STUDIES ON THE EFFECTS OF STEROIDAL ESTROGENS IN THE DWARF FRENCH BEAN, PHASEOLUS VULGARIS L. cv. CANADIAN WONDER

A thesis submitted to the University of Glasgow
in candidature for the degree of Master of Science

by

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January 1987

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ACKNOWLEDGEMENTS

I wish to express my sincere thanks to my supervisor, Professor John R Hillman for his guidance and encouragement over the last two years and for his continued interest in my work. I am grateful to the Department of Agriculture and Fisheries for Scotland for providing a postgraduate studentship and to Dr A M M Berrie for the use of the research facilities in his Department and also for his advice on the preparation of this thesis.

My thanks are also due to the following people: Drs J J Milner and J T Knowler for many helpful discussions concerning the work carried out on the effects of estrogens at a molecular level, to Mr Eoin Robertson for the preparation of material for microscopical analysis, to Mr Alec Ewing for the continued supply of seedlings and to Mr Norman Tait for photographic assistance.

Following a move to the Scottish Crop Research Institute, Professor Hillman extended the use of the research facilities of the Institute to me, and in this respect I gratefully acknowledge the following people: Drs J McNicoll and R J Killick for devising the results presentation for statistical analysis, to Mr Denton Prior for technical assistance in the processing of material by sample oxidation and to Mr Thomas Geoghegan for photographic assistance.

Finally, I wish to thank my family and my friends at Garscube for their continued patience and support.

UNITS, SYMBOLS, FORMULAE AND ABBREVIATIONS

Standard chemical formulae and certain SI units have been used throughout this thesis. The following widely accepted units have also been used, but are not, sensu stricto, SI units.

Quantity	Unit	Symbol	SI equivalent or related unit
Concentration of a substance	molarity	M	mol where $1M = 1 mol dm^{-3}$
Volume	litre	l	m^3 where $1l = 1 dm^3 = 10^{-3} m^3$
Time	day	d	second(s)
	hour	h	
	minute	min	

Abbreviations

A	absorbance
ABA	abscisic acid
AES	automatic external standard
ATP	adenosine triphosphate
BMV	brome mosaic virus
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CEPHA	2-chloroethyl phosphonic acid
cpm	counts per minute
cv.	cultivar
2-D	two-dimensional
DES	diethylstilboestrol

DMPP	dimethylallyl pyrophosphate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
E_1	estrone
E_2	estradiol
E_3	estriol
E_1-S	estrone-3-sulphate
E_2-S	estradiol-3-sulphate
EDTA	ethylenediaminetetraacetic acid
eg	exempli gratia
et al	et alia
FPP	farnesyl pyrophosphate
GA_s	gibberellins
GA_3	gibberellic acid
GC-MS	combined gas chromatography-mass spectrometry
GLC	gas-liquid chromatography
GPP	geranyl pyrophosphate
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
ie	id est
IPP	isopentenyl pyrophosphate
IR	infra red
LDP	long day plant
mRNA	messenger ribonucleic acid
MVA	mevalonic acid
NAD	nicotinamide adenine dinucleotide
NADP(H)	(reduced) nicotinamide adenine dinucleotide

	phosphate
Nmglc	N-methyl-D-glucamine
oligo(dT) cellulose	oligodeoxythymidylic acid cellulose
poly(A)$^+$RNA	poly adenylated ribonucleic acid
ppm	parts per million
PPO	2,5-diphenyloxazole
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDP	short day plant
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SK and F $7997A_3$	SK and F (tris-(2-diethylaminoethyl)-phosphate trihydrochloride)
TCA	trichloroacetic acid
TEMED	NNN'N' tetramethylethylenediamine
Ti-plasmid	tumour-inducing plasmid
TLC	thin-layer chromatography
TMV	tobacco mosaic virus
Tris	Tris (hydroxymethyl) aminoethane
UV	ultra violet
v/v	volume to volume
w/v	weight to volume
w/w	weight to weight

Additionally, the trivial names for steroids have been employed throughout this thesis rather than the IUPAC approved terms.

SUMMARY

In this thesis various aspects of the physiology and biochemistry of steroidal estrogens in Phaseolus vulgaris have been studied in an attempt to achieve some understanding of the mode of action of these steroid hormones in plants. Three main lines of investigation were followed.

Firstly, the effects of estrone-sulphate and estradiol-sulphate on root initiation were examined in primary leaf cuttings. Confirmation was obtained that application of estrogen-sulphate conjugates, by wick feeding, significantly reduces adventitious root formation. This effect was manifest after six days treatment and was consistently more pronounced with application of estradiol-sulphate. Microscopical examination of the petioles of primary leaf cuttings and the bases of hypocotyl cuttings treated with estrone-sulphate and estradiol-sulphate confirmed the induction of anomalous cell development in the vascular tissues and in the areas normally associated with adventitious root production. These zones of anomalous cell development could first be observed five days after commencement of estrogen treatment.

Secondly, the uptake and distribution of exogenous estrogens by hypocotyl cuttings and by primary leaf cuttings was examined using ^{14}C labelled estrone and estradiol. Estrone and estradiol behaved physiologically in the same way. Uptake of radioactivity by hypocotyl cuttings appeared to be, to some degree, selective, but uptake by primary leaf cuttings was apparently passive. The subsequent distribution of radioactivity in both hypocotyl and primary leaf

cuttings was very restricted, and to a great extent remained unaltered during the treatment periods employed. These results are considered in the light of previous work on the metabolism of steroidal estrogens in Phaseolus vulgaris.

Thirdly, the effects of estrogen-sulphates on root initiation and cell development in primary leaf cuttings were used as the basis of an experimental system for investigating the mode of action of estrogens in plants at the molecular level. An in vitro protein synthesising system derived from wheat germ was used to translate Poly $(A)^{+}$RNA prepared from the petioles of estrogen-treated and control cuttings. The separated polypeptide products of translation were compared but, at the level of resolution employed, it appears that estrogen treatment does not induce either quantitative or qualitative changes in the mRNA populations of the responding tissue.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The angiosperms are complex, highly differentiated organisms which have evolved mechanisms enabling them to survive the rigours of a terrestrial environment. They must have the capacity to adapt to and exploit the changing conditions of that environment such that orderly and organised growth and development can occur. Such organised growth and development results from the integration of many internal processes within and between the individual cells, tissues and organs of the plant. This requires strict spatial and temporal control and co-ordination of these processes. The mechanisms underlying these processes are many and complex, but essentially can be divided into two main groups: firstly, control by physical or field forces about which little is known, and secondly, control by chemical processes about which much more is understood.

Chemical control of plant growth and development is provided by the plant hormones (phytohormones) or plant growth substances. The sequence of publications which led to the concept of plant hormones and which culminated in the isolation of the first hormone from a higher plant can be traced back to 1758: Duhamel du Monceau noticed that swellings and root formation occurred above but not below ring wounds on stems. To account for this type of observation, Sachs (1882, 1893) proposed that organ-forming substances or calines such as root-forming substances (rhizocalines) and flower-forming substances

were produced by the plant and were distributed in a polar manner. Many years were to elapse, however, before root formation was associated with auxin, a phytohormone derived from the shoot (Thimann and Went, 1934; Thimann and Koepfli, 1935) and instead it was the study of tropic movements in plants which led to the first definite knowledge of this type of hormone. Darwin and Darwin (1880) showed that the regions of perception of phototropism in coleoptiles and geotropism in roots were spatially separated from the regions of response and concluded that some type of 'influence' was transmitted from the site of perception to the site of action where the changes in the growth pattern which resulted in organ- curvature were induced. Boysen-Jensen (1911,1913) was able to demonstrate that the 'influence' involved in the phototropic response in oat coleoptiles was water-soluble and that its transport did not require the living continuity of the protoplasm, as it could pass through non-living matter such as gelatine but not through material impervious to water such as mica. These observations led Boysen-Jensen to suggest that the influence was of a chemical nature. Further to this Paál (1919) established that if the coleoptile tip was excised and replaced asymmetrically, the coleoptile curved even in the dark and so proposed that the influence was a growth substance secreted by the coleoptile tip which in darkness moved symmetrically down the coleoptile, but if the plant was illuminated unilaterally moved asymmetrically. Despite this, it was not until 1928 that the chemical messenger was isolated: Went (1928a, 1928b) placed excised coleoptile tips on agar and then positioned the agar asymmetrically on freshly decapitated coleoptiles. The growth substance was secreted into the agar, and the coleoptile curvature which resulted was proportional to the number of tips or to the time they were on the agar. The term 'auxin', which is derived

from the Greek auxein meaning 'to increase', was proposed by Kögl and Haagen-Smit (1931) for the growth substance involved.

The term 'hormone', which is often used to describe growth substances in plants, was first adopted by animal physiologists to describe chemical messengers carried in the blood stream from a site of production to a distant, distinct site of action where a characteristic response is induced (Starling, 1905). Without embarking on a discussion here of whether or not the plant growth substances act in the strictest sense as hormones per se, most authorities agree to the existence of five major types of compound functioning in the control of growth and development in plants: these types of compound being the auxins, the gibberellins (GAs), the cytokinins, abscisic acid (ABA) and ethylene.

However, many other naturally occurring compounds are known to influence the pattern of plant growth, and these substances include phenolics, vitamins, cyclitols, brassinolides, short-chain fatty acids and the steroidal oestrogens. Since the 1930s the study of steroidal oestrogens (more commonly spelt estrogens) in animals has been an active area of research due to their role in the expression of sexuality and also a possible involvement in abnormal cell development, but the study of these substances in plants has been largely neglected and presently there is insufficient evidence to warrant their classification as plant growth regulators. Nevertheless, studies dating back to the 1920s indicate that the physiological and biochemical status of steroidal estrogens in plants should be reassessed.

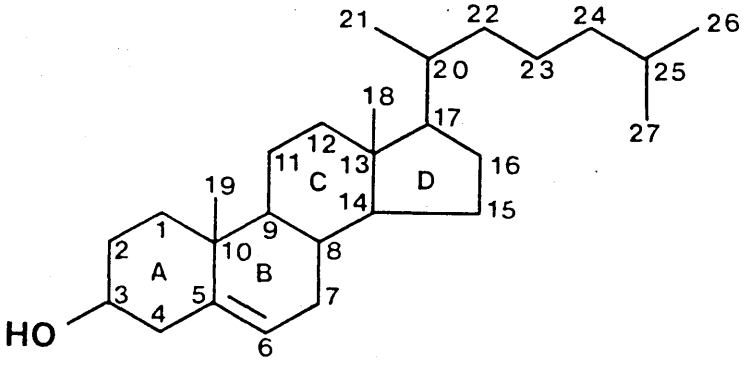
1.2 The Biochemstry of Steroids

Steroids, which are named from the Greek 'stereos' meaning solid,

belong to a class of compounds called the terpenoids. Terpenoids are widely distributed in the plant kingdom: they contribute to the aroma of plants, can act as plant growth regulators (eg gibberellins) and may be involved in host - parasite interactions (eg rishitin) or membrane development (sterols) and in photosynthesis (phytol and carotenes). Terpenoids are all derived from one main intermediate compound, mevalonic acid (MVA), which has the systematic name (3R) 3,5, dihydroxy-3-methyl pentenoic acid.

Steroids are of wide natural occurrence and are probably present in all living organisms. The parent and prototype of an almost infinite number of steroids is cholesterol. The cholesterol molecule is a condensed four-ring system with three 6C rings (A, B and C) and one 5C ring (D). All steroids are numbered as in cholesterol (Figure 1). General information on the biochemistry of steroids is to be found in Fieser and Fieser (1959).

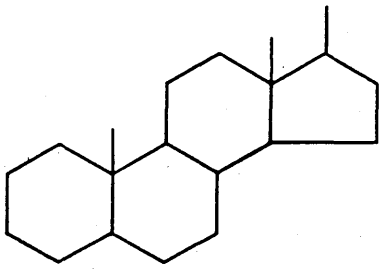
Figure 1 **The ring structure and numbering of cholesterol**



Removal of the hydroxyl group at the C3 position and reduction of the double bond between C5 and C6 converts cholesterol into the fully saturated compound cholestane which gives rise to the parent compounds

of several important steroid series (see Figure 2). Steroids have structures based on the perhydro-1,2-cyclopentanophenanthrene skeleton (Figure 3).

Figure 3 **The perhydro-1,2-cyclopentanophenanthrene skeleton**



Steroidal estrogens are compounds which induce a typical estrus response in mammals based on the bioassay of Allen and Doisy (1923). The main steroidal estrogens are estrone (E_1), formally called estrin, folliculin or theelin, estradiol (E_2) and estriol (E_3), formally called theeol. The structure of these estrogens and some of their conjugates are given in Figure 4.

1.3 The Occurrence of Steroidal Estrogens in Plants

A preliminary report of the occurrence of substances in plants capable of inducing estrus in animals appeared in the literature as early as 1925: Loewe found that estrogenic activity was present in female willow flowers. Later, more expanded reports stated that estrogenic activity was also present, although to a lesser extent, in the leaves of Althea rosea, stems of Impatiens parviflora, and ovaries of Nuphar luteum (Loewe and Spohr, 1926; Loewe et al, 1927). In the same period estrogenic activity of varying degrees was reported to be present in Beta vulgaris seeds, parsley roots, potato tubers, cherries and plums (Dohrn et al, 1926) and in rice and oat seed embryos (Fellner, 1926).

Figure 2 Steroid compounds arising from cholestane

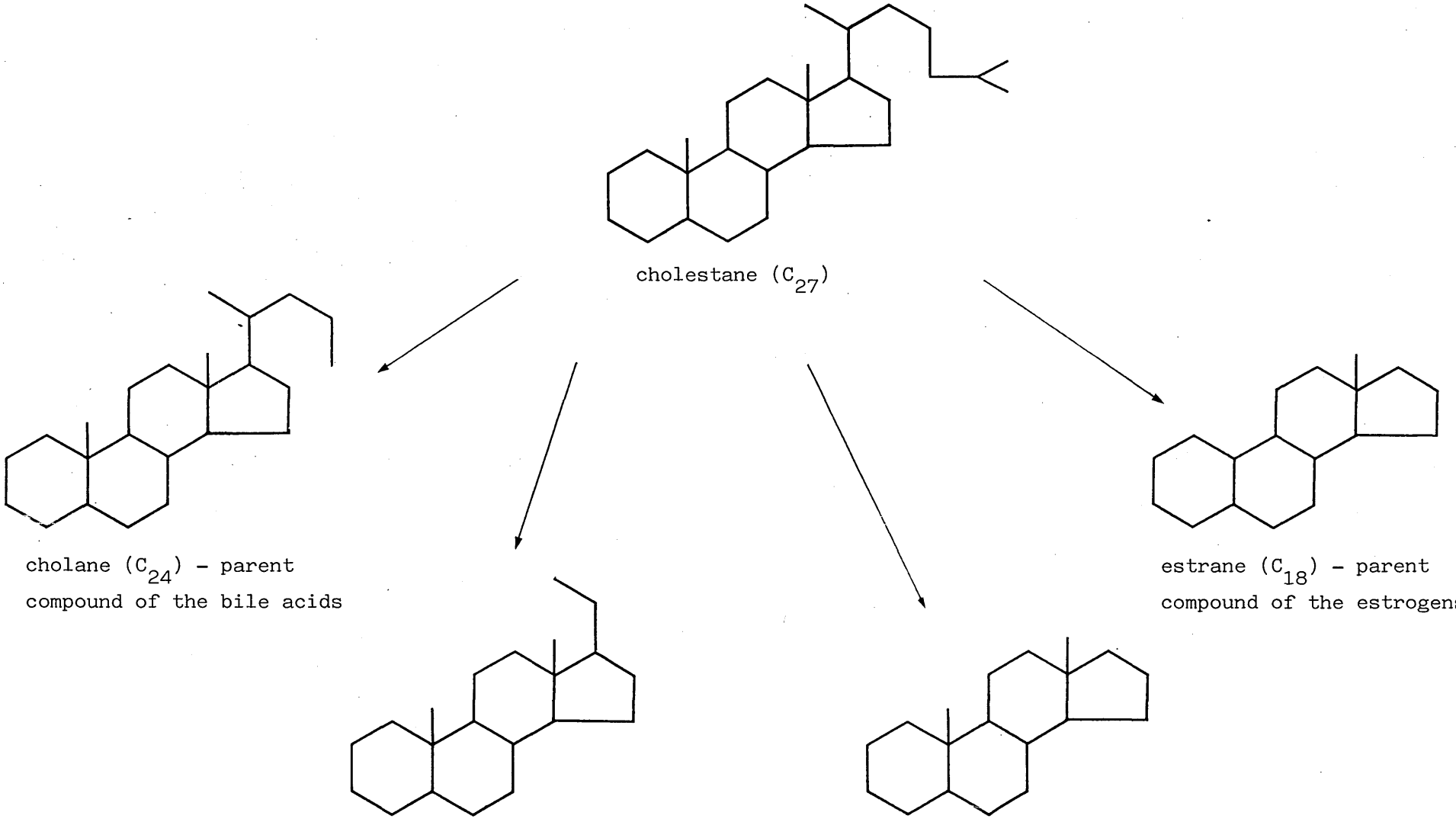
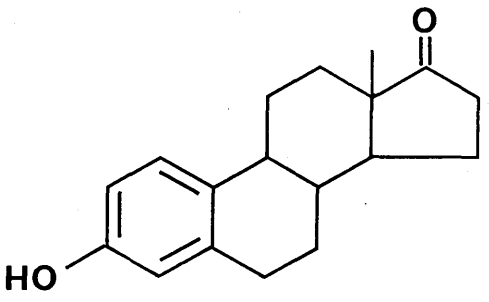
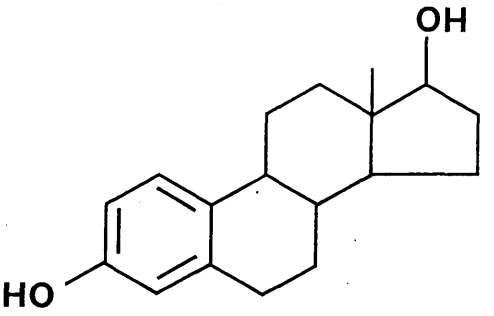


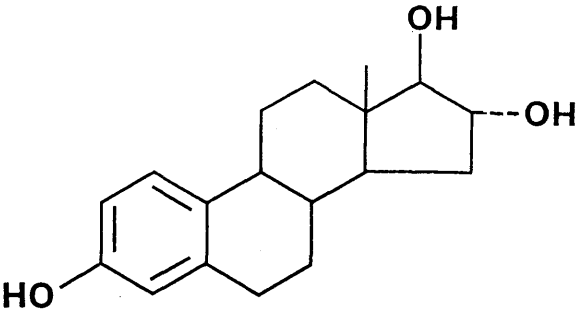
Figure 4 Structural formulae of the main steroidal estrogens and some of their conjugates



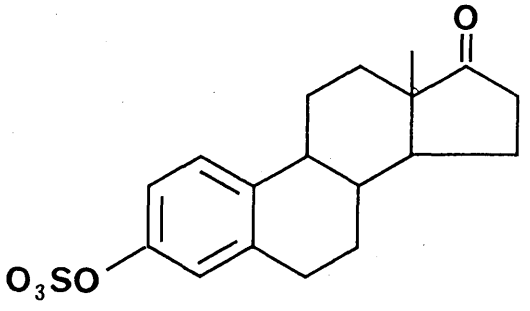
estrone



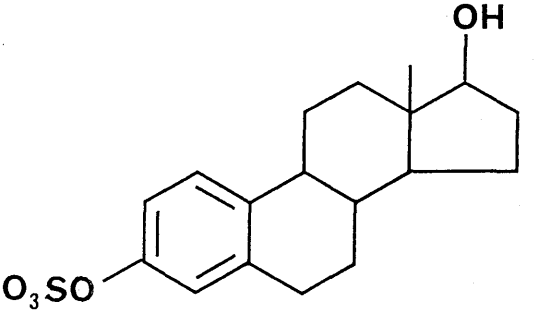
estradiol



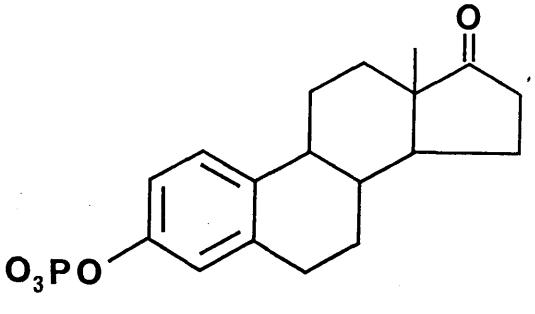
estriol



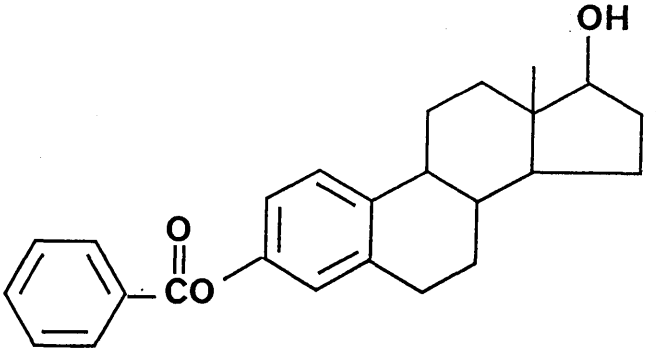
estrone-3-sulphate



estradiol-3-sulphate



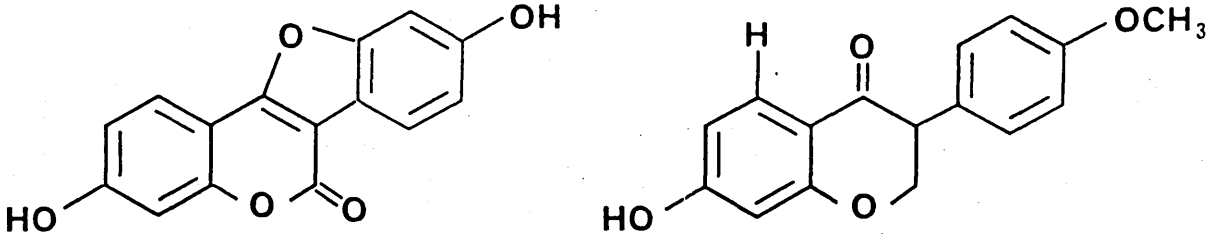
estrone-3-phosphate



estradiol-3-benzoate

In all the above cases the presence of estrogens was demonstrated using the ovariectomised mouse bioassay (Allen and Doisy, 1923) and since that time many plant extracts have been examined using this method and a large number of these have been shown to contain estrogenic activity (reviewed by Löve and Löve, 1945; Bradbury and White, 1954; Hewitt, 1980). These include extracts of Elaeis guineensis (palm) kernel (Butenandt and Jacobi, 1933), female willow flowers (Skarzynski, 1933) and the seeds of Prunus armeniaca (Awad, 1974). However, as pointed out by Hewitt (1980), bioassays, despite often having a high degree of sensitivity, are relatively unspecific and in addition any response which is elicited may be due to the activity of other compounds since the applied compound is subject to metabolism within the assay system. Furthermore, problems arise in that plants contain non-steroidal compounds known as phytoestrogens which show similar biological activity to steroidal estrogens (Cheng et al, 1953). These compounds, which are mostly isolfavanoids such as coumestrol and formononetin (Figure 5), are competitive inhibitors of estradiol in animals although binding to estradiol receptors is weaker (Shutt and Cox, 1972; Newsome and Kitts, 1975). Due to the presence of these phytoestrogens, the results obtained using the bioassay procedure are dependent on the degree of purification of the extract.

Figure 5 Structural formulae of two phytoestrogens



coumestrol formononetin

The first isolation of an estrogenic substance from plant material was in 1933: Butenandt and Jacobi isolated 8mg of estrone from pressed palm kernel and in the same year Skarzynski obtained 7mg of a pure crystallizate of estriol from 65kg of willow flowers. Identifications of the isolated compounds were made not only by bioassay but also by analysis of a series of physical and chemical properties including melting point, mixed melting point, optical rotation, derivitisation and ultra violet (UV) absorption spectroscopy.

Jacobsohn and his co-workers (1965) repeated Butenandt's work but were unable to detect the presence of estrone in palm kernel and concluded that more direct evidence was required to demonstrate the presence of estrogens in plants. In an attempt to clarify this question, Heftmann's group, employing the techniques of thin-layer chromatography (TLC) and infra-red (IR) spectroscopy, reinvestigated reports of estrogenic activity in date palm (Hassan and El Wafa, 1947) and in pomegranate (Sharaf and Nigm, 1964). Estrone was found to be present in both the seeds and pollen of date palm (Heftmann et al, 1965; Bennett et al, 1966) and in the seeds of pomegranate (Heftmann et al, 1966). The occurrence of estrone in pomegranate was later confirmed by Dean et al (1971) using the extremely sensitive technique of competititve protein binding, although the level detected differed by a factor of 4,000 so that Heftmann's estimates may have been high.

Colour tests such as the Kober colour reaction have also been used as a means of identifying estrogens in plants. This was the method used by Kopcewicz (1971a) together with TLC to investigate the presence of estrogens in Phaseolus vulgaris at various stages of development. Estrogens appeared at the time the flower bud emerged and thereafter reached two maxima, firstly during the period of flower bud development and then at pod formation. Although the presence of

estrogen-like substances was detected in the stems and roots, the largest amounts were found in the leaves. The long day plants (LDP) Hyoscyamus niger and Salvia splendens displayed synthesis of estrogens beginning as the flower bud emerged and reached maximum levels at the time of flower development, only in inductive light conditions (Kopcewicz, 1972a). Three estrogen-like substances were found in each of the plants but all of these compounds differed from known standards and from each other, although it was claimed by the author that one of the compounds extracted from Hyoscyamus was similar to estrone. Similarly, Kopcewicz (1972b) found that estrogens were first detectable in the short day plants (SDP) Perilla ocymoides and Chenopodium rubrum during inductive conditions at the period of induction of flowering and reached a maximum at the time of flower development. Two estrogen-like compounds were extracted from Perilla and one from Chenopodium, but as with the LDPs these compounds differed from known standards and from each other, although one from Perilla and estrone were similar.

Interestingly, Amin et al (1969), used colour tests and UV and IR spectroscopy to demonstrate the presence of estrogenic substances in the roots of Glossostemon bruguieri (moghat) and in the pollen grains of the Egyptian date palm: the former plant is commonly used in Egypt as a hot beverage after childbirth and the latter is used to promote fertility in women.

When assessing the above reports, however, it must be borne in mind that colour tests are not always suitable for plant extracts as the results can be affected by artifacts from acid hydrolysis (Adlercreutz et al, 1967).

In all the afore-mentioned cases the methods used to identify steroidal estrogens have been equivocal and the results obtained

varied depending on the extraction and purification procedures employed. Additionally, as with any compound present in low concentrations, the possibility that at least some of the identified substance is attributable to contamination from external sources must be taken into consideration.

These problems concerning isolation and identification of estrogenic substances, together with published failures to isolate estrogens from plants (Jacobsohn et al, 1965; Van Rompuy and Zeevaart, 1975, 1976, 1979) raised serious question as to whether or not these substances are biosynthesised by plant tissues, and as a result the physiology of steroidal estrogens in higher plants has been largely neglected or assumed to be of little significance (Heslop-Harrison, 1972).

Nevertheless, the first case of successful labelling of an identified steroidal estrogen from ^{14}C MVA was reported in 1977 (Young et al). Incorporation of biosynthetic label is of prime importance in demonstrating endogencity of a metabolite in an organism and eliminates the possibility that the identified compound arises merely by contamination. Young and his co-workers demonstrated the incorporation of radioactive isotope from the precursers, (3RS)-2^{14}C MVA, 4^{14}C estrone and potassium 6,7-^{3}H estrone sulphate into estradiol by the dwarf French bean, Phaseolus vulgaris cv. Canadian Wonder. Evidence of unlabelled estradiol in extracts of seeds and vegetative and flowering plants using a modified radioimmunoassay (RIA) method, combined gas chromatography - mass spectrometry (GC-MS) and chromatographic techniques was also presented (Young et al, 1977, 1978). Subsequently it has been demonstrated using a tissue homogenate supplied with various co-factors that metabolism of estrone occurs and evidence from TLC, column chromatography on lipadex, radio gas/liquid chromatography (GLC) and crystallisation to constant

specific activity suggests that estrone and 17-β estradiol are interconvertible in plant tissues (Young et al, 1979). Thus it would appear that this particular class of mammalian sex hormone is present in plants as well as in animals.

The driving force behind the search for estrogens in plants was the concept of parallel mechanisms of control of growth and development in plants and animals. Thus, the biosynthesis and the physiology of estrogens in animals will be briefly discussed here.

1.4 Steroid Biosynthesis in Animals

The classical pathway for steroid biosynthesis in animals is well documented (eg Goad and Goodwin, 1972) and begins with the biosynthesis of MVA from three molecules of acetic acid. The acetic acid molecules are in the form of acetyl co-enzyme A and all the intermediates involved are bound to co-enzyme A. The required enzymes are present in both cytoplasmic particles and the soluble fraction of mammalian and avian liver. Six molecules of MVA are required in the subsequent synthesis of squalene.

The first step in the biosynthesis of squalene is the formation of isopentenyl pyrophosphate (IPP) from MVA. MVA is sequentially phosphorylated with ATO in the presence of Mg^{2+} or Mn^{2+} ions. The first phosphorylation is catalysed by mevalonate kinase and gives rise to 5-phosphomevalonate. This compound is then converted to 5-pyrophosphomevalonate in a reaction catalysed by phosphomevalonate kinase. 5-Pyrophosphomevalonate then undergoes decarboxylation and dehydration resulting in the production of IPP.

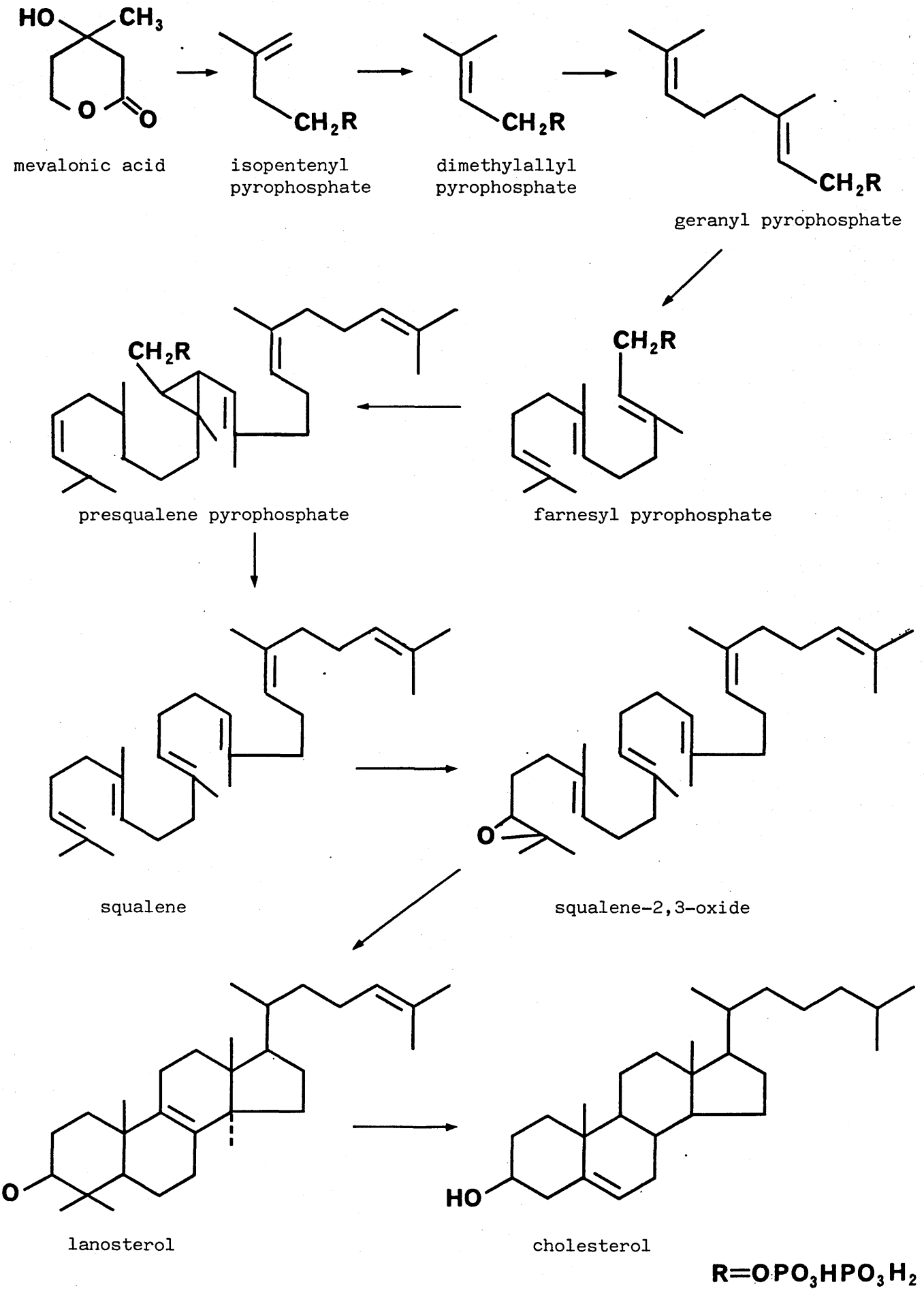
Some of the IPP is rapidly isomerised to dimethylallyl pyrophosphate (DMPP) under the influence of isopentenyl pyrophosphate isomerase. There then follows a condensation reaction involving further molecules

of IPP and which requires the presence of farnesyl synthetase. This reaction gives rise initially to geranyl pyrophosphate (GPP) and subsequently to farnesyl pyrophosphate (FPP).

Finally two molecules of FPP undergo a head to head condensation to give rise to the C_{30} compound squalene. This reaction requires a microsomal squalene synthetase and occurs in the presence of NADPH. Squalene is not formed directly in the reaction but rather via the formation of the intermediate presqualene pyrophosphate.

The cyclisation of squalene to lanosterol marks the beginning of an aerobic phase in steroid biosynthesis which requires molecular oxygen, NADPH and microsomes. Squalene is first oxidised to squalene-2,3-oxide which undergoes a protoninitiated cyclisation to give rise to lanosterol. The conversion of lanosterol to cholesterol involves three types of changes: the methyl groups at C14 and C4 are removed, the double bond at C8(9) transfers to C5(6) and the double bond at C24(25) is saturated by the addition of H_2. The various steps in the biosynthesis of cholesterol from MVA are shown in Figure 6.

Cholesterol is the biosynthetic precurser for the steroid sex hormones including the steroidal estrogens. Conversion of cholesterol to the sex hormones involves structural modifications which includes the successive degradation of the side chain and appropriate oxidation of both the side chain and the basic skeleton. The first step in the degradation of cholesterol is the enzymatic formation of pregnenalone. This conversion involves a series of mixed function oxidases and requires molecular oxygen and NADPH. Pregnenalone can then be converted to progesterone in a reaction catalysed by a 3 hydroxy steroid dehydrogenase system found in the microsomal fraction of the adrenal glands. Oxidation of C3 occurs and there is transfer of the double bond at C5(6) to C4(5). This enzymatic degradation is


HO
CH3
O
O
CH2R
CH2R
CH2R
mevalonic acid
isopentenyl pyrophosphate
dimethylallyl pyrophosphate
geranyl pyrophosphate
CH2R
CH2R
presqualene pyrophosphate
farnesyl pyrophosphate
squalene
O
squalene-2,3-oxide
HO
HO
lanosterol
cholesterol
R=OPO3HPO3H2

irreversible. Very little progesterone can be detected at any given time due to its rapid metabolism.

The synthesis of the C_{19} androgens can proceed along either of two pathways. The first pathway involves the oxidation of progesterone to 17α hydroxyprogesterone followed by the loss of C20 and C21 as acetic acid. This gives rise to androstenedione and these reactions take place in the gonadal tissues. The second pathway occurs in the adrenal tissues and involves hydroxylation at C17 of pregnenalone so that 17α hydroxypregnenalone is formed. Loss of the acetate group results in the formation of dihydro-epiandrosterone. The C3 hydroxyl group of this compound is then oxidised and androstenedione is again produced. Testosterone is biosynthesised by reduction of the C17 carbonyl group of androstenedione. The biosynthetic pathway from cholesterol to testosterone is illustrated in Figure 7.

The final stages in the degradation of cholosterol gives rise to the C_{18} estrogens. Their formation results from the removal of the angular methyl groups at C19 of androgenic compounds which occurs with the aromatisation of ring A. The various stages in the aromatisation of androgens to estrogens requires NADPH and oxygen. An outline of the steps involved is given in Figure 8.

In most animals estrone and estradiol are interconvertible, with equilibrium usually in favour of estrone. The reversible reaction is catalysed by a 17β hydroxysteroid dehydrogenase in the presence of NAD or NADP. The biosynthesis of the main estrogens is outlined in Figure 9.

1.5 Proposed Biosynthetic Pathway for Estrogens in Plants

The exact pathway for the biosynthesis of estrogens in plants has not yet been fully elucidated, but it is possible that the pathway is

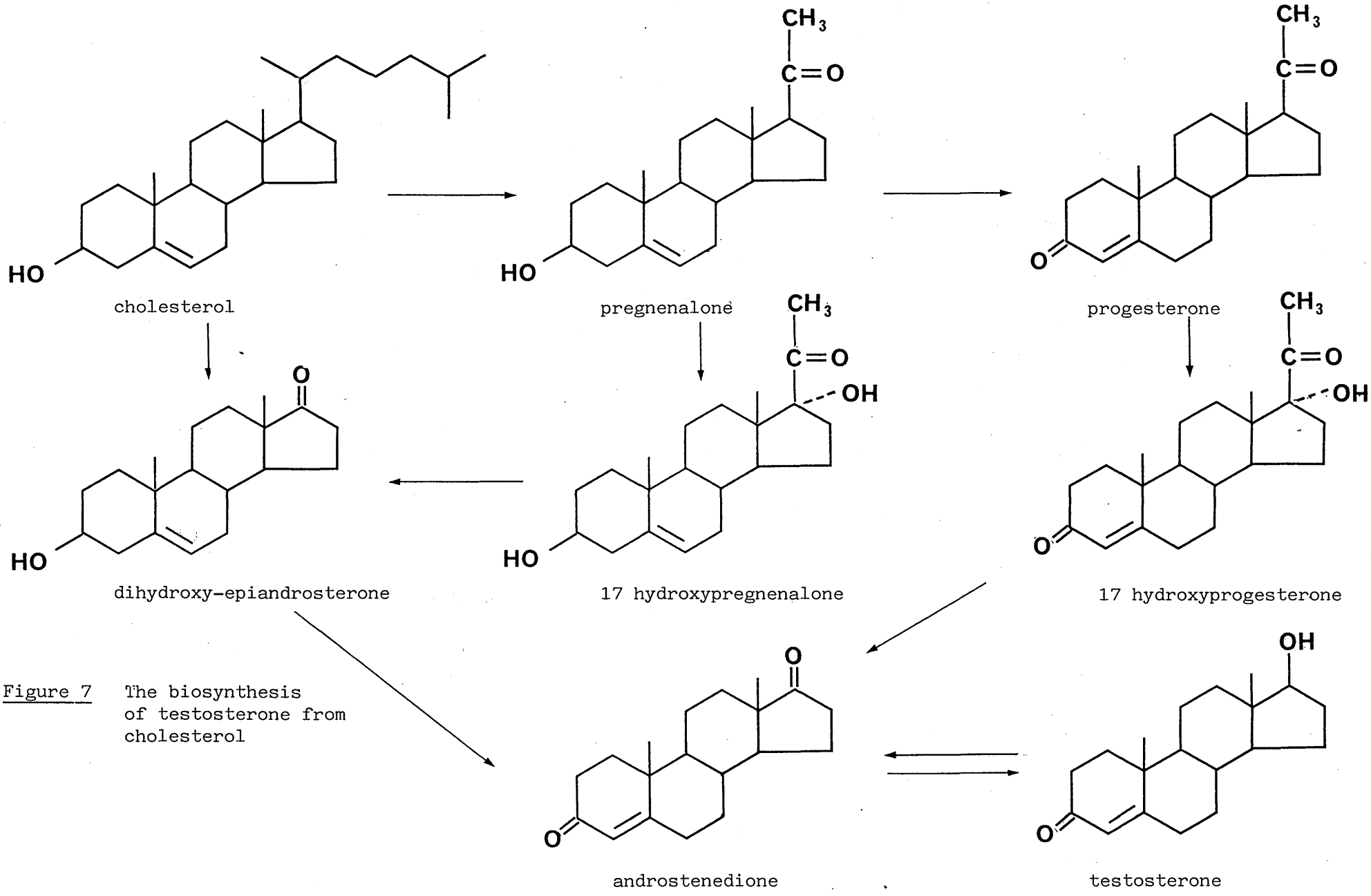
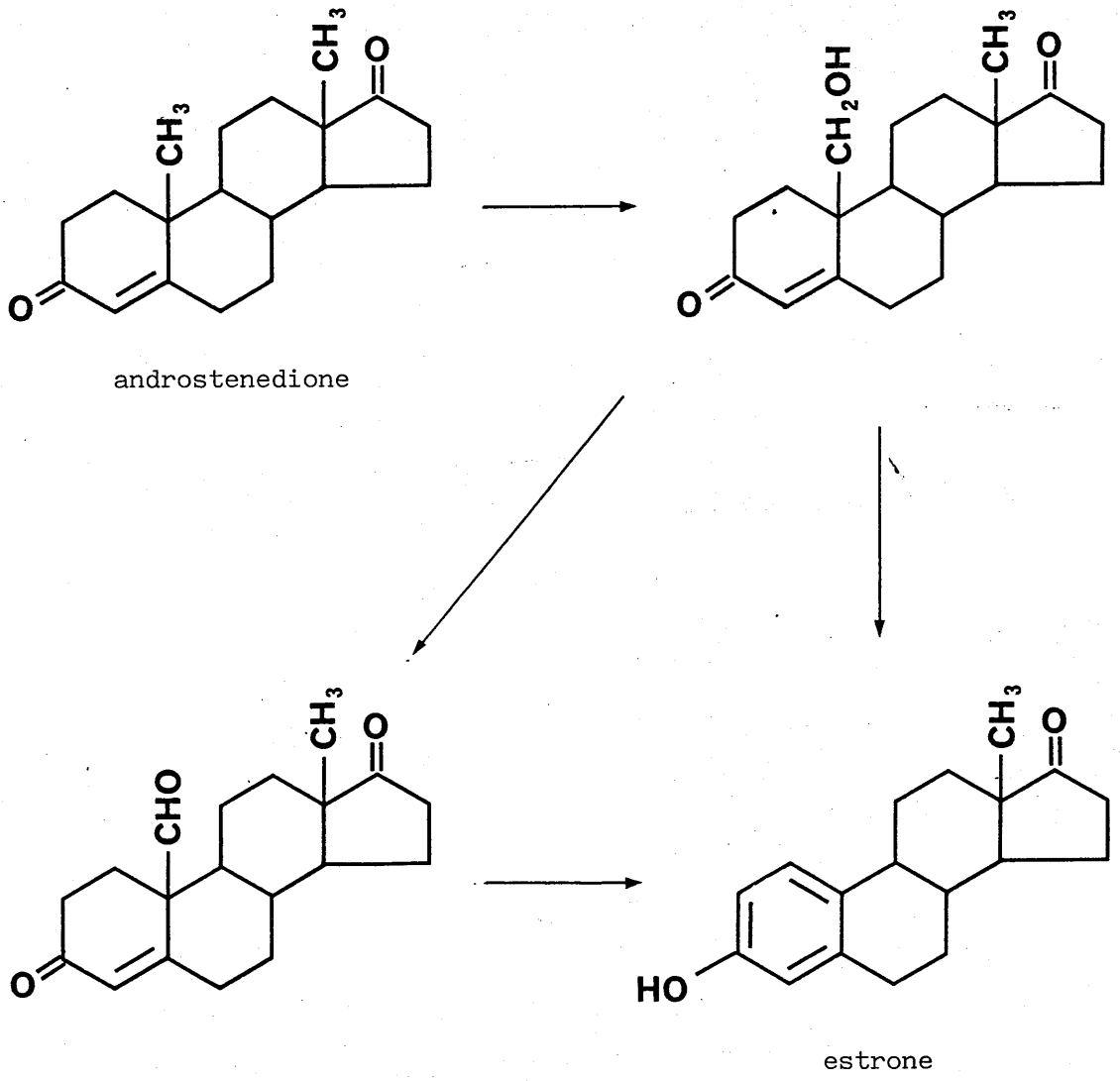


Figure 7 The biosynthesis of testosterone from cholesterol

Figure 8 The aromatisation of the androgens to the estrogens



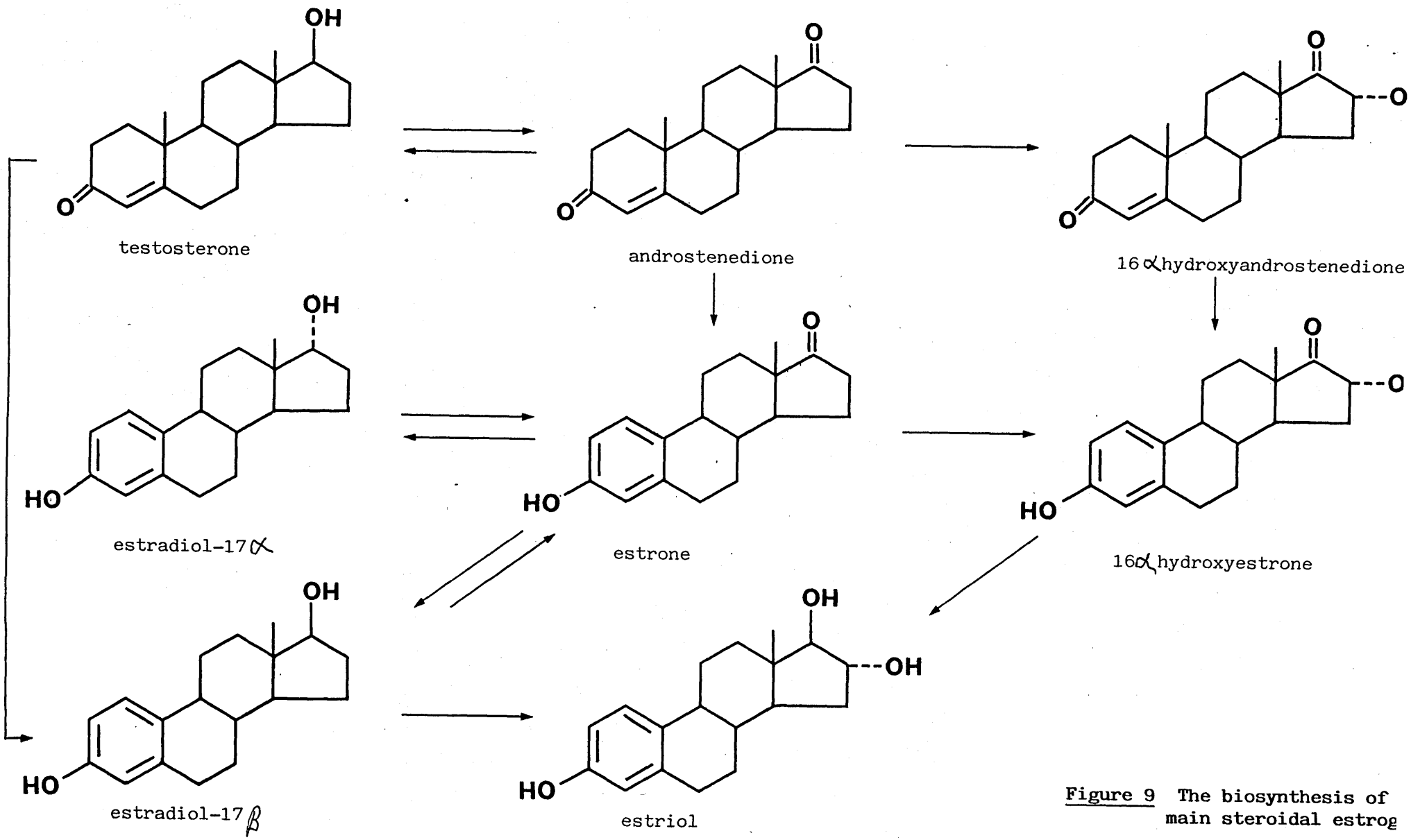


Figure 9 The biosynthesis of main steroidal estrog

analogous to that found in animal systems since the intermediates found in the animal pathway are also found in plant tissues (Grunwald, 1978).

The enzymes mevalonate-kinase and phosphomevalonate-kinase which convert MVA to 5-pyrophosphomevalonate have been shown to occur in a number of plant tissues including Cucurbita pepo (Loomis and Battaile, 1963), Ipomoea batatus (Oshima-Oba and Uritani, 1969), Kalanchoe crenata (Thomas and Stobart, 1970), Pisum sativum (Pollard et al, 1966) and Phaseolus vulgaris (Rogers et al, 1966). The further conversion of 5-pyrophosphomevalonate to IPP has been observed using cellfree systems prepared from Pisum sativum (Pollard et al, 1966), Hevea brasiliensis latex (Chesterton and Kekwick, 1968), Ipomoea batatus (Oshima-Oba and Uritani, 1969) and Kalanchoe crenata (Thomas and Stobart, 1970). Additionally, the synthesis of DMPP, GPP and FPP has been demonstrated using the cell-free system from pea (Pollard et al, 1966).

In animal systems the next intermediate formed in the biosynthesis of steroids is squalene. Germinating seeds of pea readily incorporated ^{14}C MVA into squalene (Capstack et al, 1962) and the labelling pattern was the same as that of squalene biosynthesised in animal tissues (Capstack et al, 1965). The involvement of FPP in squalene formation has been shown in preparations from tomato and carrot: the reaction required NADPH as co-factor as in the analogous reaction in animals (Beeler et al, 1963). Cell-free systems which catalyse the complete sequence of steps from MVA to squalene have been obtained from tomato and carrot (Beeler et al, 1963) and from tobacco culture (Benveniste et al, 1970). Using the tobacco system, Benveniste and his co-workers (1970) demonstrated that the enzymes involved in the pathway as far as FPP were soluble while the synthesis of squalene from FPP required

microsomes.

Cyclisation of squalene in plants is also thought to proceed by mechanisms similar to those operating in animals: the incorporation of 1-^{14}C acetate into squalene-2,3-oxide has been demonstrated with a tissue culture of Nicotiana tabacum (Benveniste and Massy-Westrop, 1967) and also using the latex of Euphorbia cyparissias (Ponsinet and Ourisson, 1968).

The intermediate formed between squalene-2,3-oxide and cholesterol in animals is the compound lanosterol but in plants it is believed instead to be cycloartenol. This belief resulted from the reports that cycloartenol was produced from ^{14}C squalene-2,3-oxide in a cell-free homogenate prepared from the leaves of Phaseolus vulgaris (Rees et al, 1968) and in tissue cultures of Nicotiana tabacum (Eppenberger et al, 1969). In neither case was there evidence of the production of lanosterol, indicating that cycloartenol was produced directly from squalene-2,3-oxide and not by subsequent modification of lanosterol. The enzyme involved in the conversion of squalene-2,3-oxide to cycloartenol in plants and to lanosterol in non-photosynthetic eukaryotes is the same. The mechanistic differences underlying the catalytic activity of the enzyme are, however, unknown (Benveniste, 1986).

The key reaction in animal steroid hormone biosynthesis is the degradation of cholesterol to pregnenalone. Cholesterol has been isolated from a number of higher plants, and its possible importance as a precurser in the formation of a range of steroids has been suggested (see Heftmann, 1975). Bennett and Heftmann (1966) isolated ^{14}C pregnenalone from the leaves of Haplopappus heterophyllum which had been fed with ^{14}C cholesterol, inferring that the enzyme required to remove the side chain from cholesterol was present. This was

confirmed by Pilgrim (1972) by demonstrating that side chain removal from cholesterol-26-^{14}C occurred *in vivo* in *Digitalis purpurea*.

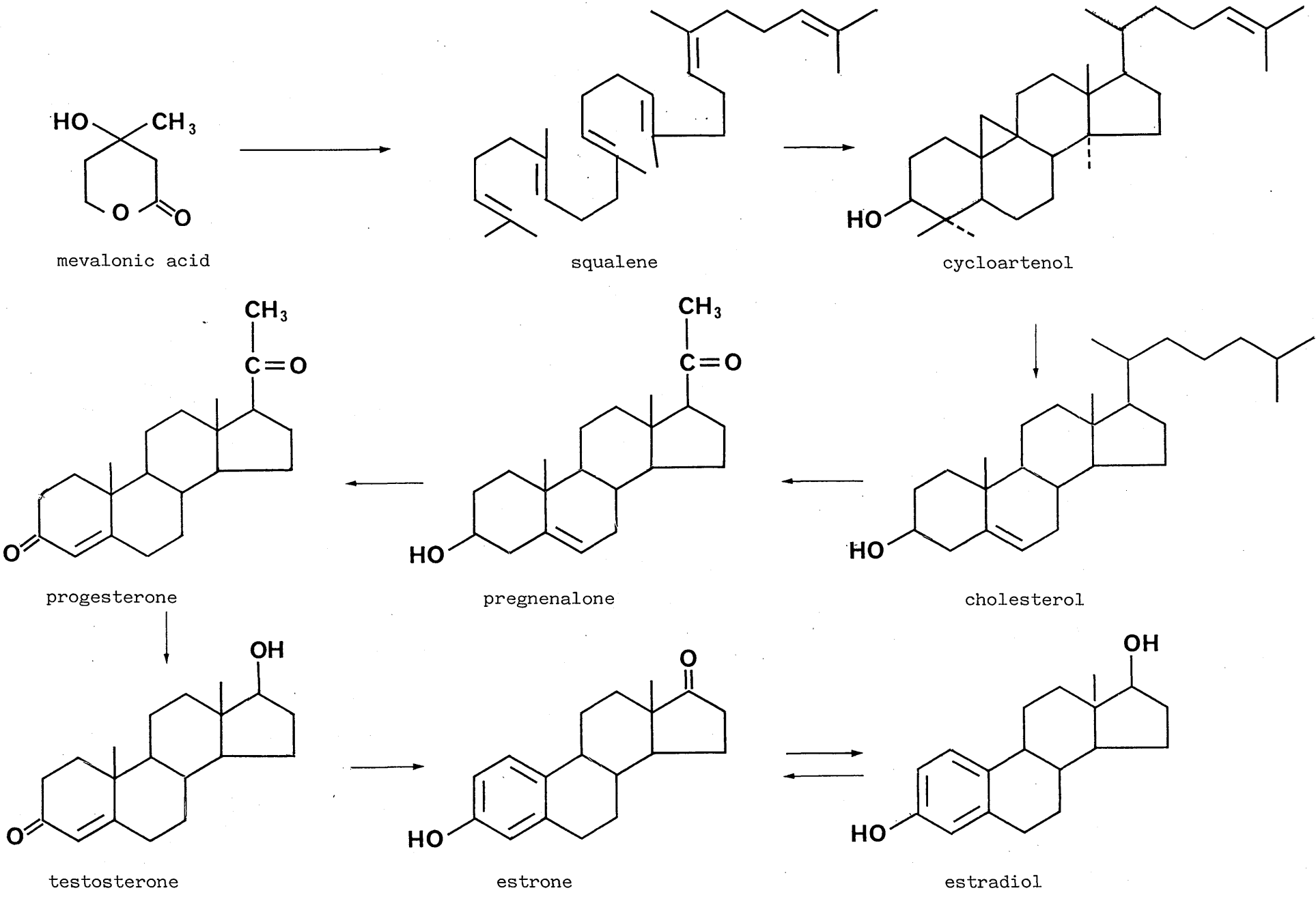
In animals the sex hormones are fromed from pregnenalone via the intermediate progesterone. Progesterone has been isolated from the leaves of *Holarrhena floribunda* (Bennett and Heftmann, 1965) and from apple seeds (Gawienowski and Gibbs, 1968) and its formation from pregnenalone has been observed *in vivo* in *Holarrhena floribunda* (Bennett and Heftmann, 1965) and in *Digitalis lanata* (Caspi and Lewis, 1967) and *in vitro* in cultures of *Digitalis purpurea*, *Digitalis lutea* and *Nicotiana tabacum* (Graves and Smith, 1967) and also in leaf homogenates of *Cheiranthus cheiri* (Stohs and El-Olemy, 1971).

Production of steroids in plants has been monitored by incorporation of label from $^{14}CO_2$ (Biddulph and Corry, 1965) and from ^{14}C MVA (Bennett *et al*, 1967; Bledsoe and Ross, 1978), but the components of the steroid fractions were not identified. The synthesis of testosterone in the leaves of growing pea plants from 4-(4-^{14}C) androstene-3,17- dione has been observed (Lin *et al*, 1979). Identification of testosterone was by TLC and co-crystallization. These workers have also studied the metabolism of (4-^{14}C) progesterone in pea using normal and reversed-phase high performance liquid chromatography (HPLC) and thus identified 11 radioactive products (Lin and Heftmann, 1981). Stohs and El-Olemy (1972) demonstrated that androstenedione was converted to 3β-hydroxy-5 -androstan-17-one and 5 -androstane- 3β,17β-diol by cell suspension cultures of *Dioscorea deltoidea*. Hirotani and Furuya (1974) reported that tissue cultures of *Nicotiana tabacum* were capable of transforming testosterone to 4-androstene- 3,17-dione and 3β-hydroxy-5 -androstan-3-one and also 4-androstene- 3,17-dione to testosterone and 17β-hydroxy-5 -androstan-3-one.

Conversion of progesterone and 4-androstene-3,17-dione to estrogens in higher plants has never been demonstrated. Nevertheless, the biosynthesis of estradiol has been demonstrated in Phaseolus vulgaris (Young et al, 1977, 1979) by incorporation of biosynthetic label from ^{14}C MVA and ^{14}C E_1 (see earlier). This implies that the enzymes required for estradiol biosynthesis are present in plants or that their synthesis can be induced by substrate application indicating that the plant possesses the genetic information for steroid specific enzyme synthesis. However, although the endogenous biosynthesis of estradiol occurred, distribution studies revealed that over 90% of the label from ^{14}C E_1 remained in the cutting base, so that the results may be anomolous. In an attempt to overcome the problem of restricted transport for the precurser ^{14}C E_1 the water soluble conjugate ^{3}H estrone-sulphate was used. In this case radioactivity moved much more readily throughout the plant and the production of ^{3}H estradiol-sulphate occurred (Young et al, 1979). In leaf discs of P. vulgaris ^{14}C E_1 was reduced to ^{14}C E_2 and ^{3}H estrone-sulphate was transformed to ^{3}H E_1 and ^{3}H E_2 with some other unidentified substances also produced (Hewitt and Hillman, 1979a). Using cell-free tissue homogenates from P. vulgaris, Young et al (1979) demonstrated that within two hours, 43% of the radioactive label from ^{14}C E_1 had been incorporated into estradiol when suitable co-factors were supplied. This rapid metabolism in homogenates has given rise to the suggestion that the much slower rates of interconversion of estrone and estradiol in leaf discs and in cuttings results from poor penetration of estrone into the plant tissue or to a compartmentalisation of the required enzymes and the applied estrogen (Hewitt et al, 1980).

A summary of the proposed pathway for the biosynthesis of estrogens in higher plants is illustrated in Figure 10.

Figure 10 Summary of the prosposed biosynthetic pathway for estrogens in plants



1.6 Physiology of Estrogens in Animals

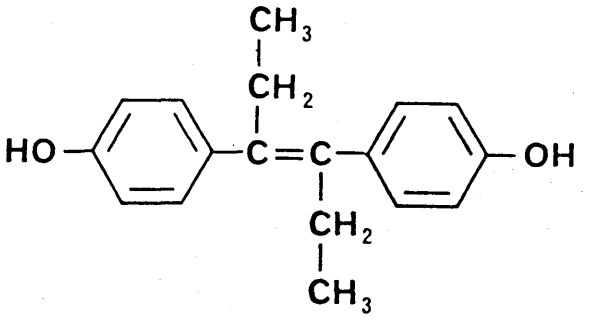
The literature concerning the physiology of estrogens in animals is vast but some generalisations can be made which are valid in the context of estrogens in plants.

Estrogens in animals are produced by the ovaries and their main function is the development of the female sex organs and secondary sex characteristics. In many of their functions estrogens act synergistically with the other ovarian hormone, progesterone. Ovarian production of estrogens varies periodically, menstrual cycle in primates, estrus cycle in the lower mammals. During the cycle, the main action of estrogens is on the growth and function of the reproductive tract in order to prepare it for a possible pregnancy. The secretion of estrogens is under the control of gonadotropins produced by the pituitary, in particular follicle stimulating and lutenising hormones. Conversely, high levels of estrogens effect a feedback inhibition of these hormones, ensuring the suppression of further ovulation. During pregnancy, estrogens, together with progesterone, maintain the uterine environment such that it is conducive to the development of the embryo and also induce mammary gland development.

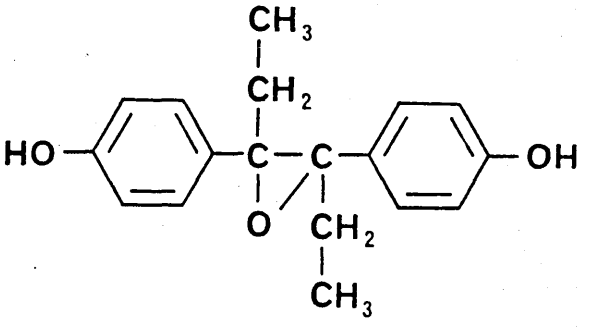
In certain non-mammalian species estrogens are involved in the promotion of synthesis of proteins required for egg production such as ovalbumin in the chick oviduct and vitellogenin in the frog liver.

Although steroidal estrogens are essential substances to animals, they have long been suspected carcinogens and indeed the synthetic estrogen, diethylstilboestrol, (DES) and two of its metabolites, DES-oxide and 2-dienoestrol (Figure 11) are among the few known steroidal carcinogens in man (Herbst *et al*, 1971; Rüdiger *et al*, 1979).

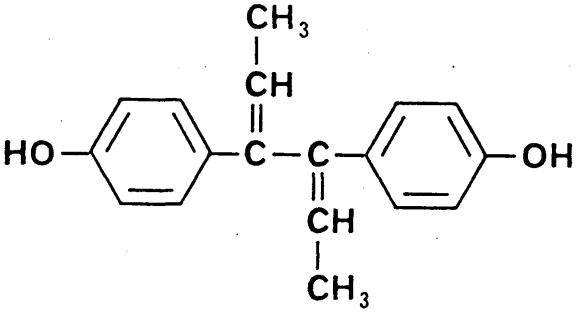
Figure 11 Structural formulae of some synthetic estrogens



diethylstilboestrol



diethylstilboestrol-oxide



2-dienoestrol

1.7 Physiology of Estrogens in Plants

Although it is now fairly certain that higher plants contain estrogens, as yet no precise physiological role, if indeed there is one, has been described. Nevertheless the effects of applying

exogenous estrogens has received much attention, and wide-ranging effects encompassing many aspects of plant growth and development have been reported. The literature concerning this subject has been extensively reviewed, earlier by Thimann (1935), Bonner (1937), Löve and Löve (1945) and Heftmann (1963) and more recently by Heftmann (1974, 1977), Young (1977), Geuns (1978), Hewitt and Hillman (1979a), Hewitt et al (1980), and Hewitt (1980). Some of the more commonly reported effects are discussed below.

1.7.1 Vegetative Development

Application of estrone was found to stimulate growth of isolated corn root tips (Fielder, 1936) and of isolated pea embryos grown on solid culture medium (Bonner and Axtman, 1937). Similarly, Helmkamp and Bonner (1953) found that application of estrone significantly enhanced growth of excised mature pea embryos. Pea was also used in a study by Kopcewicz (1969a), the results of which showed that application of 0.1µg of E_1 and E_2 had the ability to increase shoot elongation in the dwarf cultivar, Cud Kelwedonu. Dwarf peas are particularly responsive to gibberellins in terms of stem elongation and application of 0.1µg of E_1 enhanced shoot growth to the same extent as 0.001µg of gibberellic acid (GA_3) (Kopcewicz, 1969b). Jones and Roddick (1977) tried to repeat Kopcewicz's experiements using tall (Alaska) and dwarf (Meteor) cultivars of pea but were unable to induce estrogen-stimulated growth or detect any interaction between exogenous GA_3 and estrone, thus raising doubts as to how widespread this response to estrogens is. These doubts were added to by the findings of Hewitt and Hillman (1979a): growth of cuttings used in rooting experiments was unaffected by estrogens applied in solution or directly to the primary leaves or apices of Phaseolus vulgaris. Additionally, no

estrogen-stimulated growth was detected when Little Marvel pea seedlings and P. vulgaris seedlings were treated with estrone, estradiol, estrone-phosphate or estradiol-sulphate applied in methanolic solution to apices.

Löve and Löve (1945) carried out an extensive investigation into the effects of estrogens in plants and found that germination of Melandrium (Silene) dioicum seeds was stimulated by E_1 and E_2. Similarly, germination in the dark was enhanced by E_2 in the seeds of Pinus silvestris (Kopcewicz, 1970a) and Pinus pinea (Martinez-Honduvilla et al, 1976). These findings, however, have been contradicted by those of Hewitt (1980): Hewitt tested the effects of steroidal estrogens on germination of P. vulgaris but found no effects, and some preliminary experiments using Grand Rapid Lettuce seeds also indicated that estrogens were without effect on germination over a range of temperatures in darkness, red light and white light.

Gioelli (1942) examined chlorophyll levels in carrot root cultures as a means of examining the effects of estrogens added to the culture medium on the processes of senescence. Chlorophyll loss is visibly the most obvious aspect of senescence, although it is only part of a very complex phenomenon. Chlorophyll levels in the carrot cultures were found to increase, indicating that senescence was delayed. Hewitt (1980) also examined chlorophyll levels in P. vulgaris plants but could not detect any differences in the chlorophyll levels of control plants and plants treated with E_1, E_2 or estradiol-sulphate.

Another aspect of vegetative development which has received attention in terms of effects of applied estrogens is the process of root initiation. A report that estrogenic substances affected rooting first appeared in the literature in 1937: Went and Thimann noted that when estrone and auxin were applied together rooting was promoted. In

1967 Fadl and Hartmann demonstrated that an unidentified rooting factor was present in the 'easy to root' plant Old Home pear, but not in the Bartlett pear which roots much less readily. The highly active root promoting substance was found in extracts of basal segments of cuttings which had been treated with indole-3-butyric acid (IBA) and which had buds present. The rooting factor was not present in cuttings without buds or cuttings with buds but which had not been treated with IBA. Fadl and Hartmann proposed that the rooting factor, which was of a phenolic nature, was a condensation product between the exogenous IBA and a phenolic compound produced by physiologically active buds of Old Home pear. In the same year, Lesham observed that root formation in broccoli curd cuttings could be stimulated by estradiol but only at 3 and 50ppm and not at 25ppm. The effects of a range of estrogenic substances on rooting in P. vulgaris, a species used extensively in the study of rooting (eg Varga and Humphries, 1974; Altman and Wareing, 1975) yielded some interesting results, (Hewitt and Hillman, 1979b, 1980): solutions of estrone, estrone-sulphate, estradiol, and estradiol-sulphate in the concentration range 10^{-6}M to 10^{-10}M had no observable morphological or anatomical effects on adventitious root formation in P. vulgaris hypocotyl, epicotyl or primarly leaf cuttings. Similarly, direct applications of estrogens to apices of hypocotyl cuttings, rooting in distilled water, were without effect. However, application by wick feeding of estrone-sulphate and estradiol-sulphate at concentrations of 10^{-4}M significantly inhibited rooting with the inhibition being almost complete in epicotyls and primary leaves.

In P. vulgaris hypocotyls and epicotyls, root initials form in the gaps in the vascular tissue. A vascular cambium develops producing xylem internally, but no tissues are formed externally. In the

petioles of primary leaf cuttings adventitious roots arise at the side of the main leaf traces as an extension of meristematic activity from the vascular cambium (Hewitt et al, 1980). In cuttings where inhibition of rooting was observed, cell division was still apparent, but this took place within the phloem of epicotyls and petioles, and in the petioles cell proliferation also took place in adjacent parenchymatous tissues. No ordering of the cell division occurred and callus-like hyperplastic tissue formation was evident. Since mitotic activity was not inhibited, Hewitt and Hillman concluded that it was the fundamental ordering processes required for differentiation into root initials which had been suppressed.

The callus tissue formation took place at the cutting base and resembled crown gall tumours induced by Agrobacterium tumefaciens (Hewitt, 1980). A possible link between estrogens and plant tumours has been made previously: Havas (1935) noted that young female rats and mice implanted with crown gall from Pelargonium or infected with A. tumefaciens reached sexual maturity before controls and that plant tumours increased in size when treated with estrogens. It has also been demonstrated that the synthetic estrogen, DES, stimulates crown gall tumour cell division in vitro (Manil, 1961).

1.7.2 Flowering and Sex Expression

In addition to the effects on vegetative development, many workers believe that estrogens are involved in the processes of flowering and sex expression. In 1937 Chouard reported that watering Callietephus sinensis with an estradiol solution induced flowering. Similarly, flowering was induced in two strains of Lemna minor by the addition of E_2 to culture media when it could not be induced by long or short days (Czygan, 1962). However, other factors were probably involved in this

response since it could not be produced at any time (Geuns, 1978). Some more evidence in support of an estrogen involvement in flowering was obtained from investigations into the effects of inhibitors of cholesterol biosynthesis on photoperiodic induction. Using the SDPs, Xanthium strumarium and Pharbitis nil, Bonner, Heftmann and Zeevaart (1963) demonstrated that flowering could be suppressed by the inhibitor (tris-(2-diethylaminoethyl)- phosphate trihydrochloride) (SK and F $7997A_3$) which is known to block the biosynthetic pathway in animals at the conversion of lanosterol to cholesterol. The inhibitor was found to be most active if applied to the leaves shortly before the inductive long night. Lesham (1967) obtained similar results using broccoli: flowering was promoted by various concentrations of steroids but inhibited by SK and F $7997A_3$. Bonner and his co-workers (1963) suggested that it was the synthesis of a flower hormone which was being affected and that this hormone was probably an isoprenoid or steroid-like compound, yet they were unable to detect any obvious differences in the MVA metabolism of induced and non-induced plants. Bledsoe and Ross (1978) obtained similar results using Xanthium strumarium plants which had been fed with ^{14}C MVA but in addition they detected modifications in MVA metabolism in plants which had been treated with SK and F $7997A_3$. When Haplopappus heterophyllus plants were supplied with ^{14}C MVA the phenolic fraction from flowering plants contained ^{14}C although the label was not associated with any identifiable estrogen (Bennett et al, 1967).

Kopcewicz reported that when estrogens were applied to apices, E_2 promoted flowering in the LDP Cichorium intybus (1970b) and that E_1 increased the number of flowers in Ecballium elaterium (1971b). Also, E_2 induced flowering in the LDP Salvia splendens under short days although to a lesser extent than an estrogen fraction isolated from a

flowering plant of the same species (Kopcewicz and Porazinski, 1974). Estrogen-like substances appeared initially as the flower bud emerged and reached maximum levels as the flower bud developed in the day neutral plant Phaseolus vulgaris (Kopcewicz, 1971a), in the LDPs Hyoscyamus niger and Salvia splendens (Kopcewicz, 1972a), in the SDPs Perilla ocymoides and Chenopodium rubrum (Kopcewicz, 1972b), and in the cold-requiring plant Hyacinthus orientalis (Kopcewicz et al, 1973). These findings led Kopcewicz to suggest that there is a link between estrogen production and the different photoperiodic and environmental requirements for flowering in different species.

Despite the above and many other reports (see Hewitt, 1980) that estrogens are involved in flowering, other reports exist that indicate that estrogens are without effect on the process (eg Havas and Caldwell, 1935; Harder and Störmer, 1935). More recently, Hewitt (1980) found that flowering was unaffected following treatment with estrogens applied to the apex, leaves or stems of whole plants and cuttings of P. vulgaris.

Hylmö first reported in 1940 that in the flowers of Spinacia plants treated with estrone, stamen development was suppressed while testosterone treatment suppressed pistil development. Löve and Löve (1945) applied lanolin paste containing relatively low concentrations of sex hormones to the cut surface of decapitated stems of Melandrium (Silene) dioicum plants and observed an increased number of female flowers after estrone and estradiol treatments, and an increased number of male flowers after treatment with testosterone. In Ecballium elaterium application of estrogens to apices increased the number of flowers produced and the female: male flower ratio, while application of androgenic compounds increased the ratio of male flowers (Kopcewicz, 1971b). Higher estrogen levels were found in

induced female plants than in control plants of Cucurbita pepo when the induced plants had been treated with the ethylene substitute, CEPHA, which is used to promote femaleness (Kopcewicz and Chrominski, 1972). Sex expression in cucumber was influenced in the female direction by treatment with either estradiol or testosterone (Gawienowski et al, 1971) although it has been suggested that the applied male sex hormone, testosterone, was first metabolized to estradiol (Geuns, 1978).

1.7.3 Effects on Endogenous Growth Regulators

Kopcewicz has tried to explain the effects of estrogens in plants in terms of their interactions with endogenous growth regulators. Stimulation of pea seedling growth by estrone was accompanied by a simultaneous increase in the endogenous GA content (Kopcewicz, 1969b). This led Kopcewicz to suggest that the increased growth was the result of high levels of GAs induced by estrogen treatment. Evidence in support of this suggestion was obtained from an investigation of flowering in Cichorium intybus (Kopcewicz, 1970b) where GA_3 was found to induce flowering in non-inductive conditions. Estrone and estradiol were also effective in inducing flowering although it was slightly later and the stems were slightly less elongated.

Kopcewicz (1970c) has also tried to link estrogen effects and changes in the endogenous auxin content: E_2 was found to increase auxin and gibberellin activity in pine seedlings and in pea. Conversely, indole acetic acid (IAA) and GA_3 apparently have no effect on the endogenous levels of estrogens (Kopcewicz, 1971c, 1972c).

Further evidence that estrogens interact with endogenous growth substances was presented by Kopcewicz: estrogens were found to have a positive effect on the endogenous cytokinin levels in bean although

they lacked influence on the content of ABA-like substances (1972d). On the other hand, kinetin increased and ABA decreased the levels of endogenous estrogens (1972c).

1.7.4 Uptake, Distribution and Metabolism of Exogenous Estrogens

It can be seen from the above reports that many discrepancies exist in the observations and conclusions of the various investigations carried out to try and establish the physiological effects of applied estrogens. As pointed out by Hewitt (1980), several possible explanations may account for these discrepancies: growth experiments involving whole plants, especially where cloned material has not been used, are expected to yield variable results. Problems concerning the variability in the purity of the estrogenic compound used may also be a contributory factor. Additionally, the early workers never really considered the uptake, distribution and metabolism of the applied compound despite the fact that details of these processs are essential for the correct interpretation of results obtained from application experiments. Studies concerning these aspects have been facilitated by the availability of radioactively labelled estrogens. When using these compounds it must be borne in mind that unless metabolism of the applied compound has been studied, it is merely the uptake and distribution of radioactivity that is being monitored. The metabolism of steroidal estrogens in higher plants was considered earlier.

Hewitt and Hillman (1979a) investigated the uptake of ^{14}C and ^{3}H labelled estrogens by leaf discs of Phaseolus vulgaris. The results obtained indicated that there was an initial rapid passive uptake of radioactivity which paralleled a rapid uptake of water as the cells regained turgor after cutting. Once the uptake of water had reduced, the uptake of radioactivity continued and it was suggested that this

was due either to an increased estrogen concentration resulting from evaporation of the medium or that the estrogens entered the cells of the leaf discs where the membranes and other lipid bodies offered environments more conducive to their hydrophobic nature. In seeds the picture was similar as again there was an initial rapid uptake of radioactivity paralleling a rapid uptake of solution, and then once uptake of solution had reduced there was continued uptake of radioactivity (Hewitt, Berry, McIntosh and Hillman unpublished, cited in Hewitt et al, 1980). Studies of the uptake of labelled estrogens using the method of wick feeding (Atallah et al, 1975) have been carried out using cuttings of P. vulgaris. The presence of radioactivity within cuttings indicated that uptake had occurred but the detailed characteristics of the process were not considered (Young et al, 1979).

Relatively little is known about the distribution of applied estrogens in higher plants in general, but detailed examination of the fate of ^{14}C estrone and ^{14}C estradiol in Phaseolus vulgaris has been made (see Hewitt et al, 1980). In these examinations labelled estrogens were applied to hypocotyl cuttings by wick feeding. The results obtained for ^{14}C estrone and ^{14}C estradiol were very similar and showed that although radioactivity was detectable throughout the cutting, over 90% of the label taken up remained in the cutting base, and radioactivity redistributed to plant parts above the primary leaves accounted for only 5% (Hewitt and Hillman, 1979a; Young et al, 1979). Metabolism experiments revealed that most of the recoverable label was contained in the applied compound (Young et al, 1979). A similar distribution pattern became apparent after applications of ^{14}C estrogens by wick feeding to epicotyl and primary leaf cuttings (Hewitt and Hillman, 1979a). These authors suggested that the restricted movement of the

estrogens within cuttings was attributable to the hydrophobic nature of the molecules: the steroid molecules may have entered the cells which provide more suitable lipid environments rather than being carried around the plant in the transpiration stream. The problems concerning the solubility of E_1 and E_2 can be partially overcome by using the water soluble sulphate conjugates. Application of ^{3}H estrone-sulphate resulted in a much more even distribution of radioactivity, with only about 50% of the label remaining in the cutting base of hypocotyls and as little as 10% in epicotyls and primary leaf cutting (Hewitt and Hillman, 1979a).

Of the label from ^{14}C estrone and ^{14}C estradiol transported past the base, relatively high levels were translocated to the auxillary and cotyledonary buds, which suggested that the meristems were acting as metabolic sinks. To try and confirm this, labelled estrogens were applied in methanolic solution to the primary leaves of hypocotyl cuttings where metabolic sinks were provided by root primordial meristems, but even after seven days less than 10% of the label applied had moved away from the primary leaves. A similar situation resulted from applying ^{3}H estrone-sulphate in the same manner. However, as the authors point out, the results did not take into account the amount of label available to the cutting or the degree and rates of uptake. Redistribution of label above the primary leaves was similar to that obtained when the estrogens were applied by wick feeding. The levels of radioactivity in the roots were found to increase just prior to and during nodule development, but this was not associated with an increase in root mass. Similar distribution patterns were obtained when estrogens were fed to whole plants by the same method (Hewitt and Hillman, 1979a).

In seeds ^{14}C estrogens taken up from solutions were for the most part

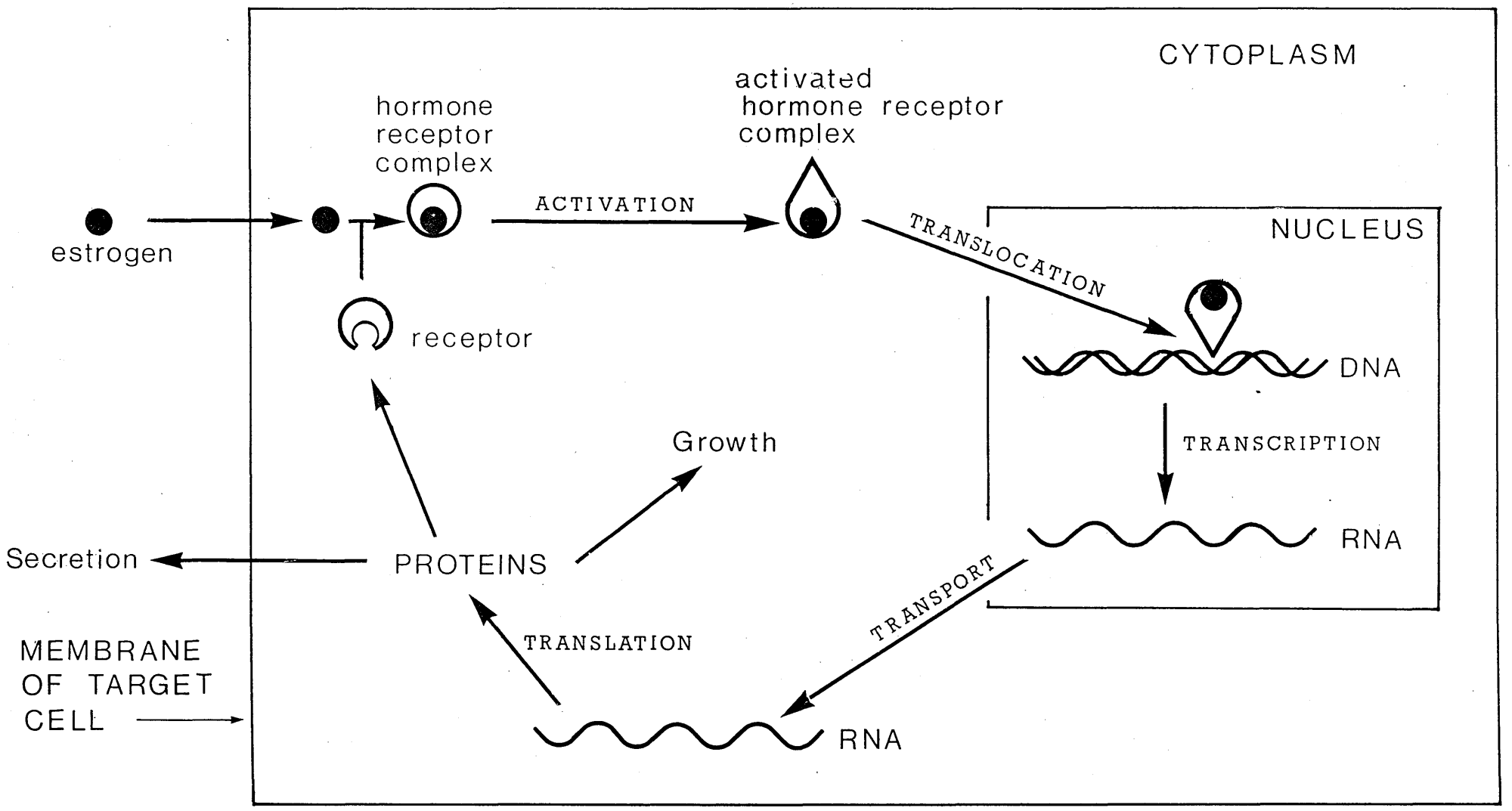
restricted to the cotyledons with little movement away from these sites during subsequent growth and development. Indeed, most label was lost on abscission of the cotyledons, although some was redistributed to the roots and hypocotyl (Hewitt et al, unpublished, cited in Hewitt et al, 1980).

1.8 Mode of Action of Steroid Hormones

In order to explain the effects of exogenously applied estrogens on growth and development, it is essential to understand the underlying biochemical processes. Very little is known about the mode of action of steroid hormones in plants, indeed very little is known about the molecular basis of action of any of the plant growth regulating substances. Nevertheless, the last two decades have seen remarkable progress towards the elucidation of the biochemical processes underlying the mode of action of steroid hormones in animals, although as yet the precise nature of many aspects of these mechanisms is not completely understood.

Steroid hormones in animals exert their effects by a simple two-step process first described by Jensen and his group in 1968 and based mostly on the estradiol receptor system. Firstly, the hormone is selectively accumulated by target cells which contain extra-nuclear proteins which have a high affinity and specificity for their particular hormone. Binding of the hormone to its receptor induces a change in the receptor to an active form, which unlike the native form has a high affinity for chromatin. The activated hormone receptor complex is then translocated to the nucleus where it binds to chromatin and causes alterations in the expression of specific genes. This picture of steroid hormone action is summarised in Figure 12.

Figure 12 The mechanism of steroid hormone action



Estrogen receptors, which are often referred to generically as estrophillin, have been most extensively studied in the rat uterus. The first estrogen receptors which were characterised by Toft and Gorski (1966) were from such tissue and were originally detected in association with radioactively labelled estradiol-17β on sucrose density gradients of low ionic strength. The sedimentation co-efficients of these receptors was 8S, but this depended on the salt concentration used during extraction and fractionation. If a salt concentration of 0.3M to 0.4M is used, a 4S receptor is isolated and this is thought to be the form existing in target cells. It contains a single polypeptide with a molecular weight of approximately 80,000, and one hormone binding site (Chamness and McGuire, 1972; Notides and Nielson, 1974). The 8S complex is believed to be an artifact of the isolation procedure and it has been suggested that it results from the association of a 4S receptor and a second non-receptor protein (Moncharmont et al, 1982).

Recently, reports have been published which indicate that estrogen receptors are of nuclear origin (King and Greene, 1984; Welshons et al, 1984). Nevertheless, binding of the hormone to the receptor, be it cytoplasmic or nuclear, results in an increased affinity of the hormone receptor complex for nuclear elements. This phenomenon has been termed 'activation' (Gschwendt and Kittstein, 1980) and is believed to be the result of an allosteric effect (Jensen et al, 1969). Receptor binding is accompanied by an increase in the sedimentation co-efficient of the receptor from 4S to 5S (Notides and Nielson, 1974). This increase is termed 'transformation' (Gschwendt and Kittstein, 1980), and a build-up of evidence suggests that it is a second-order reaction involving another factor as well as the hormone-receptor complex (Yamamoto and Alberts, 1972; Yamamoto, 1974; Thampan

and Clark, 1981). This protein bound to estrogen-receptor complexes but not to estrogens alone, and it caused activation and translocation to the 5S receptor form which then bound uterine nuclei.

Possibilities for the nuclear component involved in binding the hormone receptor complex have been reviewed by Knowler and Beaumont (1985) and include the nuclear envelope, histones, non-histone proteins, deoxyribonucleic acid (DNA), ribonucleo proteins, and the nuclear matrix. Most attention, however, has been centred on the DNA of activated genes and a possible role for chromatin proteins.

Once binding of the hormone receptor complex to the nucleus has occurred, there is stimulation of transcription. Although as yet the mechanisms underlying the changes in genetic activity are not understood, it is thought that a number of nuclear elements, including enhancer elements, repetitive elements and nuclear matrix, are involved (see Knowler and Beaumont, 1985). Transcriptional effects have been studied in a number of animal systems (reviewed by Knowler and Beaumont, 1985). These include the mammalian uterus where transcriptional effects lead to the activation of the entire protein synthesising apparatus of the cell.

It is possible that plant hormones in general may have modes of action similar to those of their animal counterparts and indeed there is some evidence that this is the case.

As described above, the first step in steroid action in animals is the accumulation of hormone by target cells. There is little doubt that target tissues, if not target cells, exist in plants, and probably the best known example is in germinating barley seeds where a signal (presumed to be a gibberellin) induces the synthesis of specific enzymes in the cells of the aleurone layer (Yomo and Varner, 1971).

Once inside the target cells, animal steroid hormones bind to receptor

proteins. Much emphasis has been placed on finding possible binding sites and investigating the nature of receptors for plant hormones. Although as yet no specific receptor molecule has been characterized, and indeed there is no direct evidence that binding to receptors occurs, binding of GAs, cytokinins, auxins, ethylene and abscisic acid to subcellular fractions has been confirmed (see Hewitt, 1980). As in many areas of plant hormone research, much interest has focused on finding binding sites for auxin, and this has been critically reviewed by Rubery (1981) with the conclusion that no receptor has been isolated with the certainty that it is the receptor protein involved in the action of the hormone. However, IAA does react with macromolecular components to exert an effect: a high affinity auxin binding site was first detected in corn coleoptile membranes by Hertel and his co-workers in 1972 and subsequently this system has been studied by several groups of workers (eg Batt et al, 1976; Ray et al, 1977). The essential features of the system are as follows:

1. Binding is of high affinity, is saturable, reversible, heat labile and associated with cellular membranes.
2. One set of binding sites seems to be on the endoplasmic reticulum (Batt et al, 1976; Ray et al, 1977; Dohrmann et al, 1978), while a second set of sites may be associated with the plasma-membrane (Batt et al, 1976) or with the tonoplast membrane (Dohrmann et al, 1978).
3. The relative affinities for various auxin analogues are on the whole what would be expected on the basis of their biological activities.
4. The binding sites can be solublised from the membrane with (Batt et al, 1976) or without detergent, and purified by conventional procedures (Venis, 1977).

Despite the above system having some of the properties expected of auxin receptors, there are many deficiencies, so that it can be by no means implied that these binding sites are firmly established as auxin receptors (see Venis, 1980).

Studies of plant hormone binding are as yet in their infancy so that it has not yet been possible for any of the plant growth regulators to satisfy fully the criteria proposed by Kende and Gardner (1976) to assess binding studies. These criteria are as follows:

1. Receptors should be found primarily in cells of target tissues and not in non-reacting zones.
2. The kinetic parameters of binding should be related to dose response parameters of the biological activity.
3. A relationship should be shown to exist between the binding specificity for different analogues of the hormone and the relative biological activity of these analogues (excepting those which act as non-competitive inhibitors).

After binding of the hormone to the receptor in animals, the hormone-receptor complex is translocated to the nucleus where gene expression and ribonucleic acid (RNA) and protein synthesis are affected. The translocation of hormone- receptor complexes to plant cell nuclei has not yet been demonstrated, but it has been well established that plant growth regulators affect synthesis of RNA and protein (Bewley and Black, 1978; Jacobsen, 1977; Jacobsen et al, 1979). Probably the most extensively used system for examining hormone action at the level of RNA and protein synthesis is the GA-induced production of hydrolytic enzymes in the aleurone layers of germinating cereal seeds. Many workers believe that hormonal control of this response is at the level of transcription and possibly translation of RNA.

Quantitive changes in the production of certain proteins synthesised during *in vitro* translation of polyadenylated RNA (poly(A)$^+$RNA) from GA_3-treated aleurones in a wheat germ cell-free system has been reported (Higgins *et al*, 1976; Higgins *et al*, 1982). The most prominent of these proteins was α-amylase, and this finding was in agreement with those of Mozer (1980); poly(A)$^+$RNA from GA_3 treated tissues was purified and the most prominent poly(A)$^+$RNA present in the tissue corresponded to translatable α-amylase messenger RNA (mRNA). These findings may have resulted from effects of GA_3 on translation or processing of precurser RNA rather than control at the level of transcription. In an attempt to distinguish between these two possibilities, Bernal-Luga and his colleagues (1981) used complementary DNA (cDNA) prepared from α-amylase mRNA and showed that there was an increase in the number of sequences which hybridise with α-amylase cDNA following GA treatment. These results were substantiated by studies using cloned cDNA complementary to α-amylase mRNA isolated from GA-treated barley aleurone layers (Chandra and Muthukrishnan, 1982; Jacobsen *et al*, 1982). These workers used the cloned cDNA as a probe to show that GA_3 treatment resulted in an increased number of sequences which produce α-amylase when translated *in vitro*.

Quantitative changes have also been observed in etiolated pea stem segments treated with IAA: within two hours IAA causes at least 3 mRNA sequences to increase in translational activity relative to initial levels and to simultaneous controls, indicating highly selective IAA regulation of mRNA levels and activities (Theologis and Ray, 1982).

It is hoped that progress in this area of hormone action research will benefit from the developing methods of recombinant DNA technology and associated DNA and RNA biochemistry.

In addition to acting on gene expression, animal steroid hormones may also exert effects by mechanisms similar to the other classes of animal hormones, ie the neuromediators and the polypeptide hormones. These hormones act at the level of the plasmamembrane influencing membrane enzymes and cation transport systems. The possibility that plant hormones also exert their effects by means of altering ion fluxes or by influencing enzymes cannot be discounted (see Baulieu et al, 1978). Clearly, much work into the mode of action of plant hormones lies ahead, but with time and improved technology, a greater understanding will be achieved.

1.9 Aims and Rationale of Project

There are three main themes to the research described in this thesis: none has hitherto been investigated in detail, yet all are highly pertinent to establishing the role of estrogens in higher plants.

1. To investigate the response of the dwarf French bean, Phaseolus vulgaris L. cv. Canadian Wonder to exogenously applied estrogens in terms of effects on root initiation and the induction of abnormal cell development.
2. To establish the details of uptake of estrogens in solution by cuttings and the subsequent distribution of the applied compound within the cutting. This aspect is aided by the availability of radiolabelled estrogens and by the technique of liquid scintillation spectrometry.
3. To try to gain some insight into the mode of action of steroid hormones in plant systems. This is carried out by examining the effects of estrogens on the RNA metabolism of responding tissues, in particular by comparing the mRNA populations of treated and control material. The mRNA populations are reflected in the

polypeptides produced during *in vitro* translation. Any quantitative or qualitative differences in the polypeptides produced from mRNA of treated material reflects similar differences in the mRNA population and may indicate that estrogen effects in plants result from changes in gene expression as they do in animals.

The plant species, *Phaseolus vulgaris*, was used as it provided relatively uniform material for experimentation purposes. It has been used by many investigators to examine chemical regulating systems in higher plants and in addition it has been shown to contain estradiol and to biosynthesise it from MVA (Young *et al*, 1977, 1979).

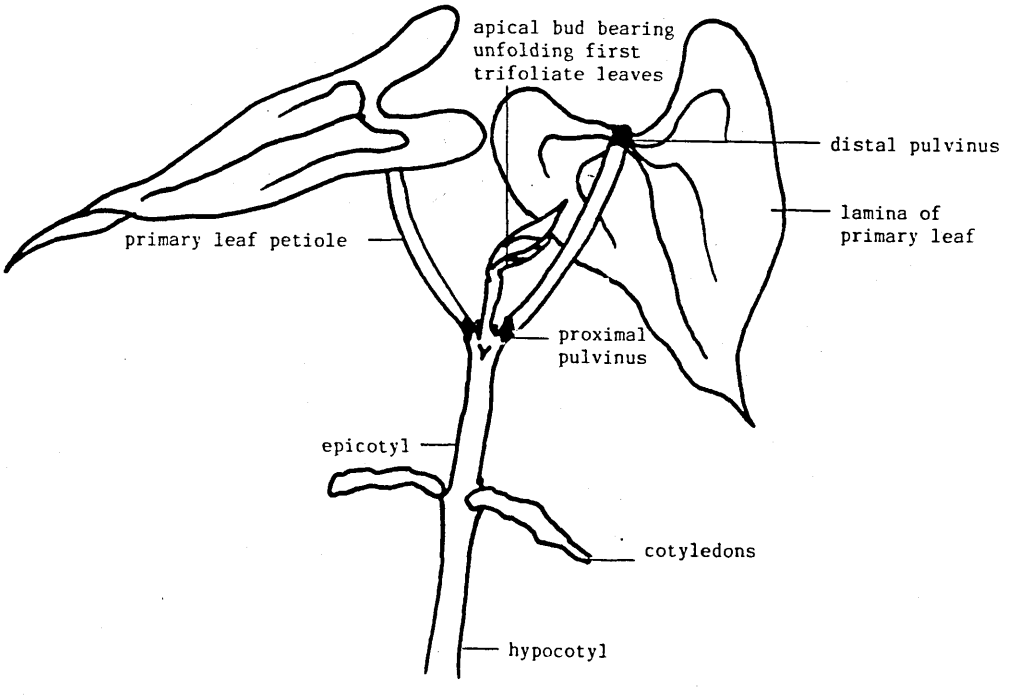
CHAPTER 2

MATERIALS & METHODS

2.1 Biological Material

The plant species used throughout these studies was the dwarf French bean, Phaseolus vulgaris L. cv. Canadian Wonder (seeds supplied by Sinclair McGill of ICI, Ayr, Scotland). The seeds were planted in 7.5cm pots in SAI (Livingston, Scotland) potting compost and grown in a heated greenhouse where the temperature was not allowed to fall below 10°C. Supplementary radiation was provided by Thorn 400W MBFR/U high pressure mercury vapour fluorescent lamps which maintained a 16h photoperiod throughout the year. Seedlings were watered as required. The morphology of a typical French bean seedling is illustrated in Figure 13.

Figure 13 **Diagrammatic representation of the shoot of a 12 day old seedling of Phaseolus vulgaris L. cv. Canadian Wonder**



2.2 Chemicals and Reagents

A list of the major chemicals used in the experiments described in this thesis is given below:

Chemical	Supplier
estrone-3-sulphate (sodium salt)	Sigma Chemical Company Ltd,
estradiol-3-sulphate (sodium salt)	Poole, Dorset, UK
indole-3-butyric acid	
Radiochemicals	
(4-^{14}C) estrone	The Radiochemical Company,
(4-^{14}C) estradiol	Amersham, UK
n - (1-^{14}C) hexadecane	
(5',5,6-^{3}H) uridine	
L - (^{35}S)-methionine	Also from New England Nuclear
	Stevenage, Herts, UK
Scintillant	
liquid scintillator Unisolve 1	Koch Light Ltd from Beveridge
	Edinburgh, UK
Molecular Biology Products	
Molecular weight markers for	Sigma Chemical Company Ltd,
proteins (described later)	Poole, Dorset, UK
High molecular weight	
standard mixture	
Dalton mark VII-L™	
Calf thymus gland DNA	British Drug House, Poole,
	Dorset, UK

All other chemicals used were of Analar or equivalent grade of purity. All solutions were made using glass distilled water. Organic solvents were redistilled before use.

2.2.1 Preparation of Phenol/Chloroform for RNA Extractions

Phenol was redistilled at 160°C to remove contaminants which cause breakdown or cross-linking of nucleic acids. 8-Hydroxyquinoline was added to a final concentration of 0.1%: this compound is an anti-oxidant and a partial inhibitor of ribonucleases (RNases). Additionally, its yellow colour provides a convenient way of identifying the phenolic phase.

Before use, redistilled phenol was melted at 70°C and saturated in buffer (1.0M Tris pH 8.0). An equal volume of chloroform was then added. Chloroform assists in removing proteins from nucleic acid preparations.

2.3 Methods

2.3.1 Physiological Effects of Applied Estrogen-Sulphate Conjugates

2.3.1.1 Preparation of Cuttings

To examine the effects of exogenously applied estrone-sulphate (E_1-S) and estradiol-sulphate (E_2-S), hypocotyl and primary leaf cuttings were taken when the first trifoliate leaf of the seedling was beginning to unfold (usually at 12 to 14 days old). Estrogen-sulphates are water-soluble and so were applied by the method of wick feeding (Atallah et al, 1975). This method involves standing cuttings in solutions and assumes that the applied compound is taken up in the transpiration stream. All cuttings were excised under distilled water to prevent xylary embolisms and the bases placed immediately into 25ml vials containing the appropriate solution. The cuttings were

supported in the vials with a plug of cotton wool. Hypocotyl cuttings were excised 30 ± 1mm below the cotyledonary node and the cotyledons were removed in air using a wet blade. Primary leaf cuttings were excised 40 ± 1mm below the leaf blade and did not include the basal pulvinus. Cuttings were then placed in a constant temperature growth room set at 25°C. Illumination of $10Wm^{-2}$ (measured using a UDT Model 40X Opto Meter, wavelength range 450-910nm) was provided by Thorn 400W MBFR/U high pressure mercury vapour fluorescent lamps. The cuttings were placed under a polythene enclosure to prevent excess transpiration.

E_1-S and E_2-S were applied in aqueous solutions at a concentration of 10^{-4}M. Distilled water and sulphate (Na_2SO_4 at a concentration of 10^{-4}M) controls were included in each experiment. The E_1-S sold by Sigma contained 35% (w/w) Tris and the E_2-S contained 67% (w/w) N-methyl-D-glucamine (Nmglc). These compounds are used to stabilise the estrogen-sulphates and their presence necessitated the incorporation of appropriate control treatments (1.7×10^{-4}M Tris and 4.0×10^{-4}M N-methyl-D-glucamine). During the treatment period, vials were refilled with the appropriate solution as required.

2.3.1.2 Microscopical Examination

After a nine day treatment period, hypocotyls and the petioles of excised primary leaves of control and estrogen treated cuttings were examined microscopically. To prepare the material for this examination, the basal 5mm of hypocotyl or petiole were fixed in 3% (v/v) gluteraldehyde in 0.2M cacodylate buffer (pH 7.0) for 12 to 16h. The material was then rinsed for 15 min, three times in 0.2M cacodylate buffer and post-fixed for 3h in 1% (w/v) osmium tetroxide in 0.2M cacodylate buffer. The fixed material was then dehydrated in two

changes (6h each) in each of (1) methylcellulose, (2) 100% ethanol, (3) propan-1-ol and (4) butan-1-ol, according to the method described by Feder and O'Brien (1968). The material was then embedded in Historesin (LKB, Sweden) and 2μm sections cut using a glass knife on an LKB III Ultramicrotome. The sections were then dried on microscope slides and stained consecutively for 1 min each in 1% aqueous acid fuchsin and 0.05% aqueous toluidine blue, again following the methods of Feder and O'Brien (1968). Sections were then examined using a Zeiss photomicroscope II.

2.3.1.3 Root Initiation in Primary Leaf Cuttings

The number of roots formed per cutting was monitored over a nine day treatment period for both control and estrogen-treated material. Generally 30 cuttings were used per treatment.

2.3.1.4 Effects of E_1-S on IBA Promotion of Root Initiation

IBA is known to promote adventitious root formation in cuttings (see Audus, 1953) so that the effects of E_1-S on IBA enhanced rooting were examined in primary leaf cuttings over a nine day treatment period. Initially the effects of IBA on rooting over a range in concentrations from 10^{-12}M to 10^{-6}M were examined in order to establish the optimum concentration at which promotion of rooting occurs. IBA was first dissolved in ethanol (0.2ml) and then applied to cuttings as an aqueous solution by wick feeding. This necessitated the use of a distilled water control containing ethanol (0.02% v/v), in addition to the normal distilled water control. Further to this, control treatments of Tris with IBA and Na_2SO_4 with IBA were required in the experiments testing the effects of E_1-S on IBA promotion of rooting, so that it could be established that any observed effects were not due

to the presence of Tris or to the sulphate groups of E_1-S.

2.3.2 Uptake and Distribution of Radiolabelled Estrogens

In parallel with the studies on the physiological effects of applied estrogens, an investigation of uptake and distribution of radioactively labelled estrogens was carried out. The labelled estrogens were supplied in organic solvent (toluene: ethanol - 9:1). Cuttings were prepared exactly as those used in the application experiments except that vials contained radioactive solutions, either (4-^{14}C) estrone (specific activity 2.03 GBqmmol^{-1}) or (4-^{14}C) estradiol (specific activity 2.07 GBqmmol^{-1}). The stock solutions of labelled estrone and estradiol were made up to 1ml with the addition of ethanol. Each vial contained 3.7KBq (10µl of stock solution) in 15ml of distilled water.

2.3.2.1 Distribution

The distribution of radioactivity in cuttings supplied with either (4-^{14}C) estrone (^{14}C-E_1) or (4-^{14}C) estradiol (^{14}C-E_2) was examined at 24h intervals over a seven day (168h) period for hypocotyl cuttings and initially at 24h intervals (until 72h) and subsequently at 48h intervals over a nine day (216h) period for excised primary leaves. Distribution of radioactivity was monitored by subdividing the cutting into 1cm segments or 0.5g portions (lamina) and determining the radioactive content of each sample (see section 2.3.2.3).

2.3.2.2 Uptake

The detailed characteristics of uptake were examined 24h and 48h after commencement of treatment with ^{14}C-E_1 to hypocotyl cuttings and 24h, 48h and 72h after commencement of ^{14}C-E_1 and ^{14}C-E_2 treatment of

primary leaf explants. The cuttings were again subdivided (into portions suitable for sample oxidation) and radioactive content analysed (see section 2.3.2.3). The volume of solution taken up by each cutting was noted and the radioactive content per unit volume of the remaining feeding solution was determined. Assuming that the radiolabelled steroid was evenly distributed, these values can be used to calculate a value for non-selective uptake of $^{14}C\text{-}E_1$ or $^{14}C\text{-}E_2$ according to the following equation:

volume of solution taken up by cutting (ml)	x	radioactivity of remaining feeding solution ($Bqml^{-1}$)	=	calculated non-selective uptake (Bq)

2.3.2.3 Analysis of Radioactivity

Methanol Extraction

Any radioactivity which became absorbed onto the surfaces of the glass vials was extracted into 2ml of methanol over a 48h period, after which time, 4ml of scintillant (liquid scintillator Unisolve 1) was added. (This method was also used for plant material in a few instances.)

Sample Oxidation

This method is based on combustion of samples in a continuous stream of oxygen. The oxidation reaction takes place in a heated furnace held at a temperature of 700°C which ensures instantaneous ignition of dry samples and minimum ignition delay for wet solids and liquid samples. The products of the combustion reaction are CO_2 and H_2O, and it is these products which contain the radioactivity, ^{14}C in CO_2 and ^{3}H in H_2O. The combustion products are trapped in scintillant and are then ready for scintillation counting. Hypocotyl material was combusted using an Oxymat IN 101 sample oxidizer (Intertechnique, France) and the scintillant used to trap the CO_2 was a solution of

0.7% (w/v) 2,5-diphenyloxazole (PPO) in toluene, phenylethylamine, methanol and water (40:33:24:5). For the experiments using leaf cuttings each sample was placed into a paper Combusto-cone (Packard, UK) and combusted in a model 306 Tri-Carb sample oxidizer (Packard, UK) for 0.3 to 1 min depending on sample weight. The resulting CO_2 for each sample was trapped in 7ml of the carbon-dioxide-absorbing agent, Carbo-Sorb (Packard, UK) and mixed with 14ml of the scintillation cocktail, Permafluor V (Packard, UK).

The radioactive content of all samples was determined using liquid scintillation spectrometry employing a Packard Tri-Carb liquid scintillation spectrometer (model 3380) for hypocotyl material and a 1219 Rackbeta Spectral liquid scintillation counter (LKB) for primary leaf material.

Since all samples were not chemically identical, it was not sufficiently accurate to use the relative counts per minute (cpm) recorded during liquid scintillation counting as a measure of the radioactivity present. It was thus necessary that a quench calibration curve was constructed to allow the efficiency of counting for each sample to be estimated and thus absolute disintegrations per minute (dpm) to be calculated.

The scintillation counters used had fully automated external standard facilities so that a calibration curve relating automatic external standard (AES) ratio to percentage efficiency was prepared using n - (1-^{14}C) hexadecane (specific activity 2.26 GBqmmol^{-1}). Each sample contained 2.6MBq and the AES ratio was depressed by the addition of increasing volumes of quenching agent. The quenching agent was provided by a crude chlorophyll extract prepared by grinding 5g of P. vulgaris leaves in methanol with a mortar and pestle.

As each sample contained a known amount of radioactivity, the

percentage efficiency was calculated using cpm recorded by the scintillation spectrometer. A graph of percentage efficiency against AES ratio was constructed and was subsequently used to estimate the percentage efficiency of counting for all samples so the absolute radioactive content was determined.

2.3.3 RNA Metabolism

2.3.3.1 Extraction of Total Cellular RNA

The procedure used to extract total RNA from the petioles of primary leaf cuttings of *P. vulgaris* was a modification of that described by Benveniste *et al* (1973). The plant material (10 to 15g) was frozen in liquid nitrogen and ground to a powder with a chilled mortar and pestle. The ground material was incubated at 60°C for 3 min in 3ml of 5 x RNA extraction buffer (0.25M NaCl, 0.025M sodium acetate, 0.005M ethylenediaminetetraacetic acid (EDTA), 5% (w/v) sodium dodecyl sulphate (SDS), pH 5.0), 12ml of sterile distilled water and 15ml of phenol/chloroform (50% v/v). The lower phenolic and upper aqueous phases were separated by centrifugation at 5,000 rpm for 20 min at 4°C in an 8 x 50ml fixed angle rotor in an MSE high speed 18 centrifuge. The aqueous phase was removed and stored on ice and the interphase and phenolic phase re-extracted in buffer. The aqueous phase was again removed after centrifugation and the pooled aqueous phases re-extracted in phenol/chloroform. RNA was precipitated in 2.5 volumes of absolute alcohol and 0.1 volumes of 3M sodium acetate pH 5.8 at -20°C overnight.

After precipitation RNA was spun down by centrifugation at 10,000 rpm for 20 min at 4°C in an 8 x 50ml fixed angle rotor of an MSE high speed 18 centrifuge. The resulting pellet was dried in a stream of nitrogen and resuspended as required.

2.3.3.2 Fractionation of Total RNA on Sucrose Density Gradients

The method used was a modification of that of Girard et al (1965) and as described by Knowler and Smellie (1973). 80-160µg of ethanol precipated RNA resuspended in 0.1ml LETS buffer (0.01M Tris pH 7.4, 0.1M LiCl, 1mM EDTA, 0.2% (w/v) SDS) was layered on to 12ml 10-25% sucrose density gradients in LETS buffer (prepared using a gradient former) in cellulose nitrate tubes. Sedimentation was for 16h at 24,000 rpm at 20°C in the TST 41.14 rotor of a Sorvall OTD-65B ultracentrifuge. The gradients were scanned using an ISCO model 185 density gradient fractionator attached to an ISCO model UA5 absorbance monitor.

2.3.3.3 Fractionation of Total RNA by Gel Electrophoresis

The main RNA fractions were separated on agarose-formaldehyde horizontal slab gels following the method of Gustafson et al (1982). 100ml of gel solution was prepared by melting 1.2g agarose in 10ml of 10 x running buffer (400mM Hepes pH 7.6, 10mM EDTA), 73.3ml H_2O and 16.7ml of 37% (v/v) formaldehyde.

Samples of ethanol precipitated RNA at a final concentration of $1mgml^{-1}$ were heated at 65°C for 10min in buffer X (deionised formamide, 10 x running buffer, 37% (v/v) formaldehyde, 5:2:1). This procedure denatures the RNA: formamide destroys the secondary structure, rendering the RNA conformationally homogeneous. The presence of formaldehyde ensures that no reformation of the secondary structure occurs. After cooling samples in ice-water, 0.1 volumes of 0.1% (w/v) bromophenol blue in 50% (v/v) glycerol was added. Electrophoresis was for 3h or until the dye front had moved just over half the length of the gel, at a power pack setting of 100V.

After electrophoresis gels were stained for 15 min in 0.2% (w/v)

methylene blue in 0.4M sodium acetate and 0.4M acetic acid and then destained in water.

Tobacco mosaic virus (TMV), brome mosaic virus (BMV) and French bean RNA samples, which were kindly provided by Dr J J Milner, were also run to provide standards.

2.3.3.4 Incorporation of ^{3}H Uridine into Ribosomal RNA

10g of petioles from the primary leaves of P. vulgaris were divided into 1cm length segments and the segments incubated at room temperature for 4h in 10ml of sterile distilled water containing 3.7 MBq of (5',5,6-^{3}H) uridine (specific activity 2.78 $TBqmmol^{-1}$). After incubation, total RNA was extracted from the material and then fractionated on sucrose density gradients using the methods described in sections 2.3.3.1 and 2.3.3.2. The sucrose gradients were scanned and 0.1ml fractions collected by attaching an ISCO model 1220 fraction collector to the gradient fractionator. The radioactive content of 0.1ml fractions was analysed after the addition of 4.0ml scintillant (liquid scintillator-Unisolve 1) by liquid scintillation spectrometry.

2.3.3.5 Purification of Poly(A)$^{+}$RNA by Affinity Column Chromatography Using Oligo(dT)cellulose

Studies on plant mRNAs have demonstrated that they have the polyadenylated region present at the 3' end as in animal mRNAs (Higgins et al, 1973; Yoshida, 1974; Key and Silflow, 1975). This has facilitated the isolation of mRNA due to the affinity of the polyadenylated region for thymidine oligomers in oligodeoxythymidylic acid cellulose chromatography (Aviv and Leder, 1972).

Ethanol precipitated RNA was spun down and resuspended in binding buffer A (50mM Hepes pH 7.5, 0.5M NaCl, 2mM EDTA, 0.5% (w/v) SDS).

Total RNA was loaded onto the column of oligo(dT)cellulose (0.15g) which had previously been equilibrated by washing with 2 x 10ml of binding buffer A. The RNA was pumped round the column by means of an Eyela MP3 peristaltic pump (Jencons) set to produce a flow rate of $0.67 ml min^{-1}$. Unbound RNA was pumped off the column and the column washed with 10ml of binding buffer A. Bound RNA was then sequentially eluted with 5ml, delivered as 1ml, 1ml and 3ml, of the low ionic strength elution buffer (10mM Hepes pH 7.5). The eluate was precipitated in 2.5 volumes of absolute alcohol and 0.1 volumes of 4M potassium acetate pH 5.5 at -20°C overnight.

2.3.3.6 Preparation of Poly(A)$^+$RNA for In Vitro Translation

Ethanol precipitated poly(A)$^+$RNA was spun down in 1.5ml aliquots at the high speed setting in an MSE Micro Centaur centrifuge. Pellets were washed in 80% ethanol, dried in a vacuum oven and resuspended in sterile Analar water. The RNA was re-extracted with an equal volume of phenol/chloroform before a final ethanol precipitation overnight. After precipitation, the poly(A)$^+$RNA was resuspended in 100-200ul Analar water.

Ribonuclease enzymes are very active in plants (Cecchini and Miassod, 1976) so that precautions to prevent ribonucleases degrading all or part of the mRNA had to be taken during extraction of total RNA and during the subsequent purification of poly(A)$^+$RNA. All glassware and solutions were sterilised and gloves were worn at all times to prevent nucleases present in the moisture of the skin from coming into contact with the preparations of RNA.

2.3.3.7 Measurement of RNA

The concentration of RNA was measured by determining the absorbance at

260nm using a Philips Pye Unicam SP8-500 UV/VIS spectrophotometer. The RNA content was calculated on the basis that A_{260} of 1 = 40ug RNA (Theologis and Ray, 1982).

2.3.3.8 Preparation of S-30 Wheat Germ Extract

The method used to prepare the wheat germ cell-free extract was based on that of Davies et al (1977).

1g of floated wheat germ was ground. With the tip of a sterile pasteur pipette to act as abrasive in a cooled mortar. After a few seconds grinding 2ml of grinding buffer (5mM Hepes pH 6.4 to 6.9, 120mM potassium acetate, 5mM magnesium acetate, 1mM dithiothreitol (DTT)) were added to the powder. After grinding for 1 min, a further 2ml of grinding buffer were added. This was repeated and the resulting slurry was transferred to an ice-cold centrifuge tube. The mortar was rinsed with a further 4ml of grinding buffer. After centrifugation of the preparation at 16,000 rpm for 10 min at 4°C in an 8 x 50ml fixed angle rotor of an MSE high speed 18 centrifuge, the supernatant (S-30) was removed and transferred to a tube containing 100µl of 500mM Hepes pH 7.6. This was mixed and centrifuged for a further 15 min at 16,000 rpm. The supernatant was removed carefully, avoiding the upper lipid pellicle and the pellet, and transferred to dialysis tubing which had previously been boiled and rinsed in Analar water. The dialysis tubing was then placed in a conical flask containing 1l of cold dialysis buffer (20mM Tris pH 7.6, 120mM potassium acetate, 5mM magnesium acetate and 1mM DTT). The flask was buried in ice and set on a magnetic stirrer. Dialysis was at 4°C and was initially overnight and then for a further 2-3h after changing the buffer.

The dialysed supernatant was clarified by a 5 min centrifugation at 5,000 rpm. The S-30 supernatant was then quick frozen in 0.5ml

aliquots and stored in liquid nitrogen.

DTT was added to the grinding buffer and the dialysis buffer immediately before use.

2.3.3.9 In Vitro Translation of Poly(A)$^+$RNA

In vitro translation incubation mixtures contained the following:

0.5 volumes S-30 wheat germ cell-free extract

0.1 volumes 'Mix 6' (30mM guanosine triphosphate (GTP), 1mM of all amino acids required for protein synthesis except methionine, 0.1M adenosine triphosphate (ATP), 0.8M creatine phosphate, 8mgml^{-1} creatine phosphokinase)

0.1 volumes 'HKMS' (200mM Hepes pH 7.6, 400mM potassium acetate, 4mM magnesium acetate, 4mM spermadine)

L-(^{35}S) methionine (specific activity 41.6 TBqmol^{-1}) was added at a concentration of 3.7 KBq per 1ul of reaction mixture.

The remaining volume was made up with sterile water.

Poly(A)$^+$RNA at a final concentration of 1 to 5 mgml^{-1} in 5µl was added to a 20µl aliquot of reaction mixture and incubated for a period of 90 min at 30°C.

2.3.3.10 Analysis of Translation Products

The incorporation of radioactively labelled methionine into proteins was analysed by removing 2 x 2µl samples from each incubation vial and mixing with 1ml H_2O and 0.5ml of a stopping/bleaching agent (IM NaOH, 5% (v/v) '100 volumes H_2O_2' and 1mgml^{-1} unlabelled L-methionine). Samples were incubated at 37°C for 15 min. The proteins present were then precipitated by the addition of 1ml of 25% (w/v) trichloroacetic acid (TCA). 10µl of 2% (w/v) bovine serum albumin (BSA) pH 7.0 was also added to act as carrier for the proteins. To ensure complete

precipitation the samples were incubated at 0°C for 15 min. Precipitated proteins were then collected on Whatman GF/C glass fibre filters (2.5cm diameter) which had been pre-soaked in 8% (w/v) TCA. The filter pads with precipitate were washed in 8% TCA and in ethanol and then dried under vacuum. Radioactivity was then assessed by liquid scintillation spectrometry after the addition of 4ml of scintillant (liquid scintillator Unisolve 1).

To observe the products of synthesis, the radiolabelled polypeptide products were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) based on the method of Laemmli (1970). The following stock solutions were used:

stacking gel buffer	0.5M Tris HCl pH 6.8
	0.4% (w/v) SDS
separating gel buffer	1.5M Tris HCl pH 8.8
	0.4% (w/v) SDS
acrylamide	25% (w/v) acrylamide
	0.66% (w/v) N,N'methylenebisacrylamide
electrolyte	25mM Tris HCl pH 8.3
	0.192M glycine
	0.1% (w/v) SDS
boiling solution	62.5mM Tris HCl ph 6.8
	2% (w/v) SDS
	10% (v/v) glycerol
	2% (v/v) 2 mercaptoethanol
	0.001% (w/v) bromophenol blue

Polypeptide products were prepared for gel electrophoresis by boiling for 2 min in 2 volumes of boiling solution. The boiling solution contains two active ingredients: SDS and 2 mercaptoethanol. Proteins

are denatured during boiling and 2 mercaptoethanol is used to break strong covalent disulphide bonds. SDS is an anionic detergent which breaks weak bonds between protein subunits and which binds to denatured proteins at regular intervals along the length of the polypeptide so that the longer the protein the more SDS will bind and thus a constant charge:mass ratio is achieved. By treating the proteins in this way the subsequent separations on gels were on the basis of molecular weight only.

12.5% acrylamide gels were used and were prepared with the following solutions:

separating gel solution	25ml acrylamide stock
	12.5ml separating gel buffer
	12.5ml H_2O
	200µl 10% (w/v) ammonium persulphate
	50µl NNN'N'-tetramethylethylenediame (TEMED)
stacking gel buffer	2.0ml acrylamide stock
	3.1ml stacking gel buffer
	7.4ml H_2O
	100µl 10% (w/v) ammonium persulphate
	25µl TEMED

10% ammonium persulphate was made up fresh on each occasion. TEMED was used to initiate co-polymerisation of the acrylamide while ammonium persulphate was used to catalyse the reaction.

Approximately 100,000cpm were loaded onto gels for each sample of translation products. The following standard proteins, which were kindly provided by Dr J J Milner and which were radioiodinated using the method of Markwell (1979), were run on each gel to provide molecular weight (MW) markers.

High Molecular Weight Standard Mixture

Protein	Approximate MW
carbonic anhydrase from bovine erythrocytes	29,000
albumin egg	45,000
albumin bovine	56,000
phosphorylase b from rabbit muscle	97,400
β galactosidase from Escherichia coli	116,000
myosin from rabbit muscle	205,000

Dalton Mark VII-L™

Protein	Approximate MW
α lactalbumin	14,200
trypsin inhibitor, soyabean	20,100
trypsinogen, PMSF treated	24,000
carbonic anhydrase from bovine erythrocytes	29,000
glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle	36,000
albumin egg	45,000
albumin bovine	66,000

After electrophoresis gels were prepared for fluorography by a procedure based on that described by Bonner and Laskey (1974). Firstly gels were washed twice (30 min each wash) in 7% (v/v) acetic acid to fix the proteins and to remove unreacted methionine. The gels were then dehydrated by washing three times for 30 min each wash in dimethylsulphoxide (DMSO). This dehydration was necessary before infiltration of the gels with the fluor PPO could occur (PPO is insoluble in water but very soluble in DMSO). Infiltration was effected by soaking the gels in 22% (w/v) PPO in DMSO at 37°C with continuous shaking for 2h. Finally excess PPO was precipitated from the gels by washing in gently running tap water overnight. Gels were dried on Whatman 3MM filter paper using a Biorad model 483 slab dryer.

Fluorography was then conducted following the methods of Laskey and Mills (1975) at -70°C using pre-exposed Fuji X-ray film. The films were developed using Kodak LX 24 X-ray developer and Kodak FX-40 X-ray liquid fixer.

2.3.3.11 DNA Assay

DNA contamination of RNA samples was determined using a spectrophotometric technique as described by Burton (1956). A standard curve was constructed using samples of known DNA concentration.

A DNA standard of 0.3mgml^{-1} calf thymus gland DNA in 3mM NaOH was mixed with an equal volume of 0.5M perchloric acid and incubated at 70°C for 15 min. A range of concentrations of DNA from 0.015 to 0.15 mgml^{-1} was prepared from the digest by appropriate dilution with 0.5M perchloric acid.

Samples of unknown DNA concentrations were digested at 70°C in an equal volume of 0.5M perchloric acid for 1h.

1ml of DNA digest (standard or sample) was then mixed with 2ml of Burton reagent (see below) and left overnight at room temperature in a dark place.

Burton reagent

A. acetaldehyde (16mgml^{-1} in H_2O)

B. diphenylamine (1.5g in 100ml glacial acetic acid and 1.5ml concentrated H_2SO_4)

Before use 0.1ml A was mixed with each 20ml of B.

Absorbance at 600nm was determined using a Philips Pye Unicam SP8-500 UV/VIS spectrophotometer. The DNA content of samples was then estimated from the prepared standard curve.

2.3.3.12 Tannin Protein Assay

Protein contamination of RNA preparations was estimated using a turbiodometric method based on that of Mejbaum-Katzenellenbogen and Dobryszycka (1959). A calibration curve was prepared using BSA standards of 0, 10, 20, 40, 60 and 80$\mu g ml^{-1}$ concentrations. Standards and samples of unknown protein content were equilibriated at 30°C for 2 min, after which time 1ml of tannin reagent (196ml 1M HCL, 20g tannic acid, 4ml phenol - heated to 80°C and filtered when cool) was added. After a further 10 min incubation 1ml of gum acacia (0.2% acacia in H_2O) was added to stabilise the turbidity. After approximately 2 min incubation at room temperature the absorbance at 500nm was determined using a Philips Pye Unicam SP8-500 UV/VIS spectrophotometer. At protein concentrations of 10-100μg per 4ml of reaction mixture the optical density is directly proportional to the amount of protein present under standard conditions.

2.4 Statistical Analysis

Statistical analyses were carried out using a Genstat V package through the Edinburgh Regional Computing Centre and the results presentation was devised by Drs J McNicol and R J Killick of the Scottish Crop Research Institute, Invergowrie, Dundee.

Standard errors of means were calculated using the following equation:

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

CHAPTER 3

RESULTS

3.1 Physiological Effects of Applied Estrogen-Sulphate Conjugates

Previous experimentation by Hewitt and Hillman (1979b, 1980) indicated that solutions of estrone, estrone-phosphate, estradiol and estradiol-sulphate in the concentration range 10^{-6}M to 10^{-10}M had no observable anatomical or morphological effects on adventitious root formation in hypocotyl, epicotyl and primary leaf cuttings of Phaseolus vulgaris. At the higher concentration of 10^{-4}M, however, estrone-sulphate and estradiol-sulphate significantly inhibited rooting in hypocotyl cuttings and almost completely inhibited rooting in epicotyl and primary leaf cuttings. Of particular interest was the further observation that treatment of cuttings with estrogen sulphates led to anomalous development of the vascular tissues.

In this first section of the results, experiments are described which were carried out to test the reproducibility of the above findings. Reproducibility is important in establishing the response as an experimental system which can be used as the basis of studies on the mode of action of steroid hormones in plants. The anatomical transformations were investigated in more detail and the effects of estrogens on rooting were compared with those of IBA.

3.1.1 Estrogen Effects on Root Initiation

Primary leaf explants were used in the rooting experiments rather than hypocotyl cuttings, as in petioles root formation occurs de novo

whereas hypocotyls possess pre-formed root initials. Particular care was taken to ensure that pulvinar tissue, linking the petiole and the node (proximal pulvinus), was not present as this is known to reduce both the number of cuttings which form roots and the number of roots per rooted cutting (Hewitt and Hillman, 1981).

The results of all the small-scale individual experiments are illustrated in Figure 14 and in Table 1, which also details the numbers of cuttings employed and the results of statistical analysis. As is frequently the case with rooting experiments, considerable variation was often observed between different batches of cuttings. This is not surprising since it was noted that even slight changes in light intensity, temperature and relative humidity can cause dramatic changes in the number of roots produced. The cultured conditions provided by carrying out the experiments in an enclosed polythene chamber within a constant temperature growth room contributed to a lessening of the variation, but despite this, in some cases within individual treatments in a single experiment and even with distilled water controls, some cuttings failed to root while others produced copious numbers of roots. These problems are further complicated by the constraints imposed by the logistics of handling large numbers of cuttings and limited growth room space. Thus a more accurate picture of the general trends is obtained by examining the collective results rather than those of individual experiments.

The effects of E_1-S and E_2-S at a concentration of 10^{-4}M were examined after a nine day treatment period. The estrogenic compounds were applied in aqueous solutions to the bases of cuttings: control cuttings were placed in distilled water. In an attempt to eliminate the possible involvement of the sulphate groups present on molecules of E_1-S and E_2-S in any response elicited by these compounds, a

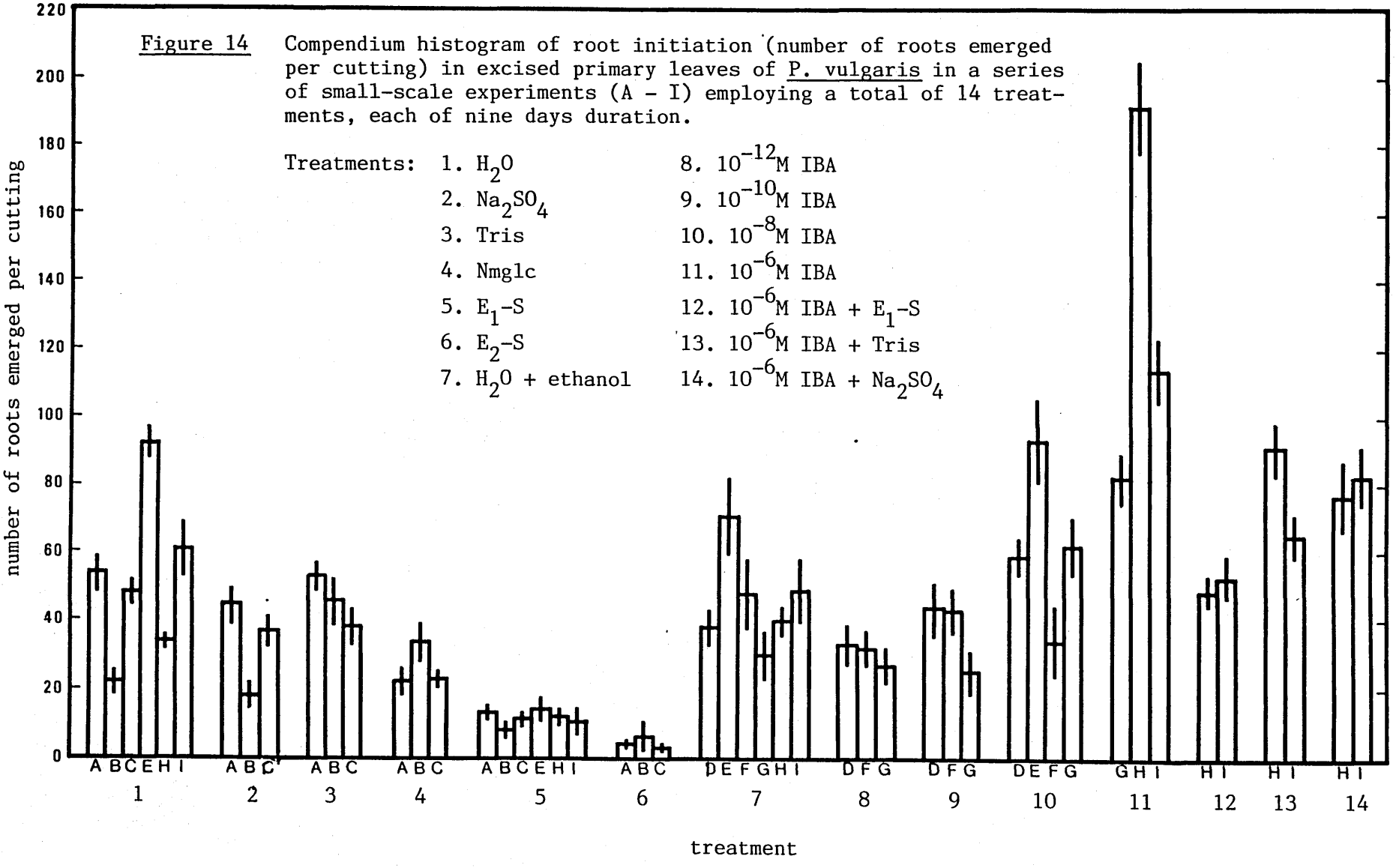


Figure 14 Compendium histogram of root initiation (number of roots emerged per cutting) in excised primary leaves of P. vulgaris in a series of small-scale experiments (A - I) employing a total of 14 treatments, each of nine days duration.

Treatments:

1. H_2O
2. Na_2SO_4
3. Tris
4. Nmglc
5. E_1-S
6. E_2-S
7. H_2O + ethanol
8. 10^{-12}M IBA
9. 10^{-10}M IBA
10. 10^{-8}M IBA
11. 10^{-6}M IBA
12. 10^{-6}M IBA + E_1-S
13. 10^{-6}M IBA + Tris
14. 10^{-6}M IBA + Na_2SO_4

Table 1 Statistical analysis of root initiation in primary leaf cuttings of P. vulgaris in a series of small-scale experiments (A - I), employing a total of 14 treatments each of nine days duration.

Experiment A

treatment	E_2-S	E_1-S	Nmglc	Na_2SO_4	H_2O	Tris
mean	3·5	12·9	22·3	44·1	53·8	54·1
number of observations	39	37	24	25	19	30
scaling factor		0·053	0·069	0·082	0·073	0·086
standard error of differences		4·03	4·6	5·02	4·75	5·15

S^2 = 308·3

Experiment B

treatment	E_2-S	E_1-S	Na_2SO_4	H_2O	Nmglc	Tris
mean	6·4	7·9	18·4	21·9	33·7	45·6
number of observations	30	30	15	15	14	14
scaling factor		0·067	0·100	0·133	0·071	0·143
standard error of differences		4·14	5·07	5·86	4·29	6·06

S^2 = 257·4

Table 1 continued

Experiment C

treatment	E_2-S	E_1-S	Nmglc	Na_2SO_4	Tris	H_2O
mean	2·6	10·7	22·7	37·1	37·5	47·7
number of observations	30	30	30	19	14	15
scaling factor		0·067	0·067	0·086	0·124	0·138
standard error of differences		3·31	3·31	3·76	4·52	4·77

S^2 = 164·5

Experiment D

treatment	10^{-12}M IBA	H_2O + ethanol	10^{-10}M IBA	10^{-8}M IBA
mean	33·4	37·0	43·5	59·4
number of observations	10	10	11	9
scaling factor		0.200	0·191	0·202
standard error of differences		8·31	8·12	8·35

S^2 = 345·4

Experiment E

treatment	E_1-S	H_2O + ethanol	H_2O	10^{-8}M IBA
mean	13·7	70·7	91·8	93·1
number of observations	13	10	9	8
scaling factor		0·180	0·211	0·236
standard error of differences		10·42	11·39	12·04

S^2 = 614·1

Table 1 continued

Experiment F

treatment	10^{-12}M IBA		10^{-8}M IBA		10^{-10}M IBA		H_2O + ethanol		10^{-6}M IBA
mean	31·8		34·3		43·0		48·4		192·0
number of observations	10		10		10		10		15
scaling factor		0·200		0·200		0·200		0·133	
standard error of differences		8·3		8·3		8·3		16·55	

S^2 = 716·3

Experiment G

treatment	10^{-10}M IBA		10^{-12}M IBA		H_2O + ethanol		10^{-8}M IBA		10^{-6}M IBA
mean	24·6		26·6		30·2		61·5		81·7
number of obserations	10		10		10		10		10
scaling factor		0·200		0·200		0·200		0·200	
standard error of differences		9·2		9·2		9·2		9·2	

S^2 = 422·9

Table 1 continued

Experiment H

treatment	E_1-S	H_2O	H_2O + ethanol	IBA + E_1-S	IBA + Na_2SO_4	IBA + Tris	10^{-6}M IBA
mean	12·4	34·2	39·5	49·1	77·5	91·1	192.0
number of observations	15	15	15	25	10	13	15
scaling factor		0·133	0·133	0·107	0·140	0·177	0·133
standard error of differences		7·55	7·55	6·75	7·74	8·70	16·55

S^2 = 427·7

Experiment I

treatment	E_1-S	H_2O + ethanol	IBA + E_1-S	H_2O	IBA + Tris	IBA + Na_2SO_4	10^{-6}M IBA
mean	9·9	48·8	53·2	60·8	65·1	83·2	114.0
number of observations	14	11	14	12	10	12	10
scaling factor		0·162	0·162	0·155	0·183	0·183	0·183
standard error of differences		9·23	9·23	9·01	9·81	9·81	9·81

S^2 = 524·7

Vertical line indicates that the means are statistically significantly different.

sulphate control (10^{-4}M Na_2SO_4) was also employed. In addition, control treatments of Tris (1.7×10^{-4}M) and N-methyl-D-glucamine (0.4×10^{-5}M) were required as these compounds are used to stabilize E_1-S and E_2-S respectively. The collective results are illustrated in Figure 15 (see also Figure 14 and Table 1 - experiments A, B and C).

A pronounced inhibition of root formation in cuttings treated with estrogen-sulphates was observed. Significantly fewer roots were formed on cuttings which had been treated with E_1-S or E_2-S than on distilled water controls. This inhibition was consistently more marked with E_2-S treatment: it is possible that the presence of Nmglc was responsible for this as in experiments A and B, although not in C (see Figure 14), this compound also inhibited rooting although to a much lesser extent than estrogen treatment. There was no consistent effect of either Tris or Na_2SO_4 on root formation.

A time-course study of root formation in both control and estrogen-treated cuttings, over the nine day treatment period employed for the experiments described above, was carried out. The number of roots which had emerged per cutting was monitored at daily intervals from one to seven days and then on the ninth day. The rooting patterns observed are shown in Figure 16a. No cuttings produced roots during the first four days of the treatment period, but by the fifth day, the bases of control cuttings (H_2O, Na_2SO_4, Tris and Nmglc) were swollen and in some cases a small number of roots were present. At this stage, cuttings which had been treated with either E_1-S or E_2-S showed no signs of root initiation. By the sixth day of treatment the inhibitory effects of E_1-S and E_2-S were obvious: fewer cuttings had rooted (Table 2) and the average number of roots formed per cutting was significantly less (Figure 16a). These inhibitory effects became more pronounced over the last three days of the rooting period.

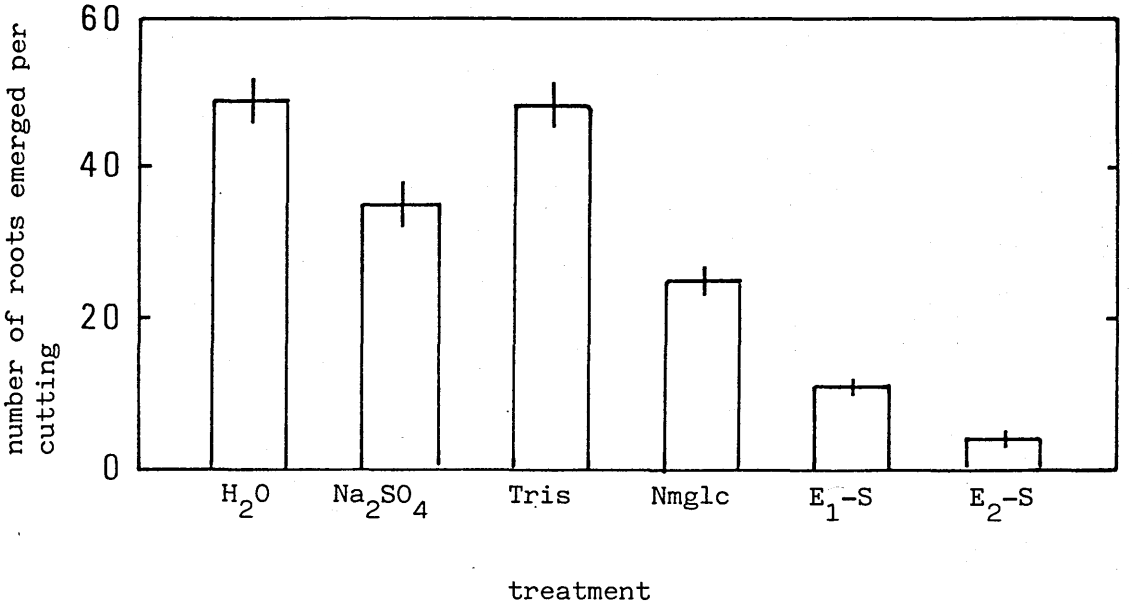


Figure 15 The effect of estrone-sulphate and estradiol sulphate and appropriate control treatments (distilled water, Na_2SO_4, Tris and N-methyl-D-glucamine) on root formation in primary leaf cuttings of P. vulgaris after a nine-day treatment period. Values shown represent the collective mean $\pm$ one standard error.

Table 2 The effect of estrone-sulphate and estradiol-sulphate and appropriate control treatments (H_2O, Na_2SO_4, Tris and N-methyl-D-glucamine) on the percentage of cuttings forming roots in primary leaf cuttings of P. vulgaris after six, seven and nine day treatment periods

	Time (d)		
Treatment	6	7	9
H_2O	76.2	93.4	96.7
Na_2SO_4	63.3	70.0	94.1
Tris	72.9	80.0	100.0
Nmglc	57.6	86.7	97.7
E_1-S	30.0	66.0	81.7
E_2-S	13.3	51.6	56.7

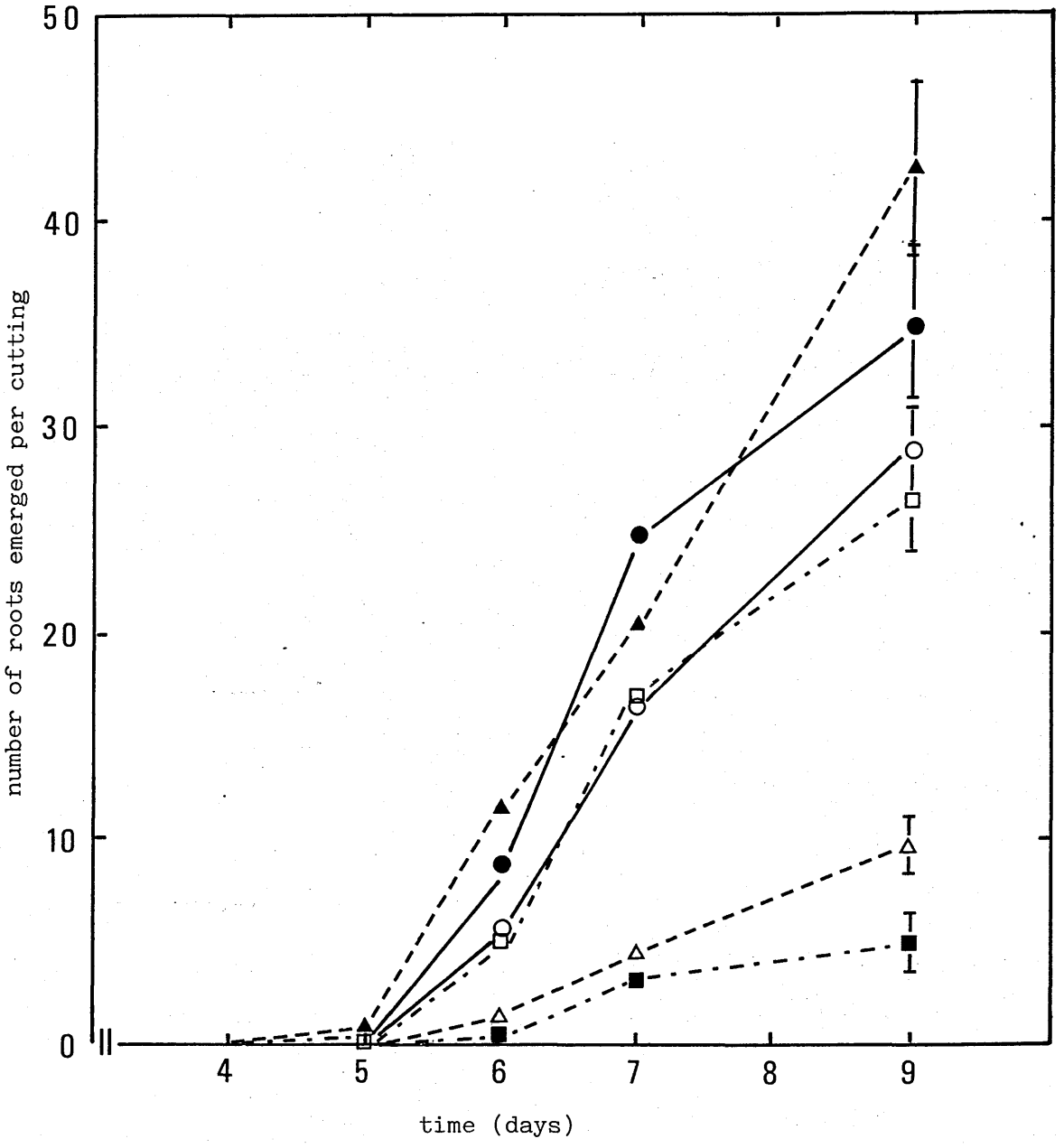


Figure 16a A time-course study of root formation in estrone sulphate (△) and estradiol sulphate (■) treated and control (H_2O ●, Na_2SO_4 ○, Tris ▲ and Nmglc □) primary leaf cuttings of P. vulgaris.

Presentation of the results as roots emerged per rooted cutting does not alter this pattern (Figure 16b). Fewer roots were formed per rooted cutting on estrogen-treated material, verifying the estrogen-induced inhibition of this process.

3.1.2 The Effects of E_1-S on IBA Promotion of Root Initiation

IBA is a well known promoter of root formation in cuttings (see Audus, 1953) and uncharacterised endogenous phenolics have been found to act as co-factors with IBA in root formation in pear hardwood cuttings (Fadl and Hartmann, 1967). On the other hand, applications of estrone together with IBA in the form of talc dusts were found to offset the promotary effects of IBA on dormant Lonicera tartarica cuttings (Grace, 1940). In order to clarify the effects of estrogens on IBA promotion of rooting, the effects of E_1-S on IBA promotion of rooting in P.vulgaris primary leaf cuttings were examined.

In order to establish the optimum concentration of IBA required to enhance rooting in P. vulgaris cuttings, the effects of a range of concentrations from 10^{-12}M to 10^{-6}M were examined after a nine day rooting period. The collective results obtained are illustrated in Figure 17 (see also Figure 14 and Table 1 - experiments D, E, F and G). Comparison of the number of roots formed on IBA-treated cuttings and control cuttings (distilled water containing enthanol: ethanol was used to dissolve the IBA before putting into aqueous solution) shows clearly that IBA at concentrations greater than 10^{-8}M promoted rooting. Under normal circumstances, root emergence in cuttings is preceded by the swelling of the cutting base, but in cuttings treated with 10^{-6}M IBA the petioles became swollen along their entire length and very often split as the many roots emerged. IBA at a concentration of 10^{-6}M was used in the subsequent experiments with E_1-S.

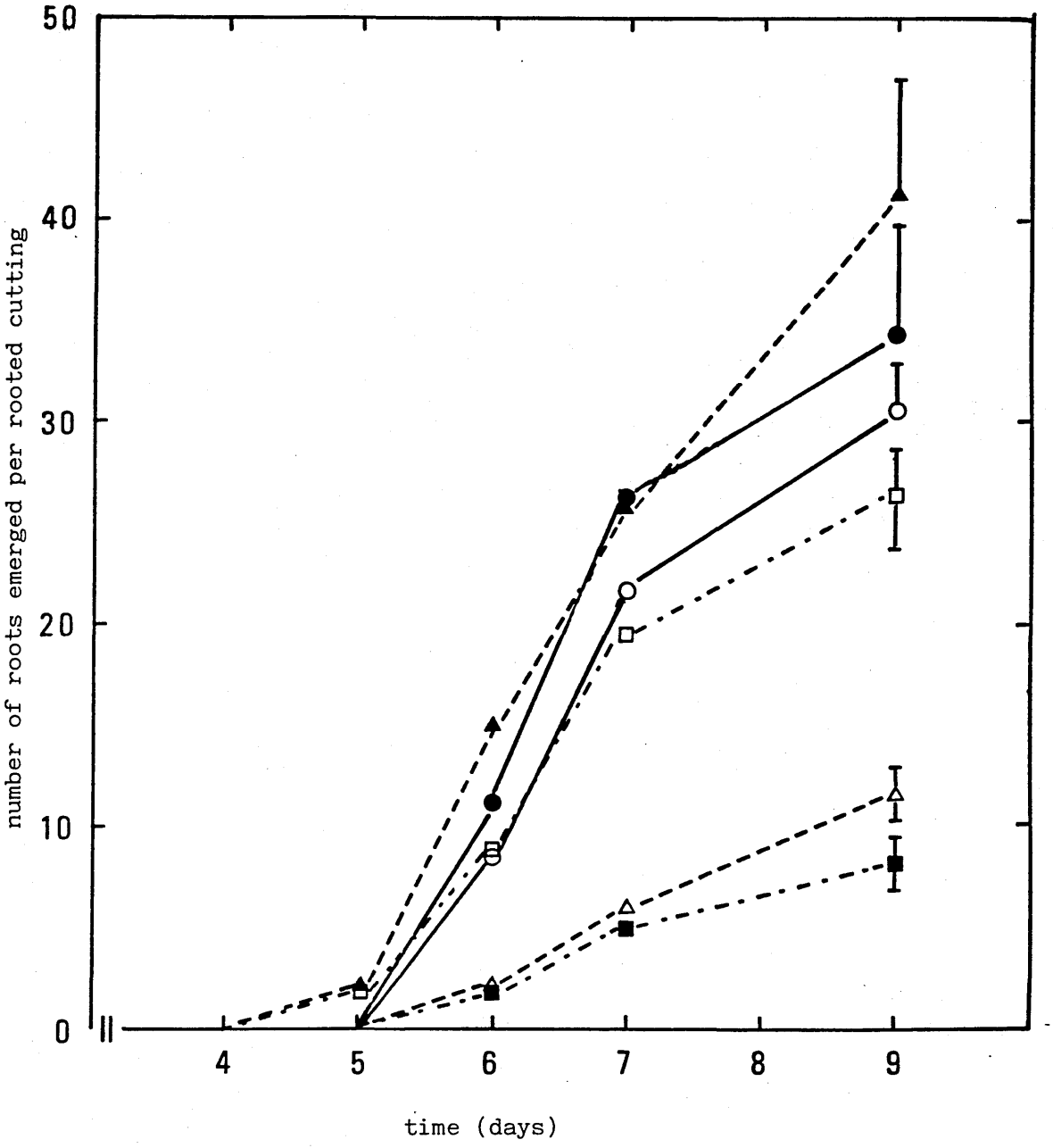


Figure 16b A time-course study of root formation in estrone sulphate (△) and estradiol sulphate (■) treated and control (H_2O ●, Na_2SO_4 ○, Tris ▲ and Nmglc □) primary leaf cuttings of P. vulgaris. The results are expressed as the number of roots emerged per rooted cuttings only.

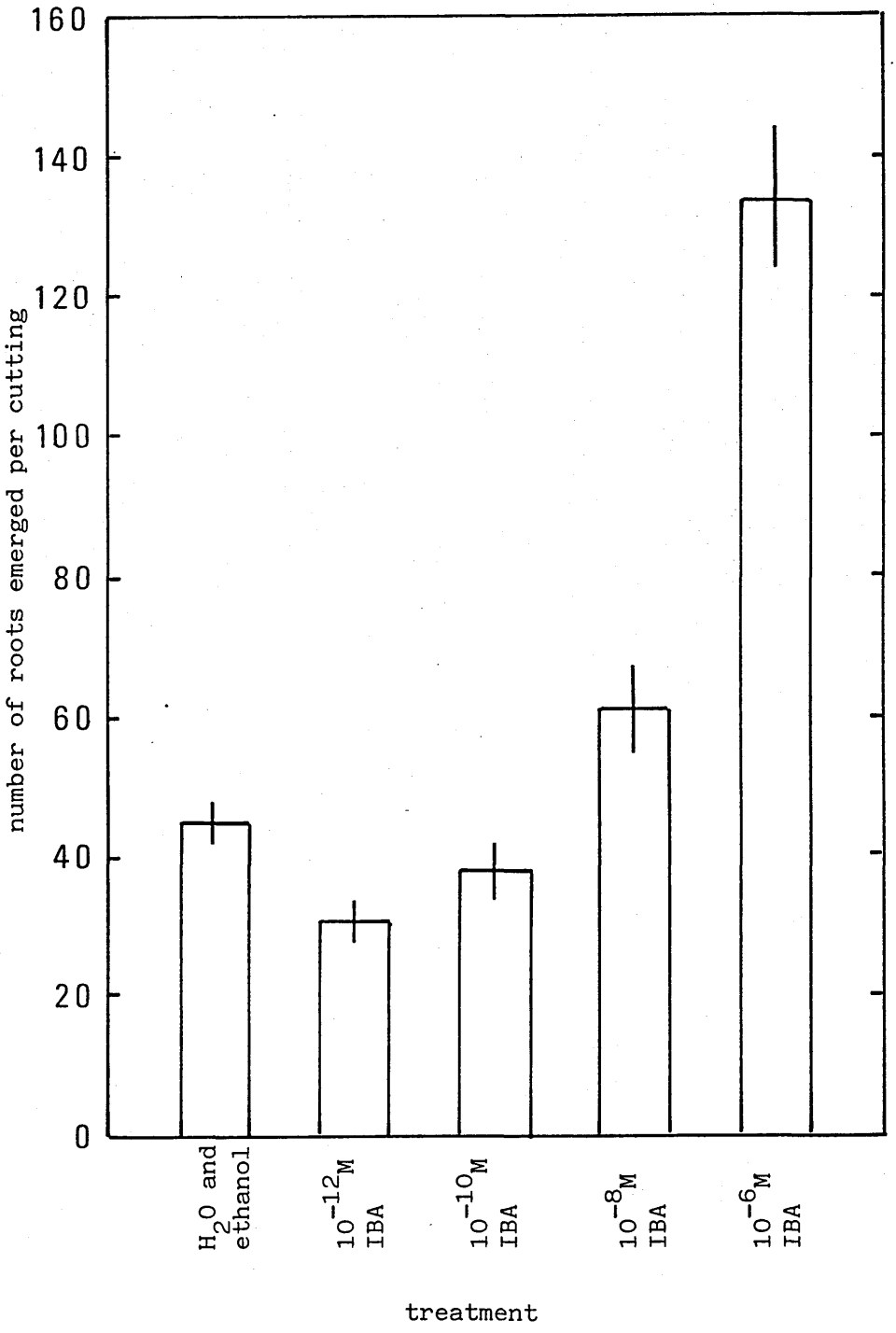


Figure 17 The effects of increasing concentrations of IBA on root formation in primary leaf cuttings of P. vulgaris after a nine-day treatment period. Values shown represent the collective means ± one standard error.

The effects of E_1-S on IBA promotion of adventitious root formation were examined after a nine day treatment period. Aqueous solutions containing E_1-S (10^{-4}M) and IBA (10^{-6}M) were applied to cuttings by wick feeding. Several control treatments were necessary and were as follows: distilled water, distilled water containing ethanol, IBA alone, E_1-S alone, IBA with Tris (1.7×10^{-4}M) and IBA with Na_2SO_4 (10^{-4}M). Examination of the collective data (Figure 18 - but see also Figure 14 and Table 1 - experiments H and I) shows that the number of roots produced by cuttings treated with solutions of IBA and E_1-S was significantly less than on those treated with IBA alone, indicating that E_1-S can at least partially overcome the promotary activity of IBA. This inhibition, however, may not be solely attributable to an effect of the steroid as significantly fewer roots were formed on control cuttings treated using IBA with Tris and using IBA with Na_2SO_4 relative to the number of roots formed on IBA treated cuttings. This is surprising since Tris alone and Na_2SO_4 alone are without consistent effect on rooting at the concentrations employed.

3.1.3 Anatomical Changes Induced by Estrogen-Sulphates

The report by Hewitt and Hillman (1979b) concerning the induction of anomalous cell development in the vascular tissues by estrogen treatment is the only published photographic evidence of such a response. A more comprehensive investigation of the phenomenon has been undertaken here using the same cultivar of French bean as that employed by Hewitt and Hillman, ie Canadian Wonder, grown under similar conditions. The following series of plates is representative of the many sections examined and illustrates clearly the main findings. The following abbreviations have been used for recognition of the various tissue types:

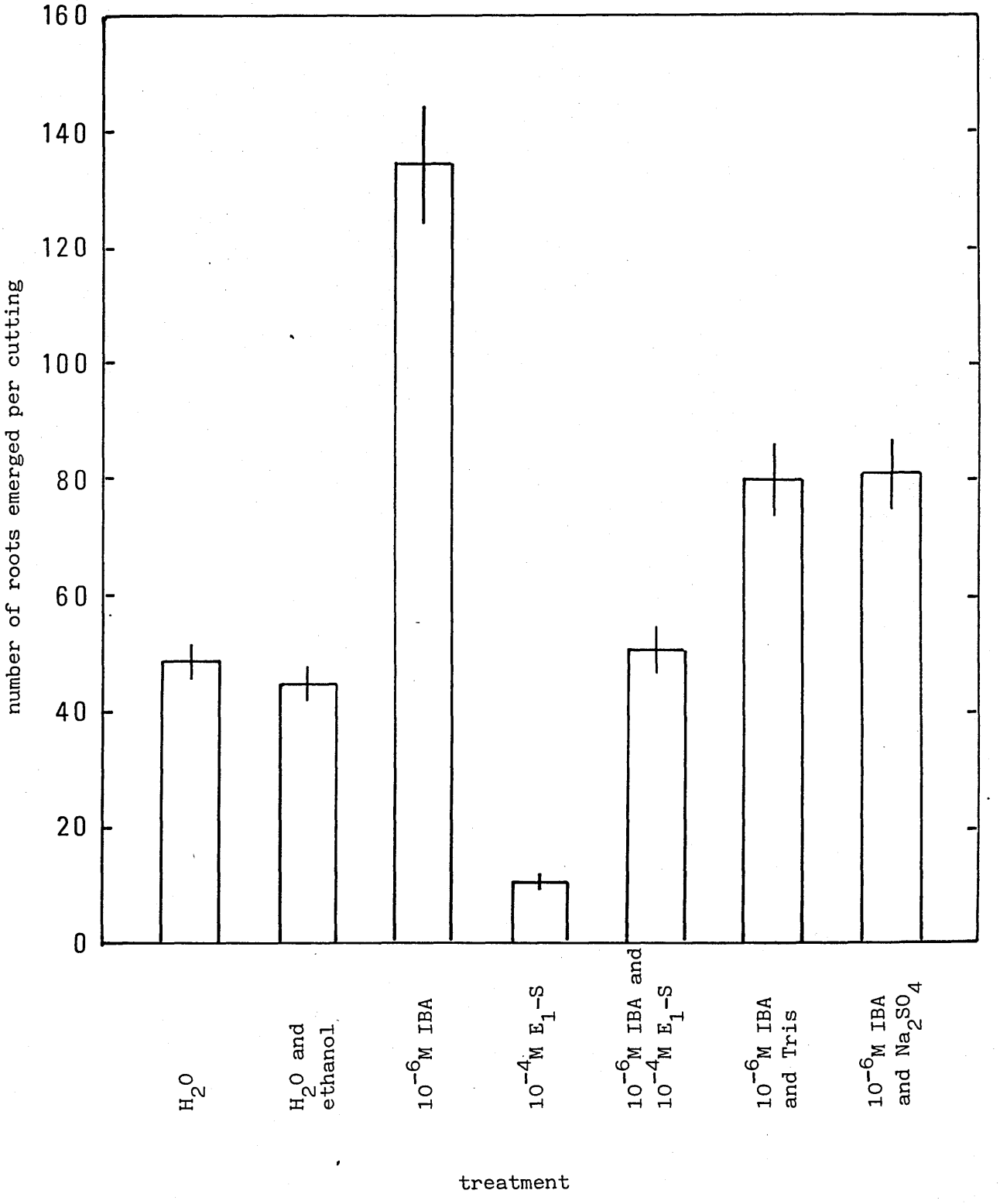


Figure 18 The effects of estrone-sulphate and appropriate controls (Tris and Na_2SO_4) on IBA promotion of rooting in primary leaf cuttings of P. vulgaris after a nine day treatment period. Values shown represent the collective means $\pm$ one standard error.

A	anomalous differentiation
C	cortex
E	epidermis
P	phloem
PCF	pericyclic fibres
Pi	pith
RI	root initial
S	secretory duct
VC	vascular cambium
VB	vascular bundle
X	xylem

Abnormal cell development was observed in sectioned material, examined using light microscopy, from the bases of hypocotyls and from the bases of petioles from excised primary leaves which had been treated with estrone-sulphate or estradiol-sulphate over a nine day rooting period. Examination of large numbers of sections from randomly selected estrogen-treated cuttings over a period of twenty months confirmed the observations of Hewitt and Hillman (1979b) and established that the response could be consistently reproduced.

In distilled water control hypocotyl cuttings, normal tissue distribution was evident, although, as is the case with any biological material, variations in the standard pattern occurred in sections from different cuttings and also in sections from the same cutting. Transverse sections from control hypocotyls revealed that the regions of the stem, containing at its base a type b transition zone (see Eames and MacDaniels, 1925), is more or less circular in outline with several ridges. At the stage used in the work described here, the epidermis bears stomata and surrounds a prominent cortex of parenchyma cells which contain chloroplasts and small groups of collenchyma cells

which are usually associated with the ridged areas. A vascular cambium is present and from this is formed a ring of secondary vascular tissues which surround the central pith. Often associated with the phloem are secretory cells, and at the inner edge of the cortex small groups of pericyclic fibres are present which form a discontinuous ring (see Plates 1a and 1b).

A feature of Phaseolus vulgaris hypocotyls is the presence of pre-formed root initials. The development of these initials was described in the Introduction: they are formed in the interfascicular regions, close to the vascular tissues, and were commonplace in the sections taken from distilled water control hypocotyls (see Plates 2a and 2b). Treatment with either E_1-S or E_2-S usually, but not invariably (see Plate 3), led to abnormal cell development in the peripheral phloem area normally associated with lateral root initials (see Plates 4a and 4b). Apparently disorganised groups of cells, akin to tracheary cells of the primary xylem, were noted in various orientations. Longitudinal sections through estrogen-treated hypocotyls did not reveal the circular outlines typical of lateral root initials. In many respects the effects of steroidal estrogens may be likened to the callusing phase in cuttings prior to lateral root initiation except that there is an internal manifestation of cell proliferation and that several cell types are involved in the response. The zones of abnormal cells were relatively discrete and similar in overall dimensions to root initials. These zones never encircled the vascular zone, they did not occur in the epidermis, the outer cortex, the xylem or the pith, and there was no evidence of 'metastases'.

None of the control treatments (H_2O, Na_2SO_4, Tris and Nmglc) induced abnormal cell development and the normal tissue distribution described earlier was always in evidence and normal vascular development and

Plate 1a

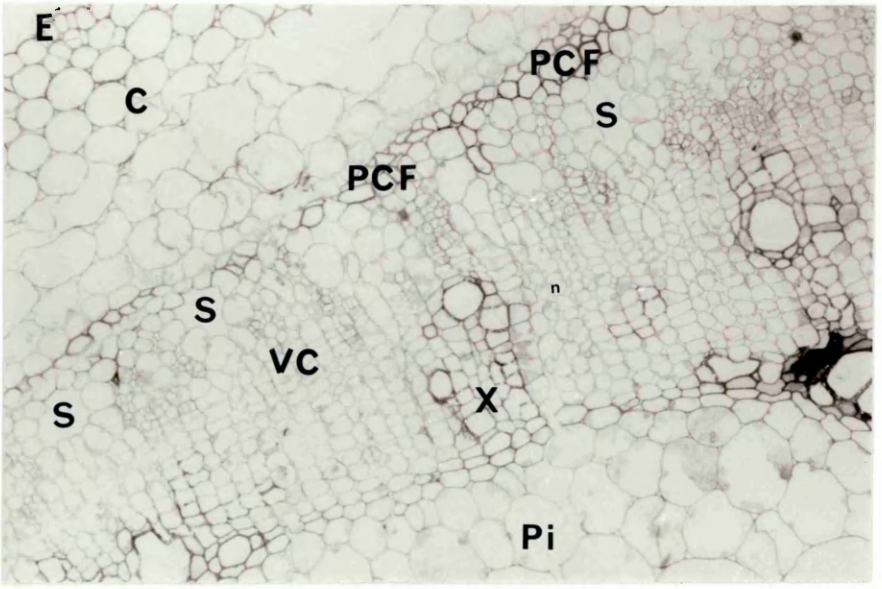
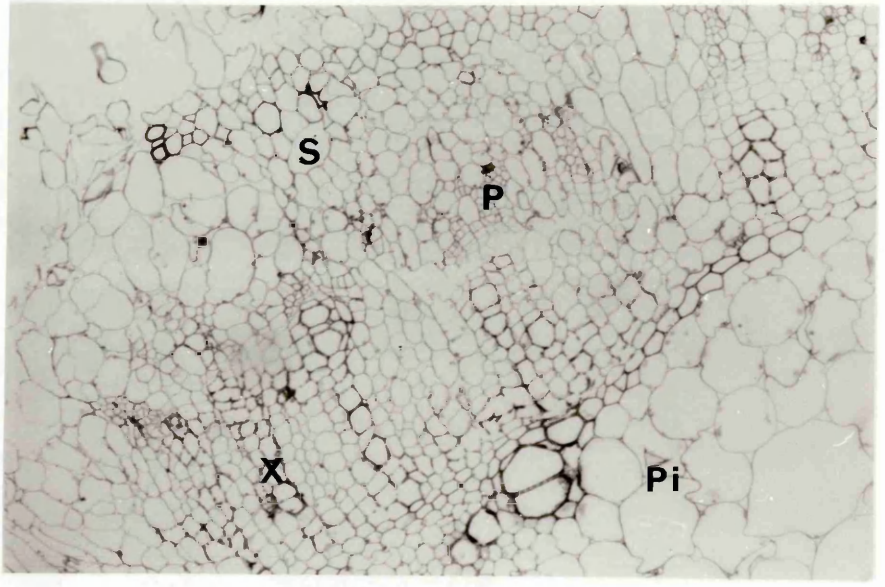


Plate 1b



Plates 1a and 1b Transverse sections through the bases of hypocotyls of distilled water control cuttings of P. vulgaris, showing normal tissue distribution.

Throughout this series of plates a standard magnification factor of x100 is used.

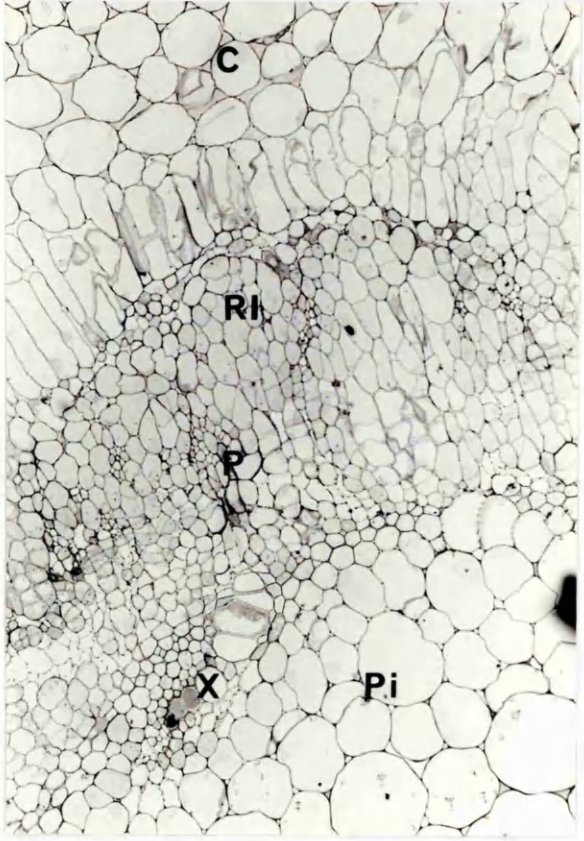


Plate 2a Transverse section through the base of the hypocotyl of a distilled water control cutting of P. vulgaris, showing a developing root initial.

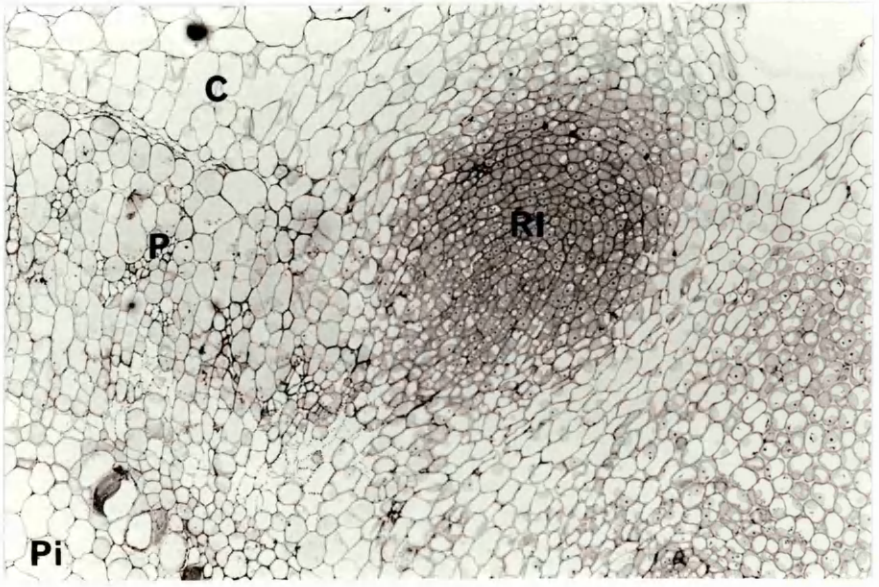


Plate 2b Transverse section through the base of the hypocotyl of a distilled water control cutting of P. vulgaris, showing a well developed root initial.

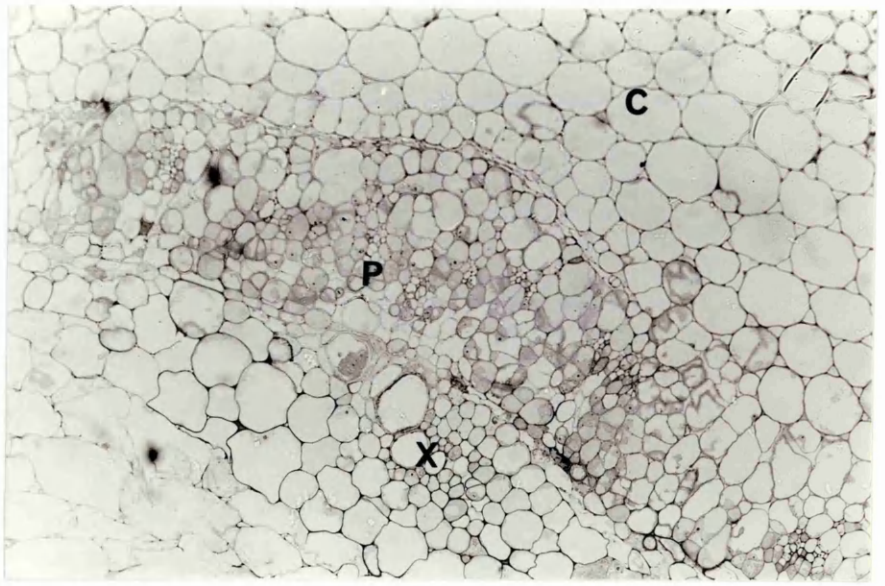


Plate 3 Transverse section through the base of a hypocotyl cutting of P. vulgaris treated with estrone-sulphate, applied by wick feeding, over a nine day rooting period. Normal tissue distribution is evident, illustrating that estrogen-induced anomalous cell development did not occur invariably.

Plate 4a

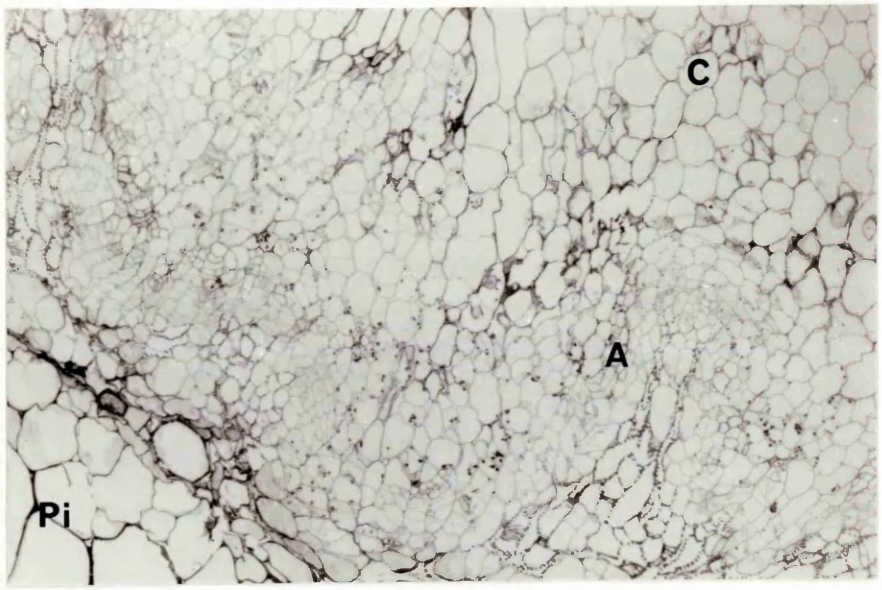
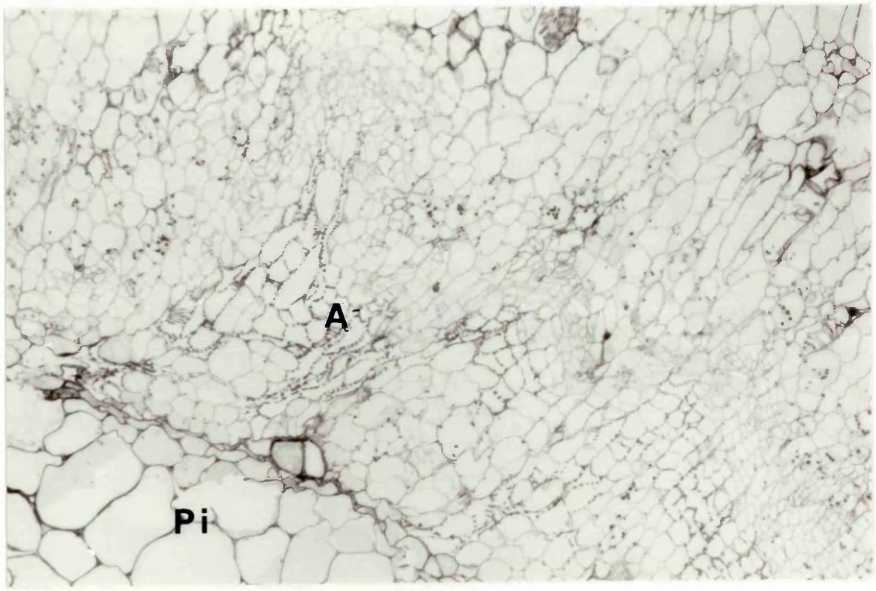


Plate 4b



Plates 4a and 4b Transverse sections through the bases of hypocotyl cuttings of P. vulgaris treated with estrone-sulphate, applied by wick feeding, over a nine day rooting period. Aberrant growth occurred in the peripheral phloem area normally associated with root initiation.

root initiation occurred (eg see Plate 5).

Anatomically the tissues of the primary leaf petiole are similar to those of the stem. Transverse sections of petioles between the upper and basal pulvinus reveal a more or less circular outline but with a pronounced adaxial groove. Stomata occur in the epidermis which overlies a chlorenchymatus cortical area. In the ridged area, especially adjacent to the groove, there are often areas of collenchyma. Several distinct, characteristically shaped vascular bundles form an open arc: the distribution of the xylem and the phloem relates directly to their arrangement in the stem with the phloem external to the xylem (see Plate 6). Lateral adventitious roots arise centrifugally in or adjacent to the zones of phloem in the vascular traces (see Plate 7). The pattern of adventitious root formation appeared to be well ordered and even in non-median sections, roots in the early stages of development could be readily seen in material from control cuttings (see Plate 8).

In petioles also, abnormal cell development was induced by E_1-S (see Plates 9a and 9b) and by E_2-S (see Plate 10) treatment. Zones of anomalous growth were noted in the outer radial portion of the phloem, most typically involving one to three vascular traces and giving rise to callus-like tissue.

Examination of sections taken from E_1-S treated primary leaf cuttings over the nine day rooting period revealed that normal tissue distribution could be observed during the first three days following commencement of estrogen treatment (see Plates 11 and 12) but by the fifth day areas of aberrant growth were visible (see Plates 13a, 13b, 14 and 15). As with hypocotyl cuttings, abnormal cell development did not occur invariably.

Treatment of primary leaf cuttings with IBA led to a massive

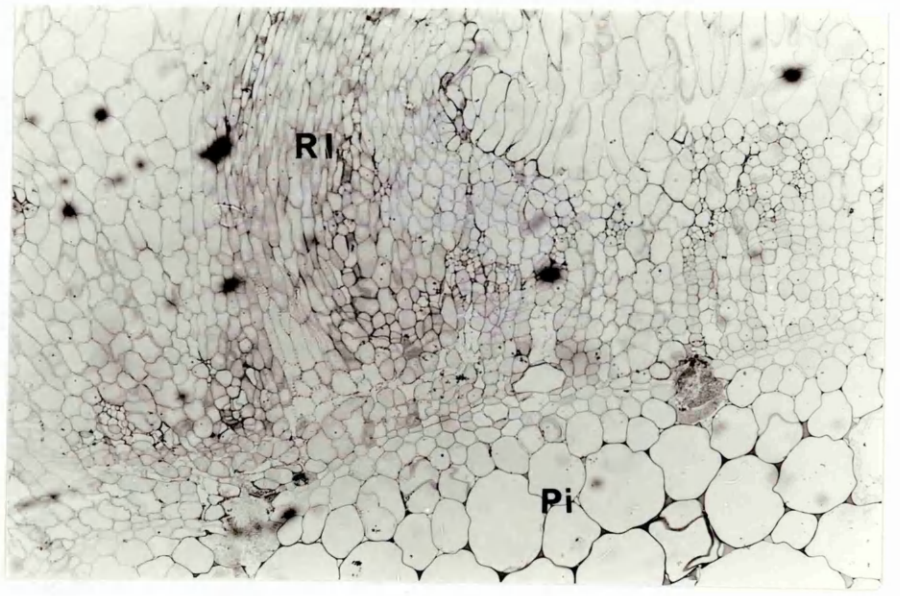


Plate 5 Transverse section through the base of the hypocotyl of a control cutting (Tris) of P. vulgaris after a nine day rooting period. Normal tissue distribution is evident and a well developed root initial is present.

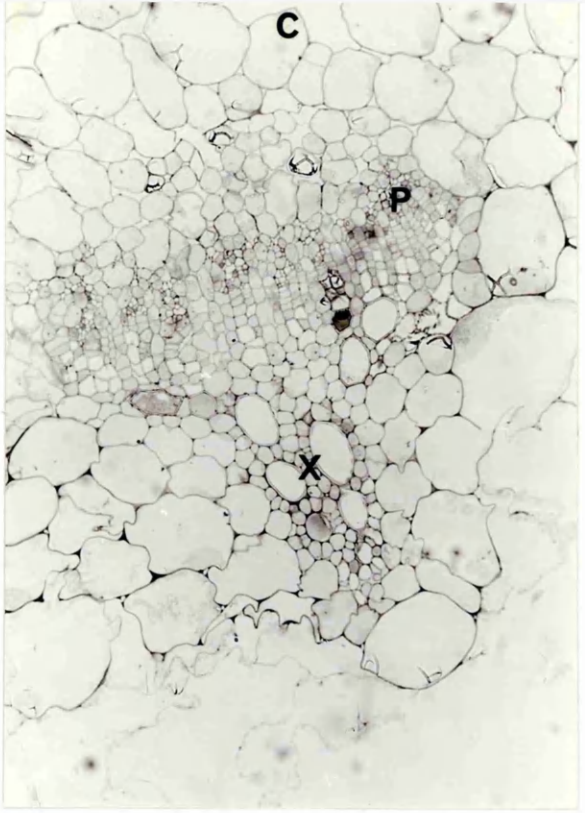


Plate 6 Transverse section through the petiole of a primary leaf cutting (distilled water control) of P. vulgaris, showing a typical vascular bundle.

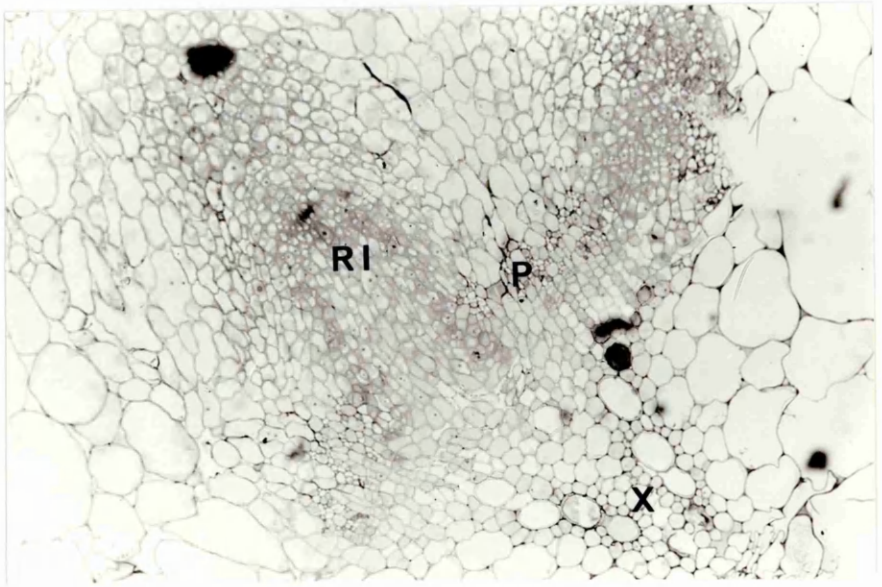


Plate 7 Transverse section through the petiole of a primary leaf cutting (distilled water control) of P. vulgaris, showing a vascular bundle and a developing root initial.

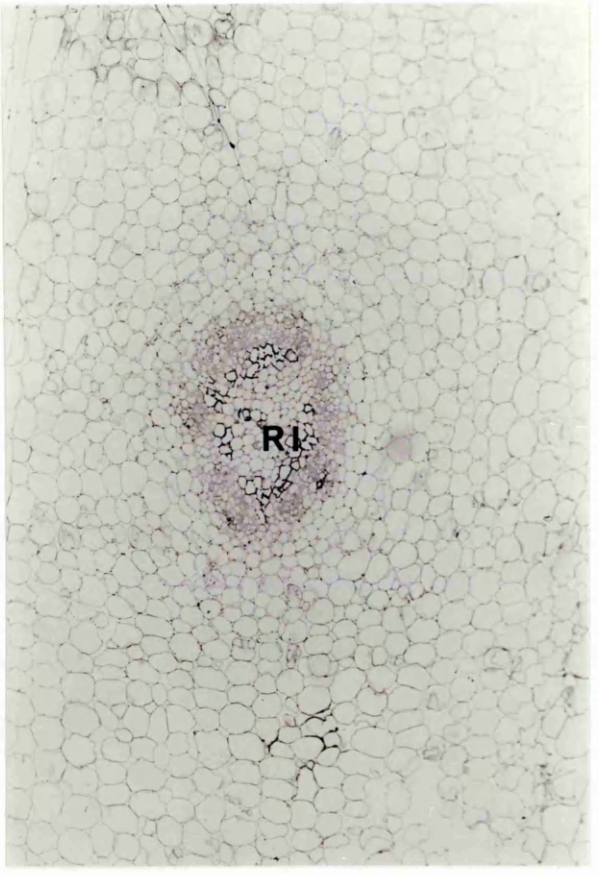


Plate 8 Radial longitudinal section through the cortical zone of a petiole of a primary leaf cutting (distilled water control) of P. vulgaris, showing a root initial.

Plate 9a

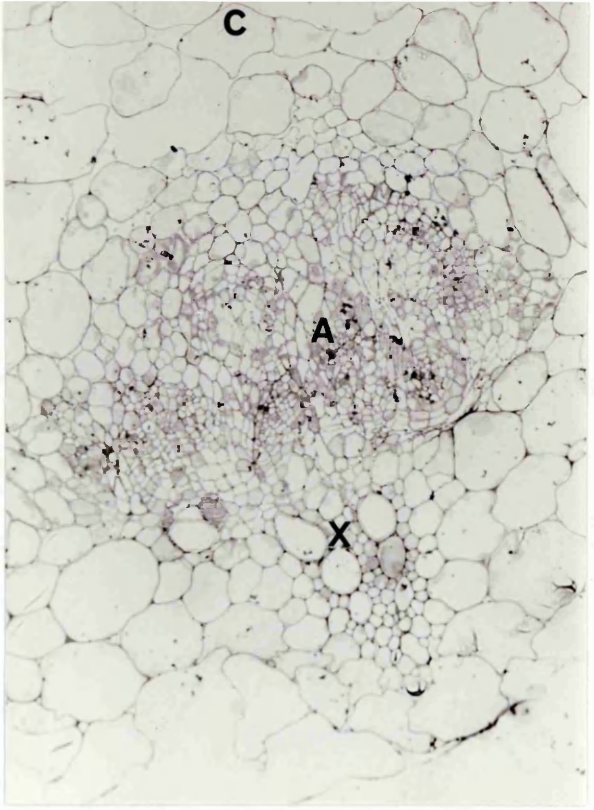
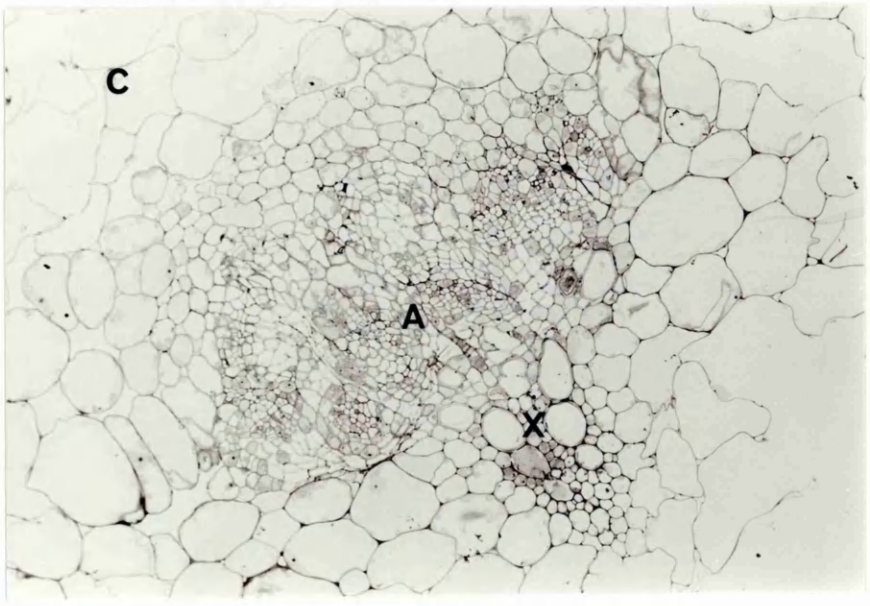


Plate 9b



Plates 9a and 9b Transverse sections through the petioles of primary leaf cuttings of P. vulgaris treated with estrone-sulphate, applied by wick feeding, over a nine day rooting period. Aberrant growth was associated with the vascular tissues.

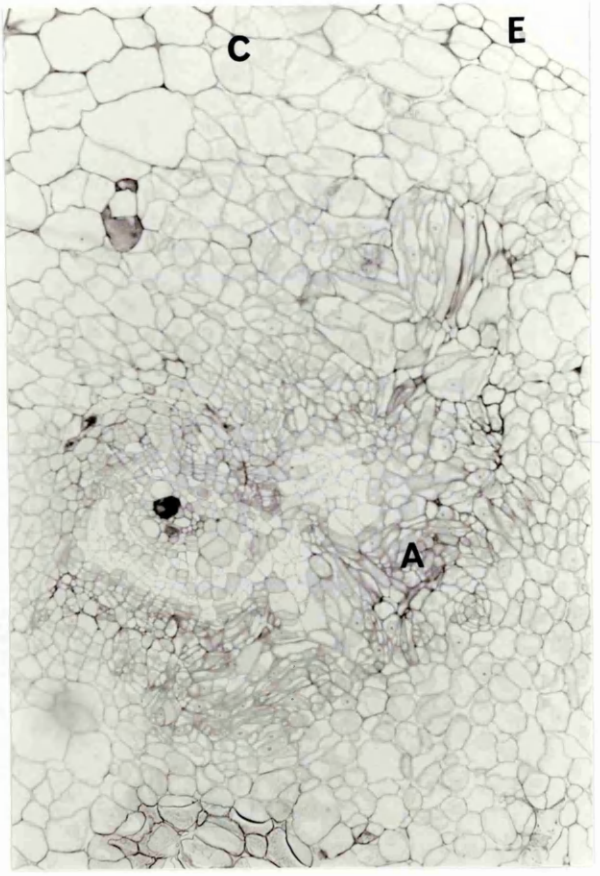


Plate 10 Transverse section through the petiole of a primary leaf cutting of P. vulgaris treated with estradiol-sulphate, applied by wick feeding, over a nine day rooting period. Areas of aberrant growth are present.

Plate 11

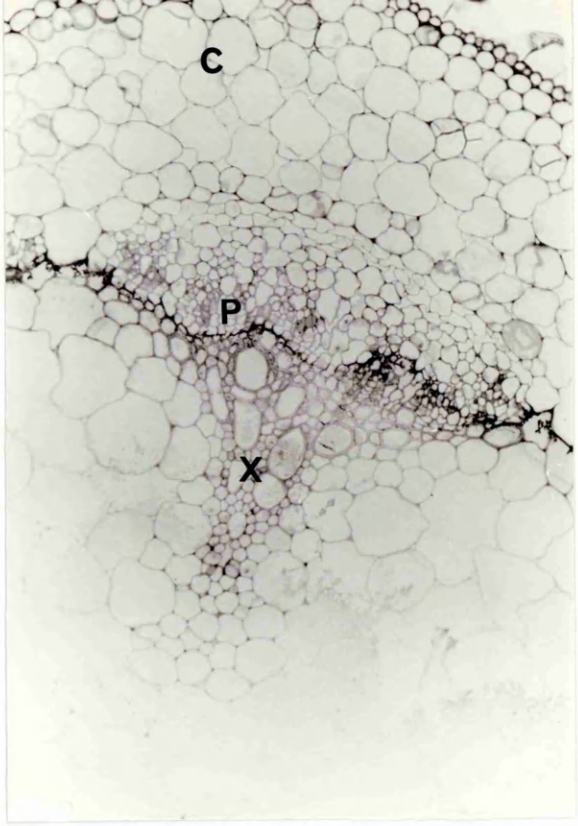
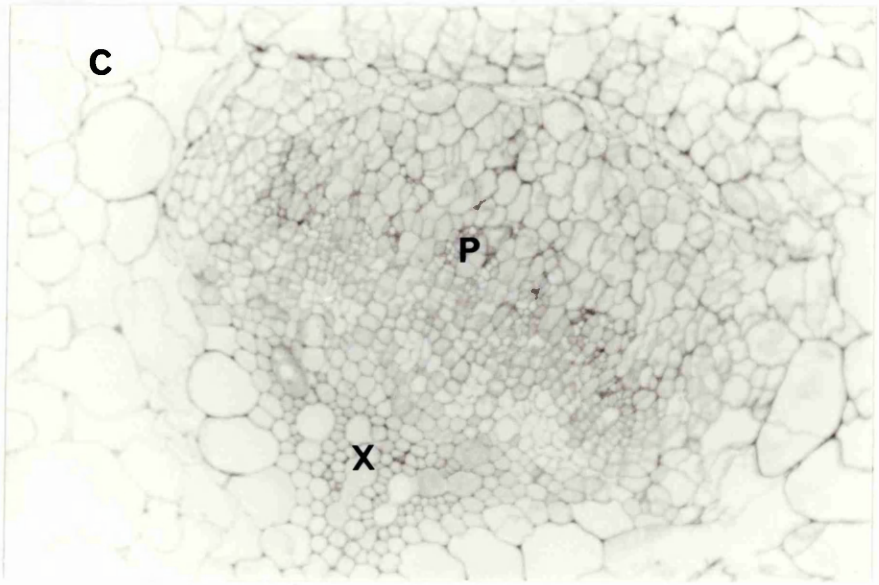


Plate 12



Plates 11 and 12 Transverse sections through the petioles of primary leaf cuttings of P. vulgaris treated with estrone-sulphate, applied by wick feeding, for one day (Plate 11) and three days (Plate 12). Normal tissue distribution is evident in both cases.

Plate 13a

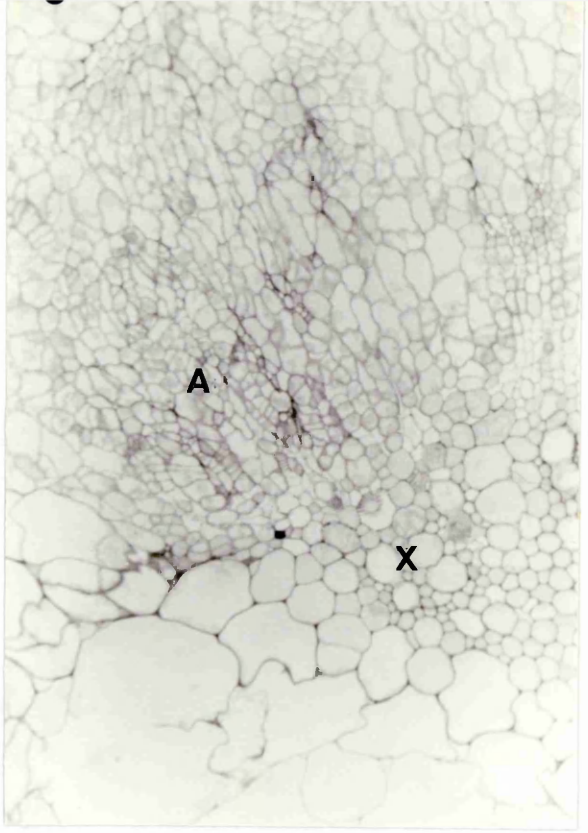
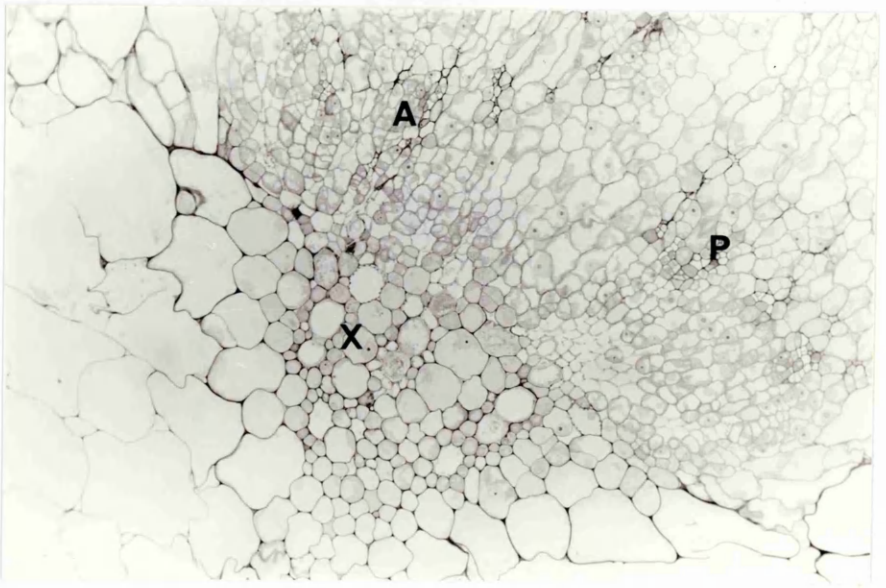


Plate 13b



Plates 13a and 13b Transverse sections through the petioles of primary leaf cuttings of P. vulgaris treated with estrone-sulphate, applied by wick feeding, for five days. Areas of aberrant growth are observable.

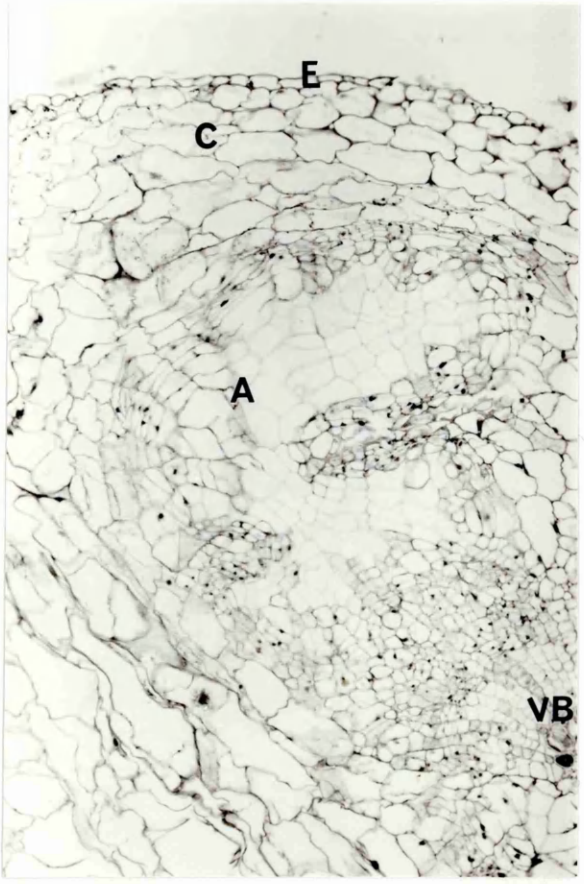


Plate 14 Transverse section through the petiole of a primary leaf cutting of P. vulgaris treated with estrone-sulphate, applied by wick feeding, for six days. Areas of aberrant growth are present.

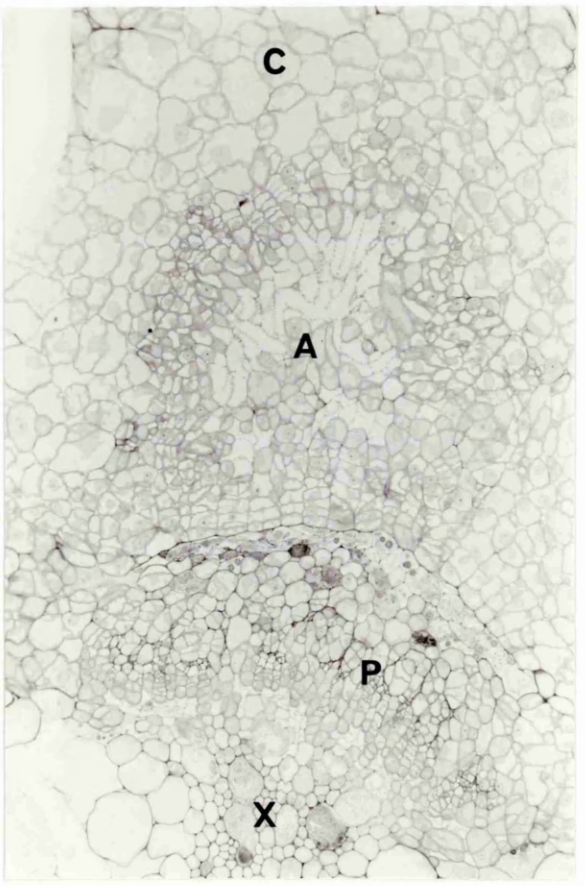


Plate 15 Transverse section through the petiole of a primary leaf cutting of P. vulgaris treated with estrone-sulphate, applied by wick feeding, for seven days. Aberrant growth is well established by this stage of the rooting period.

enhancement of adventitious root production (see Plate 16), splitting of the petiole and callus formation. Although E_1-S treatment reduced the enhancement effect (see section 3.1.2), there was no evidence in the sections examined that treatment of cuttings with IBA and E_1-S together led to the formation of abnormal cells (see Plate 17).

3.2 Uptake and Distribution of Exogenous Estrogens

In reviewing the literature concerning the effects of exogenously applied estrogens in plants, it becomes evident that there are many inconsistencies and contradictions in the reported results. As mentioned in the Introduction, one of the possible explanations for this is the misinterpretation of results, arising from a lack of information on uptake, distribution and possible metabolism of the applied hormone.

The uptake, distribution and metabolism of estrogens in cuttings of Phaseolus vulgaris was described in the Introduction, but can be summarised as follows: whilst uptake has been observed, the details of the process are unknown. Subsequent distribution of radioactivity is very restricted and as the bulk of the label remains associated with the applied compound, this reflects restricted distribution of the applied estrogen and not that of one of its metabolites. The experiments described here were designed to characterise more fully the details of uptake and the distribution of radioactivity from solutions of labelled estrogens throughout a treatment period parallel in length to those used in the application experiments (seven days for hypocotyl cuttings and nine days for primary leaf cuttings). Radio-labelled estrogen-sulphates are not commercially available, so that uptake and distribution of exogenous estrogens was monitored using unconjugated (4-^{14}C) estrone and (4-^{14}C) estradiol.

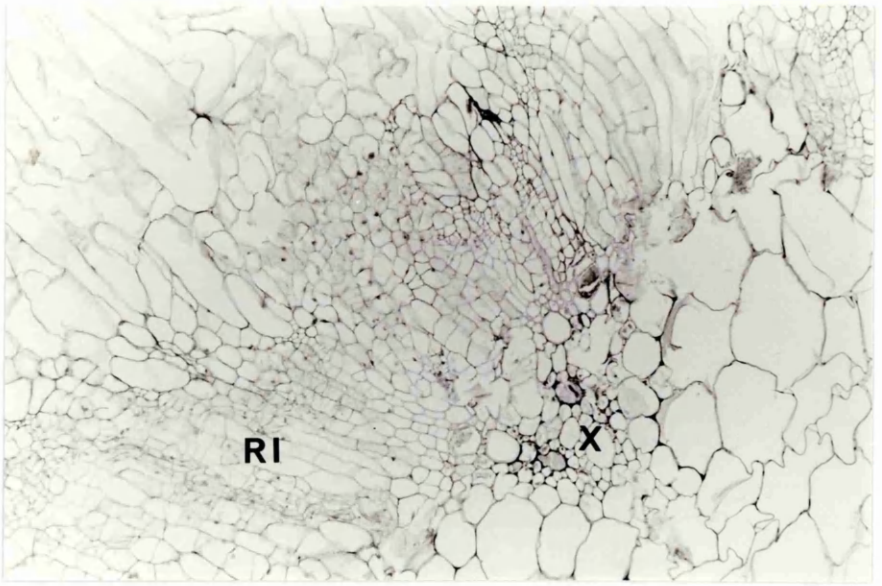


Plate 16 Transverse section through the petiole of a primary leaf cutting of P. vulgaris treated with IBA, applied by wick feeding, over a nine day rooting period. Massive root initiation occurred.

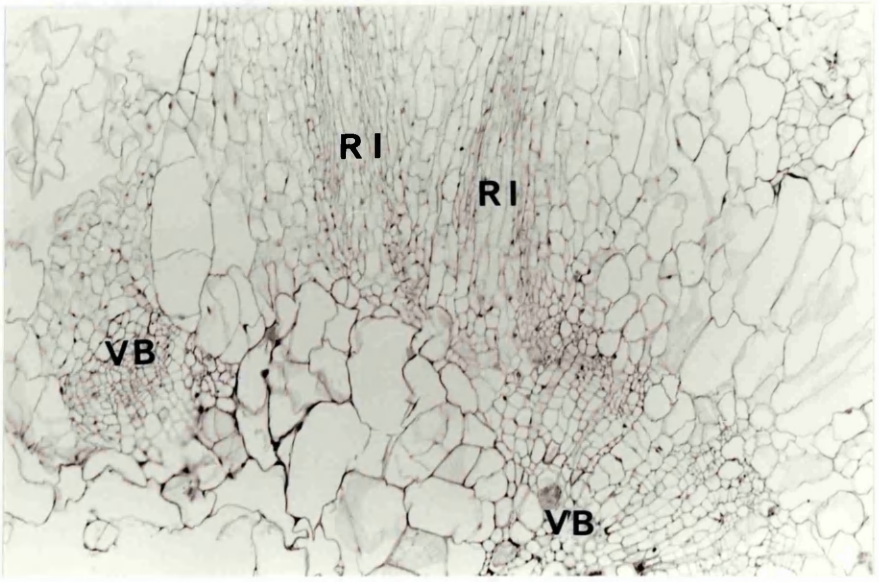


Plate 17 Transverse section through the petiole of a primary leaf cutting of P. vulgaris treated with IBA and estrone-sulphate, applied together by wick feeding, over a nine day rooting period. Normal root initiation is evident.

Before considering the results of these experiments, it was necessary to establish whether or not radioactivity was absorbed onto the surfaces of the glass vials in which cuttings were placed. At the end of an experiment the feeding solution was removed and any radioactivity which had been absorbed onto the surfaces of the vial extracted into methanol (see section 2.3.2.3). On average 101.5 ± 7.0Bq became absorbed onto the glass. This value, however, was consistent throughout the experiments and was inconsequential as the radioactivity would not have been available to the cutting for uptake anyway and so was discounted.

3.2.1 Hypocotyl Cuttings

As in previous studies (Young et al., 1979; Hewitt and Hillman, 1979a) uptake of radioactivity by hypocotyl cuttings from solutions of $^{14}C\text{-}E_1$ and $^{14}C\text{-}E_2$ was observed. In general, an increase in the label content during the seven day rooting period occurred (Figures 19 and 20).

A more careful examination of uptake at 24h and 48h after the initial application of labelled estrogen was carried out using $^{14}C\text{-}E_1$. The volume of solution taken up by each cutting and the radioactive content of the remaining solutions were noted and these values were used to calculate a value for non-selective uptake (see section 2.3.3.2). These calculated values for non-selective uptake were compared with actual uptake (Figure 21). The average actual uptake 24h after application (1492 ± 169Bq) exceeded the value calculated for non-selective uptake (1174.8 ± 244Bq), indicating that there was some degree of selectivity. Examination of the results obtained for individual cuttings, however, casts doubt over this conclusion as in only half the cases was selectivity noted (see Table 3a). After 48h, average actual uptake (1751.5 ± 113Bq) again exceeded the value

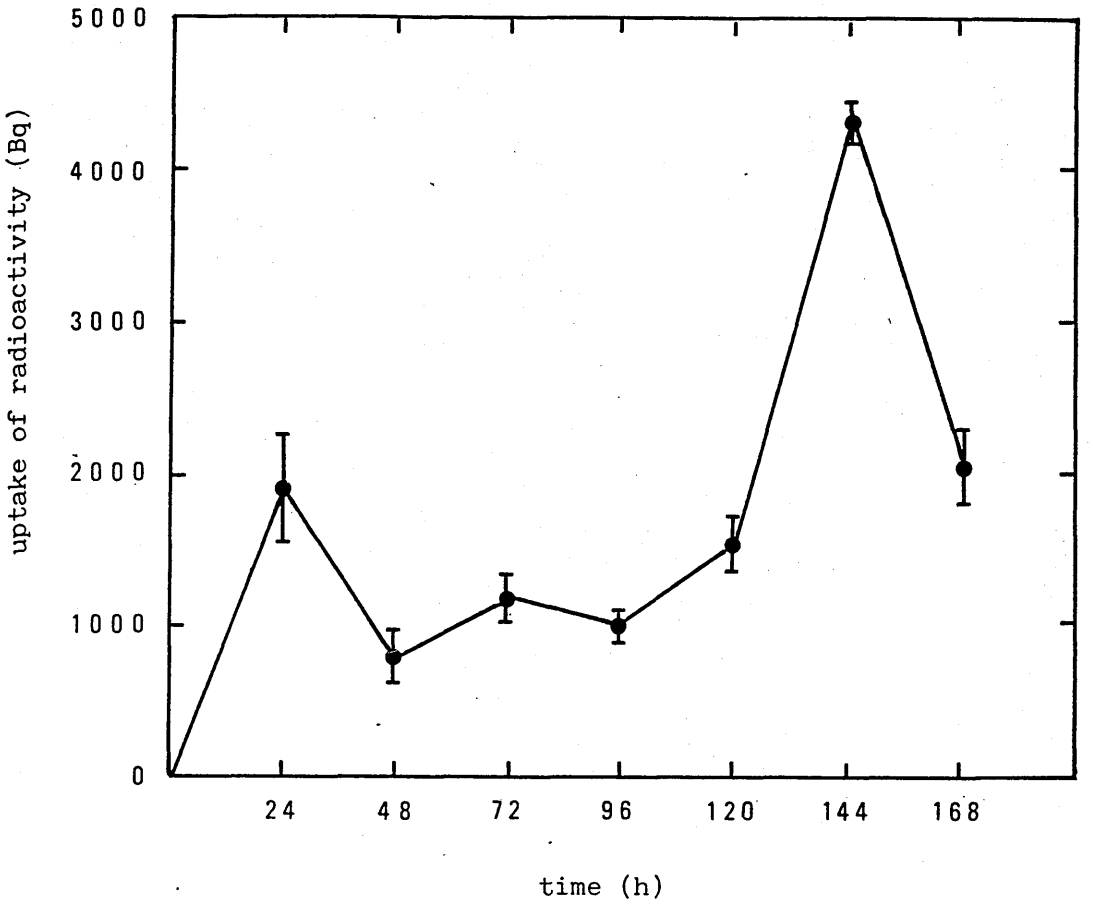


Figure 19 Uptake of radioactivity from solutions of (4-^{14}C) estrone by hypocotyl cuttings of P. vulgaris as a function of time. Values shown represent the means of five individual cuttings ± one standard error.

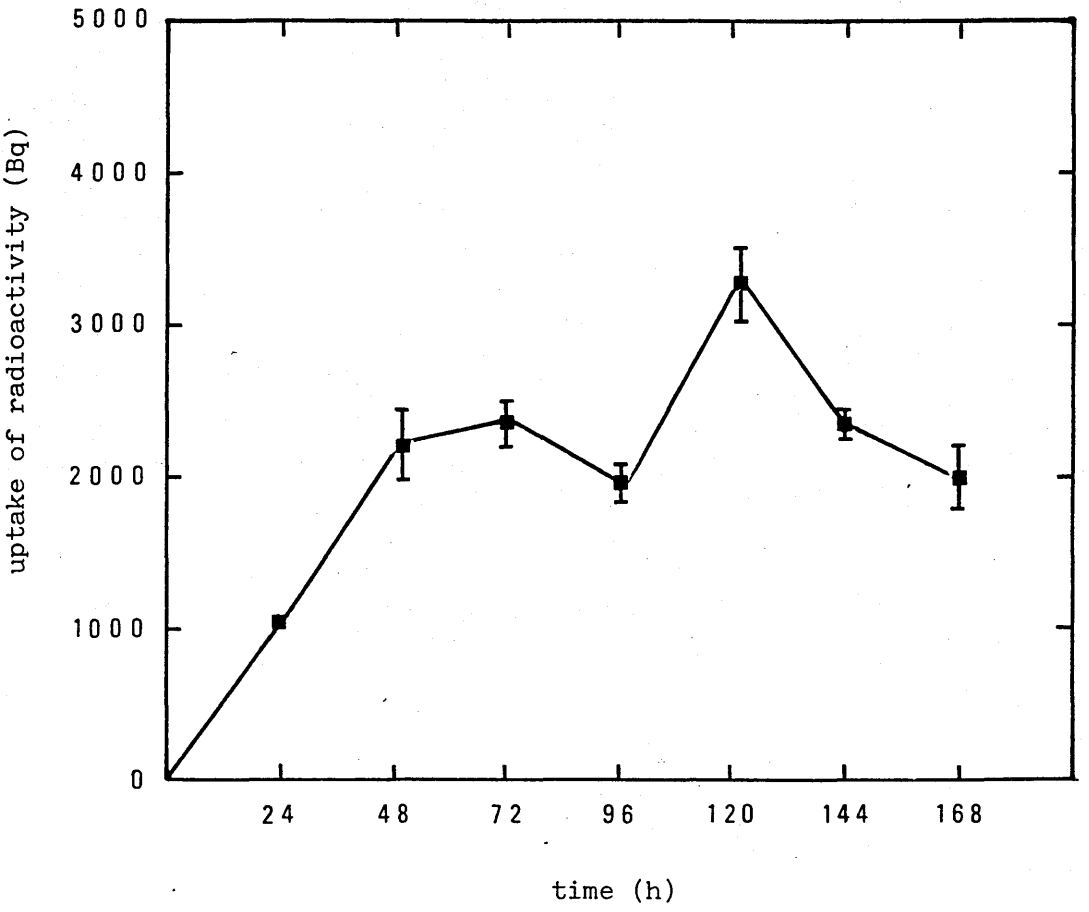


Figure 20 Uptake of radioactivity from solutions of (4-^{14}C) estradiol by hypocotyl cuttings of P. vulgaris as a function of time. Values shown represent the means of five individual cuttings ± one standard error.

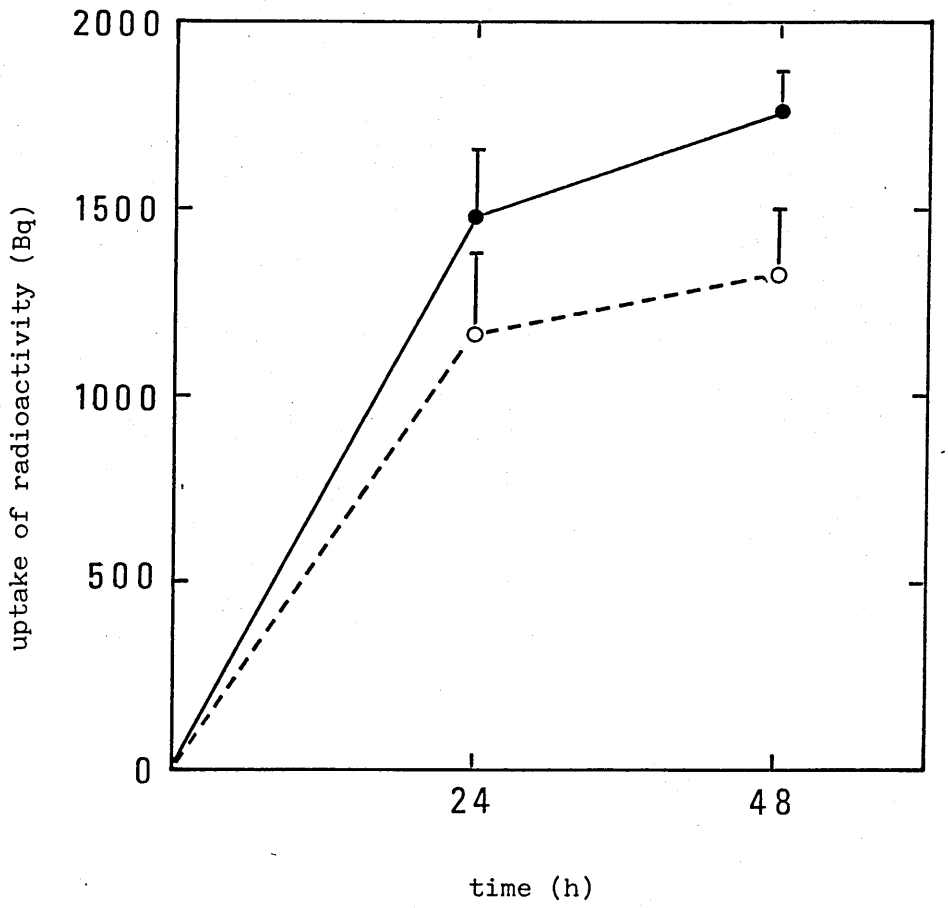


Figure 21 A comparison of calculated non-selective (broken line) and actual uptake (unbroken line) of radioactivity from solutions of (4-^{14}C) estrone by hypocotyl cuttings of P. vulgaris during the first 48h of a 168h treatment period. The values shown represent the means of four individual cuttings $\pm$ one standard error.

Table 3 A comparison of calculated non-selective uptake and actual uptake of radioactivity from solutions of (4-^{14}C) estrone by hypocotyl cuttings of P. vulgaris 24h (a) and 48h (b) after commencement of treatment.

(a) 24h

Cutting	volume of solution taken up (ml)	Bqml^{-1} of remaining feeding solution	calculated non-selective uptake (Bq)	actual uptake (Bq)
1	7.5	244	1832.5	1649.9
2	4.0	163	655.3	1875.7
3	4.7	238	1121.7	1115.5
4	4.5	242	1089.8	1327.4
average	5.2 ± 0.8	221.8 ± 19.6	1174.8 ± 244	1492.0 ± 169.0

(b) 48h

Cutting	volume of solution taken up (ml)	Bqml^{-1} of remaining feeding solution	calculated non-selective uptake (Bq)	actual uptake (Bq)
1	4.3	222	955.3	1636.3
2	9.0	205	1848.0	1999.2
3	6.3	209	1319.9	1870.5
4	6.2	180	1116.0	1499.9
average	6.5 ± 1.0	204 ± 8.8	1309.8 ± 194.3	1751.5 ± 113.0

calculated for non-selective uptake (1309.8 $\pm$ 194Bq), again indicating that selective uptake of the labelled compound had occurred. In this case, closer examination of the data obtained reveals that the collective results closely reflect the pattern of uptake for each individual cutting (see Table 3b). Thus it can be concluded that there was a trend towards selective uptake of radioactivity from solutions of $^{14}C\text{-}E_1$ which was manifest 48h after application.

The distribution of label at 24h intervals was examined and the results are given in Table 4 ($^{14}C\text{-}E_1$ experiments) and Table 5 ($^{14}C\text{-}E_2$ experiments). The general distribution patterns of radioactivity taken up from solution of $^{14}C\text{-}E_1$ or $^{14}C\text{-}E_2$ were essentially very similar and surprisingly the distribution pattern evident after the first 24h period remained unchanged throughout the entire seven days (168h). In general, 50 to 60% of the total radioactivity sampled for each cutting remained in the basal 1cm hypocotyl segment and over 90% remained below the cotyledonary node. Of the remaining radioactivity, most was present in the epicotyl, although some (less than 1%) was detected in the petioles and laminae of the primary leaves. In cuttings treated with $^{14}C\text{-}E_2$ no label travelled to any plant part above the first leaf node, but in those treated with $^{14}C\text{-}E_1$ very low levels of activity were present in the axillary buds at the first leaf node, in axillary buds above the first leaf node, in the internodes, in trifoliate leaves and in the apex. In all these cases, however, the radioactivity which had travelled beyond the first leaf node accounted for only less than 3% of the total sampled.

Table 4 **Percentage distribution of radioactivity taken up from solutions of (4-^{14}C) estrone by hypocotyl cuttings of *P. vulgaris* during a seven day (168h) treatment period.**

Plant part	Time (h) 24	48	72	96	120	144	168
hypocotyl segments							
1 (base)	48.8	61.5	66.3	87.6	35.0	25.0	53.9
2	35.5	17.7	14.9	6.4	33.7	40.0	18.9
3	3.8	7.5	9.3	0.5	13.6	16.8	13.1
4	6.7	4.7	7.0	1.9	10.0	10.5	8.4
total for hypocotyl	94.8	91.4	97.5	96.4	92.3	92.3	94.3
epicotyl	4.6	4.6	3.1	2.4	7.2	9.6	4.2
primary leaves	0.6	1.3	0.2	0.7	0.3	0.5	0.3
first axillary buds	ND	0.3	0.1	0.1	0.1	ND	ND
*internodes	ND	0.9	<0.1	0.1	<0.1	ND	0.1
*trifoliate leaves	ND	0.8	<0.1	0.1	<0.1	ND	0.1
*axillary buds	ND	0.3	ND	ND	ND	ND	ND
apex	ND	0.3	ND	0.2	<0.1	0.1	ND

The percentage values shown represent the means of five individual cuttings.

ND - not detected

*totals for parts above the first leaf node

Table 5 **Percentage distribution of radioactivity taken up from solutions of (4-^{14}C) estradiol by hypocotyl cuttings of *P. vulgaris* during a seven day (168h) treatment period.**

Plant part	Time (h) 24	48	72	96	120	144	168
hypocotyl segments							
1 (base)	60.1	60.1	53.5	51.3	60.0	62.8	47.0
2	18.8	16.3	21.0	20.6	17.0	19.4	23.1
3	10.6	9.5	11.5	14.2	10.1	10.0	15.9
4	5.5	7.4	8.0	6.6	8.1	5.0	9.9
total for hypocotyl	95.0	93.3	94.0	92.7	95.2	97.2	95.9
epicotyl	4.9	5.5	5.9	7.0	4.6	1.8	3.4
primary leaves	ND	0.3	0.4	0.6	0.3	0.2	0.5
plant parts above first leaf node	ND	ND	ND	ND	ND	ND	ND

The percentage values shown represent the means of five individual cuttings.

ND - not detected

3.2.2 Primary Leaf Cuttings

In parallel with the application experiments, uptake and distribution of radioactivity from solutions of $^{14}C\text{-}E_1$ and $^{14}C\text{-}E_2$ was monitored for a period of nine days (216h). As with hypocotyl cuttings, the results obtained using $^{14}C\text{-}E_1$ and $^{14}C\text{-}E_2$ were very similar although there was consistently greater uptake of radioactivity from solutions of $^{14}C\text{-}E_2$. The label content of cuttings increased during the treatment period, with a gradual decrease in the rate of uptake with time (Figure 22: $^{14}C\text{-}E_1$ and Figure 23: $^{14}C\text{-}E_2$). A more careful examination of uptake during the first 72h of the treatment period was made at 24h intervals. Again, a value for non-selective uptake, taking into account water uptake, was calculated (see section 2.3.2.2) and these values compared with the values measured for actual uptake of radioactivity. The results obtained are shown in Figure 24 for experiments where $^{14}C\text{-}E_1$ was used, and Figure 25 for $^{14}C\text{-}E_2$ experiments. Inspection of the data shows that there was no statistical evidence of either selective uptake or active inhibition of uptake by leaf cuttings at 24h, 48h or 72h after commencement of treatment. This differs from uptake by hypocotyl cuttings which appears to have some degree of selectivity. It may simply be that hypocotyl cuttings have more sites which can absorb and/or adsorb the lipophilic steroid molecules.

The distribution of radioactivity from solutions of labelled hormones within primary leaf cuttings was examined, initially at 24h intervals (up to 72h) and subsequently at 48h intervals throughout the treatment period. As was the case with hypocotyl cuttings, distribution of radioactivity from solutions of either $^{14}C\text{-}E_1$ (Table 6) or $^{14}C\text{-}E_2$ (Table 7) was very restricted, and this restricted distribution was evident throughout the entire 216h period. Of the total radioactivity

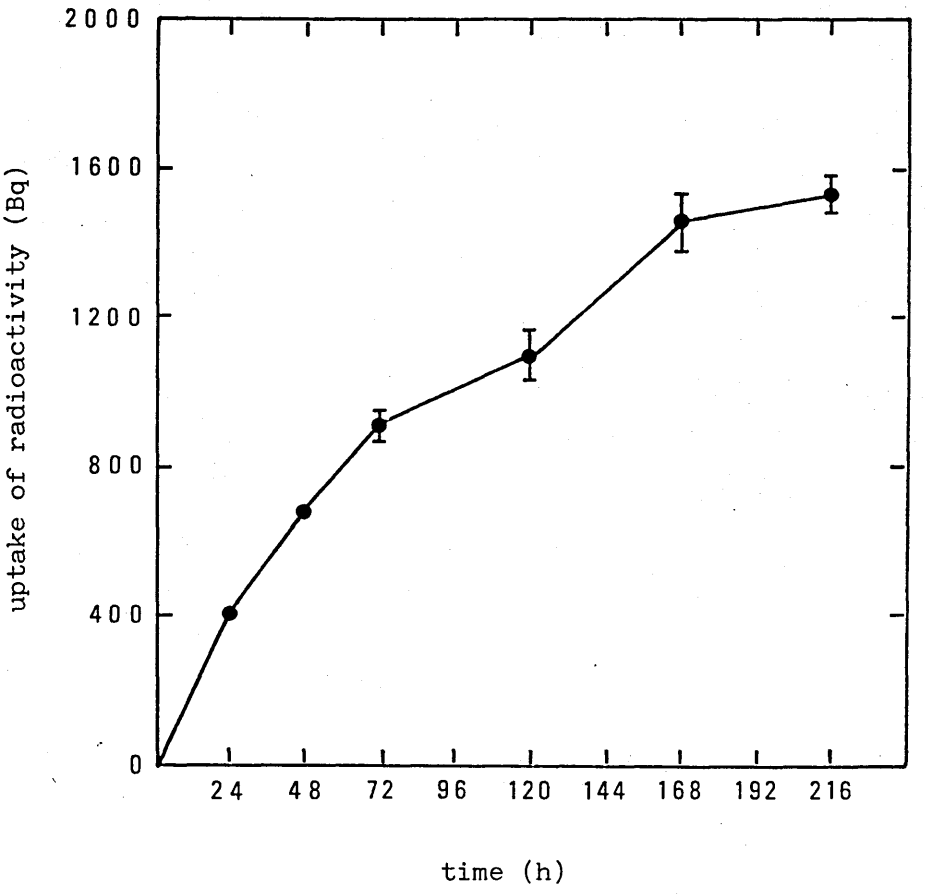


Figure 22 Uptake of radioactivity from solutions of (4-^{14}C) estrone by primary leaf cuttings of P. vulgaris as a function of time. Values shown represent the means of ten individual cuttings ± one standard error.

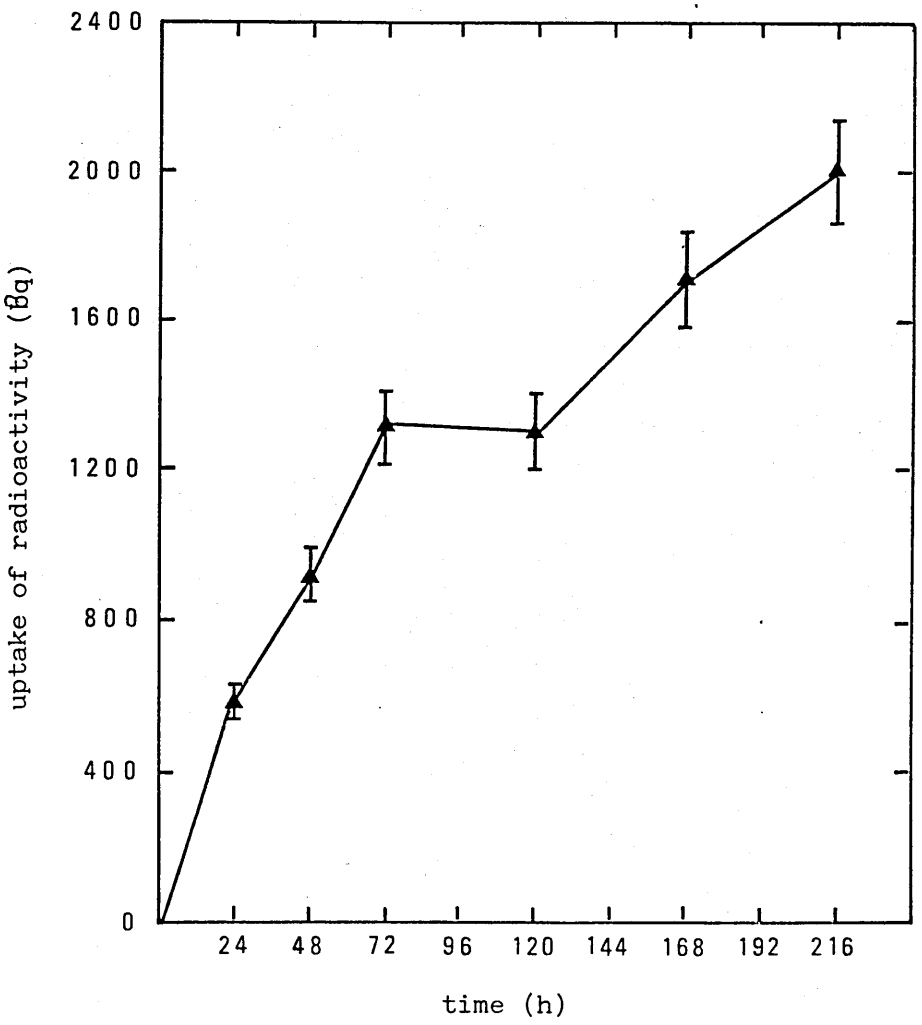


Figure 23 Uptake of radioactivity from solutions of (4-^{14}C) estradiol by primary leaf cuttings of P. vulgaris as a function of time. Values shown represent the means of ten individual cuttings ± one standard error.

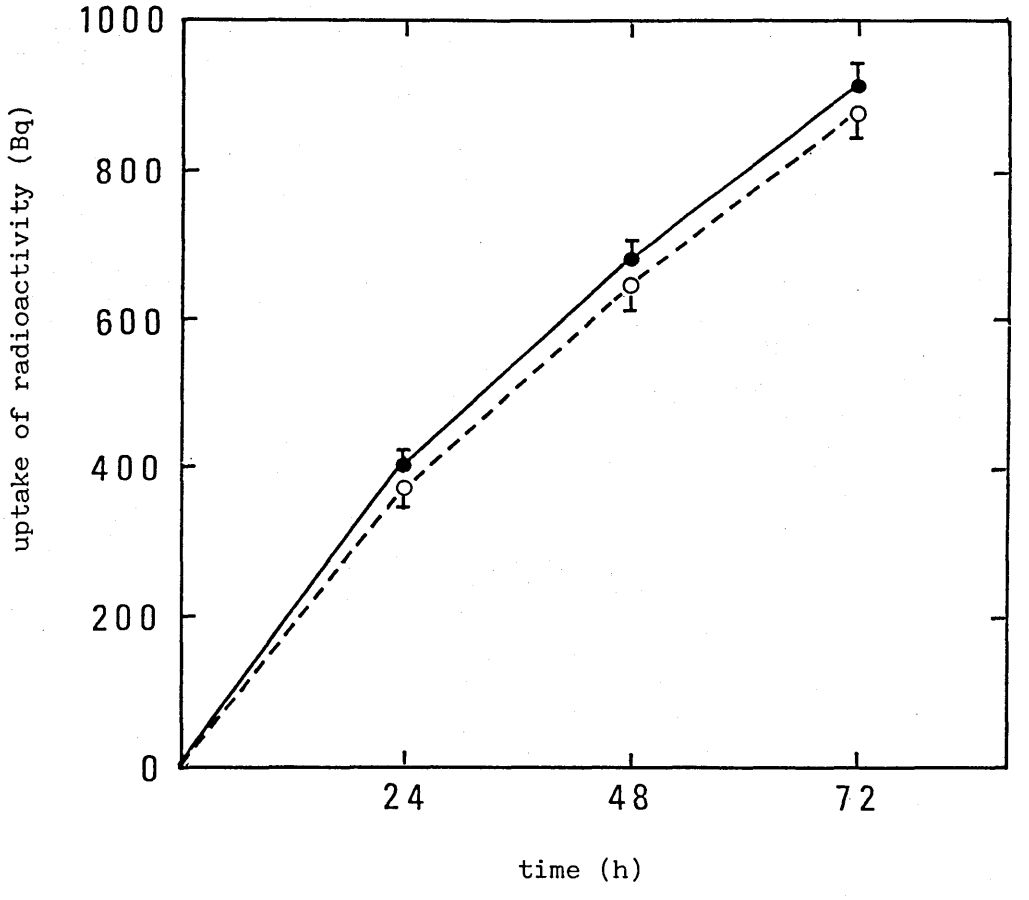


Figure 24 A comparison of calculated non-selective uptake (broken line) and actual uptake (unbroken line) of radioactivity from solutions of (4-^{14}C) estrone by primary leaf cuttings of P. vulgaris during the first 72h of a 216h treatment period. Values shown represent the means of ten individual cuttings $\pm$ one standard error.

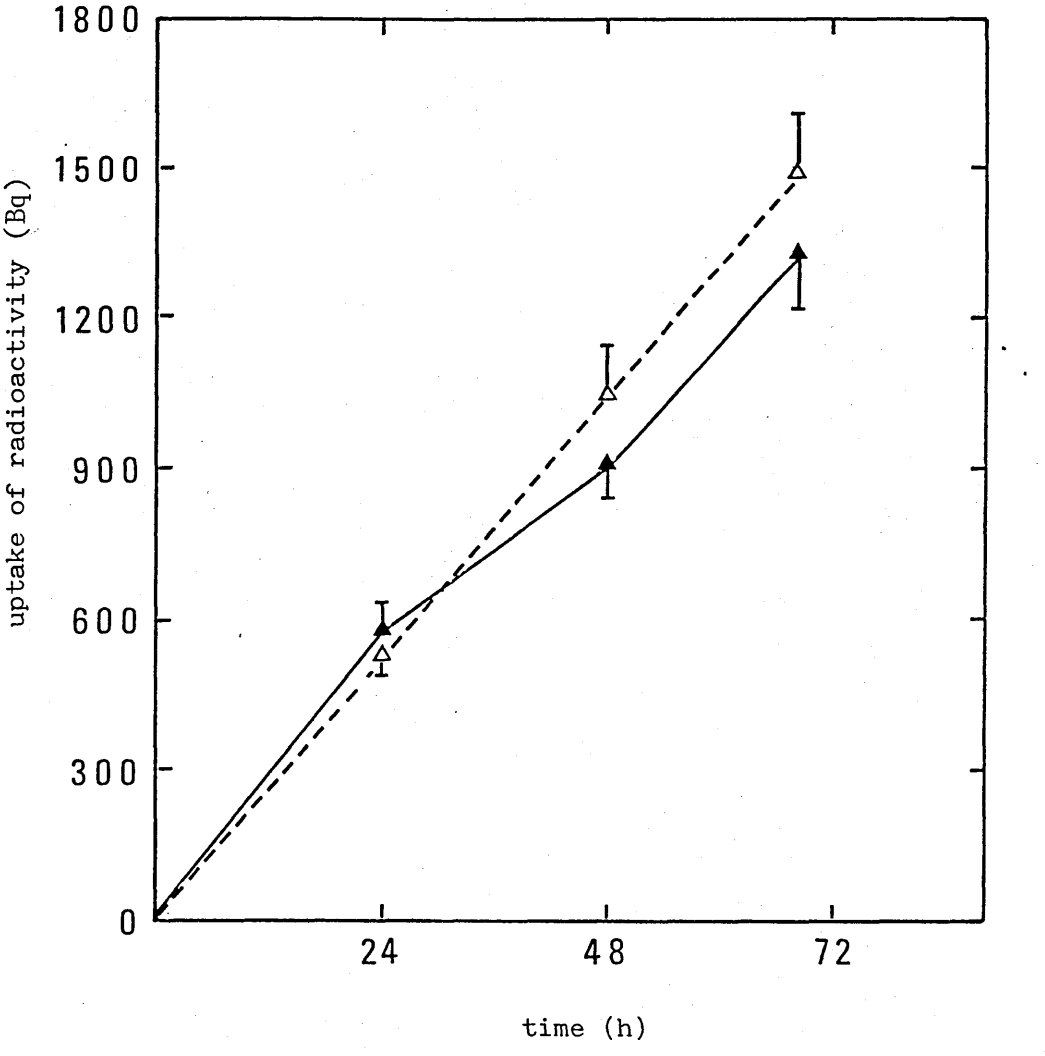


Figure 25 A comparison of calculated non-selective uptake (broken line) and actual uptake (unbroken line) of radioactivity from solutions of (4-^{14}C) estradiol by primary leaf cuttings of P. vulgaris during the first 72h of a 216h treatment period. Values shown represent the means of ten individual cuttings $\pm$ one standard error.

Table 6 **Percentage distribution of radioactivity taken up from solutions of (4-^{14}C) estrone by primary leaf cuttings of P. vulgaris during a nine day (216h) treatment period.**

Plant part	Time (h) 24	48	72	120	168	216
petiole segments						
1 base	68.2	66.9	70.2	70.0	62.9	64.5
2	15.0	14.3	13.0	14.0	10.3	7.9
3	4.7	5.8	5.3	5.8	3.1	4.5
4	3.3	3.9	3.0	2.9	3.2	2.1
total for petiole	91.2	90.9	91.5	92.7	79.5	79.0
lamina	8.8	9.3	8.4	7.4	6.5	6.1
roots	-	-	-	-	19.6	15.0

Percentage values shown represent the mean of ten individual cuttings.

*****

Table 7 **Percentage distribution of radioactivity taken up from solutions of (4-^{14}C) estradiol by primary leaf cuttings of P. vulgaris during a seven-day (216h) treatment period.**

Plant part	Time (h) 24	48	72	120	168	216
petiole segment						
1 (base)	69.8	58.0	65.5	74.5	66.4	59.3
2	11.9	20.1	14.1	8.3	9.3	9.0
3	4.8	6.6	6.1	3.8	3.6	3.9
4	4.0	4.1	3.7	5.8	2.2	2.0
total for petiole	90.5	88.8	89.4	92.4	81.5	74.2
lamina	9.4	11.0	10.8	7.7	9.3	9.0
roots	-	-	-	-	12.7	16.7

Percentage values shown represent the mean of ten individual cuttings.

taken up by cuttings, most remained in the petiole: 60 to 70% was restricted to the basal 1cm segment bearing the cut edge and there was a marked diminution of activity towards the upper petiole. Less than 10% of the label travelled into the lamina, even although this presents the largest surface area. When small numbers of adventitious roots had developed (at 168h and 216h) they accumulated around 20% of the total label taken up, mostly at the expense of the basal 1cm segment of the petiole.

3.3 RNA Metabolism

It is known that steroid hormones in animals exert their effects by alterations in gene expression. This involves changes in the protein and nucleic acid metabolism of target cells. It may be that the steroidal estrogens present in plants have a similar mode of action. Thus a study of the effects of estrogens on the RNA metabolism of responding tissues of P. vulgaris primary leaf cuttings, in terms of effects on rooting and the induction of anomalous cell development, was carried out.

3.3.1 Isolation and Analysis of RNA

Total RNA from the petioles of excised primary leaves was successfully extracted on a routine basis using the phenol/chloroform extraction procedure described in the Materials and Methods (section 2.3.3.1). Although yields of RNA varied from as little as 1mg to as much as 7mg, in general 3 to 5mg of RNA was extracted from 10 to 15g of plant material.

The integrity of the RNA was established by separating the main ribosomal fractions on sucrose density gradients. Some examples of the traces obtained by scanning the gradients for UV light absorbance

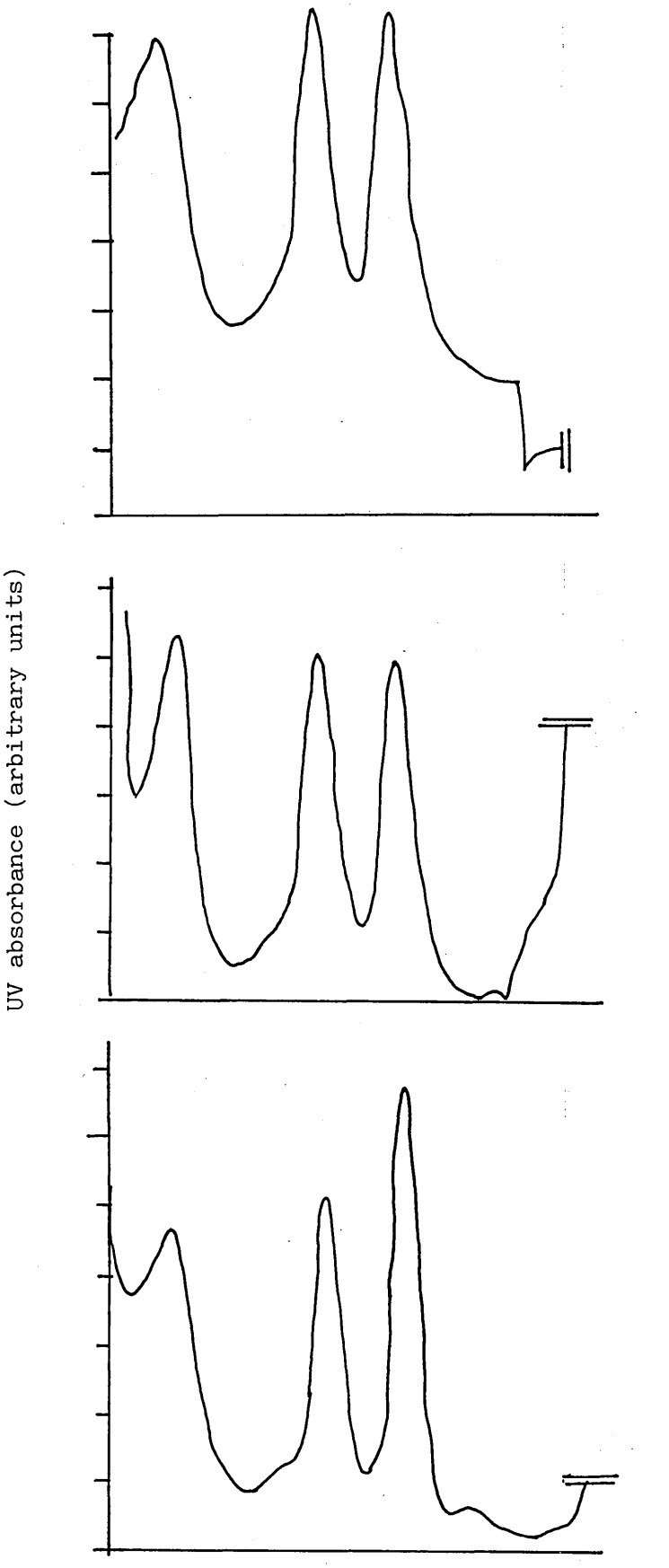
after sedimentation are given in Figure 26 and clearly show two main peaks of absorbance corresponding to light (18S) and heavy (27S) ribosomal RNA. A third peak in absorbance at the top of the gradients was also present and corresponded to a soluble phase present in the total RNA preparations. This fractionation pattern was confirmed by separating the RNA by electrophoresis (Plate 18). In addition to separating 27S and 18S RNA (bands A and C), 5S and 5.6S RNA were also resolved (band G). Furthermore, the light and heavy ribosomal RNA of the chloroplasts (bands B and D) and transfer RNA (band H) were also detected. Bands E and F were always present, but it is unclear what they represent.

Normal turnover of the RNA was demonstrated by incorporation of ^{3}H-uridine (uridine is the only nitrogenous base unique to RNA) into the two main ribosomal fractions during a 4h incubation period preceding extraction of total RNA (Figure 27). A third peak of incorporation was also detected and this corresponded to the soluble phase of the RNA preparations.

Some investigators (eg Misra and Bewley, 1985) use the bulk total RNA for <u>in vitro</u> protein synthesis rather than isolating mRNA. This has the advantage that the ribosomal RNA present in the total RNA preparation affords protection to the mRNA against degradation by contaminating ribonucleases but, on the other hand, efficiency of translation may decrease. Thus poly(A)$^{+}$RNA was isolated from total RNA by affinity column chromatography using oligo(dT)cellulose. Generally, the yields of poly(A)$^{+}$RNA represented between 1 and 2% of the yield of total RNA.

On some occasions, problems were encountered with the oligo(dT) cellulose columns becoming blocked. A possible explanation for this is that total RNA preparations contained contaminating proteins and/or

petioles of primary leaf cuttings of P. vulgaris had been sedimented by centrifugation.



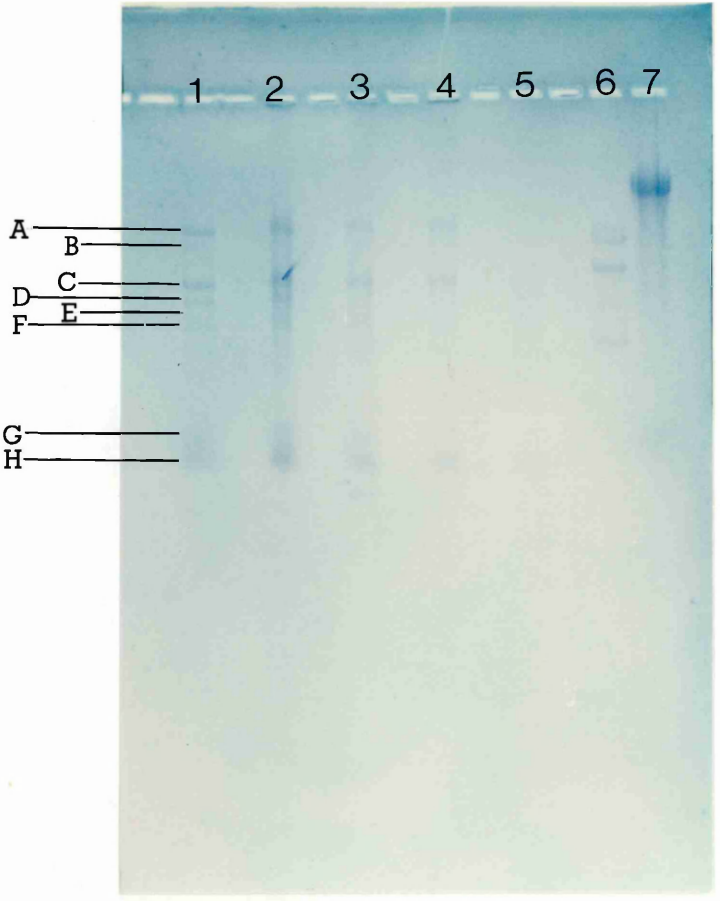


Plate 18 The separation of total RNA from the lamina (tracks 1 and 2) and the petioles (tracks 3 and 4) of excised primary leaves of P. vulgaris by agarose-formaldehyde gel electrophoresis. Bean RNA (track 5), TMV RNA (track 6) and BMV RNA (track 7) were also separated to provide standards. The main ribosomal fractions (bands A and C), the ribosomal RNA of the chloroplasts (bands B and D), 5 and 5·6S RNA (band G) and transfer RNA (band H) were resolved. It is not clear what bands E and F represent.

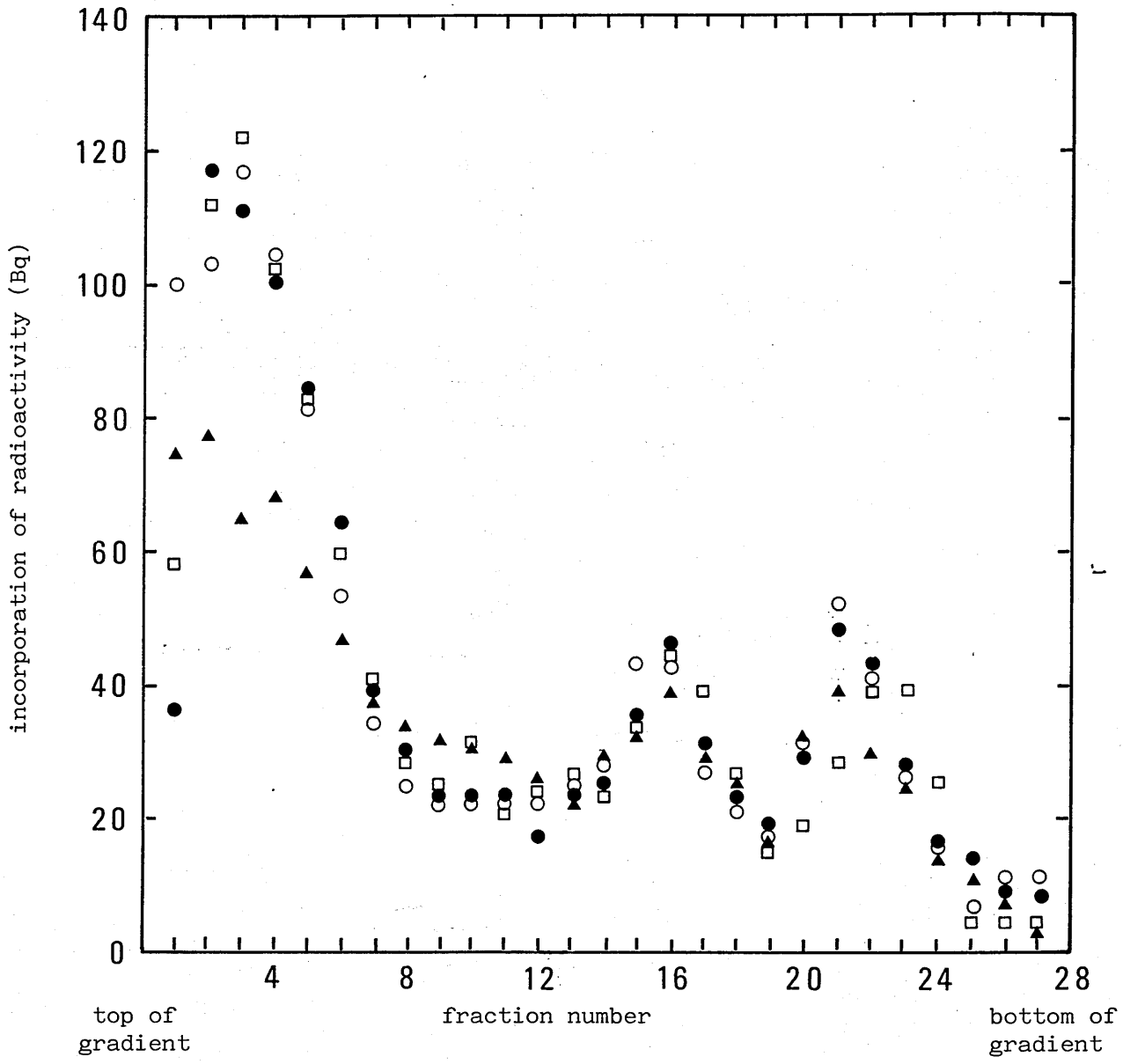


Figure 27 The incorporation of ^{3}H uridine into the ribosomal fractions of RNA during a 4h incubation period preceeding extraction and sedimentation of RNA on sucrose density gradients. The RNA was extracted from segments of the petioles of primary leaves of P. vulgaris. Incorporation into 4 samples is illustrated.

DNA. In order to investigate this possibility, the protein and DNA contents of total RNA preparations was assayed at various stages of the extraction procedure; (1) first aqueous phase, (2) the aqueous phase obtained after re-extracting the first phenolic phase and interphase, (3) the aqueous phase obtained after re-extracting the pooled aqueous phases and (4) post-column preparations (DNA assays only).

The protein content of samples was estimated using the tannin protein assay (Mejbaum-Katzenellenbogen and Dobryszycka, 1959). Very little protein was present in the RNA samples loaded onto columns (see Table 8). This is not surprising since the phenol extractions employed for isolating the RNA from the plant tissue would have removed most of the protein and special care was taken to avoid the interphase where proteins collect.

The DNA content of samples was estimated spectrophotometrically using a diphenylamine assay (Burton, 1956). The results (Table 8) indicate that in all cases less than 20µg DNA per 1g of plant tissue was present in total RNA preparations. No DNA was detected in post-column preparations.

In view of these results, it was not deemed necessary to incorporate any additional steps into the extraction procedure to deal with DNA and protein contamination. At any rate, the poly(A)$^+$RNA obtained without the use of proteinases and deoxyribonucleases provided samples which retained the same degree of translational activity over a period of 12 to 18 months, indicating that little degradation had taken place.

Table 8 **The DNA and protein content of RNA preparations at different stages of the extraction procedure.**

Stage of extraction procedure	DNA content $\mu g\ g^{-1}$ plant tissue	Protein content $\mu g\ g^{-1}$ plant tissue	RNA yield $\mu g\ g^{-1}$ plant tissue
1st aqueous phase	3.4 ± 0.2	4.9 ± 1.9	177.0 ± 27.1
2nd aqueous phase	8.1 ± 3.1	18.9 ± 9.4	146.9 ± 15.2
pooled aqueous phases	8.6 ± 2.3	11.7 ± 8.5	335.6 ± 36.2
poly(A)$^+$RNA preparation	ND		4.7

Values shown represent the means of four different preparations assayed at each stage.

ND - not detected

3.3.2 In Vitro Translation of Poly(A)$^+$RNA

A series of poly(A)$^+$RNA extracts were made from the petioles of control (H_2O and Na_2SO_4) and estrogen-treated (E_1-S at concentrations of 10^{-4}M and 0.65 x 10^{-4}M and E_2-S at concentrations of 10^{-4}M and 0.33 x 10^{-4}M) primary leaf cutting of *P. vulgaris*.

After *in vitro* translation of the RNA, incorporation of ^{35}S methionine into the newly synthesised polypeptides was monitored. Generally, the poly(A)$^+$ samples had 40 to 70 times greater incorporation than background samples to which poly(A)$^+$RNA had not been added. Translation products were separated by SDS-PAGE and visualised using fluorography. The polypeptide products were separated alongside high and low molecular weight markers (see section 2.3.3.10), TMV RNA translation products (only in some cases) and a water blank which was

used to gauge background radiation in the wheat germ translation system. During the period of this research, at least three individual samples of each type of poly(A)$^+$RNA were translated *in vitro* several times and the polypeptide products carefully scrutinised.

A total of 17 gels were run and clear banding patterns were observed (see Plate 19). The standard exposure time for fluorographs was 24h, but in many cases this period was not of adequate length for the presence of the higher molecular weight proteins to be revealed. A longer exposure time of 48h, on the other hand, led to severe over-exposure of the lower molecular weight proteins. Despite these problems, careful comparison of polypeptide products revealed that there were no differences, either of a quantitative or qualitative nature, in the products arising from translation of poly(A)$^+$RNA from control or estrogen-treated plant material (see Plate 20).

A spectrum of proteins were produced which ranged from molecular weights of below 20,000 to above 66,000. The majority of these polypeptides had molecular weights less than 45,000, but between four and six major bands were visible between 45,000 and 66,000, and three major bands were visible above 66,000 (see Plates 19 and 20). A disadvantage of using the *in vitro* system derived from wheat germ is the production of lower molecular weight polypeptides: their origin may be due to processes such as premature termination, degradation of the RNA, hidden breaks in the isolated RNA or to proteolytic activity. The polypeptide products of translation of poly(A)$^+$RNA reflect the mRNA populations and thus it can be concluded that quantitative or qualitative differences in the mRNA populations of control and estrogen-treated tissues, at the level of resolution employed for separation of the translation products, do not exist.

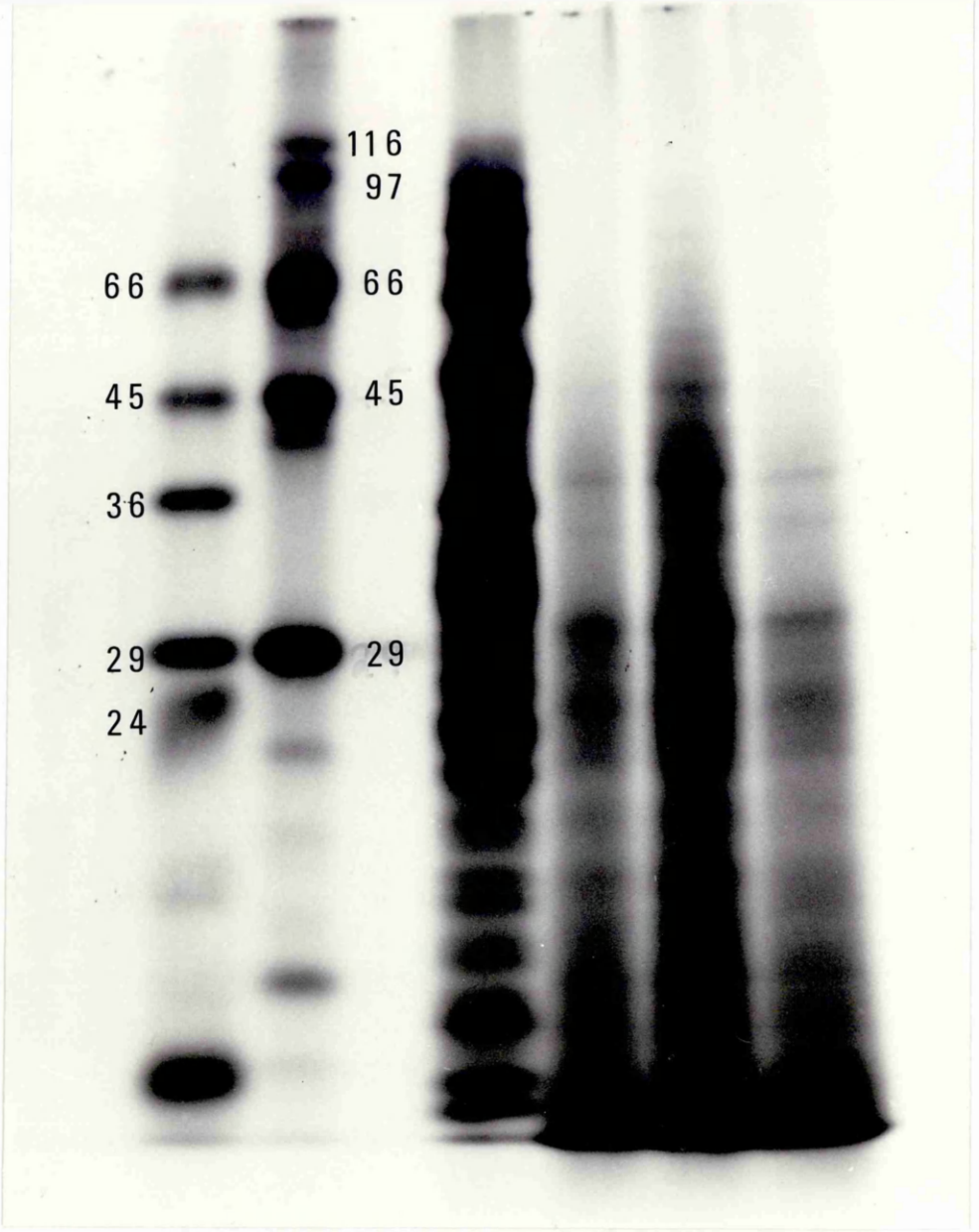


Plate 19 In vitro translation products of poly(A)$^+$ RNA extracted from TMV (track C) and from the petioles of control (H_2O - track D and Na_2SO_4 - track F) and estrone-sulphate treated (track E) primary leaf cuttings of P. vulgaris. Poly(A)$^+$ RNA was translated in a cell-free system derived from wheat germ and the products of translation were separated by SDS-PAGE and visualised by fluorography. The positions of molecular weight markers are shown in tracks A and B (molecular weight x10^{-3}).

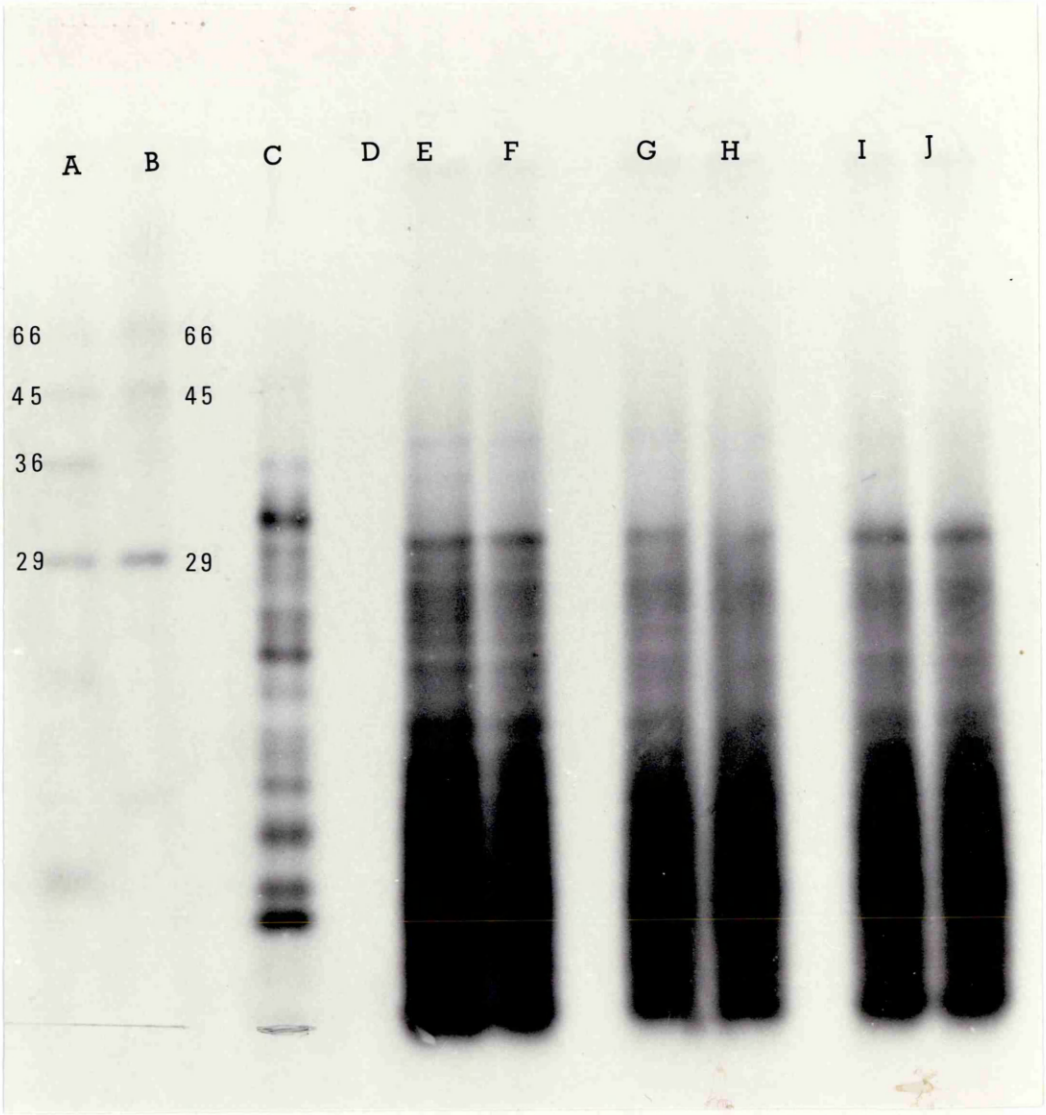


Plate 20 In vitro translation products of poly(A)$^+$ RNA extracted from TMV (track C) and from the petioles of control (H_2O - track E and Na_2SO_4 - track F), estrone-sulphate (tracks G and H) and estradiol-sulphate treated (tracks I and J) primary leaf cuttings of P. vulgaris. Poly(A)$^+$ RNA was translated in a cell-free system derived from wheat germ and background radiation in the system was monitored in track D: no poly(A)$^+$ RNA was added to the incubation mixture. The products of translation were separated by SDS-PAGE and visualised by fluorography. The positions of molecular weight markers are shown in tracks A and B (molecular weight x10^{-3}).

CHAPTER 4

DISCUSSION

Much is known about the biochemistry, physiology and mode of action of steroidal estrogens in animals, but although it now seems certain that plants also contain these hormones, little is known about their physiological role and even less about how they exert their effects. Before embarking on an investigation of the mode of action of steroidal estrogens in higher plants, it is essential that a definitive reproducible response is established as the basis of an experimental system. In this respect, following the observations of Hewitt and Hillman (1979b, 1980), the responses of primary leaf cuttings of Phaseolus vulgaris L. cv. Canadian Wonder to applied estrogens were selected for further study.

The ability of estrogen-sulphate conjugates (estrone-sulphate and estradiol-sulphate at concentrations of 10^{-4}M) to induce abnormal cell development whilst simultaneously reducing adventitious root formation was confirmed.

In the rooting experiments much variation in the number of roots produced by cuttings was noted, but despite this, even the results of the small-scale individual experiments revealed the inhibitory effects of the applied estrogen relative to distilled water controls. Additional controls of Na_2SO_4 (discussed later), Tris and N-methyl-D-glucamine (stabilising agents for E_1-S and E_2-S respectively) were also tested. Comparison of the rooting patterns of these control cuttings and distilled water controls indicated that for the most part

the observed inhibition of root formation was due to the steroid molecules themselves, although the presence of N-methyl-D-glucamine in solutions of E_2-S may have contributed to the more pronounced inhibition of rooting after treatment with this estrogen. A time-course study of the response showed that the inhibitory effects of estrogens were readily manifest by day six of the treatment period.

It is well known that indole-3-butyric acid enhances root formation in cuttings. The particular concentration required to elicit this effect in P. vulgaris primary leaf cuttings was found to be at levels greater than 10^{-8}M. Application of 10^{-4}M E_1-S together with effective concentrations of IBA(10^{-6}M) resulted in a retardation of the enhancement effect, further confirming the inhibitory influence of estrogen-sulphates on the process of root initiation and also confirming the findings of Grace (1940) that estrone offsets the effects of IBA. It would be interesting to carry out a complete interaction analysis of these two hormones, varying the concentration of each, following the recommendations of Drury (1969, 1970), although whether or not the results would assist in interpreting the effects of estrogens is somewhat debatable in the absence of a greater understanding of the mode of action of either estrogens or IBA at the molecular level.

In a similar approach to that of Hewitt and Hillman (1979b, 1980), the effects of 10^{-4}M estrone-sulphate and 10^{-4}M estradiol-sulphate and all the appropriate control treatments at the anatomical level were examined by analysis of a large number of transverse sections using light microscopy. In addition to examining sections from the petioles of excised primary leaves, sections from the basal regions of hypocotyl cuttings were also inspected. Zones of abnormal cell development were observed in sections from hypocotyls and petioles of cuttings which had been treated with E_1-S or E_2-S. The areas of

aberrant growth, which were detectable by day five of the treatment period (just prior to visible inhibition of rooting) were never extrinsic but were associated with the outer phloem, ie more or less the pericyclic region, and the inner cortex. The affected areas appeared callus-like or tumour-like and resembled tissues associated with Agrobacterium tumefaciens infections in tobacco (Van Lith Vroom et al, 1960). Estrogen effects on plant tumour growth have been reported before (discussed in the Introduction) but in the cases reported herein, although no tumour-like tissues were observed in sections from any of the control cuttings, these observations do not give a direct link between estrogen treatment and plant tumours. Nevertheless, it is possible to speculate that estrogen treatment and A. tumefaciens infections affect the same processes. An interesting line of research in the future would be an investigation of the possibility that the as yet unidentified genes on the Ti-plasmid modify the estrogen metabolism of the host plant.

Rooting in hypocotyl cuttings was not examined here, but it has been previously reported that estrogen treatment significantly inhibits rooting in hypocotyl cuttings of P. vulgaris (Hewitt and Hillman, 1979b, 1980), and in Pisum sativum L. cv. Meteor (Hewitt and Hillman, unpublished). In both these cases inhibition of rooting was not as pronounced as in primary leaf cuttings. The responding regions in both species contain pre-formed root initials and so it would seem that growth of existing root primordia is not affected by estrogen treatment. This suggests that the inhibitory influence of estrogen-sulphates is exerted at or before root initiation. Cells produced by active meristems such as those in growing adventitious roots are already committed. Those meristematic cells which give rise to either callus or to adventitious roots are derived from cells of the

hypocotyl or petiole which have been induced to become meristematic as a result of treatment with estrogen-sulphates or IBA. The key phase, therefore, would appear to be when parenchyma cells dedifferentiate and become redetermined as initials, and it is at this stage that the estrogen-sulphates most probably exerted their effects.

A possible and feasible explanation of the observed effects is that disruption of the dedifferentiation-redetermination processes or alternatively disruption of the first or early cell divisions occurred. Clearly, though, dedifferentiation took place in the presence of estrogen-sulphates, but the new initials did not function correctly. This was perhaps more evident in the primary leaf explants where areas outwith the vascular tissues were also affected. Under normal circumstances, parenchyma cells centrifugal to sclerenchymatous bundle caps undergo cell division, followed by cell expansion and then death to produce a gap allowing unhindered adventitious root growth towards the epidermis. In estrogen-sulphate treated cuttings, cell division continued to occur so that it seems more likely that the effects were elicited before cell division, during the dedifferentiation-redetermination phase.

Artifacts arising from the fixing, embedding and sectioning procedures must be considered when assessing the results of applications of estrogens at the anatomical level. Nevertheless, systematic analysis of large numbers of sections substantiates the observations of Hewitt and Hillman (1979b, 1980) that a steroidal-estrogen induced aberration of cell development takes place in P. vulgaris cuttings. Whether or not there are different types of aberrant development awaits more detailed histological study. It is not possible to state that all the aberrant zones are identical - they are not - but perhaps this is not unexpected in a complex of tissues.

The production of hyperplastic tissues in plants following applications of steroidal and synthetic estrogens is not a new discovery: abnormal cell division and mitotic disturbances (Faller, 1942; von Euler and Perje, 1945) and tumour-like tissue formation (Jakowska, 1948) have been reported as side effects of estrogen treatment. Cell division has been affected by estrogens, both positively (eg Orth, 1934; von Euler and Perje, 1945; Löve and Löve, 1945) and negatively (eg Buetow and Levedahl, 1958) and it has been suggested that plant cells not yet irreversibly determined are most susceptible to estrogen effects (Weyland et al, 1949). Additionally, atypical cleavages were found in estradiol-treated Fucus distichus embryos (Pollard, 1969), an effect also apparent in sea urchin embryos, where estradiol was thought to act at the mitotic spindle in a manner similar to colchicine (Agrell, 1954). Colchicine has been found to inhibit root formation in excised petioles of Phaseolus vulgaris by arresting cell division, but no abnormal tissues resulted (Oppenoorth, 1978) so that it is unlikely that the observations reported here are due to a colchicine-like effect of estrogens.

In the experiments described in this thesis, the method employed for applying the steroid hormones was that of wick feeding which involves standing cuttings in aqueous solutions. This constitutes a major problem in that to obtain aqueous solutions of estrone or estradiol at physiologically effective concentrations requires the presence of organic solvents at phytotoxic levels, and for this reason a large part of the work was carried out using water-soluble estrogen-sulphate conjugates. The rationale justifying the use of these conjugates rather than the highly insoluble unconjugated forms was previously detailed by Hewitt (1980). Na_2SO_4 was used as a control for the sulphate group and was found to be ineffective in modifying

adventitious root formation relative to distilled water controls and in no case was anomalous cell development observed in sections from Na_2SO_4 control cuttings. Although this would suggest that the effects of estrogen-sulphates are not attributable to the presence of the attached sulphate groups, the possibility cannot be fully discounted and in the future some attention must be paid to the existence and activity of sulphatase enzymes capable of removing the sulphate group from the conjugated compound. In addition, the degree of dissociation of the inorganic salt (Na_2SO_4) and the organic salt (estrogen-sulphate) were unknown and consequently Na_2SO_4 may not be deemed to be a proper control for the conjugated sulphate.

Radiolabelled estrogen-sulphates are not commercially available, and their custom synthesis is both complex and costly. Consequently, it was not possible to monitor their uptake and distribution by cuttings and so (4-^{14}C) estrone and (4-^{14}C) estradiol were used instead. From the results obtained it appears that in hypocotyl cuttings and in primary leaf cuttings estrone and estradiol behave physiologically in the same way. Strictly speaking, the results reported herein merely represent the uptake and distribution of radioactivity from solutions of labelled E_1 and E_2, but on the basis of previous work which demonstrated that the bulk of the label remained associated with the applied estrogen in P. vulgaris cuttings (Young et al, 1977), these results must be assumed to be an accurate indication of the uptake and distribution of the applied estrogen.

Although uptake of ^{14}C-E_1 and ^{14}C-E_2 by primary leaf cuttings apparently was passive in the aqueous feeding solution, there was some evidence that uptake of ^{14}C-E_1 by hypocotyl cuttings was selective. This selectivity may be a manifestation of the incorporation of the lipophilic steroid molecules into membranes or their adsorption onto

cuticular or non-polar sites. Distribution of radioactivity taken up from solutions of ^{14}C-E_1 or ^{14}C-E_2 by hypocotyl and primary leaf cuttings was very restricted: in general more than half the label taken up remained in the basal 1cm segment of the cutting bearing the cut surface and as much as 90% remained below the cotyledonary node of hypocotyl cuttings and in the petioles of excised primary leaves. Although the label content of cuttings increased during the treatment periods, the relative distribution of radioactivity remained, for the most part, unchanged. The method of wick feeding which was employed for application of the labelled steroids assumes that the applied compound is taken up and distributed during transpiration. Transpiration undoubtedly took place as indicated indirectly by the substantial water uptake, and yet very low levels of radioactivity were present in the leaves of hypocotyl cuttings (less than 1%) and less than 10% of the total label taken up was present in the lamina of primary leaf explants. This is surprising since the lamina are rapidly expanding areas and are the main sites of transpiration. In primary leaf cuttings by day seven of the treatment period small numbers of roots were present and these roots contained around 15 to 20% of the total radioactivity present in the cutting, mostly at the expense of the basal 1cm segment of the petiole. These results highlight the poor transport of steroidal estrogens in plant material, and in view of this it is not surprising that the areas of aberrant growth induced by estrogen treatment are confined to the zones of application.

Studies concerning the mechanisms underlying the hormonal control of plant growth and morphological and biochemical differentiation have tended to concentrate on the initial interaction of the hormone with cellular components and the possibility of selective binding, their metabolism patterns and their effects on the anatomy, physiology and

biochemistry of treated plants. More recently attention has focused on the possibility that hormones exert their effects by bringing about alterations in gene expression. Each somatic cell in a multicellular organism has the same genetic potential yet these cells can differ widely due to the expression of different parts of the same genetic complement. The many and wide-ranging effects of phytohormones in general and more particularly the differences between the organised structure of adventitious roots and the callus-like tissues observed here could be explained in such terms.

Modification of gene expression must involve changes in the protein and nucleic acid metabolism. Investigations at this level have led to the accumulation of a considerable amount of evidence which indicates that selective changes in the levels of specific mRNA sequences can be induced by all the classical types of plant growth regulators, ie GA_s and ABA (eg Higgins et al, 1982), cytokinins (eg Tobin and Turkaly, 1982) and ethylene (eg Christofferson and Laties, 1982). These findings together with the knowledge that estradiol in the rat uterus brings about qualitative and quantitative changes in the mRNA populations (Aziz et al, 1979) were the driving force behind the approach adopted here to investigate the mode of action of steroidal estrogens in higher plants.

Samples of mRNA from both treated (E_1-S and E_2-S) and control (H_2O and Na_2SO_4) cuttings were translated in vitro using a cell-free system derived from wheat germ. The products of translation were separated using SDS-PAGE and visualised using fluorography. The resulting fluorographs revealed a complex pattern of polypeptides reflecting a complex population of mRNA sequences but obvious differences, either of a qualitative or quantitative nature, were not observed. This is perhaps not surprising since the zones of anomalous growth were small

in volume so that undoubtedly the possibility of detecting small but biologically significant changes in the mRNA population was decreased. Additionally, the resolving capacity of the SDS-PAGE system employed was very limited so that future study must involve a more thorough analysis of the translation products using more sophisticated techniques of separation which have greater resolution such as 2-D gel electrophoresis using isoelectric focusing in the first dimension. If changes in the mRNA populations of responding tissue induced by treatment with estrogens could be detected, then the underlying mechanisms can be characterised. Specific cDNA clones of the hormone-regulated mRNA would be required to distinguish between the possibilities of the steroids eliciting their responses by altering the processing or translational activity of specific mRNAs or by causing alterations at the level of transcription.

Although the modification of differentiation by steroidal estrogens observed here is potentially a very useful research device, it is nevertheless very complex. Perhaps a more productive approach to the advancement of the understanding of the role of steroidal estrogens in plants would be the investigation of a simple biochemical estrogen induced reaction such as the activity of an enzyme in a uniform tissue. Unfortunately, no such biochemical reaction has yet been characterised and thus a careful study of the range of effects of estrogens in plants, extending previous work, would be beneficial. This may, however, be completely the wrong pathway of investigation to follow and perhaps the phenomenon of hormonal heterophylly, whereby compounds present in plants have an important regulatory role in animals (Burdette, 1974), should be considered as a possible explanation for the presence of mammalian sex hormones in plants. Nature, like God, moves in a mysterious way her wonderous works to perform.

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