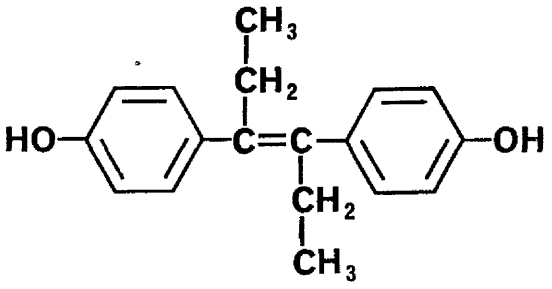
Family	Torulopsidaceae			
	Candidia albicans	oestradiol (low)	growth, no effect	Refai and El Sherif (1974)
		oestradiol (high), ethyinyl oestradiol	growth, inhibition	
		oestriol, oestradiol-benzoate	growth, no effect	Neumann and Kaben (1971)
	C. tropicalis	oestradiol	growth, inhibition (slight)	Tarbet *et al.* (1953)
	Mycoderma bordettii	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	Torulopsis glabrata	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	T. monosa	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	T. rotundata	folliculin, dihydro-folliculin-benzoate	growth, no effect	Weber (1936)
	T. utilis	oestradiol	growth, inhibition	Jefferson and Sisco (1961)
Family	Rhodotorulaceae			
	Rhodotortula glutinis	folliculin, dihydro-folliculin-benzoate	growth, stimulation	Weber (1936, 1937)
	R. suganii	folliculin, dihydro-folliculin-benzoate	growth, stimulation	Weber (1936, 1937)
Class	Deuteromycetes			
Order	Moniliales			
	Microsporum fulvum	oestradiol	growth, no effect	Tarbet *et al.* (1953)
	Trichophyton rubrum	oestradiol	growth, stimulation	Chattaway *et al.* (1962)

Very many effects can be attributed to oestrogens in fungi and often these appear to be species or even strain specific. Many fungi require an exogenous sterol supply (Hendrix, 1970), e.g. *Pythium periplocum* for the induction of the sexual phase. Sterol-induced sexual reproduction was inhibited partially by 10^{-3}mol m^{-3} and completely by 10^{-2}mol m^{-3} oestradiol, with still higher concentrations also inhibiting growth (Hendrix and Guttman, 1968). The effectiveness of oestradiol later was found to vary between strains (Hendrix and Guttman, 1969). Originally it was thought that the primary requirements for sterols would be as membrane components and incorporation of oestradiol would have altered membrane permeability characteristics: oestradiol has polar groups at C-3 and C-17 whereas sterols have a non-polar hydrophobic side chain at C-17 (Hendrix and Guttman, 1968). However, oestradiol has been found to inhibit pregnenolone-induced meiois in an amphibian, *Rana pipiens*, probably by binding to the enzyme 3β-hydroxy-Δ^5-steroid dehydrogenase / isomerase and preventing conversion of pregnenolone to progesterone. The inhibition could be counteracted by supplementation with progesterone (Spiegel *et al.*, 1978). If the promotion of sexual reproduction in *P. periplocum* required a sterol metabolite, oestrogens may have acted by inhibiting a similar enzyme system. Support for this comes from two observations; while cholesterol and cholestanol promoted formation of oogonia in *Phytophthora cactorum*, only the former sterol permitted the completion of the stages of meiosis prior to sexual fusion with antheridia (Elliott and Sansome, 1977) and 3β-hydroxy-Δ^5-steroid dehydrogenase /isomerase can utilize cholesterol but not cholestanol.

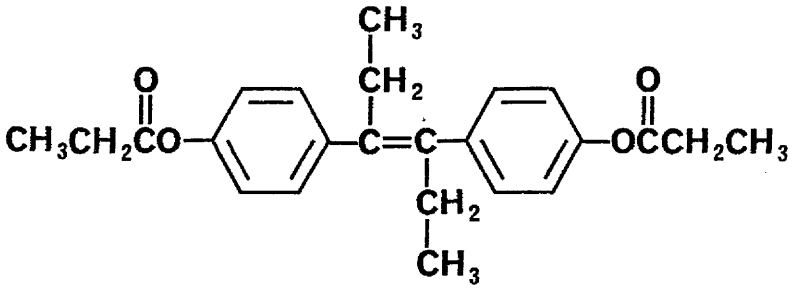
Control of fungal and bacterial growth is of great importance in medicine. Again, results obtained have been inconsistent: natural oestrogens have been reported to promote growth in bacteria (e.g. Tata, 1934: Moursi, 1966) or to have no effect (e.g. Portes *et al.*, 1939: Faulkner, 1943). Also, in eight pathogenic fungi, only the ethinyl derivative of oestradiol (68) was inhibitory (Fox *et al.*, 1957). Synthetic oestrogens, however, such as hexoestrol (71) and DES inhibited both fungal and bacterial growth (Faulkner, 1943: Heinemann, 1947: Fox *et al.*, 1957: San Clemente and MacKenzie, 1957).

These oestrogenic substances have also been used to promote fattening in livestock (McLachlan and Dixon, 1976) but this has now been banned in the USA due to accumulations of biologically active metabolites

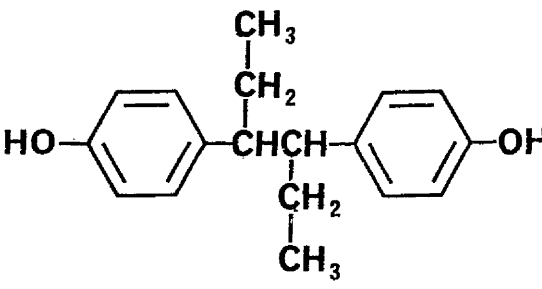
Fig.10. Structural formulae of non-steroidal oestrogens, brassinolide and ecdysone



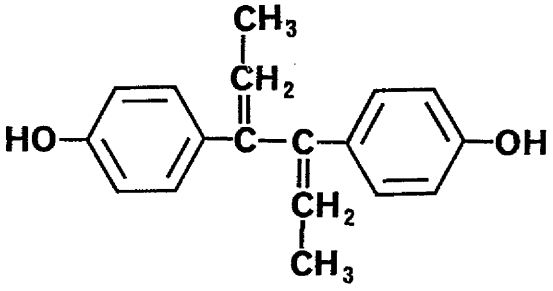
(69) DES



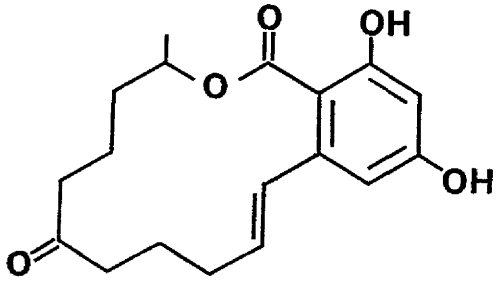
(70) DES-dipropionate



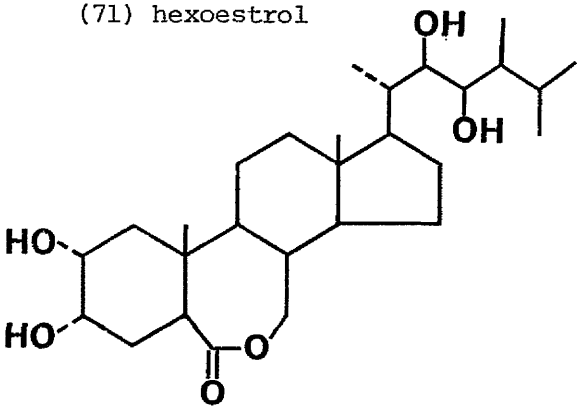
(71) hexoestrol



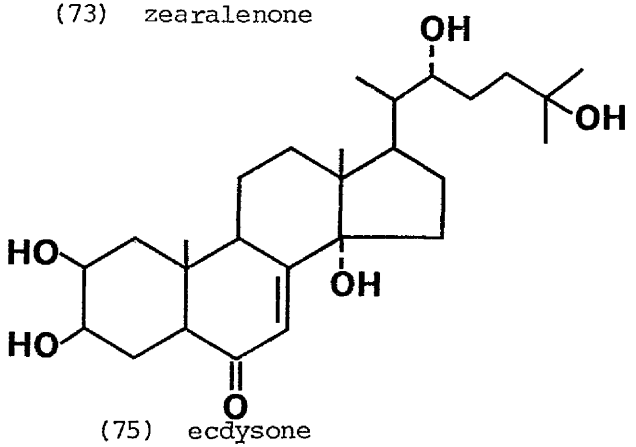
(72) dienoestrol



(73) zearalenone



(74) brassinolide



(75) ecdysone

in treated animals (Rudiger *et al.*, 1979). The amounts involved, however, were small and it is likely that man is exposed to higher levels of naturally occurring non-steroidal phyto-oestrogens in food (Verdeal and Ryan, 1979).

Most of the DES and hexoestrol administered to animals was excreted unaltered, and the potential dangers of using fertilizers from oestrogen-treated animals have been raised (Ferrando and Valette, 1976). Although uptake of DES by alfalfa was demonstrated (Ferrando and Valette, 1976), previous work had indicated that the levels involved would not be detrimental to consumers. Crop plants were found to retain some hexoestrol activity (Glascock and Jones, 1961): uptake from the soil by roots was low, from 0.06 to 0.4 per cent, depending on the soil construction and the species. When sprayed on plants, 3 to 70 per cent was retained, and *c.* 50 per cent of that was metabolised within a few hours (Glascock and Hewitt, 1963). Similarly, using as a fertilizer base urine and faeces from a steer injected with ^{14}C-DES, radioactivity was found in all parts of the crop plants grown. Only radish roots and lettuce leaves, though, had any oestrogenic activity and this was not enough to affect humans (Hacker *et al.*, 1967).

Plants, therefore, can take up and metabolise synthetic oestrogens. The effects of these compounds, particularly DES, have been investigated, mostly in economically important species (Table 4), and a wide range of effects have been found.

The most notable aspect of DES physiology has been its emergence as a useful tool in studies of plant ionic relations, and it is now widely used as a selective inhibitor of plasmalemma ATPase. This inhibition was found to be non-competitive (Balke and Hodges, 1979b) and it is possible the non-steroidal oestrogen, zearalenone (73), produced by *Fusarium* spp., acted in the same way on the plasma membrane ATPase of *Zea mays* and *Beta vulgaris* (Vianello and Macri, 1978). The way is clear to investigate the possible effects of steroidal oestrogens on ATPases: oestradiol inhibited rat uterine Mg^{++}-activated ATPase activity, but the time course suggested a selective effect on protein synthesis rather than a direct interaction (Karmaker, 1969). DES on the other hand, from the kinetic data presented, seemed to inhibit rat uterine ATPase non-competitively (Robinson, 1970) and in yeasts, exogenous respiration (i.e. uptake of glucose from the medium) was inhibited more efficiently by DES than steroidal oestrogens (see Table 4).

Table 4. Effects of non-steroidal oestrogens

Plant	oestrogen-analogue	effect	reference
Angiosperms			
Class Magnoliaiae			
Subclass Caryophyllidae			
Order Caryophyllales			
Family Chenopodiaceae			
Beta vulgaris	DES	sugar content, increase: dry weight, increase	Maska (1950)
	zearalenone (F-2)	Rb^+ uptake, inhibition	Vianello and Macri (1978)
Subclass Dilleniidae			
Order Violales			
Family Passifloraceae			
Carica papaya	DES-dipropionate (70)	sex expression, no effect	Ghosh and Sen (1975)
Order Capparales			
Family Cruciferae			
Cardaomine hirsuta	DES-dipropionate	tumours, induction	Weyland (1948)
Lepidium sativum	DES, hexoestrol	growth, inhibition	Zollikofer (1942)
	DES	growth, increase	von Euler (1946)
	DES-dipropionate	mitosis, inhibition	Ariesan *et al.* (1973)
Raphanus sativus	DES	growth, increase	Zollikofer (1941, 1942)
Subclass Rosidae			
Order Rosales			
Family Leguminosae			
Pisum sativum	DES	growth, increase	Fults and Payne (1947)
Vicia faba	DES	H^+/K^+ exchange, inhibition	Delrot and Bonnemain (1979)

Table 4 continued.....(1)

Subclass	Asteridae			
Order	Polemoniales			
Family	Solanaceae			
	Datura tatula	DES	alkaloid content, no effect	Beal *et al.* (1954)
	D. stramonium	DES	mitosis, inhibition	Manil (1961)
	Lycopersicon esculentum	DES	yield, no effect	Strong (1947)
		DES, hexoestrol	parthenocarpic fruit set, induction	Swarbrick (1945)
	Solanum tuberosum	F-2	Rb^+ uptake, no effect	Vianello and Macri (1978)
Order	Asterales			
Family	Compositae			
	Callistephus sinensis	DES	flowering, no effect	Emmerich (1942)
	Cichorium intybus	DES	flowering, induction	Margara and Tourand (1967)
Class	Liliatae			
Subclass	Commelinidae			
Order	Cyperales			
Family	Gramineae			
	Avena sativa	DES	growth, stimulation	Zollikofer (1941, 1942)
			germination, increase	Zollikofer (1942)
	(roots)	DES	K^+ uptake, inhibition	Balke and Hodges (1975, 1977, 1979a,b,c)
			K^+ efflux, increase	Balke and Hodges (1975, 1979a)
			Cl^- uptake, inhibition	Balke and Hodges (1977, 1979a)
			plasma membrane ATPase activity, inhibition	Balke and Hodges (1977, 1979b,c)
			mitochondrial ATPase activity, no effect	Balke and Hodges (1977, 1979c)
			oxidative phospharylation, inhibition	Balke and Hodges (1977, 1979c)
		DES-dipropronate, dienoestrol (72) hexoestrol	K^+ uptake, inhibition (slight)	Balke and Hodges (1979a)
			K^+ uptake, inhibition	Balke and Hodges (1979a)

Table 4 continued.....(2)

Avena sativa (roots) continued	DES-dipropionate	Cl^- uptake, no effect	Balke and Hodges (1979a)
	dienoestrol	Cl^- uptake, inhibition (slight)	Balke and Hodges (1979a)
	hexoestrol	Cl^- uptake, inhibition	Balke and Hodges (1979a)
	DES-dipropionate	plasma membrane ATPase activity, inhibition (slight)	Balke and Hodges (1979b)
	hexoestrol	plasma membrane ATPase activity, inhibition	Balke and Hodges (1979b)
	dienoestrol	plasma membrane ATPase activity, no effect	Balke and Hodges (1979b)
Hordeum vulgare	hexoestrol	growth, inhibition : mitotic disturbance	von Euler and Perje(1945)
(roots)	DES	plasma membrane ATPase activity, inhibition	Winter-Sluiter *et al.* (1977)
Triticum aestevum	DES (low), hexoestrol	growth, stimulation (roots): growth, no effect (coleoptiles)	Hinton and Morgan (1960)
	DES (high)	growth, inhibition (roots)	Hinton and Morgan (1960)
Zea mays (coleoptiles)	DES	plasma membrane ATPase activity, inhibition	Pesci *et al.* (1978): Beffagna *et al.* (1979): Tognoli *et al.* (1979)
		plasma membrane KC-binding activity, inhibition	Pesci *et al.* (1978): Tognoli *et al.* (1979)
		H^+ extrusion, inhibition	Marrè (1978)
(roots)	DES	K^+ uptake, inhibition	Marrè (1978): Lin (1979): Lin and Giaquinta (1979)
		Pi uptake, inhibition (indirect)	Lin (1979): Lin and Giaquinta (1979)
		plasma membrane ATPase activity, inhibition	Lin (1979): Lin and Giaquinta (1979): Marrè *et al.* (1980)
		H^+ extrusion, inhibition	Colombo *et al.* (1978): Lin (1979): Lin and Giaquinta (1979)

Table 4 continued.....(3)

Zea mays (roots) continued	DES	3-O-methyl glucose uptake, inhibition	Colombo *et al.*(1978)
	F-2	growth, inhibition: Rb^+ uptake, inhibition: H^+ extrusion, inhibition: plasma membrane ATPase activity, inhibition: membrane potential, depolorisation	Vianello and Macri (1978)
Subclass Liliidae			
Order Liliales			
Family Liliaceae			
Allium cepa	DES	germination, inhibition: mitosis, no effect: tumour-like tissue, induction	Jakowska (1948)
	DES	mitosis, increase frequency	Weyland (1948)
Hyacinthus orientalis	DES	flowering, increase	Cortesi *et al.*(1957)
Pteridophytes			
Class Filicophyta			
Subclass Leptosporangidae			
Order Filicales			
Family Polypodiaceae			
Aspidium fliximas	DES-dipropionate	mitosis, increase	Weyland (1948)
Algae			
Class Charaphyceae			
Order Charales			
Family Characeae			
Chara corallina	DES	ATPase content, decrease	Keifer and Spanswick (1978a, 1979)
		membrane potential, depolorisation	Keifer and Spanswick (1978a,b, 1979)
		membrane conductance, decrease	
		K^+ uptake, inhibition	Keifer and Spanswick (1978b)
Class Chlorophyceae			
Order Volvocales			
Family Polyblepharidaceae			
Dunaliella parvum	DES	glycerol excretion, inhibition	Hewitt *et al.* (unpublished)

Table 4 continued.....(4)

Fungi				
Class	Ascomycetes			
Order	Sphaeriales			
Family	Surdariaceae			
	Neurospora crassa	DES	plasma membrane ATPase activity, inhibition	Slayman *et al.* (1977): Bowman *et al.* (1978)
Order	Aspergillales			
Family	Aspergillaceae			
	Aspergillus niger	DES	growth, inhibition	Jefferson and Sisco (1961)
Order	Saccharomycetales			
Family	Saccharomycetaceae			
	Saccharomyces cerevisiae	DES	endogenous respiration, increase exogenous respiration, inhibition	Shacter (1949,1953a): Dirscherl and Geissler (1960): Romo *et al.* (1967)
		DES	glucose fermentation, inhibition:	Shacter (1953a)
		DES (high)	endogenous respiration, inhibition	
		DES	p-chloromercuribenzoic acid synergism on endogenous respiration, increase: exogenous respiration, decrease	Shacter (1953b)
		DES	O_2 uptake, increase O_2 uptake (ethanol, acetate added), inhibition: O_2 uptake (glutamate added), no effect: myosin ATPase activity, inhibition	Salmony (1956)
		DES-dipropionate	endogenous respiration, increase: exogenous respiration, inhibition	Dirscherl and Geissler (1960)
		DES	growth, inhibition	Kaufmann and Ahmad (1967)
		DES	glucose uptake, inhibition: endogenous and exogenous respiration of homogenate, increase	Romo *et al.* (1967)

Table 4 continued.....(5)

Family	Torulopsidaceae			
	Candida albicans	DES	growth, inhibition	Refai and El Sherif (1974): Yotis and Haan (1978)
		DES-dipropionate	growth, inhibition	Yotis and Haan (1978)
	Cryptococcus neoformans	DES	growth, inhibition	Refai and El Sherif (1974)
	Torulopsis utilis	DES	growth, inhibition	Jefferson and Sisco (1961)

Despite the many experiments in which oestrogens did not elicit effects and despite the many failures to confirm previous findings, the body of evidence weighs towards the view that oestrogens can have effects in plants. The mode of action has never been investigated though Kopcewicz has attempted to explain the effects of oestrogen applications in terms of endogenous growth regulators.

Oestrone raised the gibberellin-activity present in pea seedlings (Kopcewicz, 1969c) and plants treated with 0.1µg oestrone and 0.001µg GA_3 had similar growth rates and contained the same levels of gibberellin-activity (Kopcewicz, 1969b). TLC of extracts followed by the lettuce hypocotyl bioassay gave two regions of gibberellin-activity in control plants. Oestradiol-treated plants contained higher activities in these zones, and a third active region was also present (Kopcewicz, 1969b). Another possible link between oestrogens and gibberellins was seen in the induction of flowering in the long-day plant *Cichorium intybus* in short days. GA_3-treatment caused flowering in all plants, oestradiol caused 85 per cent to flower and oestrone 55 per cent, the oestrogen-treated plants flowering 10 days after GA-treated plants. No control plants flowered (Kopcewicz, 1970a). Parenthetically, the effects of gibberellins on flowering of long-day plants which are rosettes under short days are indirect: gibberellins cause bolting which is then followed by flowering, whereas in natural development they occur simultaneously (Zeevaart, 1976).

Soaking in oestradiol increased germination of *Pinus sylvestris* seeds, and it was thought that induction of endogenous growth regulator biogenesis such as of gibberellins or auxins, could have been involved (Kopcewicz, 1970b). Subsequently, oestradiol-treatment was found to increase auxin- and gibberellin-activities in pine seedlings, with the same also true in *Pisum sativum* (Kopcewicz, 1970c). Indeed, the three active regions on chromatograms of extracts of oestrone-, oestradiol- and oestriol-treated pea seedlings displayed higher auxin activity in the *Avena* straight growth test than extracts of controls (Kopcewicz, 1970d).

Cytokinin activity in bean was increased by oestrone and oestradiol, but abscisic acid-activity was unaffected (Kopcewicz, 1972d). The reverse experiments, where the effects of plant hormones on oestrogen levels were examined, have already been discussed. Two points must be raised concerning these experiments: all determinations of extract components were by bioassay, and no attempts were made to characterize extract components. Also, alterations in endogenous hormone levels could have

been related to the growth or developmental stages of the plants and not directly attributable to oestrogen effects.

In fact, whether any effects of exogenous oestrogens are specific and relate to physiological roles for endogenous oestrogens is a matter for debate and until such times as sufficient informations regarding natural levels and turnover, and distribution and metabolism of exogenous oestrogens are at hand this issue will not be resolved.

4. Aims and Rationale of the Project

Many aspects of plant growth and development have been shown to be modified by application of steroidal oestrogens, yet plant physiologists in general are loath to accept the possibility that these substances could be plant hormones. The main cause of this has been the paucity of substantiating evidence after effects have been found: oestrogens have been partially characterized only in few of the plants used in experiments, and the uptake, distribution and metabolism of the applied substances have not been investigated.

In undertaking a more detailed study of plant responses to exogenous oestrogens it was best to use a plant known to contain steroidal oestrogens. As the dwarf French bean, *Phaseolus vulgaris*, had been shown to contain oestradiol and to synthesise the substance from mevalonic acid (Young *et al.*, 1977, 1979) it was chosen as the experimental material, and the endogeneity of oestrogens confirmed.

Also with *P. vulgaris*, uniform material for experiments is readily obtainable.

In the study of oestradiol synthesis, Young *et al.*, had used cuttings. These took up the applied substances during transpiration and once they had formed roots they were potted out for future harvesting (Young *et al.*, 1977, 1979). Bearing this, and the reported effects of oestrogens on root formation in mind, the main aspect selected for study was adventitious root initiation, a system already well documented in the dwarf French bean (e.g. Humphries, 1960: Varga and Humphries, 1974: Altman and Wareing, 1975: Friedman *et al.*, 1979).

The effects of oestrogens on growth and flowering were examined and experiments on senescence of leaf discs were also carried out. For each experimental system the results were viewed in relation to the uptake, distribution and metabolism of the applied substances. The

oestrogens used were oestrone and oestradiol, which are sparingly soluble in water, and the water-soluble conjugates oestrone-phosphate (4), oestrone-sulphate and oestradiol-sulphate. These conjugates had not been used previously in physiological studies in plants.

MATERIALS AND METHODS

1. Plant Material

The dwarf French bean, *Phaseolus vulgaris* L. cv. Canadian Wonder, was used throughout. Seeds (supplied by Daggs Ltd., Glasgow, U.K.) were potted in a compost of sand:loam:peat (1:1:1) in pots or boxes and raised in heated greenhouses until required. Supplementary irradiation was given by Thorn MBFR/U high pressure mercury vapour lamps to maintain a 16h photoperiod (16h light followed by 8h dark each 24h) throughout the year, ($10 Wm^{-2}$, 293 ± 5 K). When plants were required for extractions or cuttings and when leaf material was needed, *c.* 50 seeds were planted per box (50 x 25mm). Seeds were planted singly in pots (100 or 150mm diam.) if experiments required whole plants.

When grown in the above conditions the dwarf French bean has a pair of cotyledons borne at the top of the hypocotyl. These "seed leaves" are abscinded later as the plant develops. The first true leaves are the paired primary leaves which are located at the node separating the epicotyl (or first internode) and the second internode. Thereafter, the leaves are not paired, but are trifoliate and can vary from 3 to 5 in number. In the axil of each true leaf and also at the cotyledons are axillary buds, usually one per leaf, although occasionally more can be present. When grown in greenhouses, it is not normal for axillary buds to show much development other than flowering, i.e. shoot formation is suppressed, and,as a rule, cotyledonary buds never develop beyond the embryonic stage.

While the growth form of *P. vulgaris* L. cv. Canadian Wonder was consistent, plant size was not and this feature is dealt with in subsequent sections.

2. Chemicals and Solutions

All chemicals and solvents used are listed in Table 5.

Table 5. Chemicals and Solvents

a) Fine chemicals

oestrone; oestradiol: bis-TMSi	Koch Light Laboratories, Colnbrook, Bucks..
oestrone-phosphate, mono-sodium salt; oestradiol-sulphate, mono- and di-sodium salts	Steraloids, New York.
oestrone-sulphate, mono-sodium salt	Sigma Chemicals, St. Louis.

b) Radio-chemicals

4-^{14}C -oestrone	The Radiochemical Centre, Amersham, Bucks..
4-^{14}C -oestradiol	
^{14}C -hexadecane	
6,9-^{3}H -oestrone sulphate, potassium salt	
^{3}H -hexadecane	

c) Bulk Chemicals

Na_2HPO_4, Na_2SO_4 (anhydrous), $MgSO_4$, NaCl, KOH, HCl (all Analar grade)	British Drug Houses (BDH)
PPO	Koch Light
$NaHCO_3$	May and Baker

d) Solvents

acetone*, chloroform*, methanol*, toluene, Triton X-100	A&J Beveridge
diethyl ether	May and Baker
acetone*, ethylacetate*	British Petroleum
chloroform*, dioxane*, benzene	BDH
cyclohexane, glacial acetic acid	Hopkin and Williams
sodium hypochlorite, Tween 80, phenylethylamine (scintillation grade)	Koch Light

(* routinely redistilled before use)

Oestrone and oestradiol are sparingly soluble in water and a water-miscible organic solvent and a solubilizing agent were required to prevent precipitation of the substances from solutions of high concentration. Thus, a 0.01 per cent Tween 80:acetone (9:1) solution was employed in the preparation of 0.1 mol m^{-3} oestrone and oestradiol. The lower concentrations, 10^{-3}mol m^{-3}, 10^{-5}mol m^{-3} and 10^{-7}mol m^{-3}, were made up by serial dilutions with distilled water, and controls for each treatment were prepared similarly from a 0.01 per cent Tween 80:acetone (9:1) solution. The 0.1 mol m^{-3} oestrone and oestradiol solutions were not used in experiments, preliminary studies having shown them to have severely deleterious effects on plant materials. On the other hand, oestrone-phosphate, oestrone-sulphate and oestradiol-sulphate could be employed at high concentrations as they are water-soluble. Solutions of Na_2HPO_4 and Na_2SO_4 in the same concentration range (0.1mol m^{-3} to 10^{-7}mol m^{-3}) served as controls for conjugate treatments.

3. Analysis of Radioactivity

The levels of radioactivity present in samples were ascertained using Packard Scintillation Spectrometers (Model 3380). Quench-correction curves, which relate the automatic external standard (AES) ratio to percentage efficiency of counting, are required for each spectrometer, scintillation cocktail and radioisotope and, for ^3H and ^{14}C, standard curves are prepared most accurately using appropriately-labelled hexadecane. Thus, the instrument readings, expressed as cpm, can be converted to Bq.

Four scintillation cocktails were used:

1) 0.4 per cent PPO in toluene for ^3H and ^{14}C samples without water

2) scintillant 1) : Triton X-100 (2:1) for ^3H and ^{14}C samples containing water (never more than 10^{-6} m^3)

3) 0.5 per cent PPO in dioxane:toluene (70:30), used in conjuction with sample oxidation for tritium

4) 0.7 per cent PPO in toluene:phenethylamine:methanol:water (40:33:22:5), used for ^{14}C-samples in oxidation.

Plastic scintillation vials ($25X10^{-6}m^3$) (A. and J. Beveridge Ltd., Edinburgh) were used throughout.

Sample oxidation (Liquid Scintillation Sample Oxidizer IN 401, Intertechnique) was employed in studies into uptake and distribution of labels from ^{3}H- and ^{14}C-oestrogens. In this technique, complete combustion of samples takes place; all H is incorporated into water, and all carbon into CO_2. Recovery of label is highly efficient (usually 99%).

Also, radioactivity-containing areas on TLC plates were detected using a RTLS 1A Panax thin-layer scanner (Panax Ltd., Redhill, Surrey, U.K.). The instrument was only used for qualitative analyses.

4. Extraction, Purification and Identification of Endogenous Oestrogens

The procedures used were based on those of Young (1977), using 1N NaOH with ^{14}C-oestradiol as an internal standard. In summary, 10 kg *P. vulgaris* seeds were extracted (internal standard *c.* 17,000 Bq ^{14}C-oestradiol). The extract was purified by TLC on 0.5mm silica gel G (Camlab, Cambridge) using chloroform:methanol (95:5). The areas of plates containing radioactivity and areas corresponding to the putative oestrone fraction were scraped and eluted. After derivatisation with bis-trimethylsilyacetamide (bis-TMSi), the eluate was analysed by combined GC-MS on an AEI MS-30 mass spectrometer equipped with a WF-055 Multipeak Monitor. The mass spectrometer was operated at a source temperature of 493K and a voltage of 70eV. Two columns for gas chromatography were employed: 1.5m 1% SE-30 at 523K with a carrier gas flow rate of $40x10^{-6}m^3 min^{-1}$ and 1.5m OV-1 at 513K.

5. Experiments

a) Senescence: Discs, 10mm diameter, were cut with a standard cork borer from surface-sterilized (alternate 10s washes x5 in 5% sodium hypochlorite and distilled water) leaves at two stages in their development: newly fully expanded leaves at their full colour (green leaves) and leaves which had become senescent (yellow leaves). To obviate bruising at cut edges, discs were cut from leaves placed on sterilized (100% methanol-washed) polythene stretched over a rubber block.

Two sets-up were used, depending on the scale of experiments. When the effect of one oestrogen on chlorophyll levels was investigated (small experiments), 50mm diameter petri-dishes lined with two pieces Whatman No. 3 filter-paper and containing 1.5×10^{-6} m^3 solution and five leaf discs selected at random were prepared. During these and all other senescence experiments the dishes were stored at 293 ± 1 K in darkness in an incubator. Three dishes for each treatment were removed after 2,4,6 and 8 days incubation and the chlorophyll levels of the discs determined.

In larger experiments, where more than one oestrogen was tested, *c.* 100 randomly selected leaf discs were placed in 125mm diameter petri-dishes lined with four pieces Whatman No. 1 filter-paper wetted with 20×10^{-6} m^3 of the appropriate solution. At 2-day intervals for 8 days five four-disc random samples for each treatment were removed and the chlorophyll contents determined.

Throughout, chlorophyll levels were calculated from absorption spectra (Unicam SP8000 UV recording spectrophotometer) of leaf disc extracts (24h in 10×10^{-6} m^3 methanol at 273 K in darkness) using the equation given in Sestak (1971):

$$\text{chlorophyll } (a+b) = 4.0\ A_{665} + 25.5\ A_{650}\ gm^{-3}$$

(from data of Mackinney, 1941).

The uptake of steroidal oestrogens was monitored using 10^{-3} mol m^{-3} ^{14}C-oestrone and ^{14}C-oestradiol (370 MBq m^{-3}) and ^{3}H-oestrone-sulphate (37 GBq m^{-3}). Five leaf discs of known fresh weight were placed in 50mm diameter petri-dishes containing 1.5×10^{-6} m^3 radioactive solution and two pieces Whatman No. 3 filter-paper. At 30 min intervals for the first 6h then hourly until 10h the discs were removed from three dishes for each treatment and weighed after any adhering surface moisture had been blotted off. Samples were also taken after 2,4,6 and 8 days. The radioactivity present in the discs was determined by scintillation spectrometry following sample oxidation. The levels of tritium and radiocarbon remaining in the dishes and filter-papers were determined directly by scintillation spectrometry. The relative water content (RWC) of discs, used in comparing uptake of radioactivity with uptake of water, was calculated after Barrs and Weatherley (1962).

$$RWC = \frac{\text{final fresh weight-dry weight}}{\text{initial fresh weight-dry weight}} \times 100$$

To investigate oestrogen metabolism by leaf discs, 125mm diameter petri-dishes containing $20 \times 10^{-6} m^3$ 10^{-2} mol m^{-3} oestrogen solutions (with 370 MBq m^{-3} ^{14}C or 37 GBq m^{-3} ^{3}H) and lined with four pieces Whatman No. 1 filter-paper were used. Five dishes with *c.* 100 discs were prepared for each treatment. After 2 days incubation the discs were removed and soxhlet extracted in methanol for 24h. Preparative radio-TLC (chloroform:methanol (99:5)) for extracts of ^{14}C-oestrone- and ^{14}C-oestradiol-treated discs and chloroform:acetic acid (99:1) for ^{3}H-oestrone-sulphate-treated discs) separated the major components of extracts. Radioactivity-containing regions of plates were scraped, eluted with acetone then methanol and the combined eluates dried down under N_2 then taken up in ether.

Radio-high pressure liquid chromatography (radio-HPLC) was utilized in further identifications for some of the eluates. Samples were run on a 50-100% gradient methanol:ammonium acetate buffer for 30 min in an ODS-hypersil reverse phase column, (Shandon). The flow rate was $10^{-6} m^3$ min^{-1}. The UV absorbance at 250 nm was monitored (chart speed 10mm min^{-1}) and the radioactivity content of the column effluent was also recorded. The radioactivity monitor was set at 10 cps with a time constant of 10. The scintillant used was as in scintillation cocktail 2) except that xylene replaced toluene.

b) Rooting: Cuttings were taken from plants at two growth stages, *viz:* when the primary leaves had opened and were expanding (hypocotyl cuttings) and when the first trifoliate leaf had opened and was expanding (epicotyl and primary leaf cuttings). In all experiments cuttings were excised under distilled water to prevent xylary embolism, and placed immediately in $25 \times 10^{-6} m^3$ vials containing *c.* $15 \times 10^{-6} m^3$ of the appropriate solution. Hypocotyl cuttings were excised 40 ± 1mm below the cotyledonary node, epicotyl cuttings $40 \pm$mm below the primary leaf node and primary leaf cuttings 30 ± 1 mm from the leaf blade.

Oestrogen treatments were applied by three methods: i) in solution in the rooting medium (wick-feeding after Atallah *et al.*, 1975), ii) in a 5 mm^3 droplet of methanol to apices, and iii) in a 10 mm^3 droplet of methanol to primary leaves. In ii) and iii) the cuttings were rooting in distilled water and applications of oestrogens were made within 30 min of cutting. When the effects of cotyledon excision were examined the cotyledons were removed in air with a wet blade before placing the

cutting in its vial. During the 7-day (hypocotyl cuttings) or 10-day (epicotyl and primary leaf cuttings) rooting periods, the cuttings were maintained under continuous illumination, or long day, or short day photoperiodic regimes at $293^{+}_{-}1$ K in incubators or environmental cabinets (long days = 16h light, 8h darkness: short days = 8h light, 16h darkness) ($7\ Wm^{-2}$).

Following the 7-day rooting period, hypocotyl cuttings were potted out and further growth and development monitored. During this period, the cuttings were kept under long days in greenhouses and served as controls for parallel experiments on uptake and distribution of ^{3}H and ^{14}C from applied labelled oestrogens. Generally, 10 cuttings per treatment were prepared for an experiment.

In the experiments on uptake and distribution of radiolabel from ^{3}H- and ^{14}C-oestrogens, labelled oestrogens were applied in the same three ways as the unlabelled compounds earlier. Here, however, only 10^{-3}mol m^{-3} (0.5 GBq m^{-3} ^{14}C and ^{3}H) oestrogens were used as rooting media and 50 µg oestrogen containing 10000 Bq ^{3}H or ^{14}C applied in 10 mm^{3} or 5 mm^{3} methanol to primary leaves or apices respectively of cuttings rooting in distilled water. Plants were sectioned at eight temporal or developmental stages: 1,4 and 7 days after cutting, then, following potting out, at the opening of each trifoliate leaf and at flowering. The effects of cotyledon excision and of different photoperiods during rooting also were examined. Plants potted out for harvesting were maintained under long days in the greenhouse. The amounts of radiolabel in plant parts were determined by scintillation spectrometry following sample oxidation.

c) Growth and development: On the completion of rooting experiments, hypocotyl cuttings usually were potted out and further growth and development monitored. When experiments were set up specifically to investigate possible oestrogen effects on growth, whole plants were used. Applications of oestrogens to primary leaves or apices in $10mm^{3}$ and $5mm^{3}$ methanol respectively were made when the primary leaves were fully expanded. The distributions of ^{14}C and ^{3}H from labelled oestrogens applied to primary leaves were investigated using sample oxidation and scintillation counting. Here, 50 µg oestrogens containing 10,000 Bq ^{3}H or ^{14}C was applied in 10 mm^{3} methanol. Throughout these experiments, plants were maintained in the greenhouses in long day conditions and the plants were not wetted during watering the pots.

d. Germination

The effects of oestrogens on *Phaseolus* seed germination and subsequent growth were also examined. Dry seeds imbibed oestrogen solutions for 2 days before being planted out. Growth and development were monitored. The distributive fates of ^{14}C from labelled oestrone and oestradiol taken up by seeds were determined by sample oxidation and scintillation spectrometry.

6. Photography and Microscopy

Segments (quarter-cylinders, 3-5mm long) of hypocotyls, epicotyls and primary leaf petioles were fixed in 3 per cent glutaraldehyde and embedded in an Epon/Araldite resin; 1.5µm and 3µm sections were stained in toluidine blue. Prepared sections were photographed using a Zeiss photographic microscope fitted with a x10 lens and a x8 eye piece, with a multiplication factor of 1.6.

7. Statistical Analysis

Standard errors were calculated using a programmed Hewlett-Packard SP/64 calculator.

The student t-test was employed to test for statistically significant differences. Differences were considered significant for $p \leqslant 0.05$.

Equations: i) standard error

$$se = \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n(n-1)}}$$

ii) t-test

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}}$$

where

$$s^2 = \frac{\sum x_1^2 - \frac{(\sum x_1)^2}{n_1} + \sum x_2^2 - \frac{(\sum x_2)^2}{n_2}}{n_1 + n_2 - 2}$$

RESULTS

The involvement of steroidal oestrogens in leaf senescence, germination, root initiation and internode growth are considered in separate sections. However, there is some overlap: in the study of germination and root initiation, some experiments were extended to allow observation of any long-term effects on growth and development. Thus, these results are not presented as internode growth experiments and similarly, although possible effects on flowering were also investigated, the results of these experiments are contained in the germination, root initiation and internode growth sections. The isolation and characterization of oestrone and oestradiol from *P. vulgaris* seeds is also reported.

1. Leaf Senescence: Effects on Chlorophyll Levels

The possible role of steroidal oestrogens in leaf senescence has been examined by considering the effects of oestrone, oestradiol and oestradiol-sulphate on chlorophyll content in leaf discs of *P. vulgaris*. Effects of steroidal oestrogens on chlorophyll levels have been found in several species (Table 2c) but no clear pattern has emerged from these contradictory reports. The measurement of chlorophyll content represents the most convenient assay of senescence in green plant tissues (Thomas and Stoddart, 1975).

Experiments were designed to investigate effects of the compounds on discs from leaves in various stages of visible senescence and were carried out as a time course over a period of 8 days. Attention was paid throughout to any possible effects on the chlorophyll *a:b* ratio.

Uptake and metabolism of labelled oestrogens by discs was also followed over 8 days.

The section is concluded with an account highlighting the problems of the calculation of chlorophyll levels.

a) Effects of steroidal oestrogens

The effects of steroidal oestrogens on chlorophyll levels during leaf disc senescence in darkness were examined over 8 days. Table 6 presents a typical set of results for oestradiol. No statistically significant effects were evident, and the same was also true when oestrone and oestradiol-sulphate were tested, even at 0.1mol m^{-3} oestradiol-sulphate, the

Table 6. Effects of oestradiol on chlorophyll content of leaf discs senescing in darkness for 8 days

leaf type	treatment	initial levels	concentration (mol m^{-3})		
			10^{-3}	10^{-5}	10^{-7}
green primary	oestradiol	15.8±0.3	11.5±0.6	11.1±0.2	10.7±1.0
	control		12.2±0.3	11.5±1.2	11.9±0.7
	distilled water		10.8±0.1		
yellow primary	oestradiol	12.8±1.1	7.4±1.2	8.7±0.1	7.8±0.8
	control		9.1±1.0	9.6±0.7	7.8±1.2
	distilled water		7.4±0.4		
green trifoliate	oestradiol	20.0±0.3	14.2±1.0	13.9±1.6	15.6±1.5
	control		12.4±1.1	13.5±0.4	14.2±1.6
	distilled water		12.1±0.9		
yellow trifoliate	oestradiol	15.0±0.3	10.7±1.2	10.4±0.9	10.4±0.6
	control		10.8±1.0	9.5±0.5	8.3±0.6
	distilled water		8.6±1.6		

Number are μg chlorophyll/mg dry weight leaf tissue ± standard error

highest oestrogen concentration used. An aspect obvious throughout the experiments was the non-uniform loss of pigmentation in individual leaf discs; indeed, whole leaves *in vivo* did not lose colour evenly.

The total chlorphyll (*a*+*b*) present in senescing leaf discs was found to decline differently when green and yellow leaves were used as starting materials (Fig.11). The chlorophyll levels in any given leaf type varied considerably between batches of plants and also seasonally. Generally though, *in vivo*, trifoliate leaves had higher chlorophyll levels than primary leaves, whether green or yellow. However, both in trifoliate and primary green leaf tissues, the reduction in chlorophyll levels was slow initially before entering a more rapid phase. Yellow leaf discs, on the other hand, lost chlorophyll more rapidly over the first 4 days, then more slowly. Oestrogens were without influence on these time courses (Table 7).

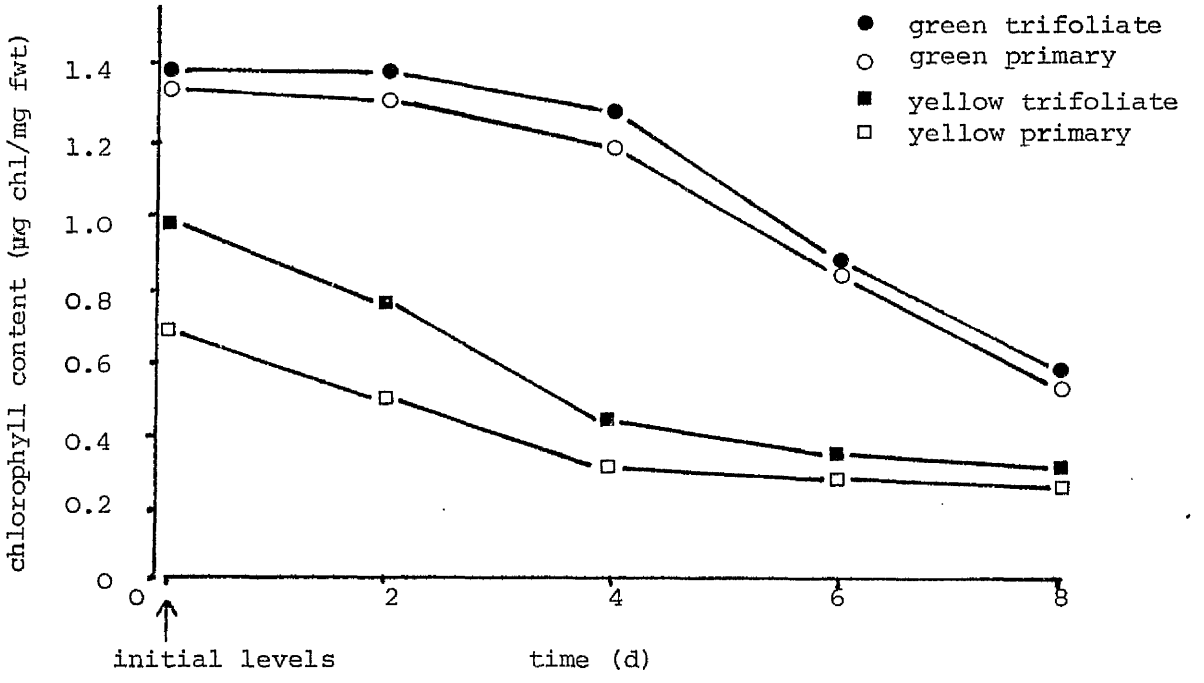
Table 7. Time course of reductions in chlorophyll content in oestrogen-treated leaf discs senescing in darkness

treatment	time (days)	concentration (mol m^{-3}) 0.1	10^{-3}	10^{-5}	10^{-7}
oestrone	2		18.0±0.6	18.6±0.4	18.2±0.6
	4		15.6±0.2	15.7±0.5	16.1±1.1
	6		13.1±0.6	12.4±0.5	14.5±0.5
	8		11.7±1.1	10.1±0.3	11.6±0.2
oestradiol	2		17.9±0.8	17.9±0.6	17.5±0.5
	4		15.3±0.9	14.9±0.5	15.3±0.5
	6		12.7±0.3	14.6±0.5	14.1±0.2
	8		10.0±0.2	11.5±0.3	10.7±0.4
control	2		16.3±0.5	18.1±0.6	16.4±0.6
	4		14.9±1.1	16.6±0.4	15.3±0.2
	6		12.4±0.4	14.5±0.5	14.0±0.6
	8		10.0±0.3	10.7±0.3	10.0±0.3
oestradiol-sulphate	2	17.8±1.0	18.1±0.9	17.0±0.6	18.4±0.7
	4	15.3±0.4	15.8±0.6	15.3±0.7	18.0±0.4
	6	12.9±0.4	12.0±0.5	13.5±0.3	15.7±0.7
	8	10.6±0.9	10.0±0.4	10.1±0.4	12.4±1.1
Na_2SO_4	2	18.8±1.1	17.3±0.4	17.6±1.3	15.7±0.4
	4	17.4±0.4	14.1±0.9	16.4±0.8	13.8±0.3
	6	14.5±0.6	12.3±0.4	13.0±0.6	11.5±0.7
	8	11.7±0.3	10.6±0.2	11.5±0.5	10.7±0.4
distilled water	2		17.7±0.8		
	4		15.5±0.7		
	6		12.4±0.4		
	8		10.7±0.1		

green trifoliate leaves; initial level 19.0±0.2

numbers are µg chlorophyll/mg dry weight leaf tissue ± standard error

Fig. 11. Time-course of reductions in chlorophyll content in leaf discs during 8 days in darkness



In a number of plants, chlorophylls *a* and *b* were broken down at different rates during senescence (Wolf, 1956). With *Phaseolus* however, the chlorophyll *a*:*b* ratio was constant within experimental materials and during experiments (Table 8) and this too was unaffected by steroidal oestrogen treatments.

b) Uptake of ^{14}C and ^{3}H from labelled steroidal oestrogens

Uptake of ^{14}C and ^{3}H into leaf discs during the first 10h incubation was investigated (Figs.12 and 13 respectively). With ^{14}C from labelled oestrone or oestradiol, uptake of solution (increase in fresh weight) and increase in radioactivity present in discs were simultaneous over the first 4h, the period when rapid uptake of solution occurred to redress water deficits (Barrs and Weatherley, 1962). Thereafter, solution uptake was diminished and uptake of radiocarbon was the more rapid. A similar situation prevailed in experiments with ^{3}H-oestrone-sulphate.

In longer-term experiments, radioactivity, especially ^{14}C, was accumulated by leaf discs over a period of 8 days (Table 9).

Fig.12. Uptakes of solution and ^{14}C (from ^{14}C-oestradiol) by leaf discs over 10h

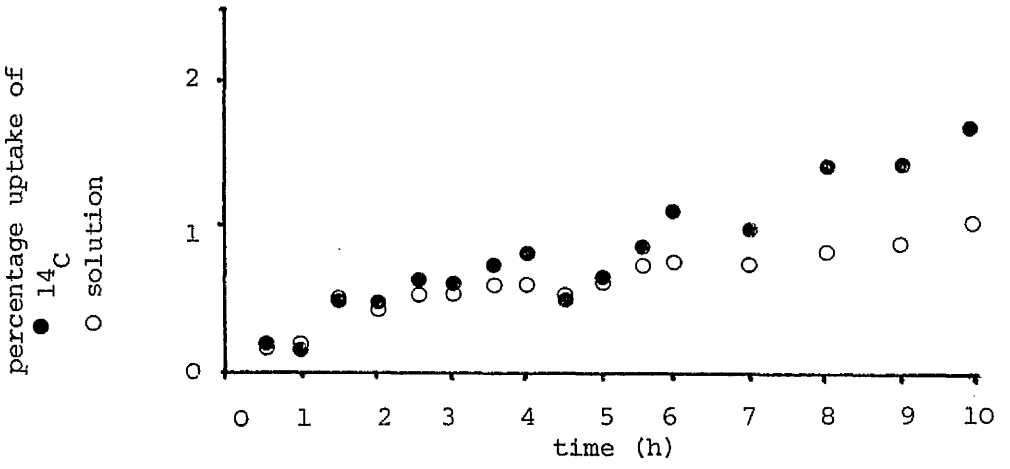


Fig.13. Uptakes of solution and ^{3}H (from ^{3}H-oestrone-sulphate) by leaf discs over 10h

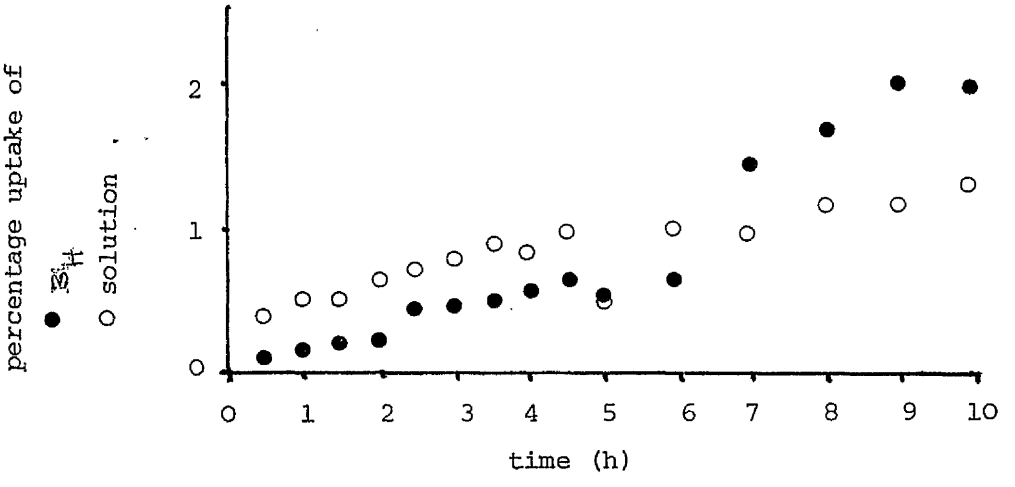


Table 9. Retention of ^{14}C and ^{3}H by leaf discs after extraction

disc type	percentage radioactivity retained after 24h soxhlet extraction in methanol		
	^{14}C (oestrone)	^{14}C (oestradiol)	^{3}H (oestrone-sulphate)
green primary	21.21	16.38	1.51
green trifoliate	21.92	17.31	0.77
dead (green primary)	1.10	0.90	0.90

all discs were incubated in radioactive oestrogens for 2 days in darkness, results up to 8 days were similar

Table 8. Effect of oestradiol on the chlorophyll *a*:*b* ratio in leaf discs senescing in darkness for 8 days

leaf type	treatment	initial levels	concentration (mol m^{-3})		
			10^{-3}	10^{-5}	10^{-7}
green primary	oestradiol	2.81±0.10	3.03±0.13	2.90±0.24	2.79±0.17
	control		2.97±0.25	2.87±0.11	2.90±0.17
	distilled water		2.82±0.12		
yellow primary	oestradiol	2.80±0.15	2.55±0.14	2.73±0.24	2.85±0.35
	control		2.96±0.20	2.91±0.35	2.78±0.38
	distilled water		2.83±0.18		
green trifoliate	oestradiol	2.81±0.09	2.58±0.12	2.78±0.18	2.85±0.17
	control		2.73±0.22	2.97±0.10	2.82±0.19
	distilled water		2.91±0.18		
yellow trifoliate	oestradiol	2.82±0.11	2.92±0.33	2.85±0.25	2.66±0.08
	control		2.92±0.20	2.92±0.32	2.64±0.09
	distilled water		2.93±0.40		

numbers are chlorophyll *a*:*b* ratio ± standard error

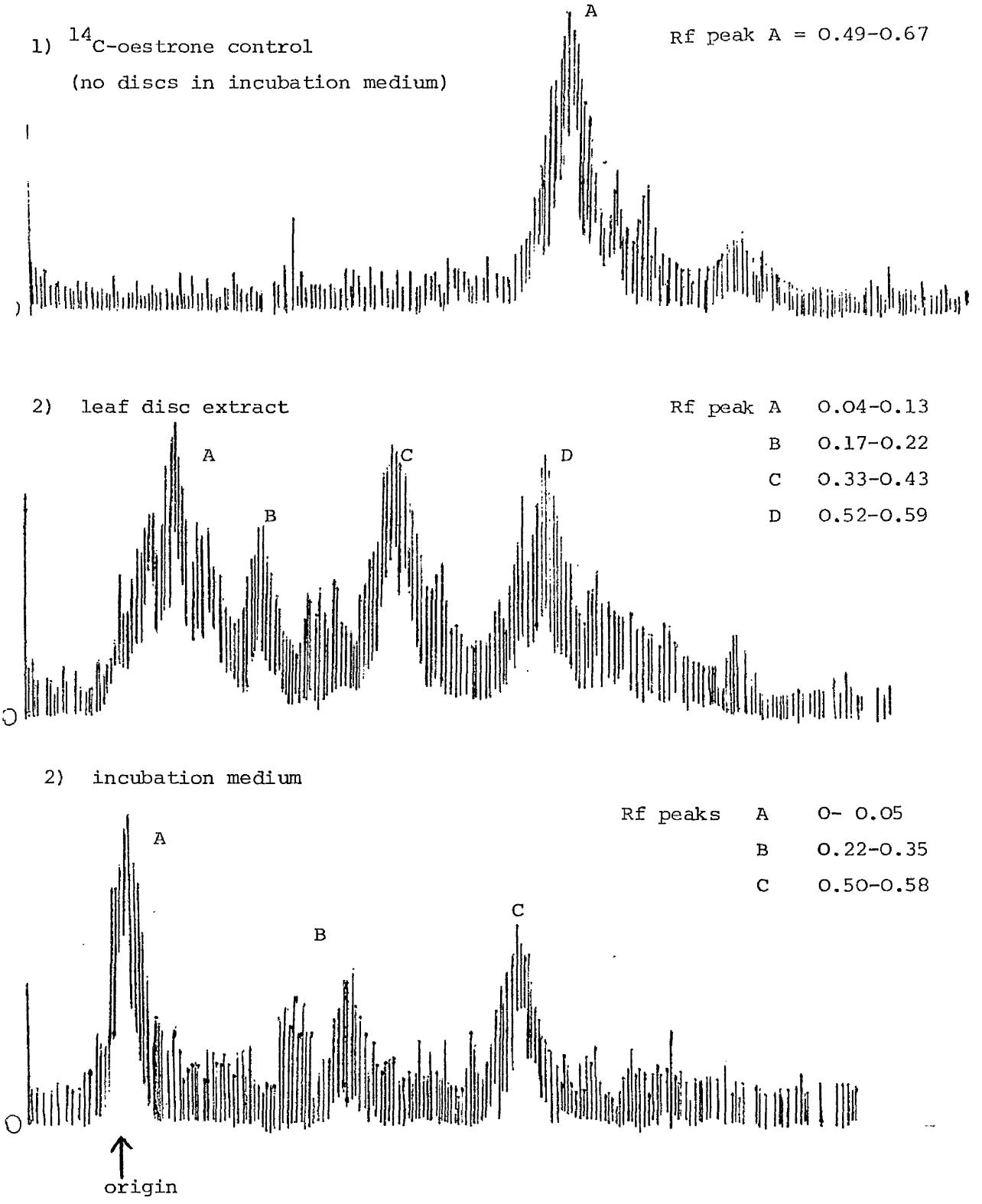
c) Metabolism of applied steroidal oestrogens

Radio-TLC and radio-HPLC studies revealed that while much of the radioactivity applied was recoverable in the applied oestrogen, some interconversion of oestrone and oestradiol hadoccurred (Fig 14). With the HPLC system used the separation of oestrone and oestradiol was slight.

d) Calculation of chlorophyll levels

All chlorophyll determinations were made using the same procedure. This was important as it allowed viable, preliminary comparisons of untreated data, similar amounts of leaf material having been used throughout. The importance of using a consistent method was underlined when the chlorophyll content was calculated from different dilutions of the same extract (Table 10). As the dilution increased, the calculated chlorophyll content rose and reading the same dilution at different sensitivities produced vastly different chlorophyll levels.

Fig.14. Metabolism of ^{14}C- oestrone by discs from green trifoliate leaves during 2d in darkness



Radio-TLC scans of plates run in chloroform:methanol (95:5)
Standard Rf values, oestrone = 0.53- 0.62, oestradiol = 0.39 - 0.45

Table 10. Calculated chlorophyll contents from different dilutions of a single extract of yellow trifoliate leaves

volume (X 10^{-6} m^3)	absorbance scale 0 - 1.0	0 - 0.2
15	133.95	-
30	139.50	-
45	148.05	96.30
90	151.20	107.10

Numbers are calculated chlorophyll contents (µg) originally present in the discs extracted

2. Germination

The effects of oestrogens in germination have already been discussed in the Introduction (see Tables 2c and 3a). In this series of experiments the effects of steroidal oestrogens on *Phaseolus* germination were investigated and, in addition, seeds imbibed in oestrogen solutions were allowed to grow to maturity and their growth and development monitored.

Uptake of radioactivity from labelled oestrogens and the subsequent distribution during growth and development were investigated.

a) Effects of steroidal oestrogens on germination

Normally, almost all *P. vulgaris* seeds germinated, and imbibition in oestrogen solutions did not affect this.

Table 11. Effects of steroidal oestrogens on *Phaseolus* germination after 2 day imbibition in oestrogen solutions

treatment	concentration (mol m^{-3}) 0.1	10^{-3}	10^{-5}	10^{-7}
oestrone-phosphate	95	-	-	-
oestrone	-	75	90	90
oestradiol-sulphate	80	90	90	-
oestradiol	-	80	100	90
Tween 80 control	-	80	-	-
distilled water		90		

numbers are percentage germination

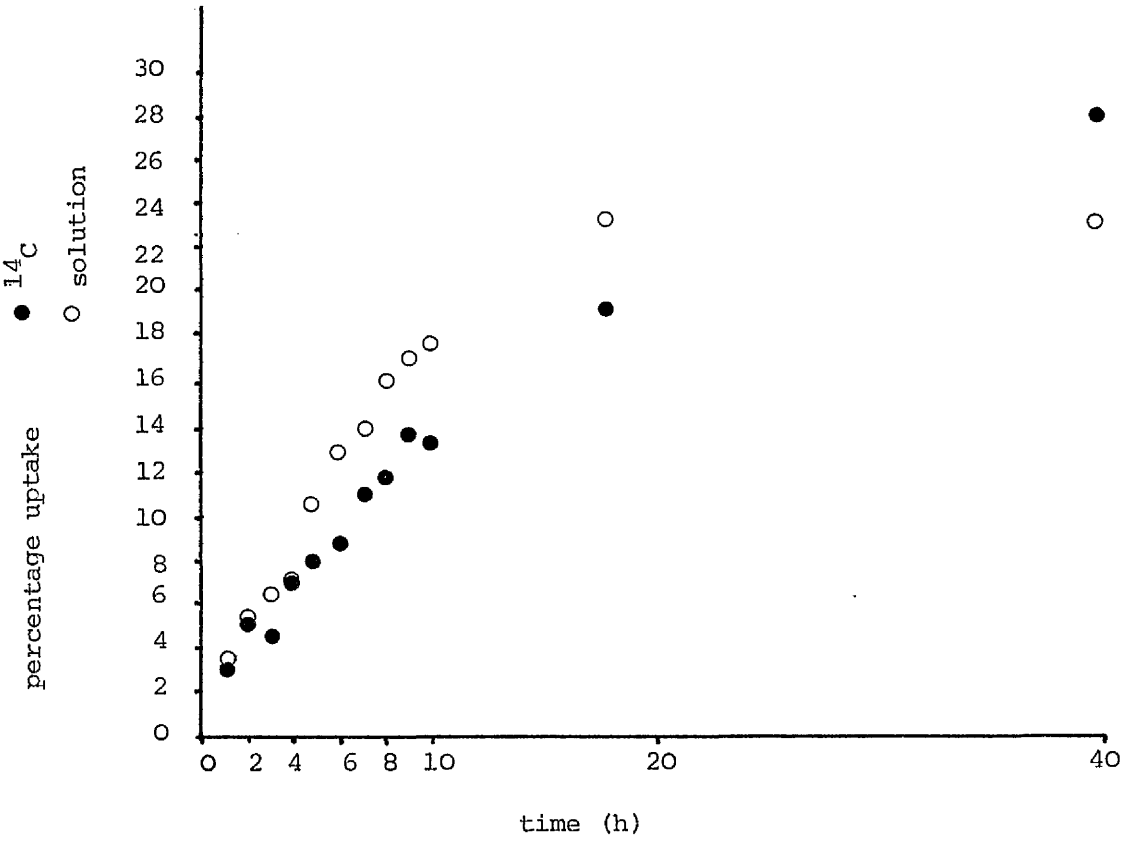
b) Growth and development of seeds imbibed in oestrogen solutions

None of the treatments affected final internode lengths (taken at flowering) or the numbers of flowers produced (Tables 12 and 13 respectively).

c) Uptake and distribution of radioactivity from labelled oestrogens

Dry seeds placed in ^{14}C-oestrone or ^{14}C-oestradiol solutions took up water (i.e. increased in fresh weight) and ^{14}C rapidly (Fig.15). As in

Fig.15. Uptakes of ^{14}C and solution by bean seeds



leaf discs, the phase of rapid water uptake was of limited duration (10 to 12h for seeds) but the radioactivity content continued to increase. Closely similar results were obtained with ^{3}H-oestrone-sulphate.

The distribution of radioactivity during growth and development was determined (see Table 14). Most of the ^{14}C taken up by seeds remained in the cotyledons, with the roots and hypocotyl receiving most of the

Table 12. Final internode lengths in bean plants grown from seed imbibed in oestrogen solutions

treatment	concentration (mol m^{-3})	internode lengths ± standard error (mm)						
		hypocotyl	1	2	3	4	5	total
oestrone	10^{-3}	65.5±2.07	46.6±1.94	57.8±3.84	31.8±2.52	50.4±5.42	56.4±3.23	371.6±17.36
	10^{-5}	62.9±1.93	53.5±2.10	54.9±3.30	32.7±2.90	44.6±4.66	38.6±4.37	307.4±11.82
	10^{-7}	57.6±2.91	52.8±2.66	64.7±3.77	26.6±2.91	34.2±5.99	34.2±3.79	262.1± 9.78
oestradiol	10^{-3}	58.6±1.62	53.8±2.41	57.8±3.87	28.4±3.03	35.9±3.07	42.6±5.09	309.1±16.83
	10^{-5}	63.2±1.91	51.6±2.29	67.9±4.10	28.6±1.97	39.8±5.14	43.3±4.51	329.6±14.21
	10^{-7}	62.4±2.12	49.6±2.33	54.3±3.21	33.6±2.17	42.2±3.91	39.8±3.10	314.1±14.85
control		63.1±2.59	51.7±3.12	50.3±2.60	24.8±2.88	49.8±4.50	56.1±3.82	307.8±15.25
oestrone-phosphate	0.1	57.9±1.87	50.6±2.55	56.6±4.13	25.0±2.14	31.9±3.09	44.0±4.79	285.9±11.82
oestrone-sulphate	0.1	56.2±2.65	52.3±2.78	60.1±4.60	30.4±2.38	41.7±4.61	41.3±7.89	297.1±15.98
distilled water		60.9±2.77	52.8±2.02	51.9±2.82	25.3±2.54	42.5±8.92	42.5±7.25	296.0±22.15

Summation of the mean internode lengths does not equate to the total internode lengths as several plants had other than 5 internodes

Table 13. Flowers formed at nodes of bean plants grown from seed imbibed in oestrogen solutions

treatment	concentration (mol m^{-3})	node number 3	4	5	6	apex	total
oestrone	10^{-3}	3.9±0.53	2.8±0.42	1.7±0.27	1.5±0.24	2.9±0.35	14.8±1.16
	10^{-5}	4.6±0.50	1.8±0.22	1.2±0.18	1.3±0.22	2.2±0.27	11.9±0.81
	10^{-7}	4.2±0.56	2.5±0.36	1.5±0.19	1.6±0.18	2.1±0.27	11.9±0.89
oestradiol	10^{-3}	4.8±0.46	2.5±0.35	1.8±0.23	1.4±0.19	2.3±0.35	14.1±1.00
	10^{-5}	4.6±0.46	2.5±0.35	1.3±0.23	1.5±0.19	2.3±0.35	14.1±1.00
	10^{-7}	4.6±0.38	1.7±0.27	1.5±0.19	1.6±0.35	2.9±0.27	13.5±0.87
control		4.5±0.49	2.5±0.38	1.5±0.17	1.5±0.15	1.9±0.30	12.5±0.87
oestrone-phosphate	0.1	4.5±0.42	2.2±0.36	1.7±0.18	1.3±0.19	3.3±0.47	13.9±1.26
oestradiol-sulphate	0.1	4.6±0.58	2.4±0.32	1.5±0.22	1.3±0.18	2.9±0.24	13.6±0.86
distilled water		4.3±0.80	3.3±0.55	1.6±0.23	1.6±0.26	2.9±0.45	14.2±1.31

Numbers are flowers ± standard error. These and the total number of flowers per plant do not correlate as some plants had other than 5 nodes

Table 14. Distribution during growth and development of radioactivity (Bq) from 10,000 Bq ^{14}C-oestradiol imbibed during germination by bean seeds

plant part	developmental stage			
	germinating	primary leaf opening	first trifoliate leaf opening	flowering
nodules				0.1
roots		34.7	67.9	80.0
hypocotyl		75.8	62.7	53.3
cotyledons	3187.7	3812.2	3512.4+	3454.4+
epicotyl		0	2.3	2.8
primary leaves	6.2	4.4	3.6	4.2
first axillary bud		0	0.1	0.1
internodes*			2.5	4.1
trifoliate leaves*			3.9	1.8
axillary buds*			0.1	0.5
apex	6.2	0.7	1.3	0.3
seed coat	482.2	154.7	122.8	72.2
radicle	39.6			
total	3721.9	4091.5	3779.5	3673.8

* totals for plant parts above primary leaf node

\+ abscinded

radiocarbon redistributed before cotyledon abscission.

3. Root Initiation

Hypocotyl cuttings were used in studies of oestrogen synthesis in *Phaseolus* (Young *et al.*, 1977, 1978) with rooted cuttings potted out until harvested at specific stages of development: the effects of oestrogens on root formation in these cuttings, however, were not investigated. The reported effects of oestrogens on adventitious root initiation in other species have been discussed in the Introduction (see Table 2c), and, in addition, endogenous phenolics (uncharacterised) have been found to act as co-factors with IBA in root formation in pear hardwood cuttings (Fadl and Hartmann, 1967).

The possible involvement of steroidal oestrogens in root initiation in cuttings was studied using oestrone, oestrone-phosphate, oestrone-sulphate, oestradiol and oestradiol-sulphate. The substances were applied to hypocotyl cuttings in three ways: 1) in solution in the rooting medium, or in a droplet of methanol to 2) the cutting apex or 3) the primary leaves. In these experiments, the influences of different photoperiods, *viz:* 24, 16 and 8h continuous illumination per 24h,and cotyledon excision were also investigated. At the completion of the rooting period, cuttings were potted out and their further growth and development monitored. The uptake and distribution of putative oestrogens was followed utilizing radioisotope techniques.

Adventitious root formation on epicotyl and primary leaf cuttings was investigated with respect to effects of steroidal oestrogens. ^{14}C-oestrone and oestradiol, and ^{3}H-oestrone-sulphate were used in the elucidation of patterns of uptake and distribution of presumed steroidal oestrogens.

a) Effect of steroidal oestrogens on adventitious root formation in hypocotyl cuttings

In the concentration range used oestrone and oestradiol were without influence on root initiation on hypocotyl cuttings (e.g. Table 15).

Table 15. Numbers of roots formed on *P. vulgaris* hypocotyl cuttings (without cotyledons) in oestrogen solutions during 7 days under continuous illumination

treatment	concentration (mol m^{-3})		
	10^{-3}	10^{-5}	10^{-7}
oestrone	31.8 ± 2.1	30.6 ± 2.0	25.2 ± 4.1
oestradiol	31.3 ± 2.7	31.2 ± 3.4	31.2 ± 2.3
control	37.8 ± 2.6	33.0 ± 0.7	36.6 ± 1.9
distilled water		30.4 ± 2.7	

numbers are roots formed $\pm$ standard error

This was true in all photoperiods whether cotyledons were present or absent. The water-soluable conjugates, oestrone-phosphate, oestrone-sulphate and oestradiol-sulphate, could be used at higher concentrations, and 0.1mol m^{-3} solutions of the oestrogen-sulphates were found to inhibit

Table 16. Root formation, bud growth and apex abscission on *P. vulgaris* hypocotyl cuttings, without cotyledons, in oestrogen conjugates for 7 days under continuous illumination

treatment	concentration (mol m^{-3})	roots formed ± standard error	bud growth (mm) cotyledonary	bud growth (mm) primary leaf	apices abscinded
oestrone-sulphate	0.1	17.6±1.8	3.3±0.7	2.2±0.3	1
	10^{-3}	33.2±0.9	0.6±0.1	1.6±0.2	0
	10^{-5}	31.0±2.4	0.3±0.1	1.8±0.2	0
	10^{-7}	31.4±2.3	0.6±0.1	1.8±0.2	0
oestradiol-sulphate	0.1	17.8±2.0	2.9±0.5	2.3±0.5	2
	10^{-3}	29.0±2.1	0.3±0.1	1.7±0.2	0
	10^{-5}	34.9±1.8	0.4±0.1	1.7±0.2	0
	10^{-7}	26.7±2.1	0.3±0.1	1.7±0.2	0
Na_2SO_4	0.1	38.3±2.7	0.3±0.1	1.7±0.2	0
	10^{-3}	33.4±1.6	0.5±0.1	1.7±0.2	0
	10^{-5}	34.7±3.4	0.5±0.1	1.8±0.2	0
	10^{-7}	32.0±2.1	0.4±0.1	1.7±0.2	0
oestrone-phosphate	0.1	39.3±4.8	0.8±0.2	2.1±0.5	0
	10^{-3}	38.0±3.0	0.5±0.1	1.6±0.2	0
	10^{-5}	38.3±5.0	0.3±0.1	1.8±0.2	0
	10^{-7}	32.3±3.2	0.4±0.1	1.7±0.2	0
Na_2HPO_4	0.1	40.0±4.3	0.9±0.1	2.1±0.5	0
	10^{-3}	37.3±2.9	0.4±0.1	1.7±0.2	0
	10^{-5}	38.0±5.1	0.4±0.1	1.8±0.3	0
	10^{-7}	29.9±3.2	0.3±0.1	1.7±0.2	0
Distilled water		36.8±2.4	0.4±0.1	1.7±0.2	0

Table 17. Bud growth in *Phaseolus* hypocotyl cuttings treated with 0.1mol m^{-3} oestrogen-sulphates during root formation

bud	time (days)	condition	treatment: distilled water	Na_2SO_4 control	oestrone-sulphate	oestradiol-sulphate
cotyledonary	7	developed*	0	0	39	46 (14)
		undeveloped	50	50	11	4
		abscinded	0	0	0	0
	45	developed	0	1	17 (11)	27 (25)
		undeveloped	50	47 (1)	5	0
		abscinded	0	2 (1)	28 (1)	21 (11)
primary leaf node	7	developed+	0	0	4	8 (4)
		undeveloped	50	50	46	42 (9)
		abscinded	0	0	0	0
	45	developed+	3	4 (2)	8 (4)	13 (12)
		undeveloped	39	38	20 (1)	11 (5)
		abscinded	8	8	22 (7)	24 (19)

Numbers are numbers of buds in that category and the figures in parenthesis indicate the number of buds involved where the main shoot apex had abscinded.

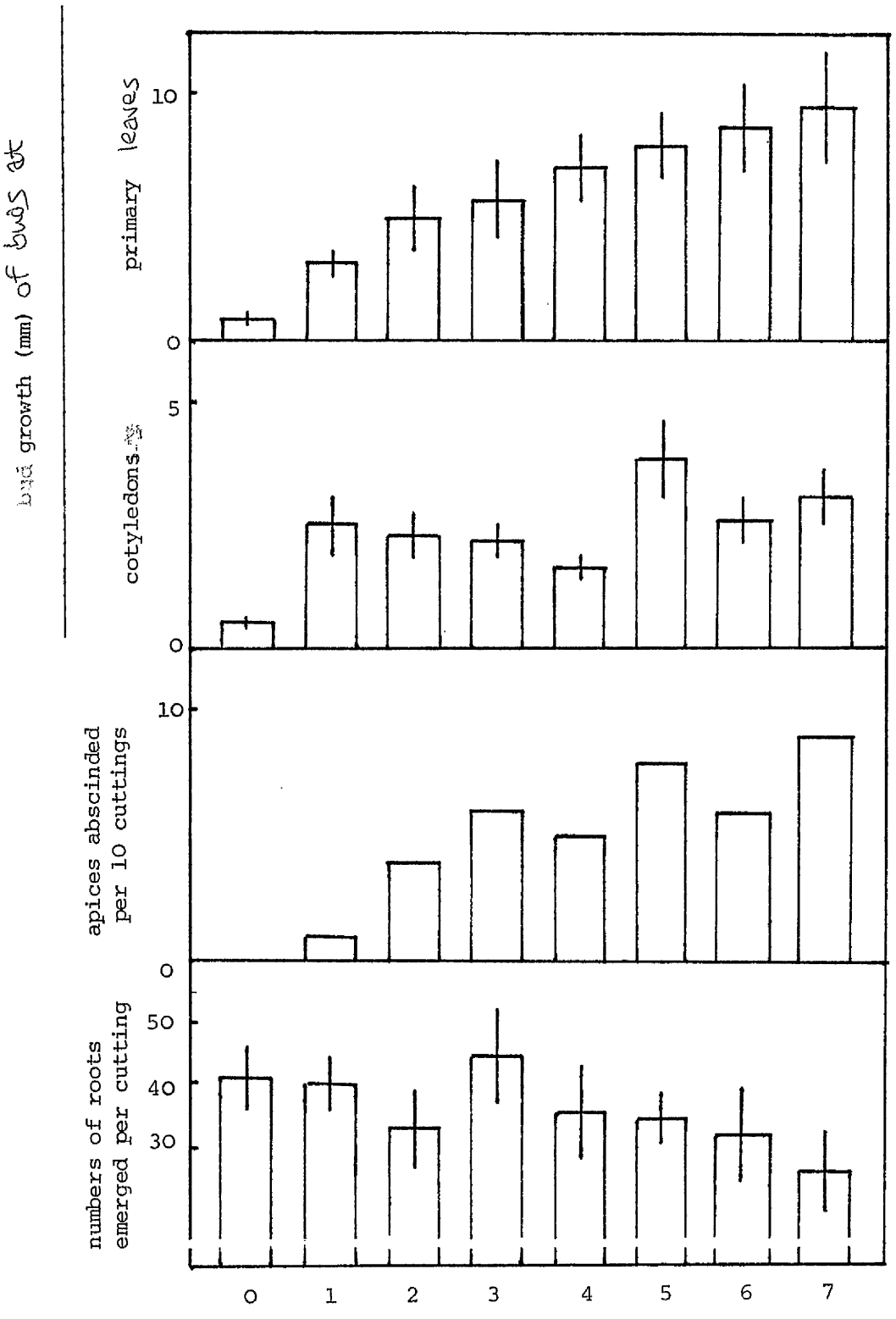
* bud size $>$ 1mm

\+ bud size $>$ 5mm

7d = end of treatment

45d = flowering (end of experiment)

Fig. 16. Effect of varying the duration of 0.1mol m^{-3} oestrogen-sulphate treatments on root formation, apex abscission and axillary bud growth in *Phaseolus* hypocotyl cuttings under continuous illumination



significantly the number of roots produced (Table 16). Also evident here were other effects: growth of buds at the cotyledonary and primary leaf nodes occurred and apex abscission took place (Table 16). These three effects could be modified by varying the time of exposure to 0.1mol m^{-3} oestrogen sulphates (Fig. 16): longer exposures increase the effects. From Fig. 16, the stimulation of bud growth and apex abscission would seem to be related. This, however, was not necessarily true (see Table 17) as, in a large-scale experiment, the full range of possible developmental types were observed (Table 18).

Table 18. Growth forms at flowering of *Phaseolus* hypocotyl cuttings treated with 0.1mol m^{-3} oestrogen-sulphates during root formation

	buds		treatment			
apex	cotyledon	primary leaf buds	distilled water	Na_2SO_4 control	oestrone-sulphate	oestradiol-sulphate
R	ND	ND	22	22	1	0
R	G	ND	0	0	13	5
R	ND	G	3	1	1	0
R	G	G	0	1	4	1
A	ND	ND	0	0	0	0
A	G	ND	0	0	3	5
A	ND	G	0	1	0	0
A	G	G	0	0	3	13

R = retained (normal apical growth)

A = abscinded

ND= not developed

G = growth (> 1mm for cotyledonary buds, > 5mm for primary leaf buds) regarded as significant

Application of oestrogens directly to the apices or primary leaves of cuttings placed in distilled water did not influence the number of roots produced in the presence or absence of cotyledons in any of the three photoperiods (Table 19).

At the end of the rooting period, the cuttings were potted out and maintained under a 16h day until flowering occurred. The internode lengths were then measured and the flowers and floral buds counted. No effects of

Table 19. Numbers of roots formed on *P. vulgaris* hypocotyl cuttings, without cotyledons, after apical or foliar applications of oestrogens

treatment	amount (µg)	site of application: apex	site of application: primary leaf
oestrone	0.1	35.7±4.1	
	1.0	30.2±2.6	31.4±4.3
	10.0	36.5±3.1	34.2±2.9
	50.0	42.1±3.2	
	100.0		30.0±1.4
oestrone-phosphate	0.1	40.9±3.0	
	1.0	37.2±4.4	35.6±5.7
	10.0	35.4±5.0	38.4±2.4
	50.0	34.8±4.6	
	100.0		30.6±2.9
oestradiol	0.1	41.9±2.9	
	1.0	37.9±3.0	37.4±4.2
	10.0	38.1±3.3	33.4±2.9
	50.0	41.4±3.1	
	100.0		37.0±7.9
oestradiol-sulphate	0.1	36.1±4.3	
	1.0	35.4±3.1	32.8±1.8
	10.0	41.5±4.6	40.5±3.2
	50.0	33.8±4.2	
	100.0		32.0±2.6
methanol		34.0±4.0	31.4±1.5
no treatment		32.4±3.2	33.0±3.9

All cuttings without cotyledons

All maintained in continuous illumination

Table 20. Final internode lengths (mm) (at flowering) of *Phaseolus* cuttings treated with oestrogens during root formation

site of application	treatment	internode					total
		epicotyl	2	3	4	5	
rooting medium	10^{-3} mol m^{-3} oestradiol-sulphate	35.4±2.62	52.6±3.53	49.1±3.15	62.4±6.31	47.1±6.36	241.4±13.59
	control	29.0±3.14	47.8±5.04	48.5±3.76	68.0±3.03	39.7±5.36	223.0±11.81
apex	10 µg oestrone	28.8±3.17	48.4±1.50	26.2±2.18	43.6±4.78	31.6±4.48	187.0±13.43
	$5mm^3$ methanol	36.0±4.95	48.6±4.50	27.6±2.18	37.8±4.28	28.5±1.50	161.4± 5.71
primary leaf	10 µg oestrone	29.9±1.50	31.9±1.64	15.4±0.93	40.3±5.69	69.3±5.90	186.7±10.75
	$5mm^3$ methanol	27.2±1.08	28.2±2.48	14.5±1.15	30.2±5.54	58.9±4.38	169.6±12.42

All cuttings were without cotyledons

All cuttings rooted under continuous illumination then grown until flowered under 16h days

Some plants had more or less than 5 internodes, therefore the mean of the total is not the total of the means

Table 21. Numbers of flowers produced on *Phaseolus* hypocotyl cuttings treated with oestrogens during root formation

site of application	treatment	nodes				apex	total
		3	4	5	6		
rooting medium	10^{-3}mol m^{-3} oestradiol	0.5±0.22	2.0±0.39	2.9±0.41	2.1±0.41	1.8±0.25	10.0±1.18
	control	0.6±0.38	1.7±0.41	3.4±0.58	2.9±0.51	2.3±0.65	11.1±1.45
apex	50µg oestrone	1.3±0.30	2.6±0.34	1.8±0.25	1.4±0.22	2.3±0.30	10.1±1.00
	$5mm^3$ methanol	0.8±0.25	2.9±0.77	3.5±0.52	1.3±0.30	2.6±0.45	10.8±1.56
primary leaf	10µg oestradiol	1.4±0.24	3.3±0.24	2.4±0.34	1.6±0.24	2.7±0.29	11.4±0.73
	$5mm^3$ methanol	1.9±0.28	2.6±0.27	2.0±0.33	1.2±0.20	2.2±0.33	10.0±0.77

Numbers are flowers ± standard error

All cuttings were without cotyledons and were maintained under constant illumination during root formation then 16h days until flowering

As some cuttings had other than 5 internodes (i.e. 4 nodes at which flowers normally were formed) the total is not the sum of the means.

the oestrogen treatments (other than 0.1mol m^{-3} oestrogen-sulphates) were observed (Tables 20 and 21).

The uptake and distribution of radioactivity from labelled oestrogens applied to cuttings were investigated over the rooting and growth periods. When radiocarbon was taken up by hypocotyl cuttings placed in $^{14}C\text{-}E_1^*$ or $^{14}C\text{-}E_2^*$ solutions, over 90 per cent of the label remained in the hypocotyl and roots (Table 22). The plant parts above the primary leaves had not

Table 22. Percentage distribution of radioactivity from ^{14}C- oestradiol and ^{3}H-oestrone-sulphate solutions taken up by *P. vulgaris* hypocotyl cuttings under continuous illumination during root formation and 16h days thereafter

	Developmental stage of cutting							
	1 day in rooting medium		7 days in rooting medium		2nd trifoliate leaf opening		Flowering	
Isotope.....	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Plant part								
Roots and nodules			14.47	10.30	11.38	2.24	8.72	5.55
Hypocotyl	95.83	60.27	84.07	48.75	81.80	38.28	86.69	58.66
Cotyledons	0.67	5.34	0.39	3.72	1.48	3.89	0.39	1.29
Buds at cotyledon node	0.13	5.81	0.16	1.48	0.11	0.42	0.24	1.77
Epicotyl	1.50	16.11	0.30	10.70	0.10	9.15	1.13	8.41
Primary leaves	1.7	8.64	0.49	24.88	4.35	45.13	2.07	23.32
First axillary buds	0.09	1.42	0.05	0.01	0.04	0.47	0.05	0.14
*Internodes					0.27	0.22	0.22	0.23
*Trifoliate leaves					0.23	0.15	0.34	0.55
*Axillary buds					0.17	0.03	0.15	0.06
Apex	0.07	2.41	0.08	0.15	0.06	0.01	0	0.01
Total (Bq)	2423.0	1632.2	4769.4	9264.0	5230.8	7771.2	7515.2	8288.4+

* Totals for parts above primary leaves

\+ All plants supplied with *c.* 10000 Bq radioactivity in solution in the rooting medium

* E_1 = oestrone E_2 = oestradiol

developed prior to potting out (removal of external ^{14}C-source) and relatively little label was found in these organs, indicating that internal redistribution of radiocarbon during growth and development had been slight. Nevertheless, it was notable that the axillary buds, internodes and trifoliate leaves consistently contained similar levels of radioactivity, and thus, taking tissue mass into account, the buds had accumulated relatively more ^{14}C than those other plant parts. The organs that abscinded (cotyledons and, occasionally, primary leaves) did not exhibit any obvious import or export of ^{14}C prior to abscission.

The distribution patterns of label from wick-fed ^{3}H-oestrone-sulphate were somewhat different (Table 22): here the radioactivity was more evenly distributed throughout the cuttings during the uptake period, with *c.* 50 per cent of the tritium taken up retained by the hypocotyl and roots. Again, however, redistribution of radiolabel to developing plant parts after potting out was slight.

At no time did the use of different photoperiods during rooting, or the excision of cotyledons alter significantly the uptake and distribution of radioactivity (Table 23).

When radioactively-labelled oestrogens were applied to the primary leaves of cuttings placed in distilled water, most of the label was retained in the primary leaves (Table 24). Unlike wick-feeding, ^{3}H was not more readily distributed than ^{14}C. The axillary buds again received comparatively more label than the other plant parts, and, additionally, the levels of radioactivity in the roots increased just prior to or during nodule development.

In cuttings which took up radiocarbon from ^{14}C-oestrone in rooting media or ^{14}C-oestrone applied to primary leaves, subsequent growth and development were unaffected. On the other hand, application of ^{14}C-oestrone to apices caused the growth habit of cuttings to be altered substantially, whereas the controls, treated with methanol or oestrone in methanol and untreated plants, displayed normal development (Table 25). The alterations at apices were manifested during development as malformed trifoliate leaves, highly developed axillary buds and aborted apices.

The excision of cotyledons from cuttings reduced significantly the number of roots produced in 8h and 24h days (see Table 26). Root mass, primary leaf mass and chlorophyll content of primary leaves all were reduced if the

Table 23. Effects of cotyledon excision and day-length during root formation on uptake and distribution of radioactivity (Bq) from ^{14}C-oestradiol in *P. vulgaris* hypocotyl cuttings

		day length (h)					
		8		16		24	
plant part	cotyledons...	+	−	+	−	+	−
roots and nodules		3418.3	1356.6	2679.8	1274.8	655.3	1989.2
hypocotyl		2623.9	6034.3	4357.7	5964.7	6514.9	5735.3
cotyledons		120.9		27.1		29.3	
cotyledonary buds		26.0	52.0	11.8	11.6	18.0	10.0
epicotyl		91.5	15.7	17.7	46.4	84.9	152.6
primary leaves		124.3	196.0	28.3	24.4	155.6	268.2
first axillary buds		97.2	93.9	11.7	9.3	3.8	2.7
internodes*		37.3	36.9	61.4	63.8	16.5	34.5
trifoliate leaves*		45.2	13.1	86.1	58.0	25.6	154.7
axillary buds*		48.6	12.6	107.4	62.6	11.3	29.1
apex		6.8	2.5	4.7	7.0	0	0
total		6640.0	7931.5	7393.7	7522.6	7515.2	8366.3

* totals for parts above primary leaf node

Table 24. Distribution of radioactivity (Bq) from ^{14}C-E_2 and ^{3}H-E_1-sulphate applied to primary leaves of *P. vulgaris* hypocotyl cuttings under continuous illumination

	Developmental stage of cutting					
	7 days in rooting medium		2nd trifoliate leaf opening		3rd trifoliate leaf opening	
Isotope ...	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Plant part						
Roots and nodules	1.8	2.5	6.0	34.3	173.3	206.7
Hypocotyl	1.4	6.5	3.3	14.0	8.0	10.2
Cotyledons	0.7	1.3	8.0	2.1	0.3	2.3
Buds at cotyledon node	2.5	0.4	12.3	1.3	6.3	1.7
Epicotyl	0.5	4.2	16.0	4.7	14.6	6.7
Primary leaves	9862.8	9897.7	9569.7	9587.2	8990.0	9615.0
1st axillary buds	2.4	19.6	15.6	50.2	5.7	32.3
*Internodes			25.3	36.7	5.2	15.9
*Trifoliate leaves			10.6	40.7	10.0	21.9
*Axillary buds			24.3	5.0	23.0	10.2
Apex	7.0	11.6	3.2	1.5	4.0	3.1
Total	9879.1	9943.8	9684.3	9777.7	9240.4	9926.0

* Totals for parts above primary leaves

Table 26. Effects of cotyledon excision and day-length employed during rooting period on *P. vulgaris* hypocotyl cuttings

Day length during rooting period	Cotyledons	Numbers of roots formed per cutting	Root mass (mg) per cutting	Primary leaf mass (mg) per cutting	Chlorophyll content* (µgChl per mg fresh weight per primary leaf pair)
Short days	+	26.7 ± 3.3	123.7 ± 15.6	536.6 ± 56.6	2.64 ± 0.10
	-	10.9 ± 2.3	94.9 ± 18.1	483.5 ± 15.9	1.90 ± 0.02
Long days	+	30.5 ± 5.6	199.9 ± 31.8	583.1 ± 51.6	3.34 ± 0.13
	-	28.7 ± 4.0	113.9 ± 16.3	513.2 ± 22.3	3.03 ± 0.14
Continuous	+	50.0 ± 3.2	242.2 ± 25.3	650.0 ± 34.1	4.57 ± 0.21
illumination	-	29.7 ± 1.9	223.8 ± 18.3	542.9 ± 17.8	3.61 ± 0.11

*Total chlorophyll = $25.5\ A_{650} + 4.0\ A_{665}$ ($g\ m^{-3}$) for extract in absolute methanol (Šesták, 1971)

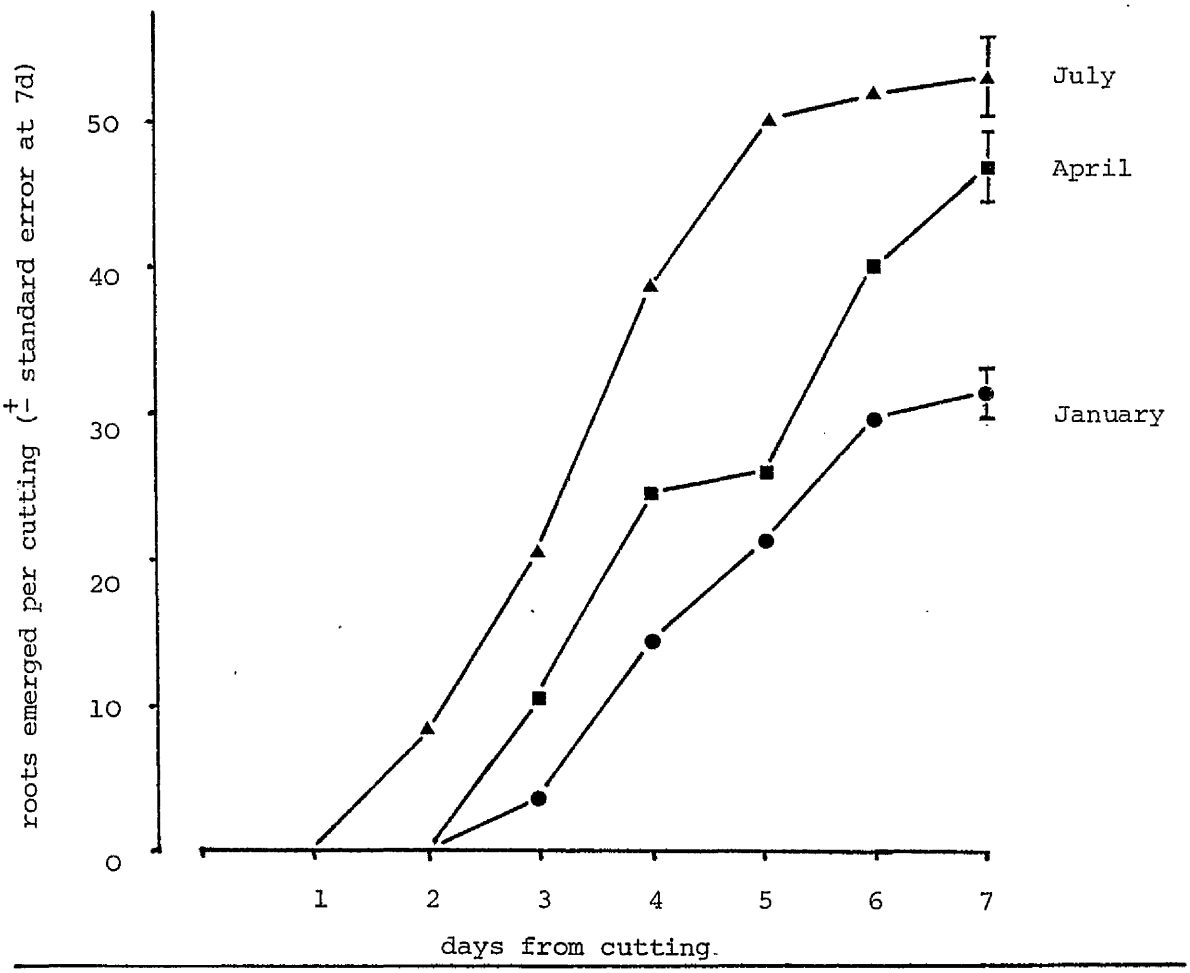
Table 25. Effects of three types of $^{14}C\text{-}E_1$ applications on growth (f.w. of specific plant parts as percentages of total plant mass at flowering) of *P. vulgaris* hypocotyl cuttings

	% total plant mass (fresh weight)			
	$^{14}C\text{-}E_1$ to apex	$^{14}C\text{-}E_1$ to primary leaves	$^{14}C\text{-}E_1$ to hypocotyl (wick feeding)	control (no treatment)
Apex	0.05	0.98	0.81	0.55
5th axillary bud	0.01	0.75	1.10	2.18
4th trifoliate leaf	11.93	0.48	0.78	0.88
5th internode	2.69	0.79	0.84	0.41
4th axillary bud	8.01	0.72	0.86	0.58
3rd trifoliate leaf	9.96	7.95	7.24	5.75
4th internode	1.62	1.31	1.05	1.33
3rd axillary bud	2.97	0.58	0.78	0.83
2nd trifoliate leaf	4.71	10.42	8.46	7.25
3rd internode	1.14	1.49	1.26	1.73
2nd axillary bud	16.85	0.77	1.73	0.76
1st trifoliate leaf	1.11	8.72	10.94	9.97
2nd internode	1.83	1.52	1.18	1.78
1st axillary bud	2.87	0.01	0.03	0.02
Primary leaves	3.57	12.66	12.25	17.17
Epicotyl	2.69	2.81	2.40	2.48
Cotyledonary buds	0.01	0.001	0.001	0.003
Cotyledons	1.04	0.32	0.46	0.55
Hypocotyl	4.61	7.69	6.75	7.38
Roots	18.35	31.20	33.14	28.34
Nodules	4.02	9.55	8.64	10.05
Plant mass (mg)	10304	11079	10272	9784

cotyledons were removed and the day length employed also affected these parameters, 24h days giving maximal and 8h days minimal levels in all.

Another recurring feature was the seasonal variation in rooting performance and time course of root formation (Fig. 17): more roots were

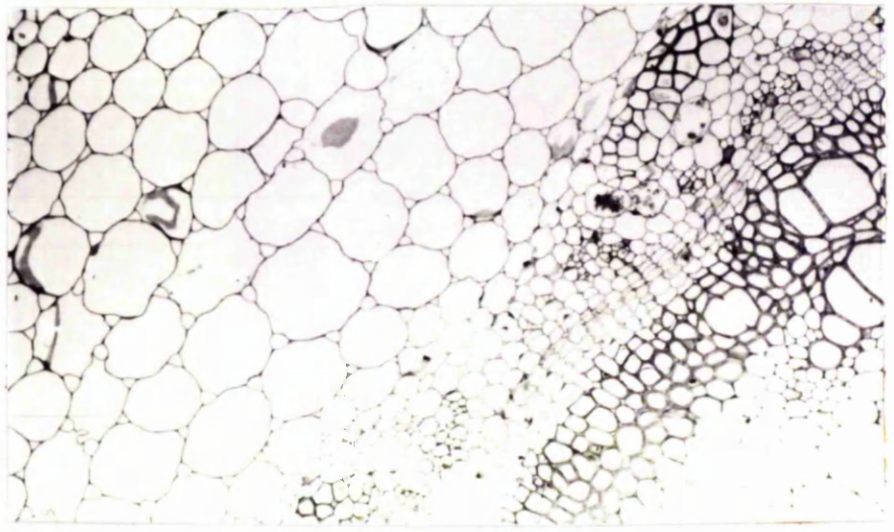
Fig. 17. Time course of root formation in *P. vulgaris* hypocotyl cuttings under continuous illumination. Seasonal variation

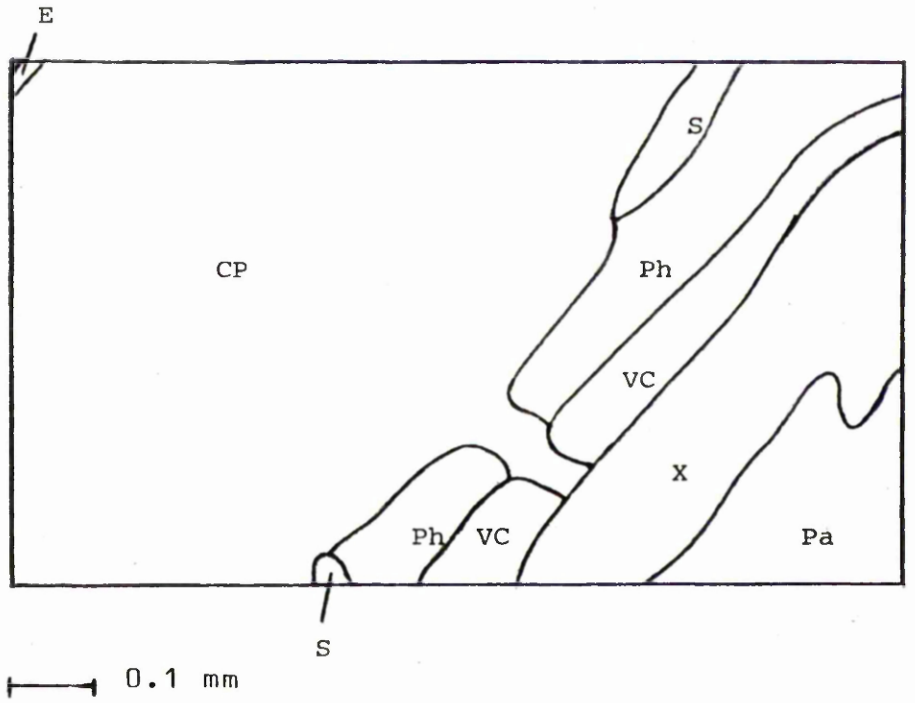


formed in a shorter time in summer than in winter.

The anatomical aspects of root initiation were studied using light microscopy. In the immature *Phaseolus* hypocotyl the vascular tissue is a ring composed of four arcs separated by four narrow gaps (Plate 1). As the hypocotyl matures the xylem becomes continuous and the vascular cambia are linked. The four regions of phloem, however, remain discrete and no sclerenchyma is found overlying the gaps. These gaps were the sites of adventitious root formation. The first stage of root initiation

Plate 1. T.S. hypocotyl





E epidermis

CP cortical parenchyma

S sclerenchyma

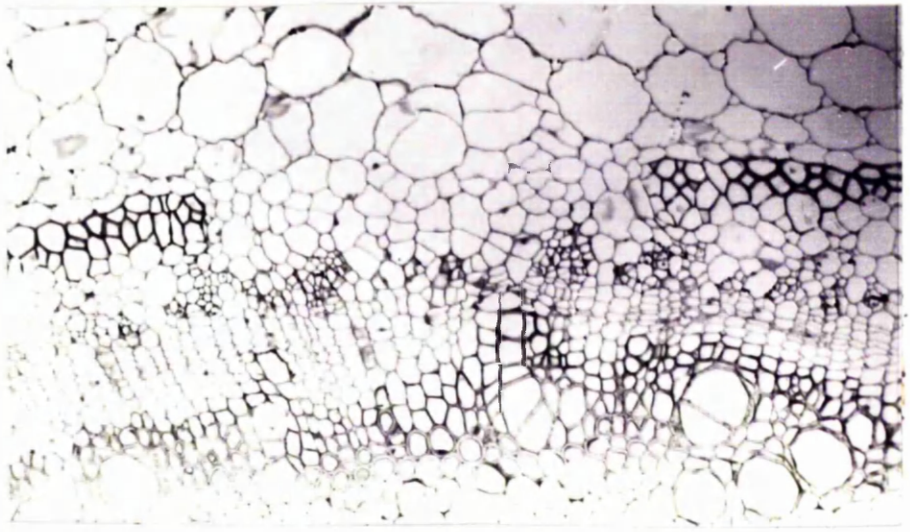
Ph phloem (containing parenchymatous cells)

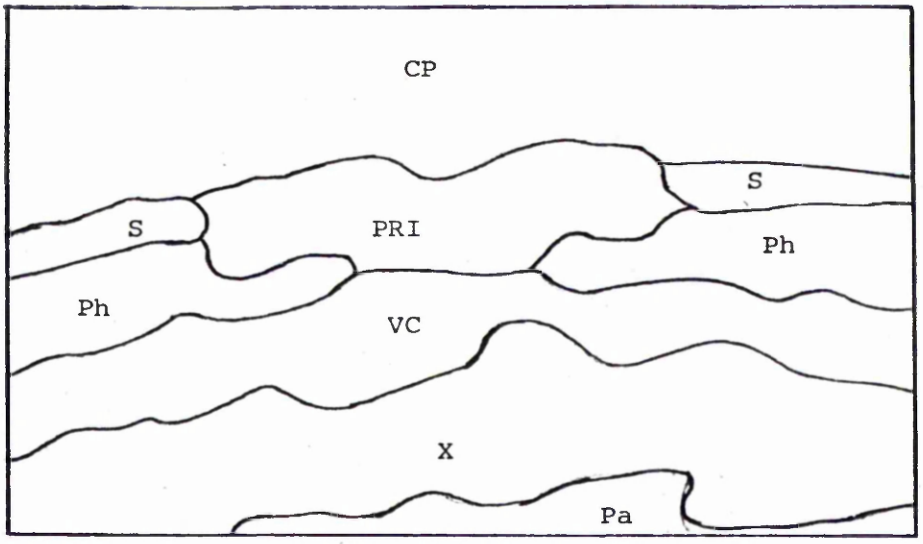
VC vascular cambium

X xylem (vessels and tracheids)

Pa parenchyma (pith)

Plate 2. T.S. hypocotyl : preformed root initial

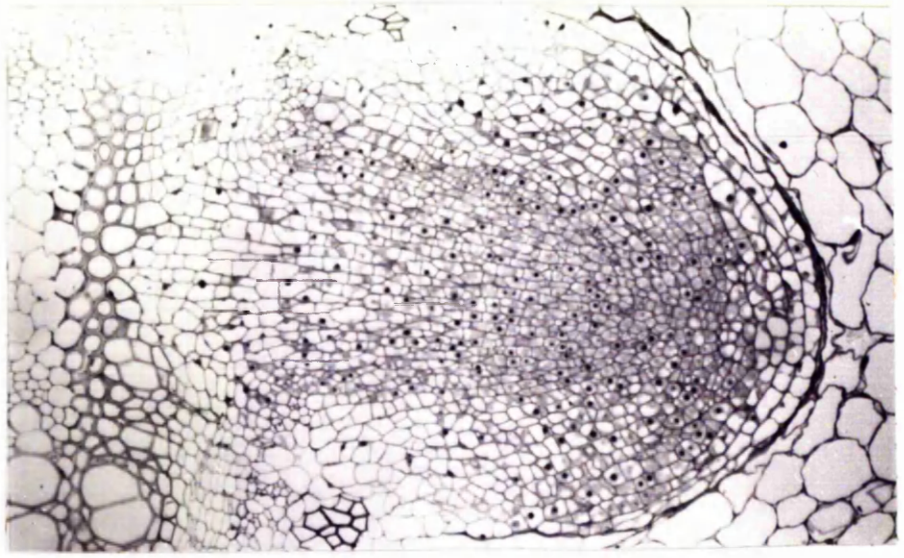


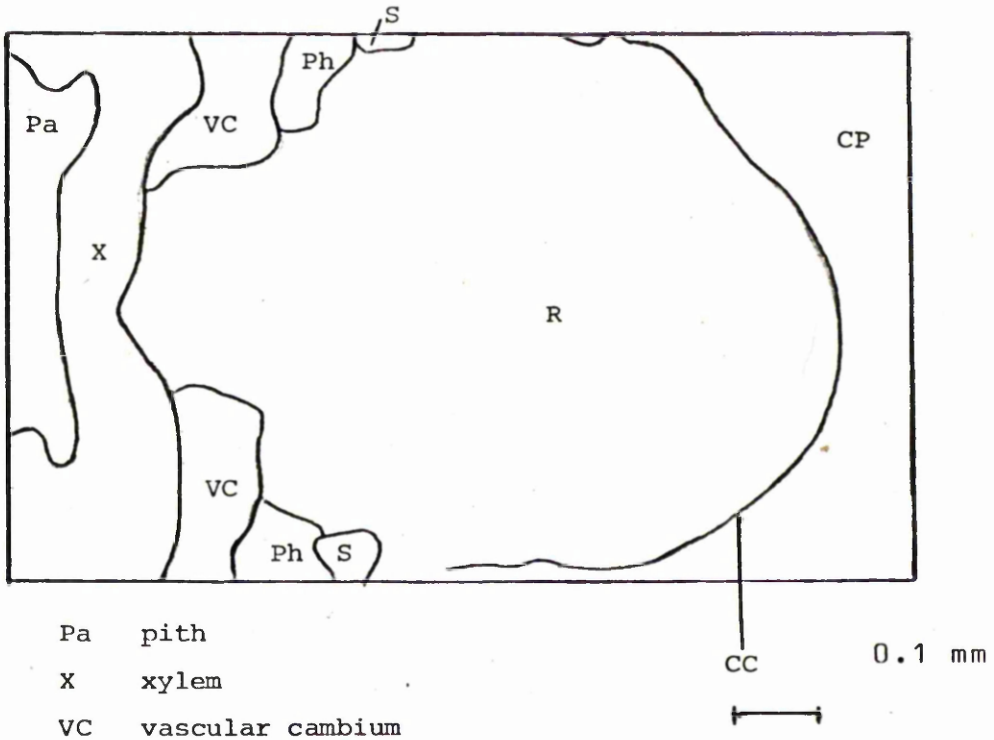


0.1 mm

CP cortical parenchyma
S sclerenchyma
PRI region containing preformed root initial
Ph phloem
VC vascular cambium
X xylem
Pa pith

Plate 3. T.S. hypocotyl : root formation





Pa	pith
X	xylem
VC	vascular cambium
Ph	phloem
S	sclerenchyma
R	root
CP	cortical parenchyma
CC	cells crushed by developing root

involved meristem formation, and this took place by division of the cortical parenchyma cells immediately centrifugal to the vascular cambium. Examination of sections from intact 9d old hypocotyls revealed that root initials were present *in vivo* (Plate 2). These can be observed in older plants as bumps on the hypocotyl and they commonly develop as adventitious roots (Plate 3) as the plant matures if they are beneath the soil surface.

The possibility that development of preformed initials had accounted for the roots produced by 0.1mol m^{-3} oestrogen-sulphate-treated hypocotyls was investigated using *P. vulgaris* epicotyls and primary leaf cuttings, both of which root readily but do not contain preformed root initials

b) Effect of steroidal oestrogens on adventitious root initiation in *P. vulgaris* epicotyl cuttings

Table 27 catalogues the effect of steroidal oestrogens on the numbers of roots produced on epicotyl cuttings maintained under continuous illumination for 10 days. Oestrone, oestrone-phosphate and oestradiol

Table 27. Numbers of roots formed on *P. vulgaris* epicotyl cuttings in oestrogen solutions during 10d under continuous illumination

	concentration (mol m^{-3})			
treatment	0.1	10^{-3}	10^{-5}	10^{-7}
oestrone		46.0±8.9	59.5±2.4	52.6±7.0
oestradiol		52.8±7.3	41.2±4.5	54.8±6.8
control		47.0±4.8	54.2±6.6	48.4±3.0
distilled water		53.6±8.9		
oestrone-phosphate	53.5±5.8	62.0±3.5	39.6±5.1	33.6±3.4
Na_2HPO_4	32.2±7.0	47.4±9.4	28.2±2.9	42.6±6.2
oestrone-sulphate	0.8±0.4	49.8±6.3	69.4±6.3	55.0±4.5
oestradiol-sulphate	0.2±0.2	49.1±2.1	43.4±2.5	52.6±7.4
Na_2SO_4	43.0±3.9	40.3±3.5	28.2±2.9	44.6±8.7

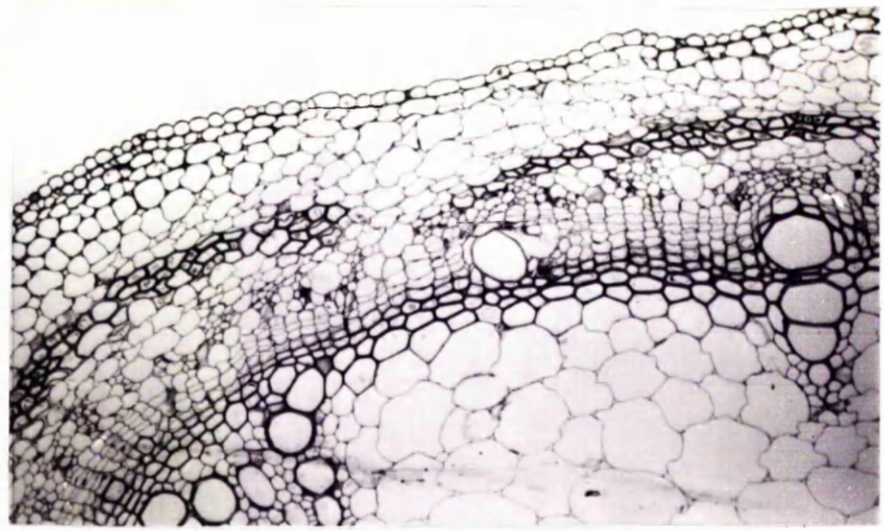
Numbers are roots formed ± standard error

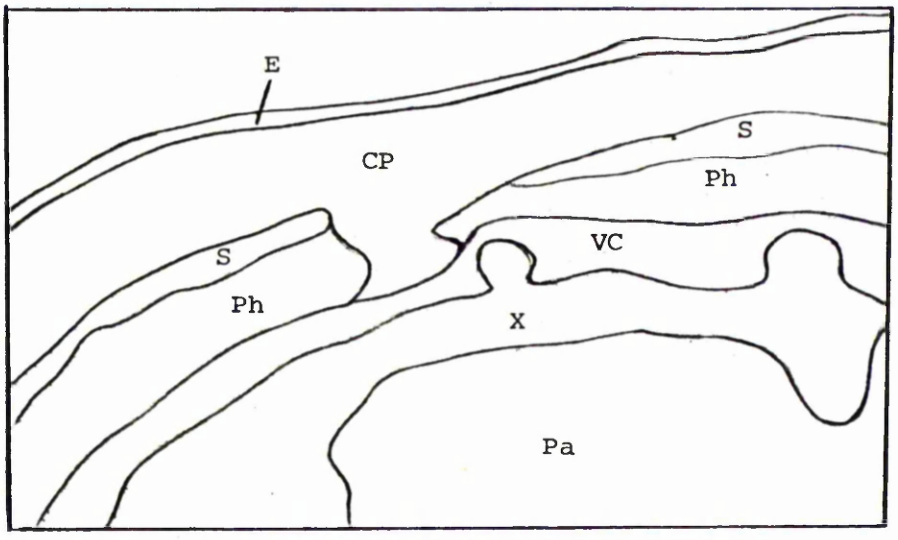
in the rooting medium were without effect over the concentration ranges investigated, but 0.1mol m^{-3} oestrogen-sulphates inhibited root formation almost completely. In these treatments a marked incurling of the young

Table 28. Distribution of radioactivity (Bq) taken up from 10000Bq ^{14}C-E_2 and ^{3}H-E_1-sulphate solutions by *P. vulgaris* epicotyl cuttings under continuous illumination

Time (h)	1		2		3		4		5		10		20	
Radioisotope	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Epicotyl	328.2	141.1	562.9	280.8	1024.6	199.6	1403.5	149.0	1373.0	320.5	5069.1	479.0	3473.9	379.6
Primary leaves	19.2	608.2	39.6	1796.0	91.5	1205.8	128.8	1304.0	119.0	1841.5	494.9	3751.7	474.0	6833.7
First axillary buds	0.4	0.4	0.8	12.5	1.5	44.1	1.2	9.2	1.2	5.5	15.8	1.0	10.6	19.4
Second internode	0.2	21.2	4.5	53.1	10.6	54.0	10.2	110.8	9.8	2.5	38.4	173.2	51.9	7.5
First trifoliate	0.0	52.0	2.4	131.9	4.8	102.5	5.5	263.0	4.5	258.5	10.4	174.3	13.2	502.1
Second axillary bud	0.0	4.4	0.6	27.3	1.5	22.5	1.2	0.7	1.2	13.9	0.3	1.5	0.8	29.8
Apex	1.2	10.4	0.8	20.6	1.7	29.7	1.2	0.7	2.1	27.8	1.6	33.1	0.8	51.4

Plate 4. T.S. epicotyl

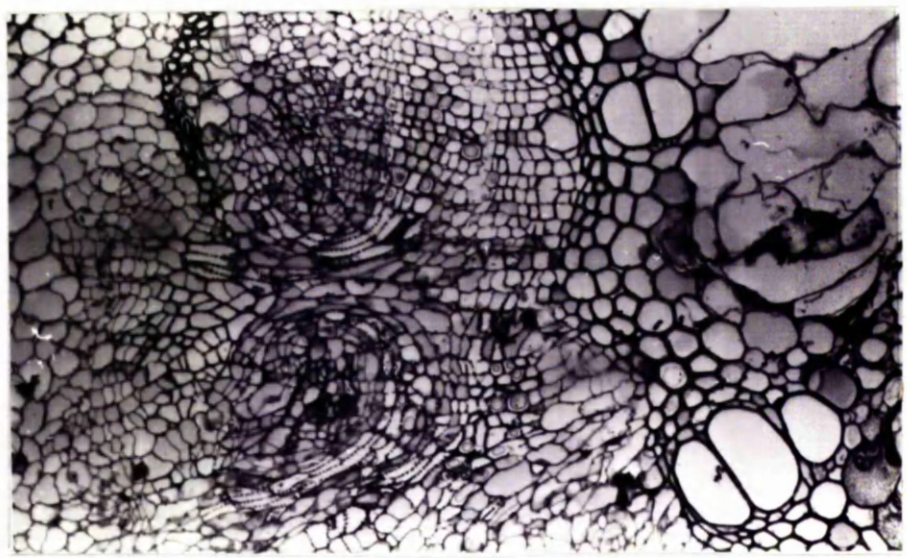


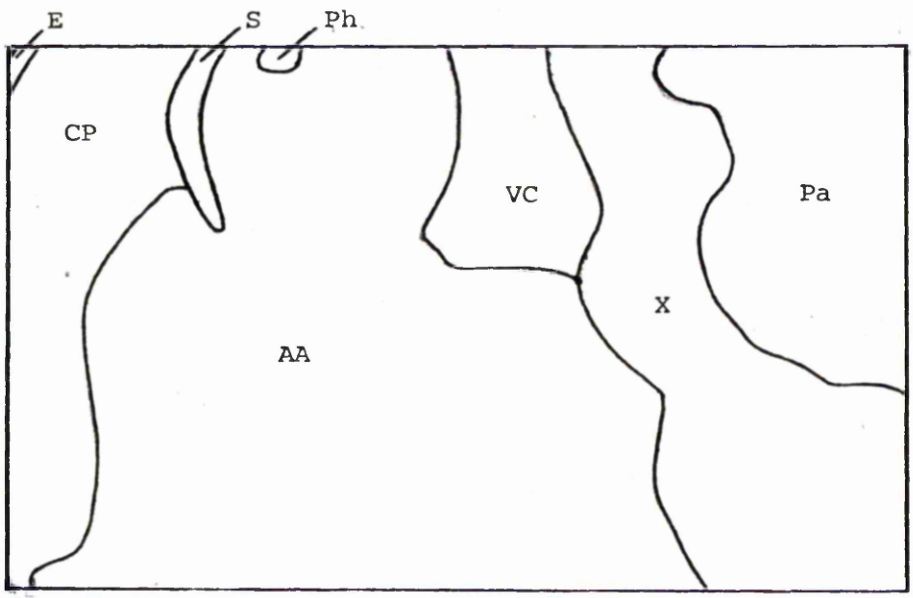


0.1 mm

E epidermis
CP cortical parenchyma
S sclerenchyma
Ph phloem
VC vascular cambium
X xylem
Pa pith

Plate 5. T.S. epicotyl : effect of oestrogen-sulphate treatments





0.1 mm

E epidermis

CP cortical parenchyma

S sclerenchym

Ph phloem

AA affected area

VC vascular cambium

X xylem

Pa pith

trifoliate leaf was evident within 24h, and axillary bud growth and abscission layer formation also occurred in most of the cuttings within the rooting period.

The uptake and distribution of radioactivity from labelled oestrogens was monitored over the first 20h incubation (Table 28). In essence, the distribution patterns were similar to those found in hypocotyl cuttings: little translocation of ^{14}C from ^{14}C-oestradiol occurred, most being retained by the cutting base, whereas the distribution of tritium from ^{3}H-oestrone-sulphate away from the epicotyl was much greater.

The anatomy of root formation in epicotyl cuttings was similar to that in hypocotyls in that root initials were formed by dedifferentiation of cortical parenchyma cells in the gaps separating regions of phloem (Plate 4). In epicotyls, however, these gaps are more numerous and give the emergence of roots a random appearance in contrast with the neatly ordered four rows in hypocotyl cuttings. In 0.1mol m^{-3} oestrogen-sulphate treated epicotyls, the formation of meristematic zones in the gaps in the phloem did not occur (Plate 5). Rather, cell divisions occurred mainly within the phloem, but there was no ordering apparent in the process and a callus-like tissue was formed.

c) Effect of steroidal oestrogens on adventitious root initiation in *P. vulgaris* primary leaf explants.

Treatment with 0.1mol m^{-3} oestrogen sulphates resulted in an almost total inhibition of root formation in primary leaf cuttings (Table 29). The presence of lower concentrations did not affect root initiation and the other oestrogens used were also without influence on the process. None of the treatments employed caused any alterations in the chlorophyll content of primary leaves and neither the leaf size not the chlorophyll content seemed to affect consistently the numbers of roots produced (Table 30).

The uptake and distribution of radiolabel from ^{14}C-oestradiol and ^{3}H-oestrone-sulphate solution by primary leaf cuttings over 20h is presented in Table 31. As in the other cuttings, translocation of radio-activity was greater with ^{3}H-oestrone-sulphate than ^{14}C-oestradiol.

Table 29. Numbers of roots formed on *P. vulgaris* primary leaf cuttings in oestrogen solutions during 10d under continuous illumination

treatment	concentration (mol m^{-3})			
	0.1	10^{-3}	10^{-5}	10^{-7}
oestrone		43.6 ± 5.7	41.8 ± 4.2	42.0 ± 5.8
oestradiol		44.6 ± 3.0	33.8 ± 7.5	46.0 ± 2.8
control		35.0 ± 8.7	43.8 ± 5.8	41.3 ± 3.4
distilled water		37.4 ± 2.2		
oestrone-phosphate	42.8 ± 3.6	40.5 ± 7.6	44.4 ± 6.3	44.5 ± 6.9
Na_2HPO_4	40.0 ± 4.3	31.8 ± 7.8	38.0 ± 8.8	46.5 ± 4.3
oestrone-sulphate	1.4 ± 0.8	38.6 ± 5.3	38.4 ± 3.7	36.9 ± 2.5
oestradiol-sulphate	1.2 ± 0.8	39.3 ± 4.8	42.0 ± 7.1	35.6 ± 2.5
Na_2SO_4	44.6 ± 6.4	34.1 ± 5.2	38.7 ± 3.8	39.4 ± 5.9

Numbers are roots formed $\pm$ standard error

Anatomically root initiation in primary leaf petiole cuttings was associated with vascular bundles (Plate 6), either two adventitious root initials arising from the sides of a large bundle or a single root from the centre of a small bundle. In 0.1mol m^{-3} oestrogen-sulphate-treated petioles (Plate 7) cell divisions occurred at the sides of vascular cambia, but rather than giving rise to root primordia (the normal course of events), the line of meristematic activity often extended, encompassing the phloem and sclerenchymatous tissues. Similar cell divisions in the parenchyma adjacent to the sclerenchyma cap of bundles also occurred in normal root development, but the meristematic activity was not continuous with the root initial itself, the divided cells tending to expand greatly then break down, providing a path for root emergence. This aspect was unexpressed in the inhibited cuttings. The majority of cellular proliferations in 0.1mol m^{-3} oestrogen-sulphate-treated petioles took place in the phloem with regions of cells resembling stacked xylem parenchyma apparent within the resultant callus-like tissue (Plate 7).

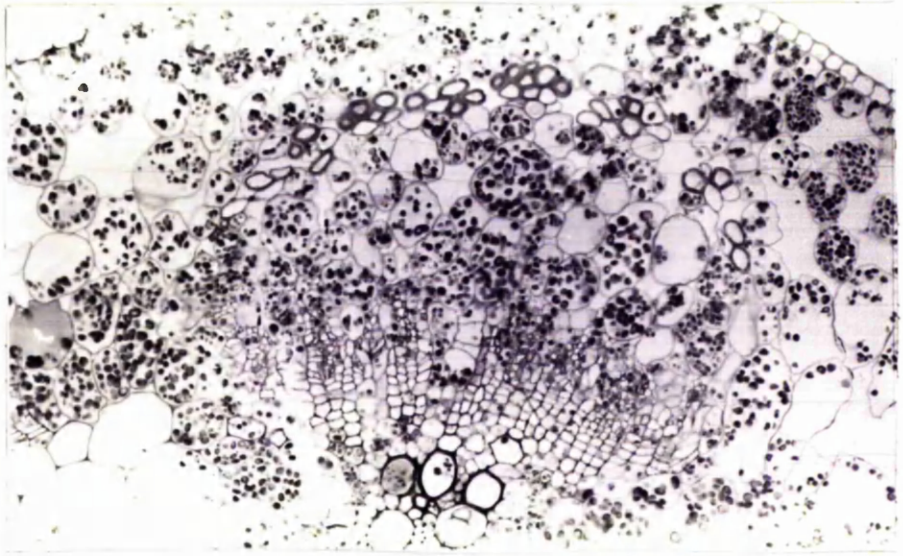
Table 30. Root formation on *P. vulgaris* primary leaf cuttings as related to leaf size and chlorophyll content

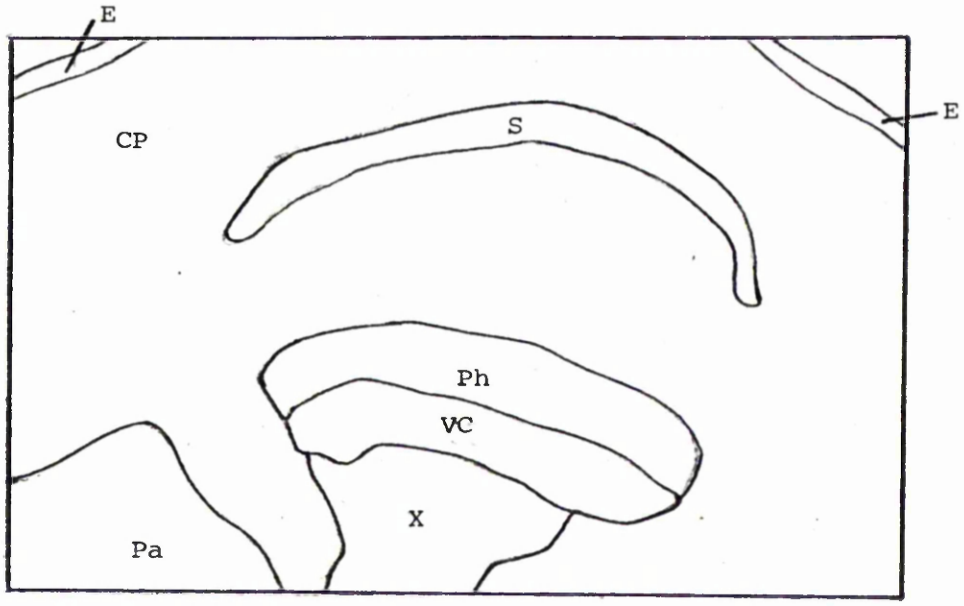
leaf size (mg)		number of leaves	number of roots ± standard error
fresh weight	700	8	19.0±3.53
	701-800	17	39.0±6.12
	801-900	21	24.2±3.78
	901-1000	19	29.7±3.84
	1001-1100	22	31.8±5.38
	1101-1200	11	39.8±6.70
	1200	1	32
dry weight	100	9	31.6±8.91
	101-140	49	31.0±2.80
	141-180	23	34.3±5.25
	181-220	13	29.7±5.32
	221-260	5	22.0±8.42
	260	1	9
chlorophyll (µg)	600	6	24.0±5.38
	601-800	22	42.3±4.89
	801-1000	24	33.1±5.17
	1001-1200	27	26.6±3.48
	1201-1400	15	21.1±4.28
	1400	6	20.0±6.45

Table 31. Distribution of radioactivity (Bq) taken up from 10000Bq ^{14}C-E_2 and ^{3}H-E_1-sulphate solutions by *P. vulgaris* primary leaf cuttings under continuous illumination

Time (h)	1		2		3		4		5		10		20	
Radioisotope	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Petiole	209.9	147.1	315.5	1222.6	812.4	119.8	695.9	142.5	1099.0	165.2	2277.4	279.4	4486.5	466.9
Pulvinus	0.9	10.2	0.3	267.7	11.3	7.1	3.7	11.1	3.2	11.1	44.0	4.9	12.8	31.3
Lamina	18.5	440.2	16.2	670.8	119.3	1142.5	55.3	1647.9	102.2	2028.5	269.2	2914.1	1151.9	5480.3

Plate 6. T.S. petiole : vascular bundle

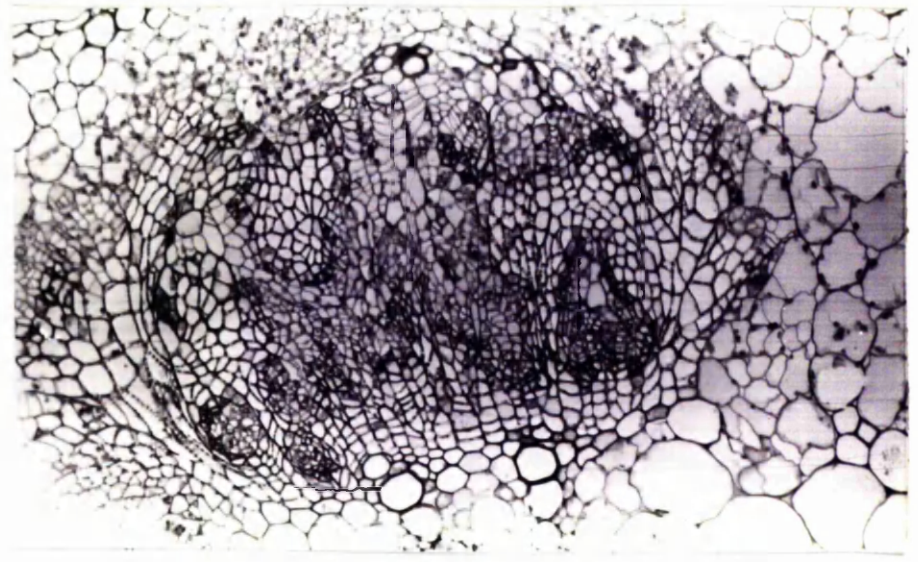


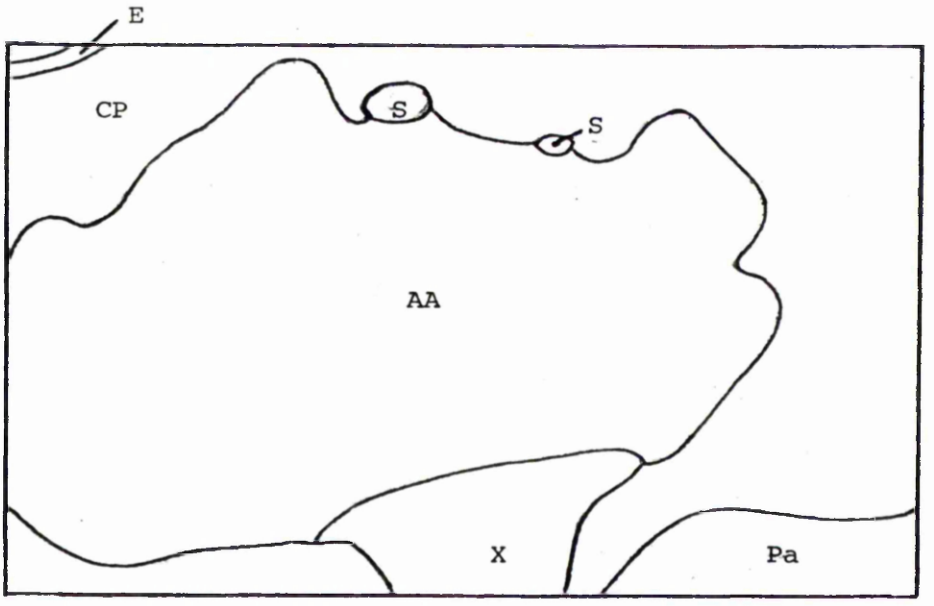


0.1 mm

E epidermis
CP cortical parenchyma
S sclerenchyma
Ph phloem
VC vascular cambium
X xylem
Pa pith

Plate 7. T.S. petiole : effect of oestrogen-sulphate treatments





E epidermis

CP cortical parenchyma

S sclerenchyma

AA affected area

X xylem

Pa pith

0.1 mm

In the course of preliminary experiments to assess the rooting performances of primary leaf explants it was noted that the presence of the proximal petiolar pulvinus (PP) generally inhibited root formation (Table 32). After 10d very few of the cuttings with intact PP had formed

Table 32. Root formation on primary leaf cuttings with and without petiolar pulvini

	plants rooted	roots per plant
+PP	5	2.2 ± 0.6
-PP	50	44.5 ± 3.1

roots, and in these the number produced was generally small. Microscopical examinations revealed that root initiation had commenced in some of the other cuttings with PP (up to 30 per cent) towards the end of the rooting period. The anatomical aspects of root initiation in cuttings with or without PP were identical. However, a major difference between the cuttings was visible within 24h : in most cuttings with PP the laminae exhibited severe desiccation and, although some recovery was made thereafter, generally only that part of the blade proximal to the petiole regained turgidity. The extent of recovery did not seem to influence the number of roots produced on those cuttings which formed roots. When cuttings with PP were placed in the rooting medium, the PP soon became fully turgid and the distal pulvini (DP), became flaccid within 20h, causing the blade to droop, Studies on the uptake of tritiated water during the first 24h after cutting showed that the ^{3}H-content of laminae was substantially reduced when PP were present (Table 33) and, using the Milburn porometer (Milburn, 1979), leaf porosity (a measure of relative stomatal apertures) was found to have a circadian rhythm (Fig.18).

Fig.18. Leaf porosity of primary leaf explants during 24h from cutting

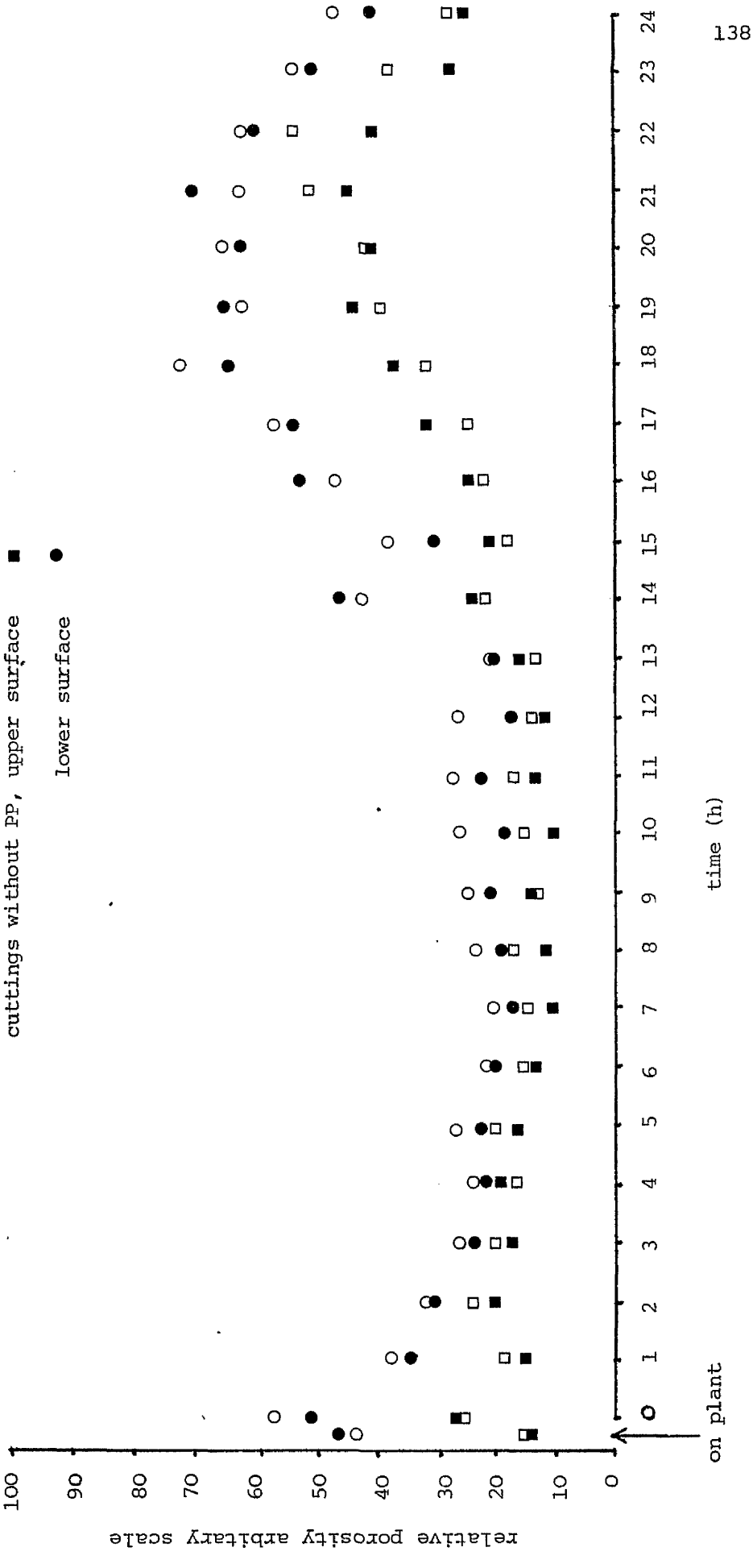


Table 33. Uptake and Distribution of ^{3}H (Bq) by *P. vulgaris* primary leaf explants with and without proximal petiolar pulvini

Time (h)	1	2	3	4	5	10	20
Plant part							
petiole	4974	2740	4148	3095	3657	3587	3321
distal pulvinis	64	168	413	199	239	316	417
lamina	760	1889	3563	3328	3871	8303	11452
solution	93802	91655	94686	92179	94132	84715	83051
proximal pulvinus	457	522	536	727	699	642	506
petiole	1966	3279	2636	2916	2583	3385	2577
distal pulvinus	35	194	143	229	342	354	103
lamina	375	2172	1247	3403	5181	4897	4338
solution	92898	96976	96487	86386	90204	84421	85886

*c.*100000 Bq $^{3}H_2O$ per explant

4. Internode extension

As well as the aforementioned experiments on growth and flowering, which were a continuation of other work with respect to oestrogens, specific studies on the effects of oestrogens on internode extension were carried out. Application of oestrogens to apices or primary leaves of 10- to 15- day old seedlings did not affect the final internode sizes (Table 34) or the numbers of flowers produced (Table 35). The sole significant and repeatable effect was the inhibition of epicotyl growth in seedlings which had foliar applications of methanol or oestrogens in methanol.

The recurrent feature of inconsistent growth patterns was also evident here: in Table 36, the internode lengths from three batches of seeds, all planted within a two week period and grown in the heated greenhouses, are given. That these differences were statistically significant gave cause for concern and highlighted the absolute requirement for adequate controls in each experiment.

Table 34. Effects of oestrogen applications to 12d old *P. vulgaris* seedlings on growth (final internode lengths at flowering) in 16h days

site of application	n	treatment	hypocotyl	internode lengths (mm) 1	2	3	4	5	total
apex	10	10µg oestrone	56.1±1.39	45.7±1.43	56.8±5.43	40.9±2.56	57.5±3.82	39.9±4.49	289.0±12.48
	10	10µg oestradiol	57.9±1.21	46.0±1.82	55.8±5.00	41.5±2.04	57.5±2.26	43.1±4.41	295.0±10.18
	10	10mm^3 methanol	56.0±1.45	43.2±1.60	54.2±3.72	40.7±2.48	60.0±3.70	43.2±5.27	294.8±14.30
	10	no treatment	58.6±1.11	43.1±1.32	57.4±3.38	42.5±2.03	58.8±2.79	37.1±3.63	300.3±10.61
primary leaf	39	10µg oestrone	61.1±1.57	50.5±1.23	60.0±2.58	49.0±2.21	38.2±1.67	85.2±5.65	407.3±12.08
	45	10mm^3 methanol	60.3±1.13	56.2±1.53	56.8±2.35	47.7±1.54	49.2±1.78	90.4±3.95	423.2± 8.20
	80	10µg oestradiol	73.8±1.34	72.0±1.59	81.9±1.95	48.2±1.27	58.9±1.95	72.1±2.58	411.5± 6.05
	33	10mm^3 methanol	72.2±1.72	66.2±2.05	79.6±3.22	45.2±2.52	57.8±4.27	75.5±4.74	402.6±11.35
	41	no treatment	74.4±1.85	78.8±2.78	82.0±2.15	44.1±2.04	57.2±2.31	68.9±3.24	415.5± 8.10

The sums of means do not equate to the [illegible] totals as there were plants with more or less than 5 internodes

Table 35. Effects of oestrogen applications to 12d old *P. vulgaris* seedlings on flower production in 16h days

site of application	n	treatment	node					
			3	4	5	6	Apex	total
apex	10	10µg oestrone	1.6±0.24	3.6±0.56	2.9±0.39	1.1±0.20	3.0±0.47	12.3±1.26
	10	10µg oestradiol	2.2±0.29	2.8±0.36	2.2±0.20	1.2±0.33	2.4±0.31	10.8±0.70
	10	10mm^3 methanol	1.7±0.30	2.8±0.42	1.7±0.21	1.5±0.34	2.4±0.40	10.1±0.55
	10	no treatment	2.3±0.37	2.2±0.25	2.1±0.23	1.4±0.37	2.0±0.42	10.0±0.49
primary leaf	39	10µg oestrone	1.8±0.15	2.3±0.16	2.2±0.17	1.5±0.13	2.0±0.15	10.9±0.56
	45	10mm^3 methanol	1.2±0.15	1.9±0.14	2.3±0.13	1.3±0.11	2.2±0.13	10.2±0.53
	80	10µg oestradiol	2.9±0.18	1.6±0.10	1.6±0.12	1.3±0.15	1.7±0.2	9.7±0.38
	33	10mm^3 methanol	3.9±0.32	1.7±0.15	1.8±0.22	1.4±0.22	1.4±0.25	10.8±0.51
	41	no treatment	2.6±0.25	1.4±0.13	1.4±0.14	1.3±0.15	1.7±0.22	9.2±0.49

Summation of mean flower numbers does not equate to the total flower numbers as several plants had other than 6 nodes

Table 36. Comparisons of internode lengths in different batches of *P. vulgaris* plants

			internode lengths (mm)					
date of planting	n	hypocotyl	epicotyl	2	3	4	5	total
3rd May 1978	33	72.2 ± 1.72	66.2 ± 2.05	79.6 ± 3.22	45.2 ± 2.52	57.8 ± 4.27	75.5 ± 4.74	402.6 ± 11.35
12th May 1978	41	59.1 ± 0.85	48.6 ± 1.12	56.6 ± 2.14	59.1 ± 1.70	83.6 ± 4.36	78.9 ± 3.69	454.5 ± 10.50
15th May 1978	45	60.3 ± 1.13	56.2 ± 1.53	56.8 ± 2.35	47.7 ± 1.54	49.2 ± 1.78	90.4 ± 3.95	423.2 ± 8.20

Summation of mean internode lengths does not equate to the total as several plants had other than 5 internodes

Table 37. Short term distribution of radioactivity (Bq) from *c.* 10,000 Bq ^{14}C-oestradiol and ^{3}H-oestrone-sulphate applied to primary leaves of 12d-old seedlings under continuous illumination

plant part	isotope...	Time (h) 1 ^{14}C	1 ^{3}H	5 ^{14}C	5 ^{3}H	15 ^{14}C	15 ^{3}H
apex		0.4	2.6	2.4	1.2	2.0	2.2
2nd axillary bud		0.5	1.3	0.4	6.0	0.5	1.5
1st trifoliate		0.4	10.9	0.5	6.2	0.7	8.9
2nd internode		1.2	7.8	0.8	9.2	1.1	2.0
1st axillary bud		0.3	2.2	0.5	2.0	0.5	2.1
primary leaf lamina		19.9	0	1.3	77.4	1.0	24.4
primary leaf petiole		0.2	0.4	0.4	4.0	0.3	0.6
1st axillary bud		0.6	3.0	1.3	5.7	0.7	2.1
primary leaf lamina		10354.2	9423.5	9800.4	9645.8	9859.8	9998.2
primary leaf petiole		0.5	10.9	0.6	6.5	2.0	2.0
epicotyl		0.6	11.3	0.8	4.6	2.0	1.2
cotyledonary buds		0.5	3.9	0.5	3.4	1.3	2.1
cotyledons		0	3.9	0	2.5	2.6	1.5
hypocotyl		1.3	4.8	1.8	4.0	2.1	3.3
roots		2.4	10.9	6.8	6.8	5.7	2.4

The uptake and distribution of radioactivity from labelled oestrogens applied to primary leaves were studied in the short term (Table 37) and the long term (Table 38). The results were similar to those obtained with foliar applications to cuttings: ^{3}H was not more readily transported than ^{14}C, and the amounts of labels translocated away from the site of application were very small.

5. Identification of Oestrogens

In the three extractions performed, significant ions were detected at m/e 416 (the molecular ion of the bis-TMSi derivative of oestradiol), m/e 342 (molecular ion of oestrone-TMSi) and m/e 285 (base ion of oestrone- and oestradiol-TMSi). Selective ion current monitoring also indicated the presence of these ions at the correct retention values.

Table 38. Distribution of radioactivity (Bq) from *c* 10,000Bq ^{14}C-oestrone and ^{3}H-oestrone-sulphate applied to primary leaves of 9d old *P. vulgaris* seedlings under 16h photoperiods

	Developmental stage of plant					
	first trifoliate leaf opening		third trifoliate leaf opening		flowering	
Isotope....	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Plant part						
Roots and nodules	8.9	5.6	10.2	2.9	9.3	4.5
Hypocotyl	0.1	0.6	1.2	0.5	0.9	0.6
Cotyledons	0.1	1.1	0.1	0.3	0.2	0.2
Cotyledonary buds	0.1	2.0	0	0.1	0.1	0.1
Epicotyl	1.8	0.3	0.3	0.1	0.8	0.3
Primary leaves	10175.6	9803.2	9998.7	10398.4	9639.3	9932.0
First axillary buds	4.6	2.3	6.6	11.8	1.3	6.6
*Internodes	1.7	2.0	5.0	6.8	3.8	8.4
*Trifoliate leaves	2.2	1.6	14.0	22.0	9.1	5.1
*Axillary buds	0.8	0.7	2.2	1.3	0.8	2.8
Apex	0.5	0.2	0.3	0.7	0.1	1.0
Total (Bq)	10196.4	9819.6	10038.6	10442.9	9665.7	9961.6

*Totals for parts above primary leaves

DISCUSSION

The results of the experiments with steroidal oestrogens and the more general observations on the plant material are discussed in separate sections. These cover senescence, germination, root initiation, growth and flowering.

1. Senescence The steroidal oestrogens tested, oestrone, oestradiol and oestradiol-sulphate, did not affect chlorophyll levels during senescence of *Phaseolus* leaf discs (Table 6). Some significant differences were found between treatments and controls, but these were not consistent, probably reflecting the varied nature of pigment loss in bean leaves. Leaves on whole plants did not lose colour evenly during natural senescence, and yellowing in leaf discs was rarely uniform. This was to be expected as senescence is an asynchronous process in many species (e.g. Moore, 1965: Treffry *et al.*, 1967: Ragetli *et al.*, 1970) and different degrees of degeneration can occur between cells in freshly excised material (Colquhoun *et al.*, 1975). The ineffectiveness of oestrogens on chlorophyll loss could not be ascribed to lack of uptake. Radioactivity from ^{14}C-oestrone, ^{14}C-oestradiol and ^{3}H-oestrone-sulphate was taken up by discs within 30min (Figs.12 and 13). This in itself did not indicate cellular uptake of oestrogens, but the continued ingression of radioactivity after water uptake had almost ceased, the retention of radioactivity after exhaustive extractions (Table 9) and the recovery of the applied substances and metabolites from discs (Fig.14) strongly suggested that oestrogens had entered cells. The initial rapid uptake was simultaneous with the rapid uptake of water (Figs.12 and 13) whereas the continued increases in radioactivity present in discs were more probably due to the concentration gradient between cells and solution. This could have been maintained by compartmentation (possibly into membranes) or metabolism of the applied substances, although binding, e.g. to cell walls, could have accounted for some of the continued uptake, particularly with oestrone and oestradiol which come out of solution readily.

Oestrogens previously had been found to affect chlorophyll levels in several species (Table 2c) but no consistent pattern emerged. This study, the first in *Phaseolus*, does not clarify the situation: if indeed oestrogens

have a role in chlorophyll metabolism, endogenous levels may already have been sufficient for the maximal response, or the exogenous substances did not penetrate to the appropriate sites. The other components of senescence potentially affected by oestrogens (see Tables 2c and 3a) were not investigated. However, some degree of parallelism in steroid function between plants and animals has been suggested (e.g. Grunwald, 1975) and similar oestrogen-induced enhancements of RNA, protein and phospholipid productions in plants to those occurring in animals clearly would be of great importance here. Sterols are known to be constituents of plant membranes (Hartmann *et al.*, 1975) and the structural configuration of steroidal oestrogens complies with the steric requirements for sterols as membrane components (Grunwald, 1978). Moreover, a number of synthetic oestrogens and naturally occurring non-steroidal oestrogens are known to affect plant membrane properties (see Table 4). Therefore, bearing in mind the highly membranous nature of chloroplasts, links between oestrogens and senescence cannot yet be ruled out.

Also, before these results on chlorophyll levels can be viewed with regard to senescence as a whole, the relevance of the assay system selected should be established. Chlorophyll loss, visibly the most obvious aspect of senescence and also the most frequently studied, is only part of a complex phenomenon which involves declining RNA and protein levels, phospholipid loss from membranes and ultra-structural changes in cell and organelle membranes (Colquhoun, 1974). These events, and the changes which may occur in plant hormone levels (Wareing and Phillips, 1978) are often assumed to take place simultaneously. There are, however, anomalies e.g. the NY mutant of *Festuca pratensis* (Thomas and Stoddart, 1975), which may demonstrate that the various processes comprising senescence are not intrinsically linked although usually closely related in a temporal sense. The situation is further complicated if protein levels are considered: while total protein declines, levels of specific enzyme activities can increase. Furthermore, the reductions in RNA, chlorophyll and phospholipids need not be due to increases in rates of breakdown as decreases in rates of synthesis coupled with normal or even decreased degradation rates would have the same net effect. Thus, senescence is not the simple process outward appearances present and any experimentation must be approached and interpreted carefully.

Reductions in chlorophyll levels can be used as a valid index of senescence, as can any of the others, so long as the limitations of each assay are borne in mind, especially when only one parameter is investigated. The main advantage of using chlorophyll levels is the simplicity of the assay. However, chlorophyll loss from the plant studied must be characterised: the two types present in higher plants, chlorophylls *a* and *b*, are known to be degraded at different rates in some species (Wolf, 1956) and this could affect results where total chlorophyll levels are calculated, masking more subtle alterations in the green pigments. Using the scanning spectrophotometer, it was possible to estimate the chlorophyll *a:b* ratio in an extract without separating the compounds.

The use of isolated leaves and more especially leaf discs permits a greater degree of control in experiments involving exogenous substances. It must be remembered though that cutting itself induces senescence (e.g. Colquhoun *et al.*, 1975) and this condition must be conpared with that in naturally senescent tissues from whole plants to allow valid interpretations of results.

A recurring feature throughout all the experimentation was the variability in *Phaseolus*. Here, considerable differences in chlorophyll levels were found between batches (e.g. Table 6 vs 7 vs Fig. 11), possibly indicating a seasonal trend. Nevertheless, within each batch trifoliate leaves always had higher chlorophyll levels than primary leaves. The time courses of chlorophyll loss in the four leaf types (Fig. 11) probably were very similar to the process *in vivo*. The slow loss of pigmentation initially in green leaves was followed by a more rapid phase, this commencing when the chlorophyll levels were similar to those in yellow tissues, in which *in vitro* senescence began with rapid chlorophyll loss.

The chlorophyll *a:b* ratio was found to vary in *Phaseolus* leaves, but the variations were not consistent (see Table 8). The true value was probably *c.* 2.8 for both primary and trifoliate leaves, no matter their condition. It was possible, however, that instrument and operator errors accounted for much of the variation encountered: the influence exerted on calculated chlorophyll *a:b* ratios by changes in absorbance can be seen in Table 39. Readings of absorbances in the 0-1.0 scale are accurate to $\pm$0.0025, but this does not account for instrument error. Further to this, large discrepancies were found between calculated chlorophyll contents

Table 39. Calculated chlorophyll *a:b* ratios using constant A_{665} and variable A_{650}

A_{665}	A_{650}	chlorophyll *a:b*
0.50	0.240	3.36
	0.245	3.06
	0.250	2.81
	0.255	2.59
	0.260	2.40

of an extract when different dilutions were used (Table 10). The consistent increase in calculated chlorophyll content using increasingly dilute solutions may have been due to differential sensitivity in the instrument, with a consistent over-estimation of absorbance with low-concentration solutions or an under estimation at high concentrations.

Applied oestrogens did not modify chlorophyll levels in bean leaf discs and certainly this does not seem a promising line for further research. Some of the other aspects of senescence, however, although less readily accessible, may be more amenable to quantification and more precise studies may be possible, especially concerning membranes.

2. Germination The steroidal oestrogens tested did not affect *Phaseolus* germination (Table 11). This was the first investigation of the effects of oestrogens on dwarf French bean germination. A wide range of species had been studied previously (see Tables 2c and 3a) but no clear pattern emerged, the range of responses being from inhibition to promotion.

P. vulgaris, like many agriculturally important species, is far from an ideal seed for germination studies: the percentage germination usually is very high, and also the seed is relatively large. Smaller seeds, e.g. lettuce, are more easily worked with in as much as greater replication, and hence more reliable results, can be achieved while using less space. Preliminary experiments (Hewitt *et al.*, unpublished) with the Grand Rapids lettuce cultivar, however, have shown oestrogens to be without influence on germination over a range of temperatures and in darkness, red-light and white light.

The uptake of radioactivity by *P. vulgaris* seeds was very similar to that in leaf discs: the initial rapid uptake of water was matched by the increase in radioactivity content. At the completion of this phase of water uptake however, uptake of radioactivity continued (Fig.15). As in leaf discs this may have been due to a concentration gradient between cells and solution.

Water uptake was not influenced by the presence in solution of oestrogens, nor, as witnessed by radicle emergence and growth, was mobilization of seed reserves. Thus, as in root initiation, differentiated tissues were not affected, and in this case the amounts of oestrogens reaching proposed sites of action probably were not sufficient to elicit responses. It is doubtful if dwarf French bean germination could be a fruitful line of research involving steroidal oestrogens.

3. Root Initiation The only treatments to have any effects on hypocotyl cuttings were 0.1 mol m^{-3} oestrogen-sulphates (see Tables 15 and 16). The inhibition of root formation, promotion of axillary bud growth and induction of apex abscission exhibited with these treatments were not found with controls or 0.1 mol m^{-3} oestrone-phosphate. The ineffectiveness of the latter compared with oestrogen-sulphates may have been due to differences in metabolism: plants contain many phosphatases but much fewer enzymes capable of cleaving the sulphate linkage (Harborne, 1977). Rapid metabolism of oestrone-phosphate, presumably producing oestrone, would have removed the oestrogen from solution by virtue of the reaction product being virtually insoluble, whereas oestrogen-sulphates, less subject to enzymic attack, remained in solution for a sufficient period of time to be effective. Support for this theory comes from the ^{14}C and ^{3}H distribution studies with wick-feeding (Table 22). With ^{14}C-oestrone and ^{14}C-oestradiol, generally less than 10 per cent of the label was transported beyond the hypocotyl base. Moreover, the uptake and distribution of radiocarbon was not affected by the photoperiod during root formation (Table 23) and, therefore, movement of the applied substances with the transpiration stream must be doubted. The cuttings, then, had not acted as the wicks implied by the name of the method, but were more similar to chromatography systems, with the hydrophobic steroids partitioning out of the aqueous phase either into cells which could provide more lipophilic environments or binding to cell walls. Indeed entry of oestrogens

into membranes could have effectively prevented their reaching possible sites of action within the cell. This probably did not happen with oestrogen-sulphates, as witnessed by the translocation of ^{3}H from oestrone-sulphate: usually less than 50 per cent of the label was recovered from the hypocotyl. The compound, therefore, remained in the aqueous phase much longer than the relatively insoluble steroids. However, the continued monitoring of radioactivity levels during growth and development of rooted cuttings revealed that little redistribution of label took place after potting out (removal of external ^{14}C- or ^{3}H-source) indicating that once the presumed oestrogens were taken up by cells, release was unlikely.

The identities of labelled substances were not ascertained in this study, previous work having shown that most of the radioactivity extractible was associated with the applied compound (Young, 1977). Metabolism of ^{14}C-oestrone to ^{14}C-oestradiol and ^{3}H-oestrone-sulphate to ^{3}H-oestradiol was also found (Young, 1977: Young *et al.*, 1977, 1979) the metabolites being extracted from leaves. There was, however, no convenient way of telling whether metabolism had occurred before, during or after the translocation of label, although leaf homogenates were found to reduce ^{14}C-oestrone to ^{14}C-oestradiol (Young *et al.*, 1979). The rapid metabolism found *in vitro* and the apparently much slower rate of turnover *in vivo* were further indications that oestrogens, when applied to plants, were compartmentalised: a likely enzyme system for oestrogen metabolism, cytochrome P_{450}-NADPH reductase, is cytosolic in plants (Murphy and West, 1969). Any sequestration of oestrogens away from the cytoplasm need not have been an active process as the compounds would readily partition from the aqueous cytoplasm into the hydrophobic environments provided by membranes.

From the distribution studies (Table 22) it was also evident that only in 0.1 mol m^{-3} oestrogen-sulphate treatments was it likely that a sufficient amount of the exogenous substance to have an effect would have reached axillary buds and apices (10 Bq radioactivity was equivalent to *c.* 10 ng oestrogen) and the ineffectiveness of direct applications of oestrogens to apices (Table 19) was not necessarily contradictory: absorption by the plant was likely, but entry into lipoid environments such as membranes or the cuticle, or binding to cell walls would have compartmentalised the applied substances. Also, the distributions of ^{3}H and ^{14}C from labelled oestrogens applied to primary leaves were markedly similar although the amounts of labels translocated varied greatly within

experiments (see Table 24). Oestrogen-sulphates, therefore, although more readily transported upwards in the xylem (during wick feeding) were not more readily translocated away from leaves, presumably in the phloem or by diffusion.

That high concentrations (0.1 mol m^{-3}) of oestrogen-sulphates were required to elicit effects did not discount the possibility that the mode of action could have been hormonal. A further possibility, however, that the oestrogens were used by buds as substrates, remained. Most plant parts synthesise their own sterols, but it is unlikely that actively-dividing cells can meet their own requirements (Grunwald, 1978) and in *Phaseolus* the apex has been found to receive at least part of its sterol requirement from young, expanding leaves (Biddulph and Cory, 1965). During growth and development of 0.1 mol m^{-3} oestrogen-sulphate-treated hypocotyl cuttings subsequent to potting out, many of the developing cotyledonary buds abscinded (see Table 17). Generally, only those buds whose first leaf had opened and was expanding were retained, and the absence or presence of the main shoot apex did not influence this. As the axillary buds of primary leaves occassionally develop *in vivo*, the same trend as with cotyledonary buds, although present, was less evident. In the light of the evidence presented by Biddulph and Cory (1965) these results could be interpreted in terms of bud self-sufficiency: after removal of external steroid source (the rooting medium) only those buds with expanding leaves, capable of steroid production, could survive. Additionally, in the distribution studies with *P. vulgaris* cuttings (Tables 22 and 24) it was noticeable that the axillary buds consistently accumulated similar levels of radioactivity to internodes and trifoliate leaves. Thus, taking the sizes of the plant parts into account, buds received relatively the most ^{14}C and ^{3}H.

On the other hand, radioactivity from labelled oestrogens applied to primary leaves of hypocotyl cuttings kept in distilled water did not accumulate in the hypocotyl, which, by virtue of root production, was a strong metabolic sink. Only at nodule formation did radioactivity levels increase in roots (Table 24) and, in *Vicia faba*, root nodules were found to have twice the sterol level of roots (Knights *et al.*, 1977). Therefore, it was possible that the oestrogens were used as substrates by

Phaseolus.

Bud growth induced by 0.1 mol m^{-3} oestrogen-sulphate treatments did not seem to be dependent upon apex abscission (see Tables 17 and 18). In fact, bud growth always occurred,while the susceptibility of apices varied during the year: apex abscission was more frequent in winter-grown seedlings than summer-grown seedlings. Fig. 16 was compiled from an experiment using winter-grown seedlings. Many cuttings in which axillary buds developed did lose their apices, but after bud growth was already well underway. In these cases the apex may have been starved of nutrients due to the influence of the new active meristems, acting as metabolic sinks.

The limited translocation of presumed oestrogens to hypocotyls from primary leaves may have accounted for the lack of influence of applied oestrogens on adventitious root formation (Table 19). Distribution of label from oestrogens applied to apices could not be investigated owing to the apparent influence of radioisotopes on subsequent growth (see Table 25). This is discussed in greater detail in the next section. A lack of oestrogens could hardly be presented as the reason for the inactivity of the substances when applied in the rooting medium. Paradoxically it was in the treatments which affected root initiation that greatest translocation of oestrogens away from the hypocotyl occurred. Removal of oestrogens from solution, whether by binding to cell walls or entering membranes could explain why applied oestrogens,other than oestrogen-sulphates, had no effects on root initiation, although present in bulk at bases of cuttings.

While the use of different day-lengths during the rooting period and the excision of cotyledons did not affect the responses to oestrogens, rooting itself was modified (Table 26). The effects of cotyledon removal, *viz:* reductions in the number of roots formed, the total root and primary leaf masses and the chlorophyll content of primary leaves, clearly underlined the importance of the cotyledons in *Phaseolus* development. The use of different day-lengths, however, gave results contrasting with those from other species. With *P.vulgaris* , the greatest numbers and masses of roots were formed under the maximal total illumination, with primary leaf mass and chlorophyll content also at maximum levels. The shortest day-length gave the lowest values for each parameter. These data suggested that photosynthates were of great importance during root

formation. They were not, however, limiting factors, although high carbohydrate levels were reported to inhibit root formation in pea cuttings (Hansen and Eriksen, 1974: Anderson *et al.*, 1975: Hansen, 1975a, b, 1976: Veierskov *et al.*, 1976). In the latter reports the cuttings were all maintained under the same irradiance level (16 W m^{-2}) during rooting, but were taken from stock plants grown under different conditions (38, 16 and 4 W m^{-2}). The results, greater rooting by the plants raised at 4 W m^{-2} than those at 38 W m^{-2}, were interpreted as indicating that the levels of carbohydrate in plants from the highest irradiance were supra-optimal for root formation (Hansen and Eriksen, 1974). This was supported by exogenous sucrose enhancing root formation only in plants raised under 4W m^{-2} and cotyledon excision reducing the numbers of roots formed by the greatest extent in plants grown at lowest irradiance (Veierskov *et al.*, 1976).

Root formation in *P. vulgaris* hypocotyl cuttings followed a seasonal pattern (Fig.17) which was related to seedling growth. Batches of seedlings were all grown in the heated greenhouses with supplementary irradiation to provide a 16h photoperiod throughout the year, but despite these controlled conditions, seedlings grown in winter could be used after 11d growth whereas those in summer were suitable after 9d. This trend was continued during root formation on cuttings: in summer-grown plants a greater number of adventitious roots were produced in a shorter time. Obviously, external influences such as greater levels of natural irradiance had affected the growth rates of the seedlings to some extent.

Viewed in relation to these findings, the results with *Pisum* could be explained in another way. When cuttings from plants grown under 38 W m^{-2} were placed under a lower irradiance (16 W m^{-2}) the level of photosynthate production would be reduced. If endogenous growth rates were still to be met, root production might be expected to be diminished. Seedlings raised at 4 W m^{-2} irradiance, on the other hand, would have a slower endogenous growth rate and photosynthesis would be increased when cuttings were transferred to the higher irradiance. From this argument, increased root production could be predicted. Indeed, Eliasson (1978) found with pea that increasing the irradiance level during the rooting period increased the number of roots formed, indicating that root production depended on the level of photosynthate production after

cutting. Unfortunately, growth rates, leaf sizes and chlorophyll levels for the different batches of pea plants were not included in the reports by Hansen and his co-workers.

Microscopical examinations of hypocotyls from intact seedlings revealed that root initials were present *in vivo* (Plate 2). These commonly developed in whole plants and those beneath the soil surface frequently formed large proportions of the total root mass. The presence of adventitious root initials must have influenced results in root initiation experiments and could possibly have accounted for the roots produced on 0.1 mol m^{-3} oestrogen-sulphate-treated hypocotyl cuttings.

P. vulgaris epicotyls and primary leaf petioles did not contain preformed root initials and the inhibitions of root formation in these were almost complete. The other oestrogen treatments had no influence on root initiation (Tables 27 and 29). In epicotyl cuttings abscission layer formation, axillary bud growth and incurling of young trifoliate leaves were also noted. These effects tied in well with the distribution of tritium from ^{3}H-oestrone-sulphate (Tables 28 and 31) but, similar to the situation prevailing in hypocotyls, much less radioactivity was retained at the site of root initiation with the treatments which inhibited root formation than with those which did not. Unlike hypocotyl cuttings, however, inhibition of adventitious rooting in petioles and epicotyls was accompanied by the induction of a hyperplastic condition (see Plates 5 and 7). Especially in petioles, the induced condition greatly resembled tumours caused by *Agrobacterium tumefaciens* (e.g. van Lith-Vroom *et al.*, 1960). This, and the other effects of oestrogen-sulphates, although not mirrored in Na_2SO_4 controls, conceivably could still have been due to the sulphate moiety. Uptake of oestrogen-sulphate followed by hydrolysis could have resulted in higher concentrations of sulphate ions being present within some cells than were possible in the controls. Also, the characteristics of sulphate and oestrogen-sulphate uptakes by cells may not have been comparable. However, while certain S-containing groups affected cell division, sulphate ions were without influence (Hammet, 1930).

The effects of oestradiol on cell division in sea-urchin embryos were similar to those of colchicine, acting as a poison on the mitotic spindle (Agrell, 1965) and the same may have occurred in *Fucus distichus* embryos whose initial cell divisions were modified by oestrogen-treatments

(Pollock, 1969). However, when colchicine was tested on *P. vulgaris* primary leaf explants, although root initiation was inhibitted, there was no induction of hyperplastic tissues (Oppenoorth, 1978).

Steroidal oestrogens have previously been found to affect plant cell division (see Tables 2c and 3a) and synthetic oestrogens also can influence this process (Table 4). Indeed the possible link between oestrogens and plant tumours is not a new concept: young female rats and mice implanted with crown gall from *Pelargonium* or injected with *A. tumefaciens* reached sexual maturity before controls and plant tumours increased in size after oestrogen administrations (Havas, 1935). DES also has been found to stimulate tumour cell division *in vitro* (Manil, 1961), but contradictory evidence also exists (e.g. Stapp, 1937). Strong support, however, came from the induction in DES-treated onions of bulb overgrowths very similar to those found with *A. tumefaciens*, yet the DES-induced overgrowths did not contain bacteria (Jakowska, 1948). Oestrogens certainly affect the genetic apparatus, causing chromosomal abnormalities (Faller, 1942) which could be carried on through at least three further generations (Weyland, 1948: Weyland, *et al.*, 1949). Thus the nuclear changes had not prevented cell divisions and the same was true when similar effects on chromosomes were found in animals (Cagianut, 1956). In the other references to oestrogen-stimulated cell divisions or miotic disturbances the nature of the tissues produced were not described, but interestingly the abnormal embryo cleavages induced by oestradiol treatments in *Fucus distichus* (Pollock, 1969) were very similar to those resulting from the same treatment in *Psammechinus miliaris* embryos (Agrell, 1954, 1955). From this it would seem that the effects of oestrogens on cell division may indeed be parallel in plants and animals. Certainly, cell division was not inhibited by 0.1 mol m^{-3} oestrogen-sulphate treatments in *Phaseolus* epicotyl and primary leaf cuttings (see Plates 5 and 7), but unlike the anatomy of root initiation, there was no ordering apparent in the process. Clearly, then, cell determination and differentiation had been seriously impaired.

The inhibition of root formation on primary leaf cuttings by the presence of the proximal petiolar pulvinus (PP) (Table 32) followed an entirely different course: very little new cambium formation took place, and when it did normal root initials were formed. Therefore it was the induction of adventitious rooting, a process which may be

controlled by IAA (see Strömquist, 1979 for discussion), which had been prevented, possibly via an inhibition of dedifferentiation and cell division.

The most obvious area in which to search for an explanation related to the desiccation of primary leaf explants with PP. The desiccation itself was brought about by a combination of factors; firstly, when explants with PP were placed in the rooting medium, the PP soon became fully turgid and afterwards the DP became flaccid, causing the leaf blade to droop. This is unlike the mechanism which brings about the normal nyctinastic movements of bean leaves (see Kiyosawa and Tanaka, 1976) where a circadian movement of ions, notably K^+, causes changes in turgor which result in DP deformation and rhythmic leaf movement (Kiyosawa, 1979). Secondly, there is a circadian rhythm of stomatal movements (see Fig.18) which occurs in parallel with the "sleep" movements of leaves: thus, when laminae are held down stomata are closed and when laminae are horizontal stomata are open. In this way, water loss at night is minimised, stomatal closure greatly reducing transpirational water loss. Thus, in these experiments, the leaf blades were in a "sleeping" position when PP were present, but stomata were not fully closed. It was after the blade drooped that it lost turgidity. In the experiments with tritiated water this began between 5 and 10h after cutting and was obvious within 20h. In explants without PP the ^3H-contents of laminae continued to increase throughout the experiments whereas cuttings with PP had no such increases (Table 33). It was likely then that water loss from the leaf was not prevented whilst water input to the laminae was restricted, possibly by conduit restriction at the swollen PP.

The rapid and severe desiccation could have reduced carbohydrate availability substantially, not only by limiting the area of leaf capable of producing assimilates but also by reducing the available carbohydrate pool through isolating reserves in the rapidly dried areas. Although the extent of recovery from desiccation did not seem to influence the numbers of roots produced on affected cuttings, the number of explants which formed roots was small and thus any trend would be difficult to observe. On the other hand, in cuttings without PP the numbers of roots produced varied considerably, even when leaf sizes and chlorophyll contents were similar (Table 30). Further evidence against carbohydrate involvement as the prime factor in root initiation was that generally less than 30 per cent of the cuttings with PP had formed root initials at the end of the 10d rooting period. While carbohydrates are essential for root development

their absolute requirement in root initiation has not been demonstrated although Eliasson (1978) has shown that pea cuttings in darkness do not form roots.

Similar arguments can be put forward for plant hormones: rapid, severe desiccation of large areas of leaves could have reduced production or isolated endogenous pools and thus greatly reduced root initiation.

Another possible response mechanism directly concerned PP: a cut-edge effect which normally precedes root initiation was not expressed in explants with PP. In this, parenchymatous cells dedifferentiate to form a callus at cutting bases, and root initials arise either within this tissue or immediately above it. Callus formation sets up a strong metabolic sink which is continued by root development, and the flow of nutrients to such sites is probably hormone-directed (Wareing and Davies, 1965). In cuttings with PP there was no basal callus formation. Therefore, the presence of PP probably prevented the establishment of a metabolic sink, and the inhibition of root formation may have been an extension of this effect.

The great variability in numbers of roots formed on primary leaf and epicotyl cuttings was apparent throughout the year and effectively concealed any seasonal variation which might have been expected after the experiments with hypocotyl cuttings. Table 30 amply demonstrates the lack of influence of leaf size and chlorophyll content on rooting capacities of primary leaves and the large values for standard errors here and in Tables 27 and 29 serve to underline the variability within a sample of primary leaf or epicotyl cuttings.

4. Internode Extension

With the exceptions of 0.1 mol m^{-3} oestrogen-sulphates applied in rooting media to hypocotyl cuttings, applications of steroidal oestrogens to *P. vulgaris* seedlings and cuttings did not affect subsequent plant growth and development. The only repeatable significant differences in internode lengths were found between epicotyl lengths of untreated control plants and all seedlings with methanol-treated primary leaves (methanol controls and oestrogens dissolved in methanol) (Table 34). This effect was not found with explants, where epicotyl growth was always greatly

reduced (Table 20 vs Tables 12 and 34) possibly due to diversion of nutrients towards root formation during the period when epicotyl extension usually takes place. No consistent differences were found when seeds germinated in oestrogen solutions were allowed to develop to maturity (see Table 12), probably insufficient amounts of oestrogens having reached growth sites (Table 14).

The effects of 0.1 mol m^{-3} oestrogen-sulphate solutions on hypocotyl cuttings have already been discussed in the section on root initiation as the effects had become obvious during the rooting period. In connection with bud development, it was suggested that the amounts of oestrone and oestradiol reaching buds were not sufficient to influence growth, with the same probably being true with oestrone-phosphate and the lower concentrations of oestrogen-sulphates. From the distribution studies (Table 22), it was clear that the same argument applied for internode extension. Similarly, the ineffectiveness of all oestrogen applications to primary leaves of cuttings could be explained in terms of amounts reaching proposed target sites being insufficient to influence growth (see Table 24).

The application of oestrogens to primary leaves of whole plants was also without effect on internode extension. The distributions of radioactivity from oestrogens applied to primary leaves of whole plants (Tables 37 and 38) were very similar to those with cuttings (Table 24) and, therefore, the same arguments are again relevant.

Lacks of oestrogens at the regions of active extension growth could not explain the ineffectiveness of direct applications to apices, either of cuttings or of whole plants (Tables 20 and 34) respectively) and the unresponsiveness of plants grown from seeds imbibed in oestrogen solutions (Table 12). In these, sequestration of oestrogens could have accounted for the lacks of effects.

Unfortunately, reliable results for distribution of radioactivity from labelled oestrogens applied to apices could not be obtained: applications of ^{14}C-oestrone to apices of cuttings resulted in greatly altered growth habits not seen in cuttings treated with methanol or oestrone in methanol or in untreated cuttings (Table 25). This implied, bearing in mind that applied oestrogens were not readily translocated, that the presence of radiocarbon at apices had resulted in modifications there which were expressed later during growth and development of cuttings and that the effects were not attributable

to oestrogens. It is also worth noting that in most other experiments involving apical applications of ^{3}H- and ^{14}C- compounds (such as GAs or IAA) much shorter time-courses have been used and thus any effects similar to those found here may have been undetected.

On the other hand, of course, endogenous levels may already have been sufficient to elicit the maximal response or the compounds may not affect growth in *P. vulgaris*.

It is doubtful if any valid comparisons can be made between experiments using different batches of plants. The growth patterns (internode lengths) of the three sets of plants in Table 36 illustrate this very well: seeds for these batches were sown over a period of two weeks and the plants kept together on the same bench in the greenhouse. Even so, there were statistically significant differences in internode lengths between batches and this suggested that even the most subtle changes in environmental parameters during particular growth phases can result in modifications of growth. However, uniformity of growth was evident within batches of plants.

5. Flowering

Flowering was not affected by oestrogen treatments (Tables 13, 21 and 35) and the numbers of flowers produced per plant were similar, both between batches and throughout the year. There were some differences between batches in numbers of flowers produced at individual nodes (see Table 35). Thus, in addition to internode elongation, slight changes in the environment could affect bud development. The timing of flowering, however, did show a seasonal trend, with flowers produced most quickly in summer (30-40d) and most slowly in winter (50-60d). It was not really possible to gauge the influence on flowering of wick-fed 0.1 mol m^{-3} oestrogen-sulphates due to the effects on bud growth and apex abscission, but at no time did oestrogens affect flowering (Tables 13, 21 and 35), even when applied directly to apices. This may reflect, again, the compartmentalisation of the applied substances, or that endogenous levels were already sufficient, or that oestrogens play no role in flowering in *Phaseolus*.

GENERAL DISCUSSION AND CONCLUDING REMARKS

The theme of parallel developments of regulatory and control mechanisms in plants and animals was stressed in the Introduction, but, of necessity, was ignored in the Results and Discussion sections. However, it is now apposite to reconsider this theory.

Steroidal oestrogens certainly occur in *Phaseolus* and probably also in many other species, although characterizations in extracts of these have been equivocal. Also, it is likely that there has been a tendancy to overestimate oestrogen levels in plant tissues (e.g. Bennett *et al.*, 1966a) due to interference during assay by extract impurities. More recent estimates using techniques with greater sensitivities indicate that oestrogens are present at the $\mu g\ Kg^{-1}$ level in fresh plant material, the same range as the generally recognised plant hormones, *viz*: IAA (e.g. Bandurski and Shulze, 1977), GA (e.g. Crozier *et al.*, 1970), abscisic acid (e.g. King *et al.*, 1977) and cytokinins (e.g. Wang and Horgan, 1978).

Biosynthesis of oestrogens from precursors has only been demonstrated in *Phaseolus* (Young *et al.*, 1977, 1979), but the significance of this should not be underestimated: previously, debators against the endogeneity of oestrogens argued that the substances could have been present as contaminants in extracts or, if the presence in plants was accepted, that they had been taken up by plants. The question of natural occurrence of oestrogens has been settled in *Phaseolus*, but in the other species, spread through most of the subclasses of the Plant kingdom, the issue remains open.

In demonstrating that oestrogens are indeed chemical constituents of plants, more weight has been added to the theory that these substances could be plant hormones. The level of credibility attached to this theory has varied over the years: in the 1920s and 1930s, auxin was thought to be the hormone controlling plant growth (Went, 1935), with oestrogens probably involved more in flowering and sexuality, but also in growth (see Introduction). The isolation of auxin from animal-hormone extracts (Kögl and Haagen-Smit, 1931) cast early doubts on such roles for oestrogens, and experiments with purified compounds tended not to yield consistent results, although very few experiments were repeated exactly in other laboratories. Thus, oestrogens fell from favour as possible phytohormones, and indeed, even recently sterols in general have been regarded as

secondary metabolites with little or no significance in plants (Heslop-Harrison, 1972: Letham, 1978). The highly selective processes in plant sterol metabolism, however, indicated that sterols were on a primary metabolic pathway and therefore would be likely to have specific functions in plants (Knights, 1973). The role of sterols as membrane components is now well established (see Grunwald, 1978, 1979) but there is little evidence to suggest that parallelisms in other sterol functions exist between plants and animals.

The occurrence of steroidal oestrogens in plants does not necessitate their functionality therein: the possibility that they are indeed by-products created by enzymes recognising erroneous substrates cannot be denied, and the concept of hormonal heterophylly must also be borne in mind. With the latter, however, the most recent estimates of steroidal oestrogen levels in plants would seem to negate the possibility (Verdeal and Ryan, 1979). Phyto-oestrogens are potentially a much more serious hazard to some animals as they can occur in far greater amounts (Shutt, 1976). An interesting comparison can be drawn with the insect-moulting hormones, steroids which can be synthesised by plants (Heftmann *et al.*, 1968: Sauer *et al.*, 1968). At least 40 such substances have been found, involving over 80 plant families (Heftmann, 1975). However, although ecdysone derivatives from bracken were active as moulting hormones when injected into locusts, the insects when fed exclusively on bracken, were unaffected, the active principals not being absorbed from the gut (Carlisle and Ellis, 1968). This ties in well with the differing effectivenesses of phyto-oestrogens in cows and sheep, whose metabolic systems cope with these substances in different ways (Shutt, 1976). Hormone heterophylly, then, if it is genuinely an evolved system, would seem to be either very specific or of limited usefulness.

The ideas of parallelism in hormone action and identity certainly have their attractions, but why should plants and animals share the same active compounds? Animals excrete significant amounts of oestrogens onto plants and into the soil. Certainly it has been shown that only slight translocation of foliarly-applied oestrogens occurs, and the question of penetration has been raised: little of these steroids may have actually entered the plant. Much of the oestrogen-content of urine, however, is oestrone-sulphate (Purdy *et al.*, 1961) and it is possible that this

might be taken up more readily from the soil by roots. By the same token, it must be remembered that degradation by microorganisms prevents the build-up of oestrogens in the soil (Grant, 1969). Therefore, although plants are often subject to "applications" of oestrogens *cf.* the early Egyptian pregnancy test (Gad-Andresen and Jarlov, 1934), significant amounts of the compounds may not be taken up. The conceptual problems caused by phyto-oestrogens remain: their inactivity in the plants which produce them may be due to compartmentalization, and, in any case, as these substances are generally very weak oestrogens, their presence need not preclude the steroidal oestrogens from physiological roles in plants, although the phyto-oestrogens could still act as anti-oestrogens.

On a more fundamental basis, if, as seems likely, the first evolutionary steps splitting the Plant and Animal kingdoms took place before the advent of multicellular organisms, the development of identical hormone systems in animals and plants would appear of necessity to have been independent. The classical definition of a hormone denies the possibility that unicellular organisms, whether prokaryote or eukaryote, can have such compounds, as active substances, within them. It should be noted, however, that algae contain auxins (Augier, 1976a), gibberellins and cytokinins (Augier, 1976b) (comprehensively reviewed in Augier, 1977b, 1978) and these substances apparently have physiological roles (Augier 1976c, 1977b,c). This may indicate, as suggested in the Introduction, that the definition of hormone is too narrow concerning the requirement for translocation.

Pheromones do not enter this category as they are active outwith their producer (see Kochert, 1978). While it is likely that unicellular algae have regulatorymechanisms at least similar to those in higher plants it is not axiomatic that hormone systems evolved in the proposed common prokaryote ancestors of plants and animals. It might be expected though, that the hormone systems of animals and plants, if not identical might show some similarities. Such a situation may arise in sex expression: in animals, oestrogens and androgens are responsible, and in plants, of the most likely counterparts, two are terpenoids, *viz*: abscisic acid and the gibberellins. Indeed production of animal sex hormones and gibberellins both involve a cytochrome-P_{450} NADPH reductase system towards the ends of the pathways (Estabrook *et al.*, 1973, and Murphy and West, 1969, respectively).

There is no irrefutable evidence that oestrogens are plant hormones. The effects of oestrogens on plant systems have been many, varied and often contradictory, but the same may also be said of the established phyto-hormones. Past studies of oestrogen effects in plants have suffered from a lack of definition in that parallel studies on the distribution and metabolism of applied oestrogens have not been made. In addition, the turnover of endogenous oestrogens and their distribution patterns have not been established in the plants used. The value of such information, however, can easily be overrated: distribution patterns of supposed hormones on an organ basis tell very little, especially if the definition of a hormone is held to be correct. Really, to be of any great value, such information should be obtained at the cellular or preferably sub-cellular level and be accompanied by details on rates of turnover and possible transport to or from plant cells. Without these, distribution studies merely indicate the amounts present at given sites at particular points in time, and no valid conclusions can be drawn even if there is a high correlation between endogenous hormone levels and some aspect of development. This is especially true if distribution is analysed at the organ level.

Viewed with regard to the necessary constraints thorough re-examinations of oestrogen effects on plant growth and development should be carried out in an effort to confirm previous findings. Of the earlier literature, the experiments of Löve and Löve (1940, 1945) are most worthy of repetition: recent attempts to repeat part of this work on a much smaller scale have yielded inconclusive results (Knights, pers. comm.). The main areas in which possible roles of oestrogens require clarification are sex expression, flowering and growth, but these may yet prove less important than the possible involvements in cell division and differentiation. Although these aspects of development have rarely been studied *per se* in plants, abnormalities of division and differentiation of cells have often been noted, apparently as side-effects of oestrogen treatments, and in this respect there may be some parallelism between plants and animals (in effect, not in function). This study in *Phaseolus* has also shown an effect of oestrogens on cell differentiation, and rooting cuttings may prove to be a very useful tool in such investigations: the system is simple and allows the study of dedifferentiation, cell division and differentiation. An extension of this part of the thesis,

involving other steroidal oestrogens and different plant species, could provide valuable information on these processes and their control. It would also be useful to compare any resultant hyperplastic tissues with *Agrobacterium tumefaciens*-induced plant tumours, and future studies should utilise tissue-culture techniques and microautoradiography.

Of the other aspects of *Phaseolus* physiology dealt with, only the release of apical dominance seems worthy of further investigation, and in this respect it is imperative that the metabolism of the applied oestrogens is closely monitored. Applied oestrogens did not affect flowering, elongation growth, germination, or senescence in *Phaseolus*. The obvious supposition from this would be that oestrogens do not influence those aspects of development, but the possibilities of inefficient penetration to required sites or that endogenous levels already were sufficient to induce maximal responses must also be considered.

While most work in plant hormone physiology will continue to be with auxins, gibberellins, cytokinins, abscisic acid and ethylene, there must always be provision for study of other groups of substances, such as steroidal oestrogens, brassinolides, fungal toxins etc., as the search for a greater understanding of hormone action in plants continues. Yet, as advances are made towards a knowledge of the precise molecular basis of hormone activity it is easy to lose sight of the place hormones occupy in the development of an organism. Before a cell can have a specific reaction to a hormone it must be receptive to it, and this receptiveness is preconditioned. Each cell in an organism has the same genetic complement and therefore has the inherent capability to be responsive to any hormone, yet responsiveness is expressed only in certain types of cell. Hormones may act by allowing the transcription of previously blocked sections of DNA, but, in animal systems at least, it is hormone-protein complexes which achieve this, not the hormone itself. Responsiveness to a hormone then is laid down in differentiation, and this is why a given hormone can have several effects in different cells. Hormones are indeed messengers and, as such, they play an important role in development. A more complete knowledge of their mode of action may lead to a greater understanding of the processes involved in cell determination, but it is unlikely that hormones are the single key to unlock the secrets of differentiation, the control of which offers virtually limitless possiblities.

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