Steroidal Estrogens in *Phaseolus vulgaris*

by

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Abbreviations

BSA - bis-trimethylsilyl acetamide

BuOH - butan-1-ol

ca - circa - approximately

Ci - Curie

cpm - counts per minute

C_6H_6 - benzene

$CHCl_3$ - chloroform

CH_3COOH - acetic acid

dpm - disintegrations per minute

E_1 - estrone

E_2 - estradiol

E_1SO_4 - estrone sulphate

Et_2O - diethyl ether

EtOAc - ethyl acetate

et al - et alia

FSD - full scale deflection

GC-MS - combined gas chromatography-mass spectrometry

GLC - gas liquid chromatography

H_2O - water

LDP - long day plant

MVA - mevalonic acid

MeOH - methanol

N - normal

NADH - reduced nicotinamide adenine dinucleotide

NADPH - - do - - do - phosphate

Pr-2-OH - propan-2-ol

PBS - phosphate buffer solution

RIA - radioimmunoassay

R_f - retention factor

SDP - short day plants

S.E. - standard error of the mean

TLC - thin-layer chromatography

TMSi - trimethylsilyl

T_r - retention time

V_r - retention volume

As far as possible S.I. units are used throughout this thesis.

The following trivial names of steroids are employed throughout the thesis for convenience.

Trivial Name	IUPAC Approved
Androstenedione	4-androstene-3,17-dione
Androsterone	3α-hydroxy-5α-androstan-17-one
Cholesterol	5-cholesten-3β-ol
Cycloartenol	4,4,14α-trimethyl-9,19-cyclo-5α,9β-cholest-24-en-3β-ol
Dehydroepiandrosterone	3β-hydroxy-5-androsten-17-one
Estradiol	1,3,5(10)-estratriene-3,17β-diol
Estrone	3-hydroxy-1,3,5(10)-estratrien-17-one
Lanosterol	8,24-lanostadien-3β-ol
	4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol
Pregnenolone	3β-hydroxy-5-pregnen-20-one
17α-hydroxypregnenolone	3β,17α-dihydroxy-5-pregnen-20-one
Progesterone	4-pregnene-3,20-dione
17α-hydroxyprogesterone	17α-hydroxy-4-pregnene-3,20-dione
Testosterone	17β-hydroxy-4-androsten-3-one

SUMMARY

This thesis presents an investigation into the presence of steroidal estrogens in Phaseolus vulgaris var. Canadian Wonder. The approach was threefold. Firstly purification methods were appraised, and it was found that partitioning ether with 1N NaOH removed ca 60% of [^{14}C]-estradiol compared with only ca 40% with 2N and 30% with 4N NaOH. Partitioning 1N NaOH against a benzene:butanol mixture was also only ca 40% efficient. Suitable preparative chromatographic systems - thin-layer chromatography and elution on a hydroxyalkoxypropyl derivative of Sephadex - were developed.

Secondly, employing the most efficient of these methods, evidence of estradiol in extracts of seeds, vegetative and flowering plants using a modified radioimmunoassay technique and combined gas chromatography-mass spectrometry was obtained. The GC-MS analyses involved one of two methods: either continuous monitoring at the collector of the ion abundance at m/e 416 (the molecular ion of the bis-trimethylsilyl derivative of estradiol) as generated in the ion source from the GLC effluent of two different columns, allowing the mass spectrometer to be used as a semi-specific detector for estradiol; or three ions (m/e 416, 342, 285) were continuously monitored in a process commonly referred to as mass fragmentography - a sensitive and selective detector system. (M/e 342 is the molecular ion of estrone trimethylsilyl ether, and m/e 285 is the base ion common to both E_1 and E_2).

In the third approach an investigation was carried out to ascertain whether the dwarf French bean could synthesise estrogens. Eight-day old seedlings were sectioned at the hypocotyl, under water, and stood in aqueous solutions of various radioactive substances - [^{14}C]-estrone, estradiol and mevalonic acid as well as [^{3}H]-estrone sulphate. Chromatographic mobilities of metabolites were compared with authentic compounds

using TLC on three solvent systems, column chromatography and radio-gas-liquid chromatography of the trimethylsilyl derivative. Samples believed to contain estradiol were diluted with non-radioactive carrier estradiol and recrystallised to constant specific activity.

Using these techniques in combination, radioactivity was incorporated into estradiol from estrone, estrone sulphate and mevalonic acid. There is also some evidence from thin-layer and column chromatography that estradiol is converted to estrone.

INTRODUCTION

The cells of higher plants and animals share a common metabolic characteristic in containing a number of closely related compounds typically referred to as the 'steroids'. These substances, so named from the Greek stereos meaning solid, belong to a class of unsaponifiable lipids called isopentanes, isoprenes, isoprenoids, terpenes or terpenoids. Steroids are compounds based on the cyclopentanoperhydrophenanthrene skeleton. Stable chemically, this nucleus is relatively unaffected in shape by substituents. The oft-attributed biochemical stability of the nucleus is based on experiments on animals fed with [^{14}C] steroids where no significant levels of $^{14}CO_2$ are obtained. Nes (1971) has suggested that some steroid molecules present in organisms today may be as old as the metabolic pathway itself (10^9 years). The steroid nucleus however does not seem to accumulate in the soil so it is likely that it is degraded to simpler substances by micro-organisms.

This hydrocarbon skeleton constitutes the basis of a vast collection of compounds which include sterols; vitamins D and analogues; sapogenins and alkaloids; bile acids and alcohols; cardiac glycosides; the pregnane derivatives including progesterone and the corticosteroids; the androgens and the estrogens.

The physiology and biochemistry of steroids, particularly in animal tissues, have been studied extensively during the past fifty years and the voluminous literature which has accumulated in this time has been authoritatively reviewed by Fieser and Fieser (1959), Grant (1969), Heftmann (1963, 1969, 1971 a and b, 1974, 1975 a and b), Goodwin (1971), Stohs and Rosenberg (1975) and Grunwald (1975).

With the advent of sensitive chemical and physiological assays and a commercial as well as social requirement for birth controlling substances, especial interest has been directed towards the steroidal estrogens.

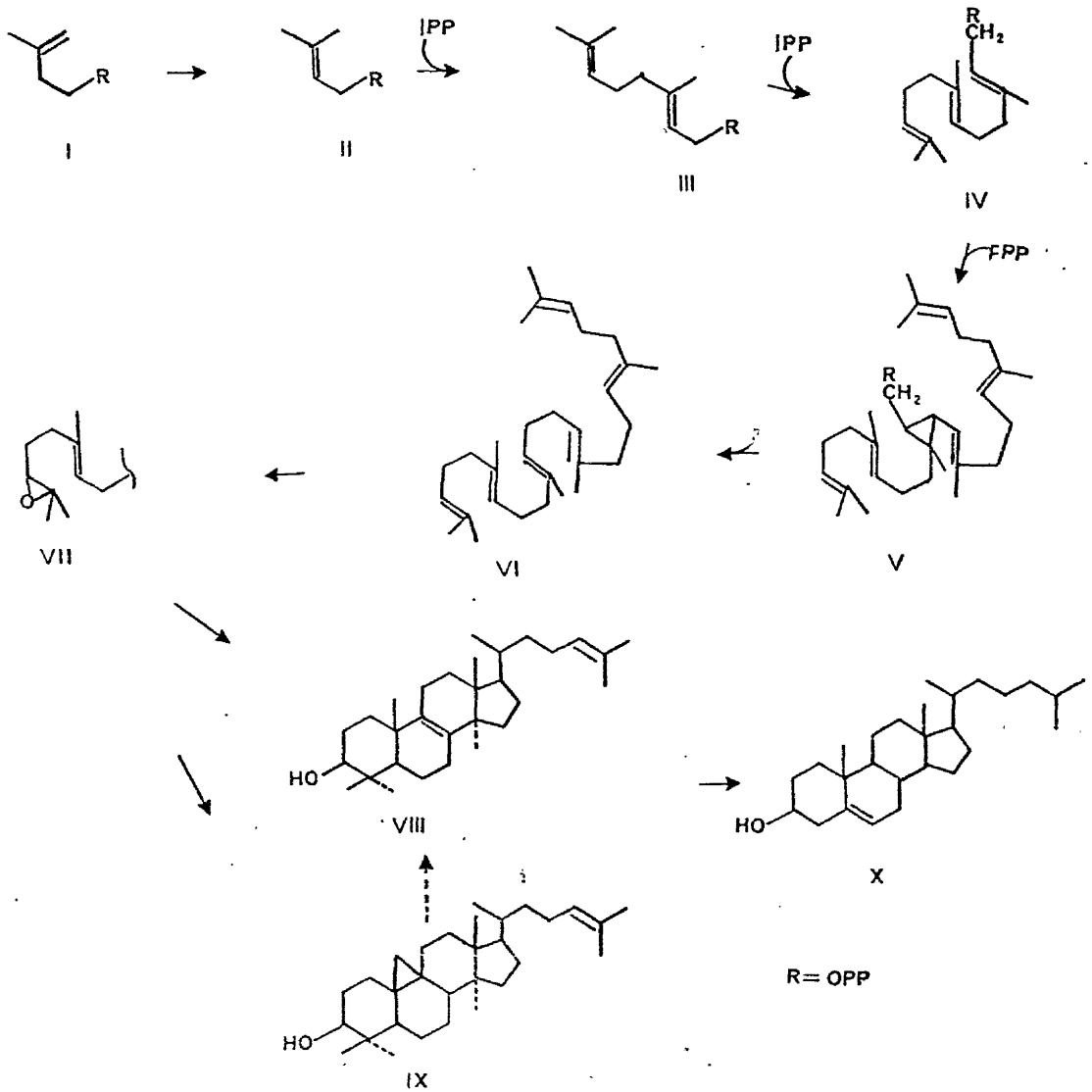
Whereas a considerable amount of effort has been expended on experimentation on animals, the plant kingdom has been largely neglected. Indeed it is frequently assumed that the autotrophic plant mirrors the biochemistry, if not the physiology, of the heterotroph. This thesis concerns an investigation on the presence and biosynthesis of steroidal estrogens in the higher plant Phaseolus vulgaris L. var. Canadian Wonder, and before introducing the current state of knowledge with regard to plants, it is desirable to review briefly the physiology and biochemistry of steroidal estrogens in animals. In view of their close metabolic relationship with the androgens and the pregnane derivatives, these substances are also considered.

Steroid sex hormones in animals

Although the discovery of steroids was first reported in 1812 with the isolation of cholesterol (X) from animal lipids, attributed to Chevreul (cited by Fieser and Fieser, 1959), the most dramatic expansions in steroid chemistry arose from the discovery of the sex hormones during 1929-1935.

The sex hormones are flat molecules with oxygen functions at either end of the nucleus. A few synthetic steroids without the oxygen moiety in ring A have biological activity on application to animals but these are thought to undergo hydroxylation at C_3 *in vivo* before reaching their sites of activity (see Grant, 1969). Stereochemistry is also important as epimers of estradiol and testosterone with their 17-HO group in the α-orientation (normally it is β-orientated) exhibit little biological activity (see Grant, 1969).

The initial stages of steroid biosynthesis involve the anaerobic elaboration of the C_6 MVA into the C_{30} hydrocarbon squalene. MVA pyrophosphate is converted to C_5 isopentenyl pyrophosphate (I) which is subsequently isomerised via isopentenyl pyrophosphate isomerase to dimethyl-


R
I
R
II
IPP
III
IPP
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CH2
IV
FPP
R
CH2
V
VI
VII
HO
VIII
HO
IX
HO
X
R= OPP

Figure 1: Biosynthetic pathway from MVA to cholesterol - a summary

I - Isopentenylpyrophosphate (IPP)

II - Dimethylallyl pyrophosphate

III - Geranyl pyrophosphate

IV - Farnesyl pyrophosphate

V - Pre-squalene pyrophosphate

VI - Squalene

VII - Squalene-2,3-oxide

VIII - Lanosterol

IX - Cycloartenol

X - Cholesterol

allyl pyrophosphate (II). This then condenses with further molecules of isopentyl pyrophosphate in the presence of farnesyl synthetase to give first geranyl pyrophosphate (III) and then farnesyl pyrophosphate (IV). Finally two molecules of farnesyl pyrophosphate undergo a head-to-head condensation to give the C_{30} squalene (VI) (see Richards and Hendrickson, 1964). This reaction proceeds under the influence of a microsomal enzyme and NADH and is now known to proceed via the intermediate presqualene pyrophosphate (V), (see Goad and Goodwin, 1972).

In the presence of oxygen and NADPH squalene is oxidised to squalene-2,3-oxide (VII) which then undergoes proton-initiated cyclisation to lanosterol (VIII).

The methyl functions at C-14 (two) and C-4 are removed and the double bond at C-8(9) transfers to C-5(6) and the double bond at C-24(25) is saturated by the addition of H_2 to give the C_{27} precursor of the sex hormones, cholesterol (X) (see Fieser and Fieser, 1959). The sequence from MVA to cholesterol is summarised in Figure 1. Structural modifications to convert cholesterol to the sex hormones include the successive degradation of the side-chain and appropriate oxidation of both the side chain and the basic skeleton (see Heftmann, 1971a).

The first stage in this degradation is the formation of the C-21 pregnanes. This is thought to be achieved by the oxidation of cholesterol to 20α-hydroxycholesterol and then to 20,22-dihydroxycholesterol which leads to the formation of pregnenolone (XI). Conversion of pregnenolone to progesterone (VIII) is effected by a 3β-hydroxy steroid dehydrogenase system which is found in the microsomal fraction of the adrenal glands and with the oxidation of the hydroxyl group at C-3 the double bond at C-5(6) transfers coincidentally to C-4(5). The enzyme responsible for this oxidation acts on a number of C_{19} and C_{21} steroids but unlike most enzymatic dehydrogenations is irreversible (see Heftmann, 1971a).

Figure 2: Biosynthetic sequence from pregnenolone to testosterone

XI - Pregnenolone

XII - 17α-hydroxypregnenolone

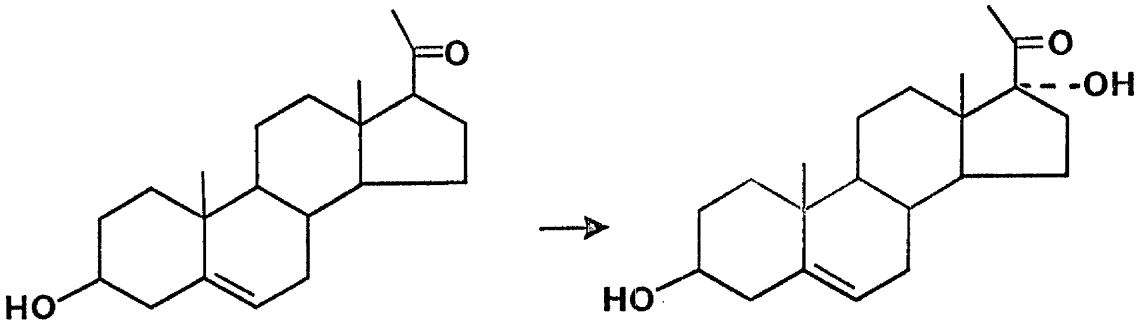
XIII - Progesterone

XIV - 17α-hydroxyprogesterone

XV - Dehydroepiandrosterone

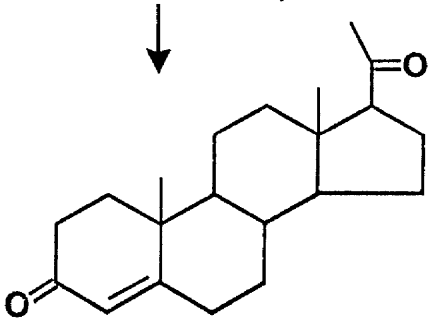
XVI - 4-Androstene-3,17-dione

XVII - Testosterone



XI

XII



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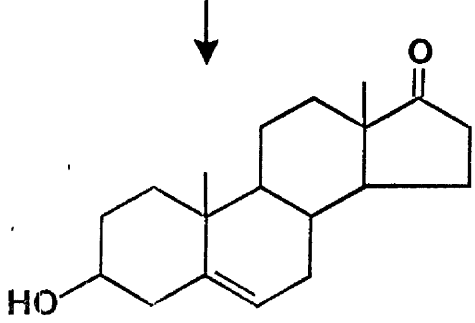

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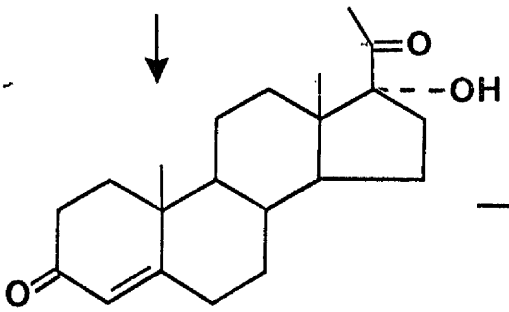

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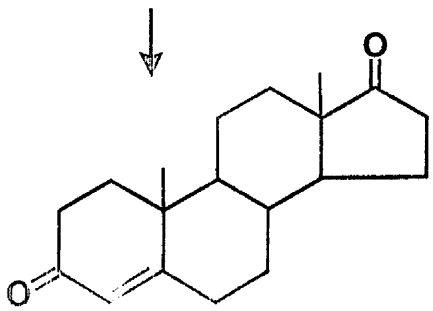
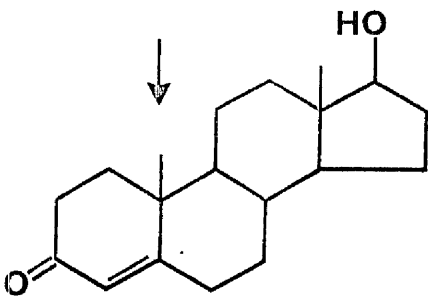


XVI



XVII

b

Progesterone is very rapidly metabolised in both animal and plant tissues and only very small amounts per unit weight can be detected at any one time.

Two methods by which the C_{19} androgens can be synthesised in vivo have been established. In the first, which takes place in the gonadal tissue, progesterone is oxidised to 17α-hydroxy-progesterone (XIV). The C-20 and 21 are lost as acetic acid leaving androstenedione (XVI), (Figure 2). The second pathway which occurs in the adrenal glands involves hydroxylation at C-17 in pregnenolone to 17α-hydroxypregnenolone (XII) which on the loss of the acetate group leaves dehydroepiandrosterone (XV). Oxidation of the hydroxyl group at C-3 by the enzyme which catalyses the conversion of pregnenolone to progesterone results in the formation of androstenedione as in the gonads. Reduction of the 17-carbonyl group gives rise to testosterone (XVII), (Figure 2).

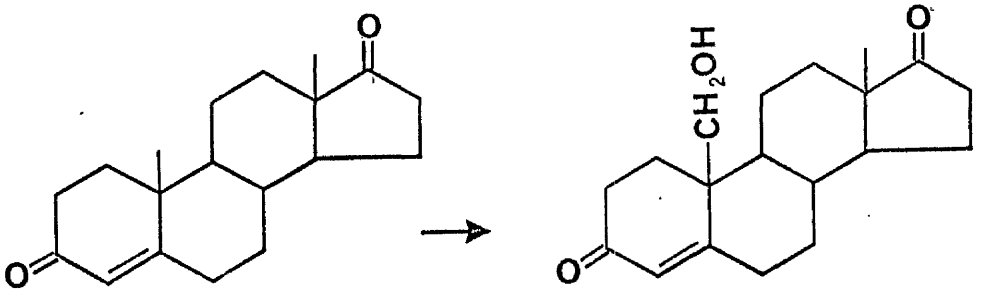
The estrogens represent the final stage in the degradative removal of the fragments from the thirty-carbon precursor lanosterol. They are derived from the androgens such as androstenedione by the removal of the angular methyl group at C-19 which occurs with the aromatisation of ring A. The known steps in the biosynthesis of estrone by the microsomal fraction of placental tissue are outlined in Figure 3. In most animal species estrone (XVIII) and estradiol (XIX) are interconvertible in the presence of transhydrogenase enzymes and NADH but normally the equilibrium is displaced in favour of estrone. The pathway from cholesterol to estradiol is summarised in Figure 4.

Physiological role in animals

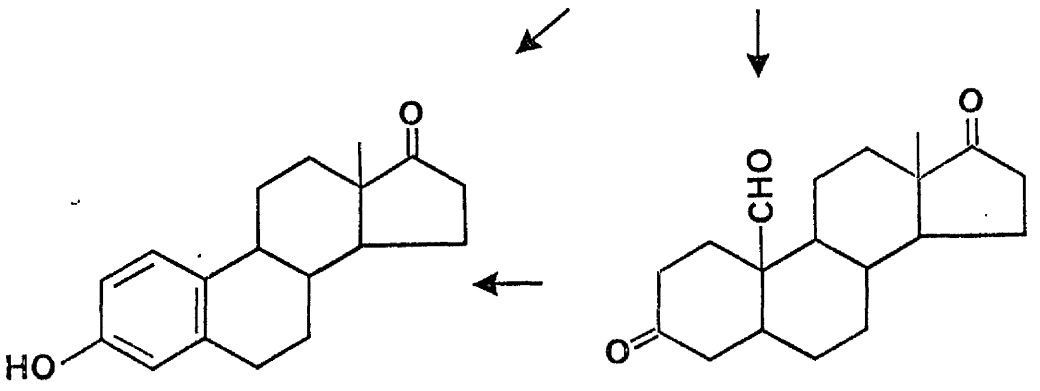
In the mammalian ovary after ripening and rupture of the follicle, there is formed a tissue which, due to the abundance of carotenoid pigment, is called the corpus luteum (see Fieser and Fieser, 1959). As early as 1932 Allen secured crude, physiologically active extracts from this tissue.

Figure 3: Presumptive pathway from the androgens to the estrogens

XVIII - Estrone



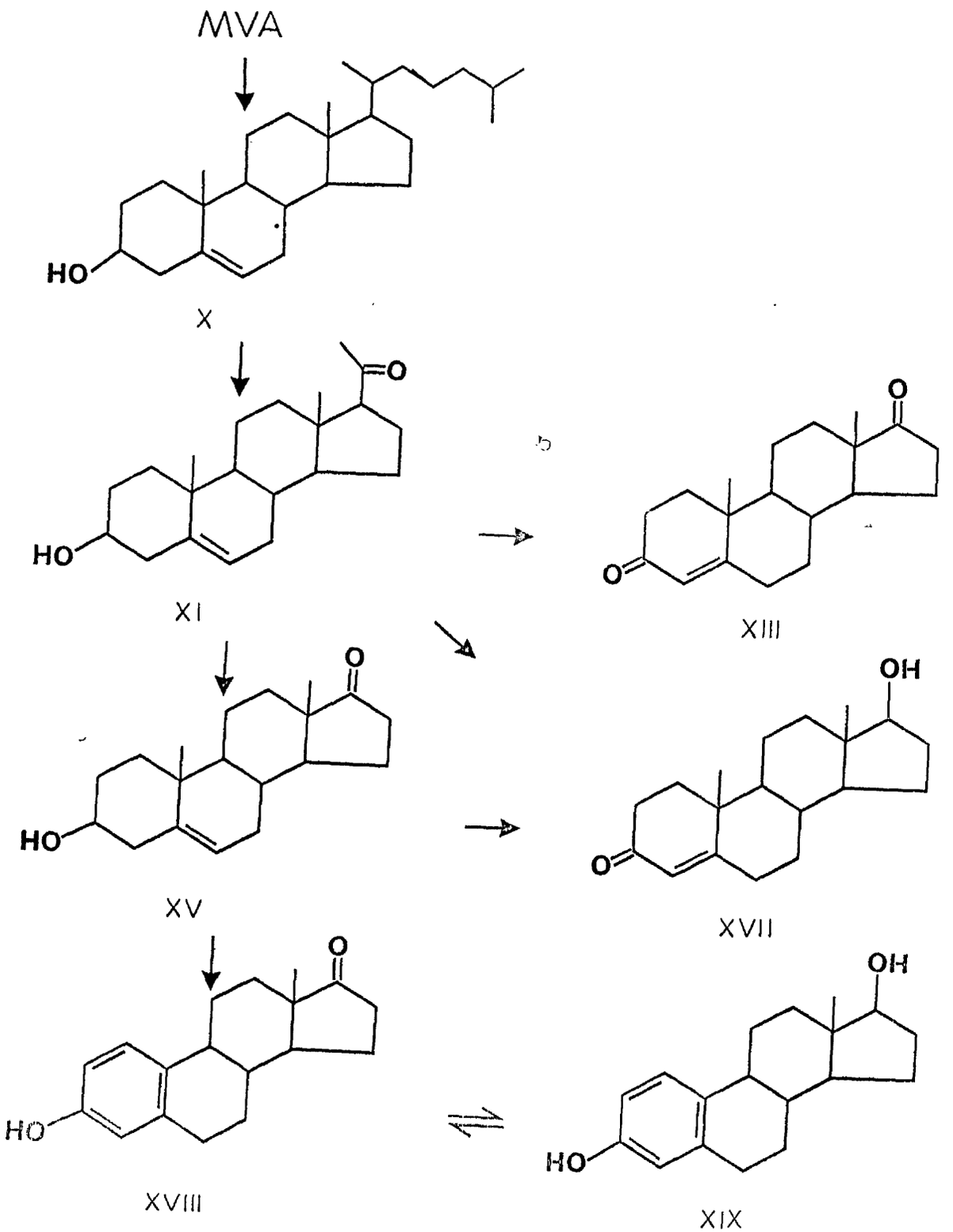
XVI



XVIII

Figure 4: Summary of biosynthetic pathway from MVA to estradiol

XIX - Estradiol


MVA
HO
X
O
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XI
O
O
XIII
O
HO
XV
OH
O
XVII
O
HO
XVIII
OH
HO
XIX

It has since been ascertained that the main function of the corpus luteum is the production of progesterone. This compound synergistically with the estrogens, prepares the uterus for the implantation and maintenance of the fertilised ovum. If no conception occurs the progesterone-induced changes regress and part of the endometrium is sloughed off during menstruation (see Heftmann, 1971a). On the other hand, if fertilisation does occur the corpus luteum does not regress and performs the following functions: 1. suppression of ovulation; 2. maintenance of uterine environment conducive to the development of the embryo; 3. inhibition of uterine motility; 4. induction of mammary gland development.

The activity of progesterone is not confined to the regulation of sexual processes. It is thought that by antagonising the effect of aldosterone on the renal tubules, progesterone leads to an increase in sodium excretion. In large doses, however, it causes sodium retention, presumably due to its conversion to deoxycortisone. Urea and total nitrogen excretion is also increased by stimulating protein catabolism (see Heftmann, 1971a). It has also been reported that progesterone may affect the central nervous system (see Grant, 1969).

The androgens stimulate the development of the male reproductive organs in utero and secondary sexual characteristics. In many species they have an organising action on neural tissues. The effects on sex morphology function and behaviour if administered before birth are usually permanent but if administered during adulthood the sex hormones stimulate certain functions only temporarily.

Androgenic activity is not confined to male reproductive processes. They induce ovulation in amphibians and stimulate the growth of the oviduct in birds and of the ovary of rats. Androgens also stimulate the growth of the female prostate and the development of the mammary glands. Their effects on non-sexual tissue are numerous, for example on increasing both

red blood cell count and the haemoglobin content of the blood. Lack of androgens due to castration leads to a decrease in the size of the kidneys and heart, a general decrease in skeletal muscle and an accumulation of subcutaneous fat.

On a more fundamental level enzyme induction by androgens is readily demonstrated in bacteria e.g. Pseudomonas testosteroni (see Heftmann, 1971a). They promote nitrogen retention by increasing protein synthesis and by decreasing the rate of amino acid catabolism. The anabolic effect of androgens on muscular development is referred to as myotropic and is not necessarily correlated with their virilising effect. The plasma concentration of testosterone may be the determining factor in the masculinisation of the male and virilism of the female. Other C_{19} androgens may be active only to the extent of their conversion to testosterone.

The other group of sex hormones, the estrogens, are almost continuously produced by the human ovaries. Although the human testes produce only small quantities of estrogens the richest source of estrogens yet found is the testes of the horse. A stallion excretes over 17 mg l^{-1} compared with only 10 mg l^{-1} in the pregnant mare (see Fieser and Fieser, 1959). However, in both men and women the concentration is usually less than 1 µg 100 ml^{-1} plasma as about half to two thirds of the circulating estrogens in the plasma are bound to proteins.

Estrogens stimulate the growth and development of the female reproductive organs and secondary sexual characteristics. During pregnancy the estrogens act with progesterone to maintain gestation and facilitate parturition. They also have some effect on salt and water retention and may raise the blood pressure (see Heftmann, 1971a).

Animal sex hormones in plants

Although the presence of estrogenically active substances had been reported in plants as early as 1926 (see Loewe and Spohr, 1926, and Dorhn,

1926, cited by Bonner, 1937), it was not until 1933 that Butenandt and Jacobi isolated 18 mg of estrone from 50 kg pressed palm kernels (see Fieser and Fieser, 1959). Skarzynski, in the same year, obtained 7 mg of a pure crystallizate of estriol from 65 kg of willow flowers (cited by Fieser and Fieser, 1959).

The quantities of hormone obtained by Butenandt compare favourably with those isolated from animal sources. For example Westerfield (cited by Fieser and Fieser, 1959) on the processing of four tons of sow ovaries, only produced 25 mg total estrogens (half of which was thought to be estradiol).

Jacobsohn (1965) repeated Butenandt's work but could not confirm the presence of estrone in palm kernels. His minimum detectable limit was 10 $\mu g\ Kg^{-1}$ and at this level he could detect no estrone and this certainly would imply that Butenandt's initial identification was incorrect. The following year, however, Bennett et al. (1966) isolated estrone from date palm pollen employing TLC and infra-red spectroscopic identification techniques. Heftmann et al. (1966) also secured the equivalent of 17 $mg\ kg^{-1}$ of estrone from pomegranate seeds. The quantitation was performed using a colour reaction and it is highly probable that this method is inaccurate for plant extracts as part of the extraction procedure produces artefacts which interfere with colorimetry and fluorimetry and may therefore influence the quantification. The occurrence of estrone in pomegranate was confirmed by Dean et al. (1971) employing the extremely sensitive technique of radioimmunoassay. However, they estimated that samples contained only 4 $\mu g\ Kg^{-1}$ and this infers that Heftmann's original estimate may have been high.

Estrone has been isolated from a number of other plant sources. Kvanta (cited in Heftmann, 1975a) identified estrone in a mixed pollen sample. Amin et al. (1969) investigated two plants for estrogenic

activity; in the first of these, Clossostemon bruguieri, the root is often used as a hot beverage after childbirth and the second, the pollen grains from the Egyptian date palm is used to promote fertility in women. The estrogenic substances were isolated as the p,p^{1} - nitrophenylbenzoate derivative. Gawienowski and Gibbs (1969) demonstrated the presence of estrone in seeds of two apple varieties. Identification was by TLC and GLC and forming the acetate derivative. From the peak areas on the gas chromatographic trace they estimated the variety Red Delicious contained 13 μg 100 g^{-1} and MacIntosh 10.1 μg 100 g^{-1}. Kopcewicz (1971) investigated P. vulgaris var. Saxa for the presence of estrogens at various stages of development. Although he found no evidence for estrogens in seeds or seedlings or in the period of rapid growth up to the formation of the second node, it was alleged that estrogens appear as the flower buds emerge reaching two maxima in the period of flower bud development and of pod formation. His results demonstrated that comparatively the greatest quantity of estrogen-like substances were in the leaves, although their presence in roots and stems was also established. The same author has associated increased estrogen levels with flower induction in both short and long day plants (Kopcewicz, 1972 a and b). In the SDP Perilla ocimoides and Chenopodium rubrum the estrogens were first detected at the period of inflorescence initiation and reached a maximum at flower development. Two "estrogen-like" substances were extracted from both P. ocimoides and C. rubrum. In the LDP Hyoscyamus niger and Salvia splendens the "estrogen-like" substances appear at the time of flower development (Kopcewicz, 1972a). Three substances were obtained from both of these plants and, although no two were similar it was suggested one substance in H. niger may be E_1 (Kopcewicz, 1972b).

The methods of identification employed by Kopcewicz were TLC and the Kober colour reaction. This method although satisfactory for animals

suffers from interference from artefacts of acid hydrolysis.

There is one report of the isolation of estradiol from plants. Awad (1974) identified by infra-red spectroscopy both estrone and estradiol from peach.

Of the other sex hormones, progesterone has been detected only in Holarrhena floribunda leaves (cited in Heftmann, 1974) and apple seeds (Gawienowski and Gibbs, 1968). It is likely, however, that as so many organisms have the ability to produce and utilise progesterone in the biosynthesis of other steroids it is reasonable to assume, as Heftmann (1975) has, that it is very widely distributed in the plant kingdom (see later).

Androstenedione, testosterone and epitestosterone have been characterised in the pollen of Pinus sylvestris (Saden-Krehule, 1971).

Although there is therefore evidence for the existence of sex hormones in plants the majority of the research carried out has been on their biosynthesis and metabolic fate both in vivo and in vitro.

Biosynthesis and metabolism in plants

The biosynthesis of squalene in plant tissues has been shown to proceed by a route very similar to that operative in animals (see Goad and Goodwin, 1972). The enzyme MVA kinase, has been isolated from a number of plants including Phaseolus vulgaris (Rogers et al., 1966 and Goad and Goodwin, 1972). Most of these studies were based on the incorporation of labelled mevalonic acid into plant tissues.

The incorporation of [^{14}C] into MVA-5-phosphate, MVA-pyrophosphate, isopentenyl pyrophosphate, dimethyl allyl pyrophosphate and, eventually, geraniol as well as farnesol was shown by Pollard et al. (1966) using a cell-free extract from Pisum sativum. Labelled geraniol has been incorporated into geranyl pyrophosphate and squalene in a homogenate of germinating peas (Van Aller and Nes, 1968).

MVA incorporation into squalene in flowering plants was first demonstrated in germinating pea seeds (Capstack et al., 1962) and has since been shown in other plants, e.g. carrot and tomato (Beeler et al., 1963), tobacco tissue culture (see Goad and Goodwin, 1972) and recently in Pinus pinea seeds (McKean and Nes, 1977). The enzyme responsible for the conversion of MVA into farnesyl pyrophosphate were in the soluble fraction, while squalene synthetase was microsomal.

The conversion of [^{14}C]-acetate into squalene-2,3-oxide has been described for tobacco tissue cultures (see Goad and Goodwin, 1972) and when phytosterol biosynthesis is inhibited in Nicotiana tabacum squalene-2,3-oxide accumulates (Reid, 1968). The latex of Euphorbia cyparissias treated with [^{14}C]-acetate has also been shown to incorporate label into squalene-2,3-oxide (Ponsinet and Ourisson, 1968). Although lanosterol has been isolated from yeasts and fungi (see Goad and Goodwin, 1972) and higher plants (Bennett and Heftmann, 1965) the intermediate from squalene to cholesterol is now thought to be cycloartenol (IX). It has also been established in a number of cases that the cycloartenol is derived from the direct cyclisation of squalene-2,3-oxide and not by any subsequent modification of lanosterol (see Goad and Goodwin, 1972).

Radioactively labelled MVA has been incorporated into the sterol fractions of a number of plant species including Pinus pinea (see Goad and Goodwin, 1972) Larix decidua (Goad et al., 1969), Salvia splendens (Nicholas, 1962) Pelargonium hortorum (Attalah et al., 1975). Bennett and Heftmann (1965a) have demonstrated the in vivo conversion of squalene to sitosterol in Pharbitis nil seedlings and geraniol has been incorporated into sitosterol in pea seeds (Baisted, 1967). As well as there being considerable evidence that squalene biosynthesis, cyclisation and conversion to sterols occurs via a similar pathway to that in animals there is also a body of literature concerned with the degradation of

sterols to the steroid sex-hormones and their analogues (see Heftmann, 1969, 1974, 1975 and Stohs, 1975). [^{14}C]-pregnenolone was isolated from Haplopappus heterophyllus leaves which had been fed with [^{14}C]-cholesterol (Bennett and Heftmann, 1966) inferring that the enzymes capable of removing the side-chain are present in higher plants. This has been substantiated by Pilgrim (1972) who demonstrated that the side-chain of cholesterol-26-^{14}C was removed *in vivo* by Digitalis lanata and by Gawienowski and Gibbs (1969) who secured [^{14}-C]-pregnenolone from [^{14}C]-cholesterol in Punica granatum. Sitosterol, which is different from cholesterol in having an ethyl group at C-24, has been metabolised to progesterone *in vivo* by D. lanata (Bennett *et al.*, 1969). The progesterone recovered accounted for 0.043% of the label added and over 10% of that recovered. This tallies up well with the percentage incorporation obtained by Bennett and Heftmann (1966) and Gawienowski and Gibbs (1969) who found 0.041% and 0.053% conversion of sterol to pregnenolone. The conversion of pregnenolone to progesterone appears to be widespread. It has been observed not only *in vivo* in Holarrhena floribunda (Bennett and Heftmann, 1965b) but also in D. lanata (Caspi and Lewis, 1967 and Sauer *et al.*, 1967) and *in vitro* in cultures of D. purpurea, D. lanata and Nicotiana tabacum (Graves and Smith, 1967) in leaf homogenates of D. purpurea and Cheiranthus cheiri (Stohs and El-Olemy, 1972). The reverse reaction of progesterone to pregnenolone was observed in D. lanata (Bennett *et al.*, 1968). Research by Stohs and El-Olemy (1971) indicated that 20α-hydroxycholesterol may be an intermediate in the transformation of cholesterol to pregnenolone. However, there appears to be some doubt about this because, although they succeeded with this side-chain cleavage in leaf homogenates of Cheiranthus cheiri and Nerium oleander, they were unsuccessful with D. purpurea and Strophanthus kombe. Pilgrim (1972) also found that cultures of D. purpurea would not remove the side-chain from cholesterol.

A great deal of research has been performed on pregnane derivatives by Stohs and colleagues (see Stohs and Rosenberg, 1975) and they have shown that progesterone is readily metabolised to compounds such as 5α-pregnane-3,20-dione and 5α-pregnan-3β-ol-20-one.

It comes as no surprise that progesterone has been isolated in only a few species.

Two pathways may be involved in the metabolism of progesterone in plants: the first would give rise to the above metabolites and the other would involve reduction at C-20 to give the corresponding 20α- or 20β- derivative. Progesterone may also be an intermediate in the synthesis of cardiac glycosides (Caspi and Lewis, 1967, Bennett et al., 1968 and Stohs and El-Olemy, 1972).

It should also be noted that progesterone is hydroxylated at C-21 in D. lanata to give the adrenocortical steroid deoxycorticosterone (Caspi et al., 1968).

Although progesterone has been converted to testosterone by species of the fungus Aspergillus (Sallam et al., 1973, El-Rafai et al., 1973) there is yet to be shown a similar conversion in higher plants. Undoubtedly, higher plants have the ability to metabolise C_{19} steroids. Androstenedione was incubated with a suspension culture of Dioscorea deltoidea and was transformed to 5α-androstan-3β-ol-17-one and 5α-androstane-3β,17β-diol (see Stohs, 1975). Slices of green bean reduced dehydroepiandrosterone to Δ^5-androstane-3β,17β-diol and pineapple slices oxidised dehydroepiandrosterone to androstenedione and also oxidised androstenediol to testosterone (Schneider, 1970) which, as far as is known, is the only report of higher plant tissues synthesising testosterone.

Estrone can be converted to estradiol in the presence of fermenting yeasts (Wettstein, 1939 cited in Fieser and Fieser, 1959) but no such

evidence exists in higher plants. Bennett and Heftmann (1967) applied [^{14}C] mevalonic acid to six plants of Haplopappus heterophyllus and harvested them at regular intervals. One plant was left for six months until it flowered. This was harvested and assayed for [^{14}C] in the phenolic fraction on TLC in a dichloromethane : methanol solvent system and monitoring by a thin-layer scanner, several peaks of radioactivity were found, two of which had similar mobilities to estradiol and estriol but these were too close to background radiation to be unequivocal.

Much of the research on sex hormones in plants implicates their presence with the sexual reproductive phase, i.e. the flowers or seeds, and indeed Heftmann (1975) suggests that we may conclude that their occurrence is not restricted to any particular plant family and that flowers and seeds are probably the best source.

Physiological role in plants

Although involved in many cellular functions undoubtedly the most important function of these substances in animals is in sexual differentiation and the regulation of sexual processes.

More interesting from the standpoint of this work is the implication of a similar role for such compounds in higher plants (Heftmann, 1966 and Kopcewicz, 1971). Unfortunately, some of this work has involved uncritical observations (Jacobsohn et al., 1965; Kopcewicz, 1971) and statements made without resource to definitive experimentation:

> " ..... it seems unlikely that they, steroids, are concerned in any definite way in sporophyte sexuality." (Heslop-Harrison, 1972); "Inhibition of flowering by SK and F 7997 could not be reversed by the application of various sterols. This suggests that sterol biosynthesis is not an essential part of floral induction." (Zeevaart, 1976).

In the older literature, reviewed by Thimann (1935), Bonner (1937) and

Heftmann (1963) there are descriptions of the possible effect of sex hormones on higher plants. Nevertheless, it should be noted that experiments in which hormones are administered to growing plants are generally accepted as being notoriously difficult to reproduce. The most interesting reports in these early accounts are those dealing with effects on sex-expression and flowering. Schoeller and Goebel (1931, 1934, 1935 cited in Bonner, 1937) found that an estrogen preparation accelerated flowering in Calla and Hyacinthus if the bulb were placed in solutions containing the hormone. However, several other authors could not repeat this observation (see Bonner, 1937). Apparently a positive response was only obtained when distilled water was utilised and not with tap water. Schoeller (cited in Bonner, 1937) also showed that the number of flowers was increased in plants of Primula, Fuchsia, Chrysanthemum and many others to which estrogens had been added. In the case of tomatoes, fruit yield was increased.

Chouard (1936, 1937; cited in Heftmann, 1963) demonstrated that when Callistephus sinensis was watered with a solution of estradiol flowering was promoted. Czygan (cited in Heftmann, 1963) reported that flowering was induced in Lemna minor when either estradiol or progesterone was added to the water. Love and Love (cited in Thimann, 1935) applied E_1, E_2 and E_2 benzoate, testosterone and its propionate in lanolin paste to the axils of Melandrium dioecum (Silene dioica). It was found that germination of seeds and cell division in root was stimulated by these sex hormones, especially the estrogens. Moreover, interesting effects on sex expression were produced by the application of sex hormones to young stems before flowering. Estrogens suppressed anther growth and influenced the plant in a female direction, whereas testosterone suppressed gynecium growth and influenced the dioecious herb in a male direction.

Bonner (1937), however, postulated that these hormones do not affect flowering directly but do so through influencing vegetative growth.

Effects on root growth have been reported for maize (cited in Heftmann, 1963), and pea (Bonner and Axtman, 1937). The growth of isolated pea embryos has been stimulated by estrone (Helmkamp and Bonner, 1953, Heftmann, 1963). Conversely, testosterone has been shown to inhibit the growth of Pisum and Lepidium sativum (see Heftmann, 1963).

It has been reported that estrogens stimulate the growth of yeasts. In addition, androgens, estrogens and adrenal steroids are growth promoters of Euglena gracilis (see Heftmann, 1963).

More recently Leshem (1967) studied the effects of animal steroids and gonadotrophins on curd cuttings of Brassica oleracea L var. cymosa. Both flower development and rooting of the curd cuttings were promoted by various concentrations of cholesterol, estradiol and androsterone. By using appropriate steroid inhibitors it was found that the stimulus for floral development and rooting appeared to be at the nucleic acid level.

Earlier, Bonner et al. (1963) succeeded in suppressing floral induction by employing inhibitors of sterol biosynthesis. On the other hand, these inhibitors are not selective and it is unwise to make any general statements on the physiological effect subsequently manifested.

Topical application of estrogens to Ecballium elaterium L, a member of the cucumber family (Kopcewicz, 1971a) increased the number of female flowers while the number of male flowers was increased by androgens. Cortisone increased the total number of flowers but did not affect the female to male ratio. Gawienowski et al. (1971) however, found that the application of both estradiol and testosterone to cucumber plant increased the proportion of female flowers that develop.

Kopcewicz (1972) has also demonstrated that the increase in female

flowers of pumpkin, Curcurbita pepo produced by Ethrel (2-chloroethyl-phosphoric acid) was due to an increase in its "estrogen" content.

Estrogens have also been implicated in photoperiodicity. Estradiol stimulated flowering in the long day plant Cichorium intybus some ten days after gibberellin treatment did (Kopcewicz, 1970). It was suggested that this may be due to an effect on gibberellin metabolism but ten days certainly seems a long time to induce this effect. The same author has also demonstrated that steroids influence growth in higher plants (Kopcewicz, 1969 a, b). In the first of these estrone, estradiol and sitosterol were shown to promote growth in Pisum sativum var. cud Kelwedonu. It is interesting to note that both testosterone and cholesterol had no effect on growth. An increase in the growth of seedlings of dwarf pea (Kopcewicz, 1969b) treated with estrone and gibberellic acid was observed 96 hours after treatment. Endogenous levels of gibberellic acid were ascertained and it was claimed that plants which did not display any differences in growth rate contained a similar quantity of gibberellin.

Other reports exist of estrogens on endogenous levels of plant hormones and vice versa (Kopcewicz, 1970a, b; 1972c). An increase of auxin content has been demonstrated in pea seedlings and pine seedlings treated with estrone, estradiol and estriol (1970 a, b). Considerable differences were found 72 hours and 96 hours after application. It was suggested (1970b) that the increase in auxin may be due to the estrogen's effect on gibberellin metabolism. Treatment of 0.1 µg of estrone, estradiol, estriol per plant in Pisum sativum seedlings resulted in increased levels of cytokinins without affecting the abscisic acid levels, growth of the seedlings was also observed (Kopcewicz, 1972c). Application of the synthetic cytokinin, kinetin, to Phaseolus vulgaris was alleged to increase the levels of estrone but abscisic acid had no effect on levels (Kopcewicz, 1972d).

Thus steroidal sex hormones have been associated with various inter-

actions within plants. Unfortunately, little of this research has been reproduced by other authors and it is unfortunate that much of Kopcewicz's work is called into question by equivocal identification procedures. It is a matter of concern that interest in the physiology and biochemistry of steroids in plants has been largely neglected save by the few authors cited in this review.

It was deemed imperative that a detailed reappraisal of steroidal estrogens in higher plants be undertaken using the dwarf French bean Phaseolus vulgaris var. Canadian Wonder. This particular plant was investigated as previous and current studies in Glasgow established the growth characteristics and uniformity of this cultivar (White, 1973; Colquhoun, 1974).

The scope of this investigation was restricted to two themes: (1) The isolation, identification and assessment of quantities in the plant. The techniques employed included combined gas chromatography - mass spectrometry (VandenHeuvel and Zachei, 1976 and Palmer and Holmstedt, 1975) and radioimmunoassay (RIA). This latter technique has also been called competitive protein binding, saturation analysis or radioligand binding (Midgeley, 1969). It is an assay in which a radioisotopically labelled substance, in this case a steroid, competes with varying amounts of unlabelled ligand for available sites on a binding agent. This agent, or antigen, is obtained by inducing an immune response to the compound of interest in an animal such as a rabbit, sheep, pig or horse. As the animal does not have an immune response to the steroid it is bound to a protein to produce a hapten. In the case of estradiol and most of the sex hormones the protein employed is bovine serum albumin. This steroid-protein conjugate is injected into the animal and after a few weeks anti-serum can be collected by performing jugular phlebotomies. In larger animals this can be repeated at regular intervals with the occasional

booster injection of hapten.

After incubating the diluted antiserum (1:20,000 - 1:30,000) with the sample and excess radioactively labelled steroid, dextran-coated charcoal is added to remove any unbound isotope. The bound fraction is then normally assayed by liquid scintillation spectrometry. Although it has finite limits of response it is extremely sensitive and levels of 10 - 200 pg of estradiol equivalent can be detected by this method.

In combination with GC-MS analysis RIA should provide unequivocal evidence for the presence of estrogens.

An important aspect of this approach included the development of suitable isolation procedures. This is dealt with in Section I of the results.

(2) The metabolism of estrogens (including $[^{14}C]$-E_1 and E_2 and $[^{3}H]$-E_1SO_4) their uptake and distribution and their biosynthesis from $[^{14}C]$-MVA was investigated and the results of these studies are dealt with in Sections II - V of the results.

MATERIALS AND METHODS

1. Plant Material

All plant materials used in these studies were obtained from the dwarf French bean Phaseolus vulgaris var. Canadian Wonder (seed supplied by Daggs Ltd., Glasgow, U.K.). Seeds were sown 50 to each box, in a compost of sand, peat and loam (1:1:1), and grown in either a heated greenhouse with supplementary radiation supplied by Thorn 400 MBFR/U high pressure mercury vapour lamps to maintain a 16 hour photoperiod throughout the year, or in a constant environment cabinet at 22-26° illuminated with 65/80 watt warm white Atlas; warm white Mazda; or daylight Philips fluorescent tubes.

The following growth characteristics were noted throughout these studies:

A: Seeds — Mature, dry seeds as obtained from the supplier.

Length: 14.9 mm (±0.31)
Width: 7.05mm (±0.22)
} (from 10 samples)

Weight: 0.43 g (mean weight of 5 x 10 samples was 4.3 g ± 0.18)

B: Plants — Two developmental stages were analysed: vegetative and flowering.

Vegetative plants (14 - 21 days old), when harvested, had no evidence of flowering. The primary leaves were fully expanded and the first trifoliate leaf was rapidly expanding. The second trifoliate leaf was usually at a very elementary stage. The overall height was of the order of 15.02 ± 2.76 cm (mean of 5 samples).

The flowering plants (30 - 35 days old) were characterised by the presence of fully formed flowers. All plants did not flower at the same time and different

B: Plants stages of flowering could be found within samples (e.g. buds, flowers, and post fertilisation bearing young fruits.) These plants possessed from 3 to 5 trifoliate leaves and had attained a height of 46.9 ± 1.97 cm. Budding plants were taken to be those which, although having buds possessed no discernably open flowers.

2. Chemicals, reagents and their purification

(a) Solvents

Solvents such as methanol and acetone were normally supplied in bulk (various suppliers) and redistilled prior to use. Diethyl ether, chloroform, benzene (May and Baker and British Drug Houses) were of technical grade and these were also routinely redistilled. Other solvents not redistilled included butan-1-ol (BDH) dichloromethane and toluene (May and Baker) which were technical grade.

(b) Bulk Chemicals

Whilst most chemicals employed were of technical grade NaCl and $NaHCO_3$ were normally Analar grade.

(c) Fine Chemicals and Reagents

Both estrone and estradiol (pure grade) were supplied by Koch Light Ltd., and bis-trimethylsilylacetamide was obtained from Sigma Chemicals Ltd.

(d) Scintillation Cocktails

(i) Toluene with 4 gl^{-1} of 2,5-diphenyloxazole (PPO)

(ii) For samples with small amounts of water, Triton X-100 was added to (i) in the proportion 1:2.

(iii) Unisolve 1 scintillation fluid (Koch-Light Ltd) was employed in experiments with a high aqueous content.

(iv) A solution of PPO (0.7%) in toluene:phenethylamine: methanol:water (40:33:22:5).

2. (d) (v) Dioxane:toluene (70:30) and PPO (0.5%).

(e) Radiochemicals

The following radiochemicals, summarised om Table 1, were supplied by The Radiochemical Centre, Amersham, U.K.

Table 1. Radiochemicals

		Specific activity	
		mCi mmol^{-1}	µCi mg^{-1}
[4-^{14}C]-estrone (E_1)	I	58	213
	II	55	202
[4-^{14}C]-estradiol (E_2)		56	204
[6,7-$^{3}H_2$]-E_2		46 x 10^3	1.69 x 10^3
[2,4,6,7-$^{3}H_4$]-E_2		91 x 10^3	334 x 10^3
[6,7-$^{3}H_2$]-E_1 sulphate		1.1 x 10^3	4.04 x 10^3
NN'-dibenzylethylenediamine-di-dl-[2-^{14}C]-mevalonate (dl-[2-^{14}C] MVA DBED salt)		22.8	42

Typically, the estrogens (except E_1SO_4) were diluted with 10% EtOH in benzene to 2-5 µCi ml^{-1}. The tritiated E_1SO_4 was diluted with distilled water to give a final concentration of 10 µCi ml^{-1} and the [^{14}C]-MVA diluted to 2 µCi ml^{-1}.

All radiochemicals were stored in a refrigerator at 4°as recommended by The Radiochemical Centre, Amersham.

3. Chromatography

(a) Thin-layer

(i) When employing TLC in the analysis of endogenous estrogens the phenolics were, typically, dissolved in a minimal volume of 10% ethanol in benzene and loaded onto 0.5 mm, 20 x 20 cm pre-prepared silica gel TLC plates (E.F. Merck, Darmstadt, Germany). The plates were pre-washed in methanol overnight and regenerated by drying at room temperature.

(ii) TLC of radioactive fractions involved loading onto either 5 x 20 cm or 20 x 20 cm 0.25 mm Sil G plates (Camlab). Several solvent systems were employed.

(a) $CHCl_3$:acetylacetone (95:5)

(b) $CHCl_3$:MeOH (95:5)

(c) $CHCl_3$:CH_3COOH (95:5)

(d) C_6H_6:Pr-2-OH (95:5)

(e) Light petroleum (40-60 b.p.):EtOAC (75:25)

(f) Pr-2-OH:$CHCl_3$:MeOH:H_2O (40:40:20:8)

R_f values for the estrogens (E_1 and E_2) were determined for solvent systems (a) - (e) and are summaried in Table 2.

Table 2. Retention factors of E_1 and E_2 in various solvent systems

Solvent system	R_f E_2	E_1
(a)	0.28 - 0.4	0.48 - 0.6
(b)	0.34 - 0.39	0.52 - 0.58
(c)	0.32 - 0.42	0.50 - 0.56
(d)	0.18 - 0.23	0.29 - 0.33
(e)	0.03 - 0.09	0.13 - 0.18

3. Chromatography

(a) Thin-layer (contd)

The plates were developed for 15 cm in the solvent saturated atmosphere of a Shandon chromatography tank at constant temperature in an incubator (21-23°)

To facilitate the identification of the relevant zones the samples were run alongside marker spots of synthetic E_1 and E_2 or [^{14}C]-E_1 and E_2.

After allowing to dry at room temperature the plates were sprayed with 0.1% dichlorofluorescein (BDH) in ethanol and examined under ultra-violet light (254 nm). Zones of interest were scraped off and the organic material recovered by eluting with five or six volumes (1 ml) of 10% EtOH in benzene in small chromatography columns. The efficiency of recovery by this method was also assessed. A known aliquot of [^{14}C]-E_2 (ca 100,000 dpm) was applied to 20 x 20 cm 0.5 mm plates along with some concentrated plant extract. After developing in solvent systems (b), (d) or (e) the zone corresponding to [^{14}C]-E_2 was obtained in the usual way. The data obtained are tabulated in Table 3.

Table 3. Percentage recovery of [^{14}C]-E_2 from TLC plates

Solvent system	% recovery	(S.E. of mean)
(a)	79.6	(±8.42) *
(b)	72.8	(±2.40) **
(e)	82.9	(±8.30) *

* - 4 replicates ** - 3 replicates

(b) Column Chromatography

A methanolic suspension of Lipidex 5,000 (supplied by Packard Instrument Co. Inc., Illinois, U.S.A.), a hydroxy-alkoxy-propyl

derivative of Sephadex, was transferred to a Buchner funnel and the MeOH removed by suction. The gel was washed with 5% propan-2-ol in benzene (4 x 100 ml) transferred to a beaker with solvent (400 ml) and allowed to swell for at least three hours. The gel slurry was then poured into a 10 mm diameter column the bottom of which was plugged with cotton wool and glass beads (40 mesh) and after eluting the excess solvent the gel was allowed to settle. This produced a column 75 cm in height.

Prior to the purification of the extracts the column was calibrated. Typically 50,000 dpm [4-^{14}C]-estrone or estradiol were added to 1 - 2 ml of a chlorophyll extract concentrate in C_6H_6:Pr-2-OH (95:5) and applied to the surface of the gel, via a Pasteur pipette, care being taken to disturb the surface as little as possible. This was allowed to drain on to the column. The vial was washed out with 2 x 1 ml volumes of the 5% propan-2-ol in benzene mixture, applied to the column and allowed to drain. The column was eluted with the benzene:propan-2-ol mixture maintaining a flow rate of 20 - 40 ml hr^{-1}. Forty samples (5 ml) were collected in a Gilson TDC 80 fraction collector, dried under reduced pressure and radioassayed by scintillation spectrometry. Each calibration was carried out three times and typical elution profiles are shown in Figure 5. The retention volumes obtained are summarised in Table 4 and the dpm above background for the replicates in Table 5. Although the compounds of interest were routinely eluted in tight bands (column efficiency was 500-600 theoretical plates) it was usual to collect samples 10 -20 ml either side of the expected elution volume ensuring that maximum amounts of estrogen were obtained for analysis.

Figure 5: Elution profile of [^{14}C]-E_1 and E_2 on Lipidex 5,000.
(benzene:propan-2-ol 95:5)

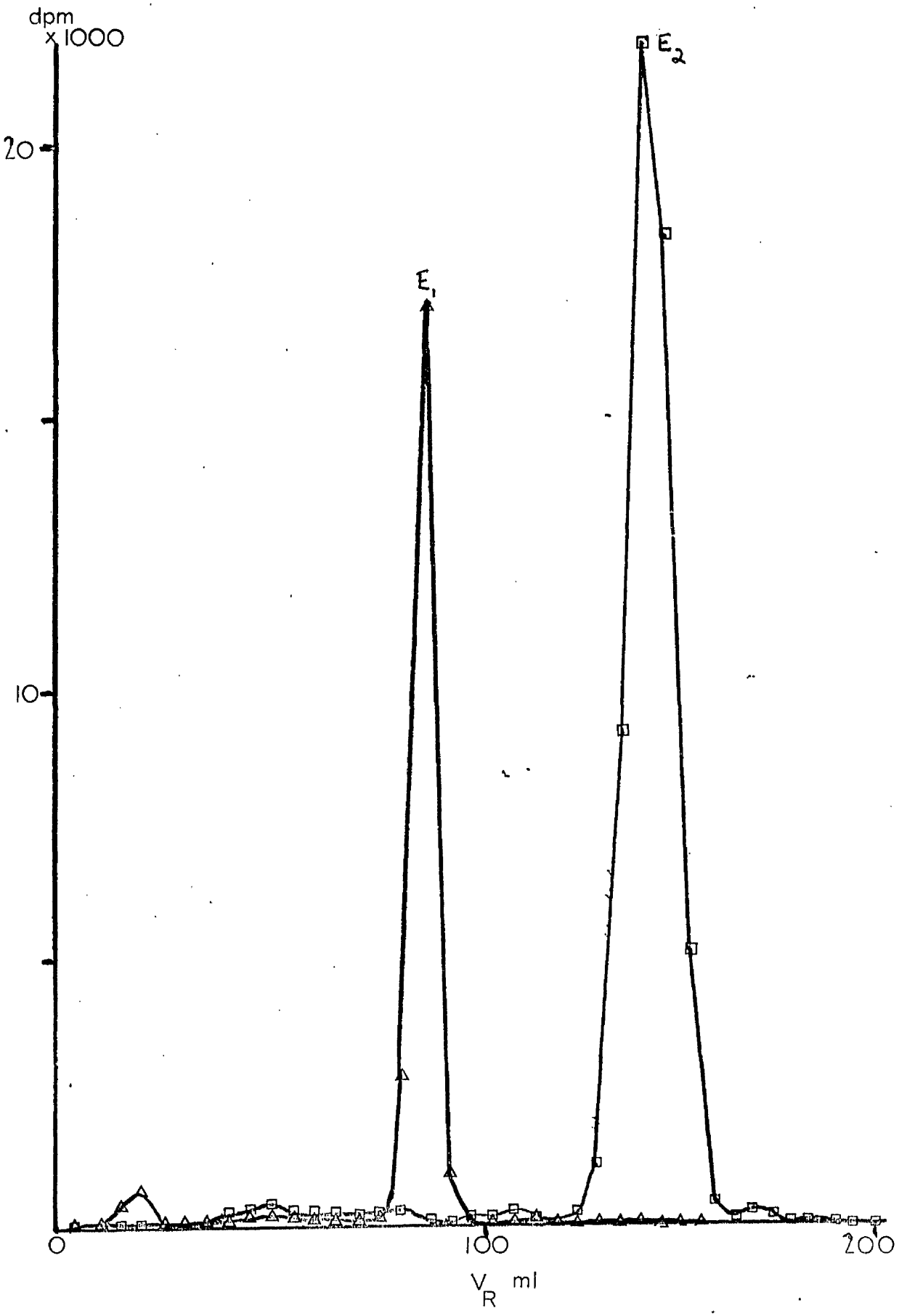

dpm
x 1000
20
10
E1
E2
0
100
200
VR ml

Table 4. Retention volume of E_1 and E_2 eluted on Lipidex 5,000

	Retention volume (ml)	S.E. of mean
E_1	81.7	±6.01
E_2	141.7	±4.41

(c) Gas Liquid Chromatography (GLC)

(i) Conventional

Initial GLC analysis involved determining the operating conditions and hence the retention times of synthetic E_1 and E_2. The instrument was a Pye 104 gas chromatograph fitted with either 5 feet 3% SE 30 or 5 feet 3% OV 101 columns. Optimal conditions were obtained with a carrier gas (N_2) flow rate of 30-40 ml and temperature of 250°.

Analysis involved injecting standard estrogens until constant retention times were obtained. Varying quantities of the compounds were injected to ascertain linearity of response, and, prior to assaying of samples, solvent blanks (BSA) were injected. From time to time standards were injected to check that operating conditions had not changed.

(ii) GC-MS

Two methods of analysis were employed.

In the first procedure the ion abundance at m/e 416 or 285 (the molecular ion and base peak respectively of the bis-trimethylsilyl ether of estradiol) was continuously monitored at the collector as generated in the ion source from the GLC effluent. This process called single-ion monitoring enabled the gas chromatograph-mass spectrometer (GC-MS) to be used as a semi-specific detector of estradiol. Two columns were used

Table 5. Dpm above background obtained from Lipidex 5,000

Sample	Estrone			Estradiol		
	I	II	III	I	II	III
1	22	2	0	7	3	53
2	32	7	0	6	28	37
3	414	6	0	5	10	31
4	719	6	0	4	5	21
5	82	27	0	58	0	15
6	61	44	0	64	15	0
7	0	63	0	63	42	0
8	0	268	156	74	104	87
9	223	283	393	174	182	164
10	227	257	184	217	242	162
11	142	156	113	148	213	165
12	121	399	70	109	147	183
13	130	14,772	51	183	149	2
14	105	24,749	115	88	176	169
15	170	561	182	124	198	169
16	2,907	331	906	121	315	177
17	17,222	66	19,153	159	131	301
18	1,242	66	25,911	233	78	146
19	140	89	1,195	92	169	123
20	91	108	133	54	228	190
21	61	106	80	88	133	288
22	134	63	112	176	106	152
23	34	28	116	185	59	81
24	31	33	89	124	99	67
25	26	33	40	88	1,286	170
26	60	31	33	70	9,733	1,575
27	73	20	23	417	23,607	9,300
28	112	13	38	3,914	17,266	21,979
29	80	12	21	14,854	3,556	18,610
30	163	18	56	20,423	414	5,217
31				8,987	263	610
32				1,328	194	277
33				264	110	231
34				201	80	128
35				156	73	68
36				89	70	52
37				78	66	48
38				62	46	60
39				48	80	56
40				57	50	28

viz: either a 5 feet 1% SE 30 operated at 250° or a 3 feet OV 210 + OV 17 column at 230°.

The second procedure was similar to the first but differed in that the accelerating voltage of the mass-spectrometer was alternated allowing the simultaneous monitoring of several ions. In this process (called mass-fragmentography or multiple-peak monitoring) the mass-spectrometer was tuned on the following ions: 416, 342 and 285 (342 is the molecular ion of E_1 TMSi and 285 is the base peak of both E_1 and E_2 TMSi).

(iii) Radio-GLC

In analysis by radio-GLC two gas chromatographs connected in series with proportional counters (4d) were employed. The first of these was a Perkin Elmer gas chromatograph fitted with a 6 feet 5% OV 101 column at 240-250° and carrier gas flow rate of 50 ml min^{-1}. The second was a Pye 104 GC equipped with a 5 feet 1% OV 17 column at 240° and 40 ml min^{-1}.

Typically the retention times of radioactive E_1 and E_2 were determined by injecting aliquots of the labelled compounds along with their non-radioactive counterparts. This was repeated until peak heights and retention times were constant. BSA was injected as a solvent blank prior to injecting samples. Samples were assayed along with their non-radioactive counterparts.

4. Radioactivity Analysis

Radioactive samples were assayed by one of the following methods:

(a) The radioactivity present on TLC was assessed by a chromatogram scanner (Panax Ltd., Redhill, Surrey, U.K.) with propane:argon (98:2) as carrier gas at 0.4 kg cm^{-2}. The chromatogram was scanned at 120 mm hr^{-1} with a slit width of 4 mm, the sensitivity was normally set at 3 cps full scale deflection and time constant at 100s. The relative amounts of radioactivity detected were transformed into a trace produced by a Servoscribe RE 511 flat-bed recorder.

(b) In certain experiments plant tissue thought to contain radioactivity was oxidised by an Oxymat IN 1401 sample oxidiser (Intertechnique Ltd., U.K.). The [^{14}C]is converted into $^{14}CO_2$ and tritiated samples to $^{3}H_2O$ trapping them in special scintillation cocktails [2d (iv) and (v)] and dispensing them into vials ready for assay by scintillation spectrometry.

(c) Liquid scintillation spectrometry utilising a Packard Tricarb Spectrometer (model 3380). Samples were corrected for quench by referring to a standard curve of efficiency versus external standard (AES) ratio which was determined by adding known aliquots of [^{3}H] or [^{14}C] hexadecane to the relevant scintillation cocktail quenched with chloroform and/or pigment. The efficiency of counting could therefore be established for a given AES ratio and plotted as a curve.

Scintillation vials were either glass (May and Baker Ltd.) or polypropylene disposable (New England Nuclear, Germany and Beveridge and Co., Edinburgh) and filled with 10 ml of one of the scintillation cocktails (2). During any assay the background radiation was determined by assessing three vials containing only scintillant. The background (cpm) thus obtained was subtracted from results before estimation of dpm. Vials (and their contents) were assayed for 2-5 min.

(d) Radio-GLC analysis of [^{14}C] was carried out by chromatographing on a Perkin Elmer connected, via a stream splitter (9:1) to a Packard proportional counter in which the [^{14}C] is converted to $^{14}CO_2$.

Tritium was analysed on a Pye 104 connected via a 1:1 stream splitter to a Panax proportional counter. The tritium is converted to $^{3}H_2O$.

Any radioactivity detected is translated into a peak by a pen recorder.

(e) In addition to the above methods chromatograms containing radiocarbon in small amounts were assayed by autoradiography. This involved placing TLC plates in contact with X-ray film for four weeks then developing them in standard X-ray film developer. The zones of fogging on the

plate due to [^{14}C] were observed.

5. Radioimmunoassay

The assay may best be divided into two sections.

(a) Reagents

(i) Phosphate buffer (PBS) : To a solution of NaCl (8.75g) and the antibiotic methiolate (o.11g) in deionised water (100 ml) were added the solutions of NaH_2PO_4 (0.5 M, 7.5 ml) and Na_2HPO_4 (0.5 M, 15 ml) respectively and the resultant solution diluted with deionised water to 1097 ml corresponding to a dilution of 0.14 M for NaCl and 0.01 M for phosphate and a pH of 7.0.

(ii) Gelatine solutions : Gelatine powder (0.5g) was dissolved in PBS (100 ml) and an aliquot (20 ml) was removed and further diluted with PBS (80 ml) to afford concentrations of 0.5 and 0.1% (w/v) respectively.

(iii) Dextran-coated Charcoal : A mixture of methanolic-washed Norit A charcoal (0.5g) and dextran T-70 (0.05g) was prepared as a suspension in PBS (200 ml).

(iv) Antiserum : An aliquot (up to 1 ml) of the frozen stock solution of antiserum (1:100 dilution of Y3/5B from Dr. B. Cook, Royal Infirmary, Glasgow) was thawed and diluted further using the 0.1% gelatine solution (final dilution 1:20,000 - 1:30,000).

(v) [2,4,6,7-^{3}H]-estradiol : Tritiated estradiol was diluted with 0.1% gelatine to give a final concentration of 10,000 - 12,000 cpm 100 μl^{-1} (50-60,000 dpm which is equivalent to ca. 690 pg ml^{-1}).

(vi) Non-radioactive estradiol : Unlabelled estradiol was dissolved in EtOH (99.99%) to give a final concentration of 1 ng ml^{-1}.

(b) The Assay

Using constant volume pipettes with disposable tips for all

pipetting operations a duplicate series of aliquots of the non-radioactive estradiol were dispensed into test-tubes (10, 20, 50, 100, 200 pg respectively) and the solvent completely removed by gently warming under a stream of nitrogen. To each tube was added a sample of the tritiated estradiol solution (100 µl) followed by an equal volume of the antiserum solution. Three pairs of control tubes were set up at the same time, all containing the same volume (100 µl) of radioactive solution. To the first of the tubes was added the diluted gelatine (0.1%, 1100 µl) in order to assess the total radioactivity employed in the assay, (T).

The specific binding by the antiserum (B_0) was estimated by adding antiserum (100 µl) to the second of the tubes (ideally, binding should be 50-60% of the estradiol present). The non-specific binding (N) (i.e. the background of the assay) was estimated by adding no antiserum to the third set of tubes. All tubes were mixed thoroughly with a 'Whirlimix' and incubated at less than 4° for at least 2 hours after which the gelatine solution (0.5%, 100 µl) followed by ice-cold dextran-coated charcoal (1 ml) was added to all the tubes except tubes T. The latter operation was performed with the tubes standing in ice and after allowing 10 minutes to elapse the tubes were centrifuged for 10 minutes at 2,500 rpm. (MSE Mistral 4L). The supernatant was decanted into scintillation vials containing scintillation cocktail (2d (ii)]. The resultant dispersions were assayed by Scintillation spectrometry.

Table 5 summarises the procedure.

Table 5. Summary of standard tubes in in Radioimmunoassay

Tube No.	E_2 (pg)	^{3}H-E_2 (µl)	Antiserum		0.1% Gel (µl)	0.5% Gel	Dextran Charcoal (ml)
1 (T)	-	100	-	INCUBATION	1100	100	-
2 (B_0)	-	100	100		-	100	1
3 (N)	-	100	-		100	100	1
4	10	100	100		-	100	1
5	20	100	100		-	100	1
6	50	100	100		-	100	1
7	100	100	100		-	100	1
8	200	100	100		-	100	1

6. Efficiency of Purification Methods

Although the age of plants for this series of experiments was not deemed to be critical, green leaf tissue only was harvested from 21-45 day-old plants.

The purification methods commenced with the extraction of leaf tissue (30 g) in boiling $CHCl_3$:MeOH (2:1) (ca 300 ml) for 15-20 minutes by which time the leaves had become colourless. The solution was decanted and evaporated to dryness under reduced pressure in a rotary film evaporator. Thereafter the residue was purified using one of two procedures:

(a) The residue was redissolved in Et_2O (ca 500 ml) and [4-^{14}C]-E_2 as internal standard was added to the liquor, (an aliquot of the standard was removed for radioassay). The organic layer was partitioned against 2% $NaHCO_3$ (4 x 200 ml) and the combined aqueous fraction backwashed with aliquots of Et_2O (2 x 250 ml). This procedure removed the carboxylic acids.

The first experiments suggested that the losses involved at this stage were negligible and these fractions were subsequently discarded without counting. The combined ethereal fractions were then partitioned against NaOH (1,2 or 4 N, 6 x 200 ml) to remove the phenols.
(3 x 2% aliquots were removed for radioassay after each washing throughout this series of experiments).
The combined aqueous phase was washed with Et_2O (2 x 200 ml) and acidified to pH<2 and allowed to cool and partitioned against Et_2O (4 x 250 ml).
(b) The residue was taken up in C_6H_6:BuOH (3:1) (ca 500 ml), as Kopcewicz, (1971), and primed with a known aliquot of radioisotope as before. The liquor was washed with 2% $NaHCO_3$ (4 x 200 ml) and the aqueous phase thus obtained washed with the C_6H_6:BuOH mixture (2 x 150 ml). The combined organic fractions were washed with NaOH (1N, 6 x 200 ml). The aqueous phase was backwashed with volumes of C_6H_6:BuOH (2 x 200 ml) acidified to pH<2 allowed to cool and partitioned against dichloromethane (4 x 250 ml). Those aliquots removed from the organic fractions were allowed to dry under vacuum before the scintillation cocktail was added and then to these vials were added 4 ml distilled water. This procedure allowed constant quenching for all the organic and aqueous samples. The vials were allowed to equilibrate in the scintillation spectrometer at ca 4° at least 1 hour before counting.
The experiments were repeated on three occasions and the methods are summarised in Figures 6 and 7.

7. Extraction and Purification of Endogenous Estrogens

Plant material was harvested at various stages of development (1) and the leaf tissue was collected by excising at the laminal pulvinus. Care was taken to harvest only those leaves which appeared healthy. Leaf tissue (250 g) was then macerated in an MSE Atco blender with $CHCl_3$:MeOH (2:1) filtered through a cellulose Soxhlet thimble (Whatmann

Figure 6: Flow diagram summarising efficiency of partitioning procedure I

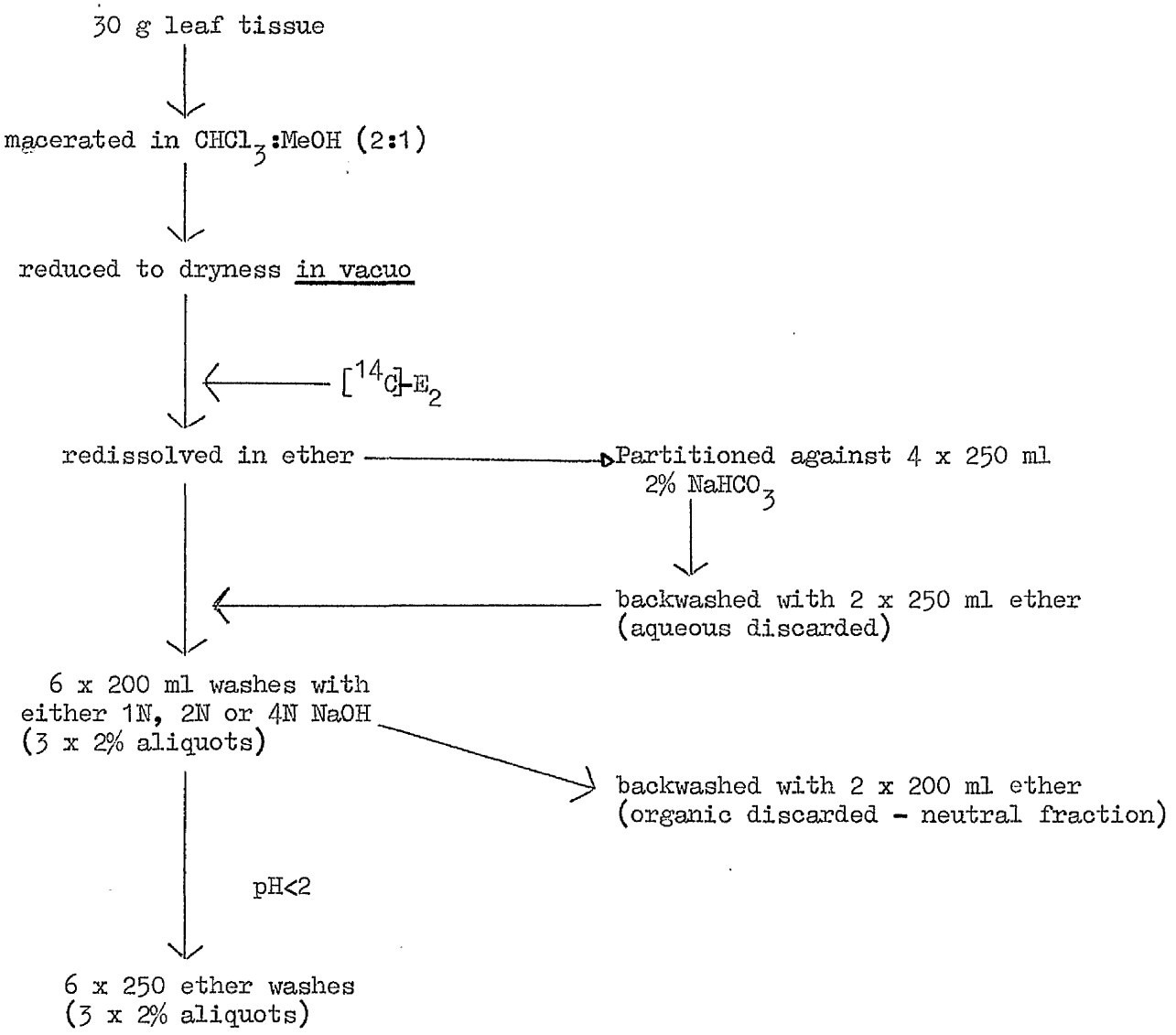
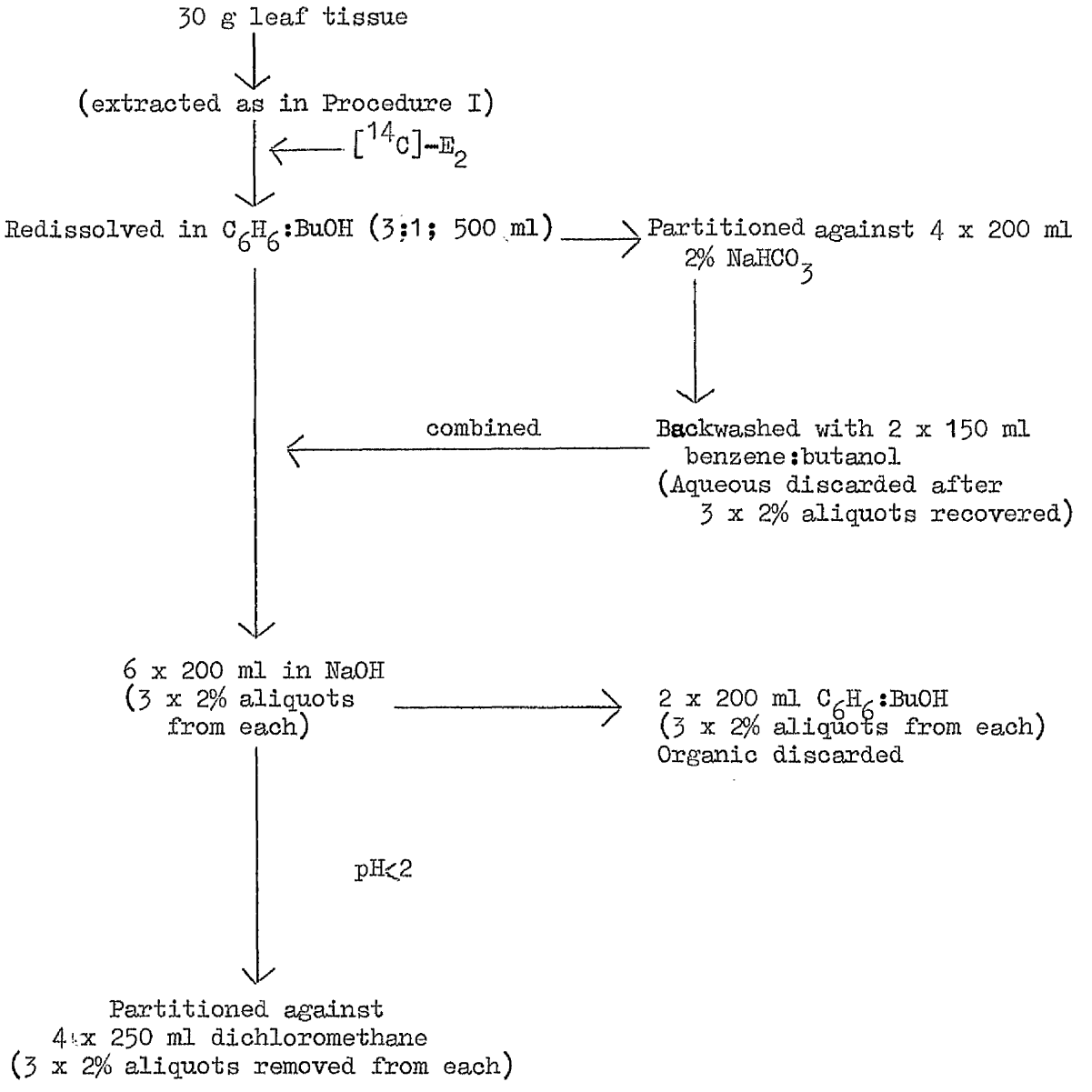


Figure 7: Flow diagram summarising efficiency of partitioning procedure II



160 x 60 mm) and solvent extracted by recycling under reflux in ca 1200 ml solvent in a Soxhlet apparatus (500 ml) for 8 hours. Seeds were normally ground dry and extracted as above.

In experiments in which the quantities of free estrogens were being compared with total estrogens it was the practice with leaf tissue to extract two samples, combine these, and after thorough mixing divide them into two aliquots. After drying in a Buchi rotary film evaporator one half was purified as in method (a) (6). The other fraction was heated under reflux with C_6H_6:HCl:H_2O (1:1:2) (400 ml) as per Vestergaard (Knights, 1970).

After cooling, the benzene layer was separated from the aqueous fraction which was subsequently washed with diethyl ether (3 x 1/3 volumes).

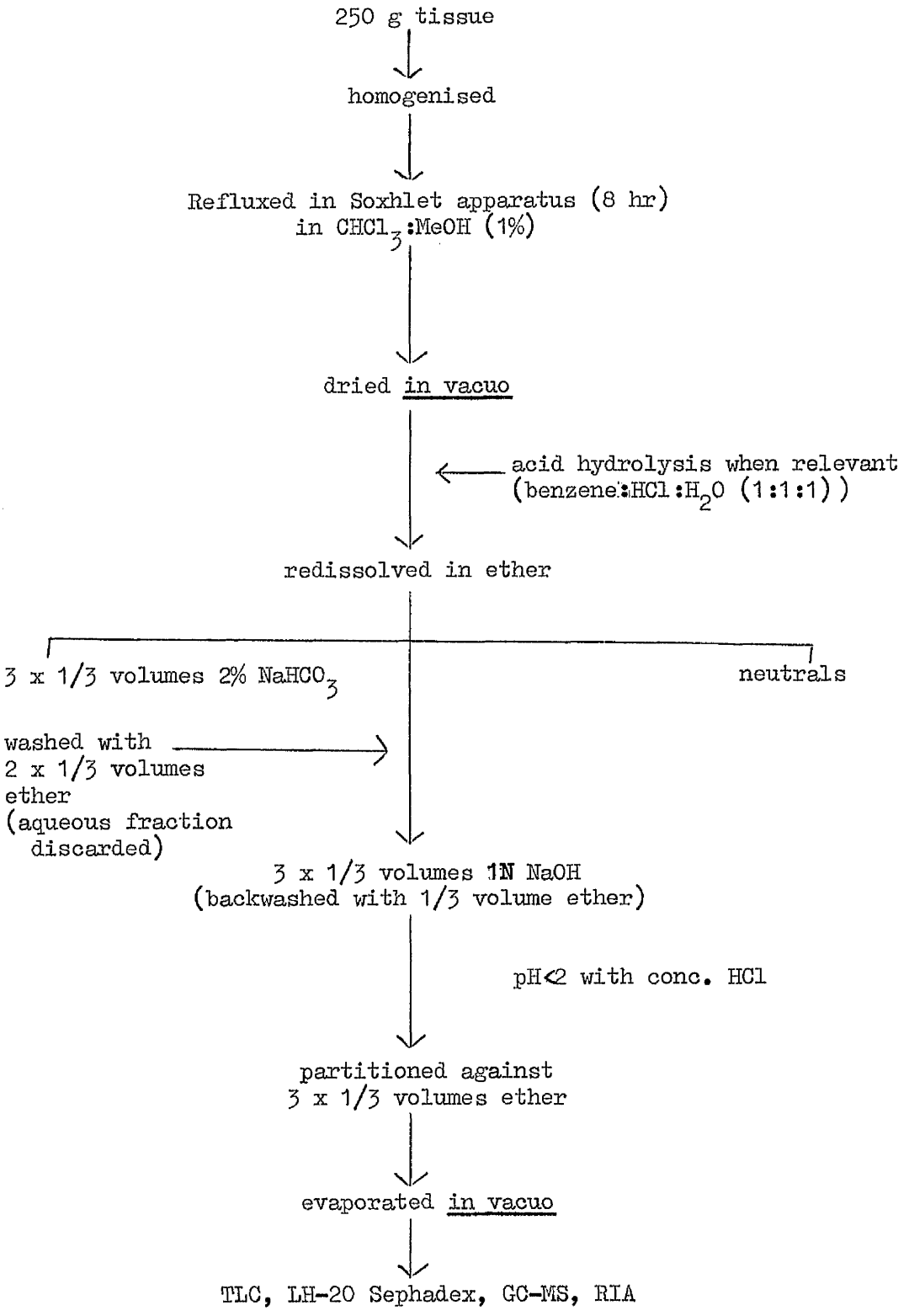
The combined organic layer was reduced to dryness in vacuo and the phenolic fraction obtained in the normal way. Initially, the phenolic fraction thus obtained was chromatographed on thin-layer and developed in solvent system (b) (2).

Zones corresponding to E_1 and E_2 were obtained and reacted with BSA to produce the trimethylsilyl ethers. Samples were then analysed by GC-MS. Subsequently, phenolic fractions were eluted on Lipidex 5,000 (2b) and either analysed by GC-MS with an aliquot removed for RIA or the entire sample assayed by RIA (Figure 8).

8. Recrystallisation of [^{14}C]-E_2

Typically 50 mg estradiol and the presumptive [^{14}C]-E_2 zone were dissolved in a minimal quantity of methanol. To this was added dropwise distilled water until a milky flocculence was obtained, which redissolved on heating. The solution was cooled and the solute allowed to crystallise. Excess solvent was decanted by Pasteur pipette and the remaining crystals dried by warming and a slow stream of nitrogen. A 2-5 mg aliquot was weighed and the radiocarbon assessed by scintillation spectrometry.

Figure 8: Flow diagram summarising extraction and purification methods employed in the investigation of endogenous estrogens



This procedure was repeated until three closely similar results were obtained.

9. The Metabolism of [^{14}C]-estrone and [^{14}C]-estradiol in whole plants

The hydrophobic compounds estrone and estradiol were solubilised in a manner similar to that of Atallah et al.(1975) : the steroids were dissolved in methanol ca 0.5 ml and two or three drops of 0.05% Tween 80 detergent. A further 0.5 ml water was added and the solution evaporated under heat and a gentle stream of nitrogen to minimal volume to ensure that little or no methanol remained. Water was then added to make up the particular concentration required (1-2 μCi ml^{-1}).

Seedlings (8-10 days old) were sectioned under water about 1 cm. above the root and stood in 0.5 ml of aqueous solutions of the radiochemicals. These were allowed to transpire for 5 days under constant illumination at room temperature. The seedlings were kept viable with distilled water during incubation and were found to possess adventitious roots at the end of this period (Figures 9 and 10). The seedlings were potted up in either 3.1/2or 4" pots containing a sand and loam mixture (1:1) and grown for 10-35 days to allow normal development to take place.

Plants were harvested when in either the vegetative or flowering stage. (1). Whole plants were homogenised and heated under reflux for 6 hours in the Sohxlet apparatus and, after drying in vacuo, the residue was purified and the phenolic fraction obtained in the usual way.

The phenolic fraction was chromatographed on thin-layer employing solvent systems (a) - (e).

Chromatograms were assayed by monitoring with the radiochromatogram scanner. Zones thought to contain estradiol were recrystallised to constant specific activity with non-radioactive carrier (8).

Aliquots of the phenolic fraction were eluted on Lipidex 5,000 and those samples expected to contain E_2 and E_1 were analysed for radiocarbon.

Figure 9: Plate showing ten-day old seedling in incubation medium

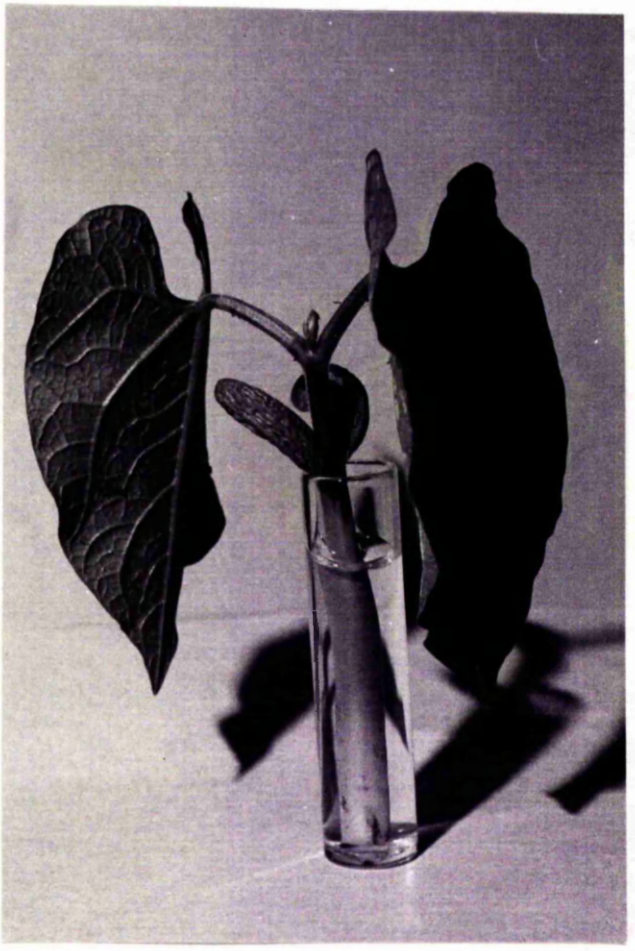
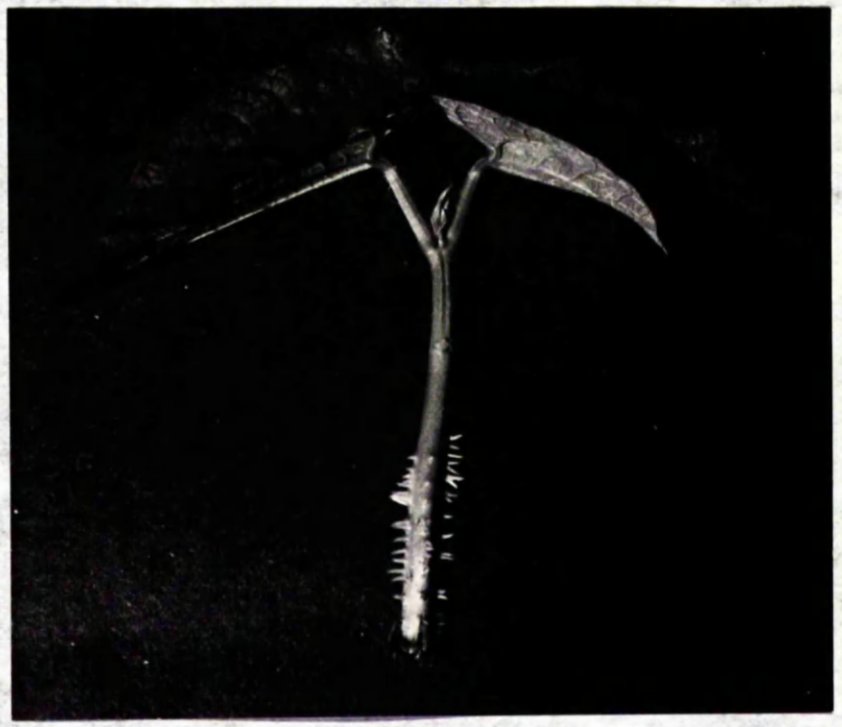


Figure 10: Plate exhibiting seedlings after five days incubation



The crude phenolic fraction remaining was reacted with BSA and assayed by radio GLC.

10. Metabolism of [6,7-^{3}H]-estrone sulphate in whole plants

Being water soluble, potassium [6,7-^{3}H]-estrone sulphate was diluted with water to 5 μCi ml^{-1} and applied to plants as above.

Whole plants, at budding and flowering, were recycled under reflux in the Sohxlet apparatus in $CHCl_3$:MeOH:BuOH (2:1:1). After extraction, the liquor was evaporated until only butanol remained. This was diluted to ca 1 litre and partitioned against NaOH (1N, 3 x 1/3 volumes). After acidifying and washing with ether (3 x 1/3 volumes) the organic fraction was partitioned against 2% $NaHCO_3$ to remove the fatty acids.

The phenolic fraction was chromatographed on thin-layer in solvent systems (b), (d), (e) and (f), and assayed using the Panax thin-layer scanner.

The trimethylsilyl ether derivative of the phenolic fraction was assayed by radio-GLC (2c (iv)).

11. Metabolism [^{14}C] MVA, DBED in whole plants

Two μCi ml^{-1} of dl-[2-^{14}C] mevalonic acid DBED salt was applied to cut stems of 10 seedlings in the normal fashion and the plants were eventually harvested when flowering.

After homogenisation, the tissue was covered with NaOH (4N) and heated under reflux for 6 hours. After cooling, the extract was diluted to 1N NaOH with distilled H_2O. The aqueous phase was washed with ether to remove the neutral fraction. The sodium hydroxide was acidified to pH<2 and partitioned against either. The ethereal fraction was subsequently washed with 2% sodium bicarbonate, removing the fatty acids and leaving the phenols.

On evaporating in vacuo the phenolic fraction was analysed on TLC in solvent systems (b), (d) and (e); by radio-GLC (2) and the pre-

sumptive estradiol crystallised to constant specific activity (8).

12. Metabolism of [^{14}C]-E_1; [^{3}H]-E_1 sulphate and [^{14}C] MVA in different regions of dwarf French bean plants

The radiochemicals were applied to the plants in the normal way and when harvested the plants were divided into roots, shoots, primary leaves, trifoliate leaves and flowers. The homogenised tissue was solvent extracted in $CHCl_3$:MeOH (2:1) and purified as in previous sections. Samples were analysed by TLC in solvent systems (b) - (e) and subsequently on Lipidex and by radio-GLC.

13. Distribution of Radiochemical

Concurrently with the metabolism studies, plants were investigated for distribution of [^{14}C] and [^{3}H] throughout the plant.

Segments of the plants were examined for the presence of radiochemical by either solvent extracting or oxidising before assay by scintillation spectrometry.

14. Statistical Analysis

Standard errors of the mean were calculated using the following equation.

$$S,E. = \pm \sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n(n-1)}}$$

where x is the individual value of each observation

and n is the number of observations.

RESULTS

I

The Analysis of Endogenous Estrogens

(a) The efficiency of partition techniques

It is an axiom that any quantitative analysis of endogenous metabolites should take into account the losses occurring during the isolation procedures. Since the introduction of suitable radioactively labelled compounds, such estimations of purification efficiency have become routine and generally accepted.

In the first series of experiments Phaseolus extracts were primed with [^{14}C]-E_2 (M and M, 6) and the amounts of radioactivity present were then calculated (Table I : 1 and 2) : here the data from three experiments have been summarised and represent the amount of radioactivity (after background radiation) at each step of the partition.

Following the preliminary partitioning it is interesting to note that the efficiency with which [^{14}C]-estradiol can be removed from solution diminishes with increasing concentration of sodium hydroxide (Table I : 1). Not only was the initial partitioning adversely affected (80.5% at 1N, 68.9% at 2N and 58.2% at 4N:line A) but the ethereal backwash also revealed the marked effect of concentration (15.4 at 1N, 23.5 at 2N and 39.6 for 4N:line B). Thus the percentages of radioactivity (corrected) remaining in the aqueous fractions were 63.3 for 1N, 48.6 for 2N and 31.6 for 4N respectively (line C). It was observed that the 2N and 4N NaOH produced large quantities of emulsion between the organic and aqueous phases during partitioning and the backwashing may have removed this emulsion leading to the subsequent loss of radioactivity from the aqueous fraction.

On acidification and partitioning with ether more than 91.5% of the radioactivity moved into the ethereal phase regardless of the concentration

of NaOH employed beforehand (line D). The overall efficiences were then calculated, and found to be 62.9% efficient for 1N compared with 44.5% for 2N and 30.5% for 4N (line E). The variation amongst the three experiments is also shown in the Table (line F).

Table I : 2 presents the results obtained from three experiments using purification method 2 - essentially that of Kopcewicz (1971). Although 1N NaOH was employed the overall efficiency was only 38.8% (line E). The initial stages of this extraction were similar to method 1 with 82.6% of the [^{14}C]-estradiol being removed in the aqueous phase (line A). On the other hand, a great deal of the benzene/butanol mixture was suspended within the aqueous fraction and backwashing removed over 40% of the radioactivity from the aqueous phase (line B). Dichloromethane appeared to be as efficient as ether (line D) and both solvents removed the bulk of the radioactivity in the first two washes. It was noted that when the aqueous fraction became colourless the radioactivity had been almost entirely removed. A further check was made on the efficiency by counting the radioactivity in the neutral fraction, but the quenching of the samples exceeded, in AES ratio, 0.05 and were thence discarded.

From these findings it was concluded that the most appropriate purification technique would be that based on 1N NaOH in Method 1.

TLC and column chromatography were selected as described in detail in M and M 3a) and b), and were seen to give recovery efficiences in excess of 75%

Table I : 1 Distribution of radioactivity (dpm) from [4-^{14}C]-estradiol using extraction procedure (1)

(Data corrected for accumulative sampling losses are given in brackets)

NaOH

		1N	2N	4N
	Total radioactivity donated to sample	109,276	112,842	130,329
	Acid partition	discarded	discarded	discarded
	6 washes with NaOH	28,933 19,967 14,550 11,217 7,739 5,589	20,322 17,683 13,039 10,880 9,117 6,761	17,772 14,160 13,239 12,044 10,211 8,370
	Combined total amount extracted in NaOH	87,995	77,802	75,796
A	% extracted in NaOH	80.5	68.9	58.2
	2 backwashes with ether	5,761 7,761	9,367 8,894	16,578 13,444
B	% radioactivity into backwash	15.4 (16.4)	23.5 (25.0)	39.6 (42.2)
	Radioactivity remaining in aqueous after backwashing (corrected for sample loss	74,473 (69,196)	59,541 (54,873)	45,774 (41,226)
C	% radioactivity remaining in aqueous phase	(63.3)	(48.6)	(31.6)
	4 washes with ether after acidification	47,761 17,806 2,489 711	33,056 11,239 5,200 711	27,761 10,034 1,589 305
	Combined total amount in ether after acidification	68,767	50,206	39,689
D	% partitioned from aqueous phase	92.3 (99.4)	84.3 (91.5)	86.7 (96.3)
E	Overall efficiency	62.9	44.5	30.5
F	Corrected replicate efficiences	(63.05, 63.8, 61.2)	(44.3, 45.2, 43.8)	(31.1, 36.7, 22.3)

Table I : 2 Distribution of radioactivity employing Method 2

(Data corrected for accumulative sampling losses are given in brackets)

	Total radioactivity of E_2 in sample	129,780
	Acid partition	discarded
	6 washes with NaOH	29,072 23,283 18,366 14,739 12,122 9,617
	Combined total amount extraction NaOH	107,199
A	% extracted in NaOH	82.6
	2 backwashes with benzene/ butanol	23,255 20,911
B	% radioactivity into back-wash from aqueous	41.2 (43.8)
	Radioactivity remaining in aqueous after backwashing	63,033 (56,601)
C	% radioactivity remaining in aqueous phase	(43.6)
	4 washes with dichloromethane after acidification	46,655 3,350 228 185
	Combined total amount in dichloromethane after acidi-fication	50,418
D	% radioactivity partitioned from aqueous phase	79.9 (89.1)
E	Overall efficiency of purification	38.8%
F	Replicate efficiencies corrected	(36.1, 45.3, 35.7)

(b) Identification of steroidal estrogens

Following selection of a suitable partition series, the next phase of the studies was the integration of these methods in a study leading to the identification of steroidal estrogens in the seeds and shoots of P. vulgaris. The techniques employed were (i) GC-MS (SIM and MPM) and (ii) Radioimmunoassay and the results from thirteen experiments have been brought together to provide this section of the results.

Phenolic fractions were obtained from three stages of development (M and M, 7) and initially purified by TLC in solvent system (b). After eluting the putative E_2 zones their TMSi ethers were analysed by GC-MS (M and M, 3) and the free steroids by RIA (M and M, 5).

(i) GC-MS analyses

All early assessments were by single-ion-monitoring (SIM) (M and M, 3). A typical trace is demonstrated in Figure I : 1 which represents the mis-named total ion current of (a) 50 and 10 ng E_2 TMSi and (b) the TMSi ether of the putative E_2 zone from seed phenolics.

It is apparent that there was a peak with a retention time closely similar to that of the standard E_2 TMSi. Similar traces were obtained from other stages of development but both the vegetative and flowering fractions contained compounds with retention times close to E_2 TMSi but resolved from it. Analyses on two other occasions revealed the presence of a compound with a similar retention time to E_2 TMSi in seeds but results from other stages of development were less clear (Table I : 3), although Figure I : 2 shows positive traces obtained from flowering and vegetative fractions.

Figure 1:1 Total ion current at m/e 416 of:

(a) 50 and 10 ng E_2 TMSi

(b) Putative E_2 zone from seeds

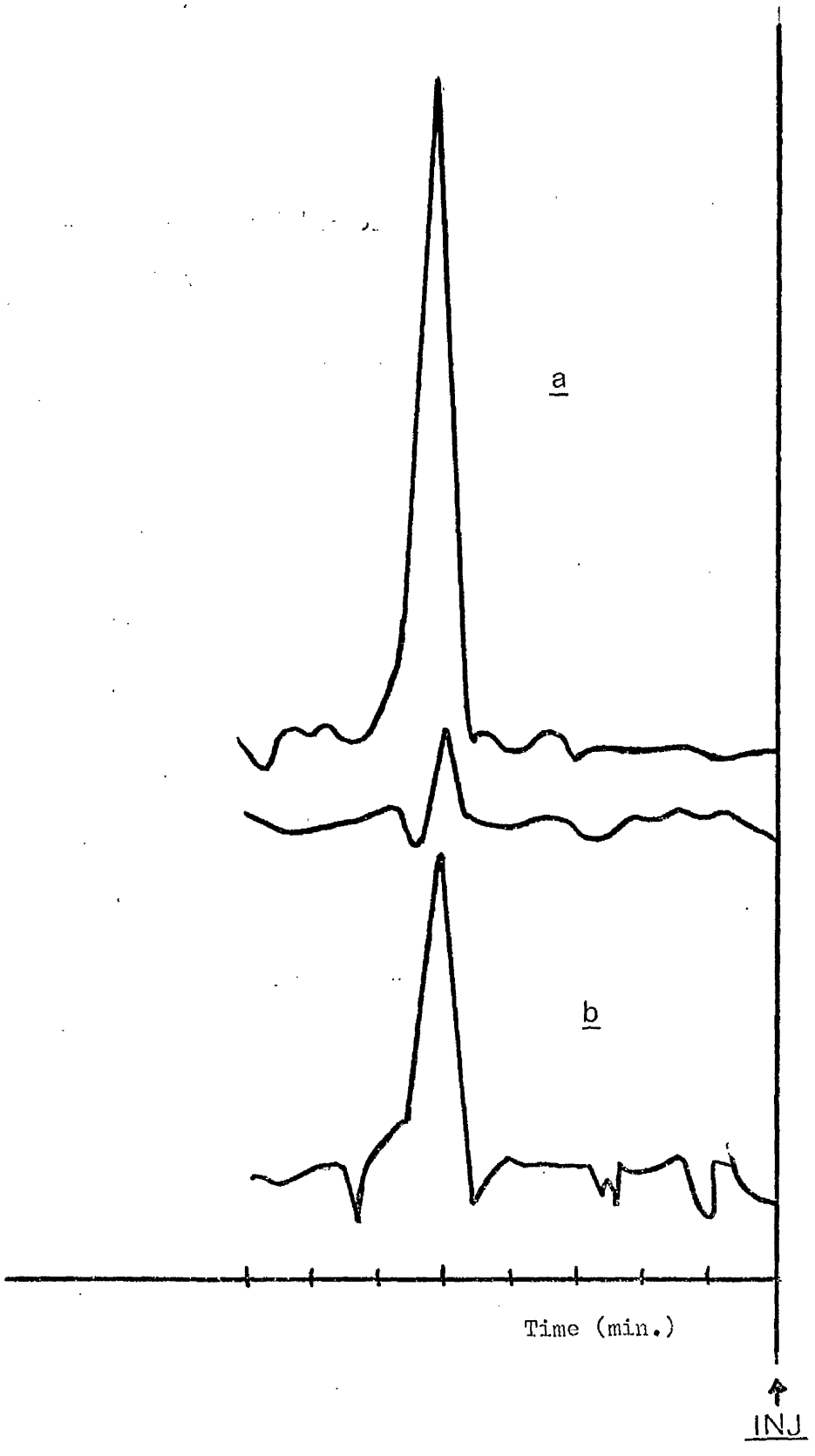

a
b
Time (min.)
INJ

Figure 1:2 Total ion current at m/e 416 of:

(a) 50 ng E_2 TMSi

(b) Vegetative fraction

(c) Flowering fraction

(d) Standard E_2 TMSi

(e) Standard E_2 TMSi

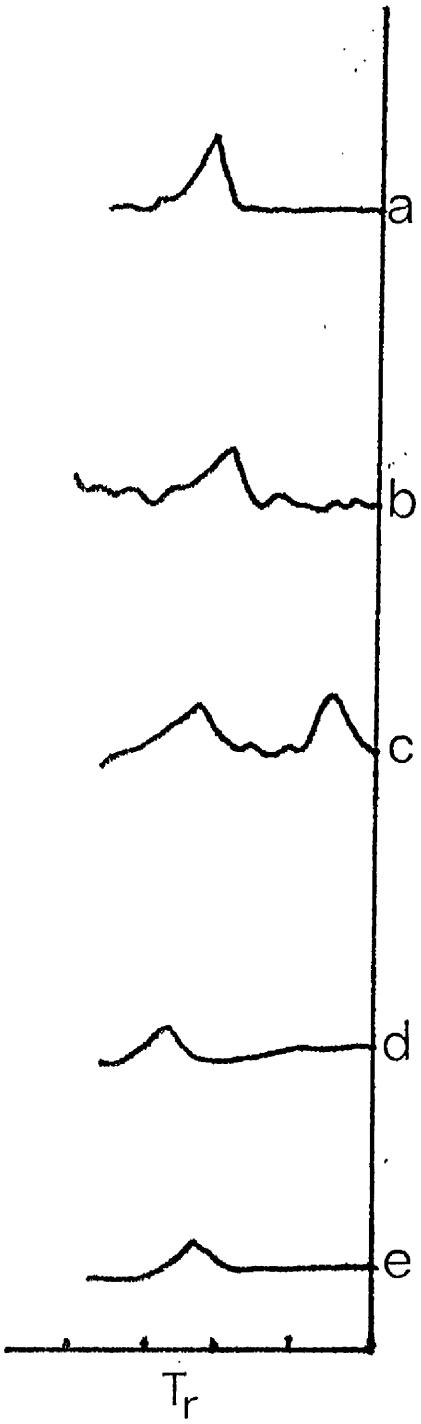

a
b
c
d
e
T_r

Table I : 3 Retention times of E_2 fractions of various stages of development (250 g tissue)

		R_T (SIM)	R_T (GLC)
Seeds	1	2.86	3.05
	*2	(3.4)**	-
	3	5.1	5.3
Vegetative	1	2.7	2.9
	*2	2.1	3.1
	3	-	-
Flowering	1	2.8	2.9
	*2	-	-
	3	-	-
E_2 TMSi (50 ng)	1	2.75	3.0
	*2	2.3 (3.4)**	3.0
	3	4.95	5.5

5 ft. 1% SE 30 column 40 ml min^{-1} 250° except * 3 ft. OV 210 + OV 17 column 15 ml min^{-1} at 230°. MS tuned on m/e 416 except ** m/e 285.

The total amount of estrogens within the samples were then compared with the free estrogens, i.e. those extractable by $CHCl_3$:MeOH (M and M, 7). Instead of TLC, the phenolics were eluted on a Lipidex column (M and M, 3b) prior to GC-MS analysis which involved the technique of MPM (M and M, 3c).

Prior to the actual analysis of the samples a series of standards were analysed and the resulting graph can be seen in Figure I : 3. To each vial, before reacting with BSA, E_1 (1 µg) was added as internal standard. The results obtained from assessing the seeds and vegetative (and their hydrolysed fractions) are summarised in Table I : 4.

Figure 1:3 Graph demonstrating relationship between estrogens and peak height in MPM analysis

E_2 peak height from m/e 285; attenuation x 3

E_1 peak height from m/e 342; attenuation x 10

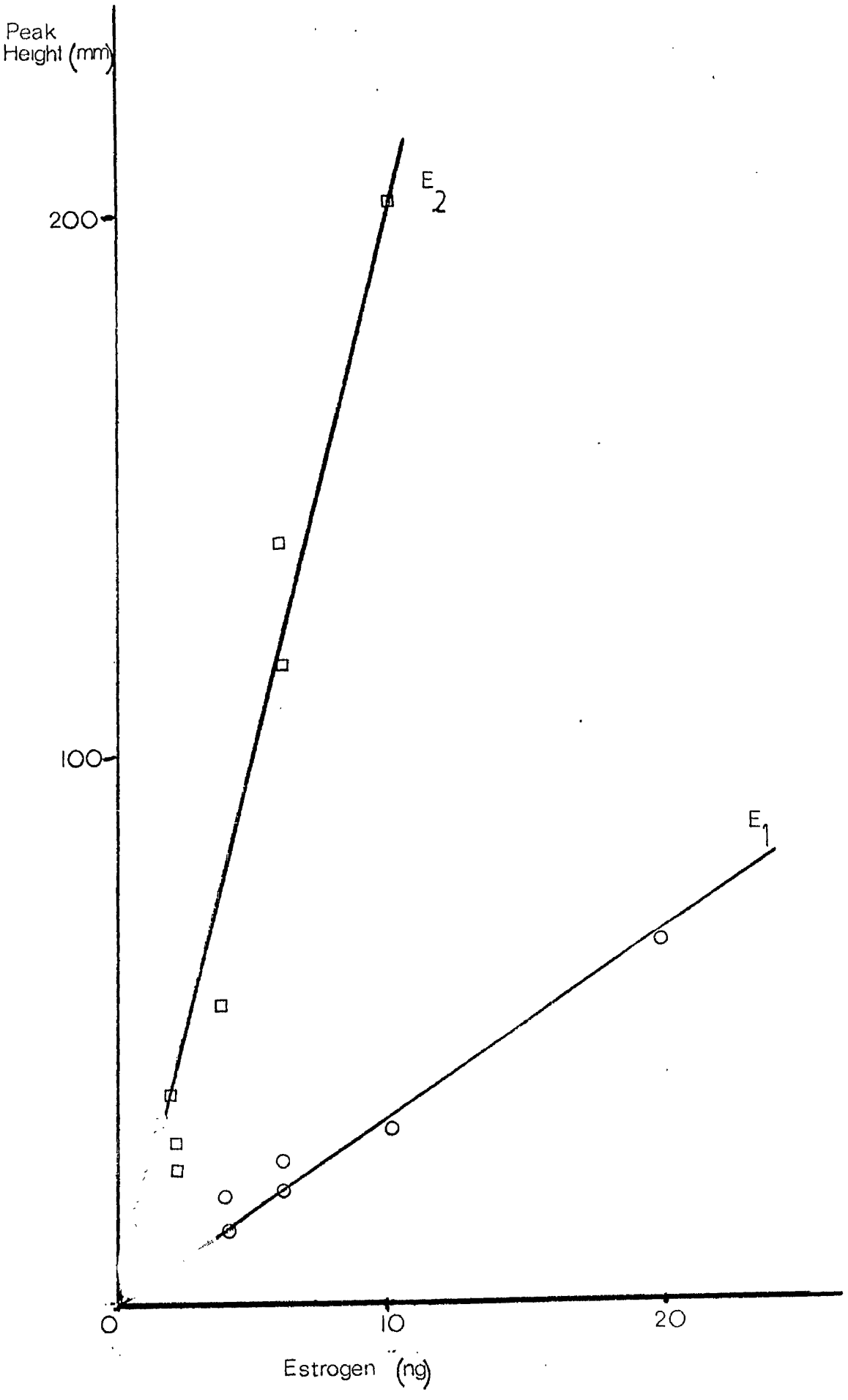

Peak
Height (mm)
200
100
E2
E1
0
10
20
Estrogen (ng)

Table I : 4 Peak heights and retention times of putative E_2 zone from MPM analysis (250 g)

	Peak Height E_1	Peak Height E_2	Weight (ng) E_1	Weight (ng) E_2	% E_2 recovered (weight ng)	Relative Retention Time of E_2 (E_1=1.0)
Seeds	170	195	1,000	256	10.93 (19.6)	0.52
Hydrolysed	183	30	1,000	36.5	24.3 (33.4)	0.54
Vegetative	113	18	1,000	35.5	4.88 (10.6)	0.54
Hydrolysed	212	43	1,000	45	16.49 (36.4)	0.54
Solvent blank	256	43	1,000	37.5	21.6 (48)	0.49

These peak heights are taken from m/e 342 for E_1 TMSi and 285 for E_2 TMSi. 5 ft. QF 1 column 240° 40 ml min^{-1}.

The relative retention time of standard E_2 TMSi was 0.52 - 0.55.

The largest amount of E_2 recovered was from the free fraction of seeds (2.34 µg Kg^{-1}) compared to the next highest, the vegetative fraction (727 ng Kg^{-1}). Both the hydrolysed fractions appeared to have little E_2 TMSi present (150.2 ng for hydrolysed seeds and 272.9 ng for hydrolysed vegetative respectively). The blank fraction was also positive with 173.6 ng detected.

In a second analysis of this type peaks with relative retention times 0.59 and 0.6 in seeds and hydrolysed seeds respectively were recorded. The relative retention of standard E_2 TMSi was 0.62. Little unequivocal evidence was found for E_2 in vegetative or hydrolysed vegetative fractions and no evidence at all in two replicates of flowering extracts (free and total estrogens).

During these MPM investigations the E_1 fraction from the Lipidex column was collected and the TMSi derivative (+ 1 μg E_2) was monitored by mass-fragmentography. Figure I : 4 and 5 demonstrate the peak trace obtained from the MPM analysis of the presumptive E_1 fractions from seeds and from flowers. Figure I : 6 represents a similar analysis of 4 ng E_2 TMSi and 10 ng E_1 TMSi. The peaks at m/e 342 in Figure I : 4 had retention times closely similar to the standard E_1 TMSi. Analysis of solvent blanks revealed no peak at m/e 342.

On repeating this investigation no estrone could be detected in other seed samples but there were peaks with similar retention times in flowering and hydrolysed vegetative fractions. These were, however, not as large as those observed in Figure I : 5.

SIM and MPM procedures have given some evidence in support of the presence of steroidal estrogens in extracts of vegetative and flowering plants and especially seeds. The quantitative aspect, nevertheless, was seen to be most unsatisfactory; revealing the inadequacy of the purification procedures.

(ii) Radioimmunoassay

The second means of identification employed for estradiol was RIA (M and M, 5). Five experiments, some of which were carried out in tandem with the GC-MS experiments are described in this section. The purification methods are as outlined for the GC-MS analyses.

The data were obtained in the form of cpm with quantification by reference to a calibration curve composed with each assay: a typical curve is exhibited in Figure I : 7. Initially 25% aliquots from the putative E_2 zones from TLC (solvent system b) were assayed (Table I : 5).

Figure 1:4 Ion current from the MPM analysis of the putative E_1 fraction from Lipidex (Seeds).

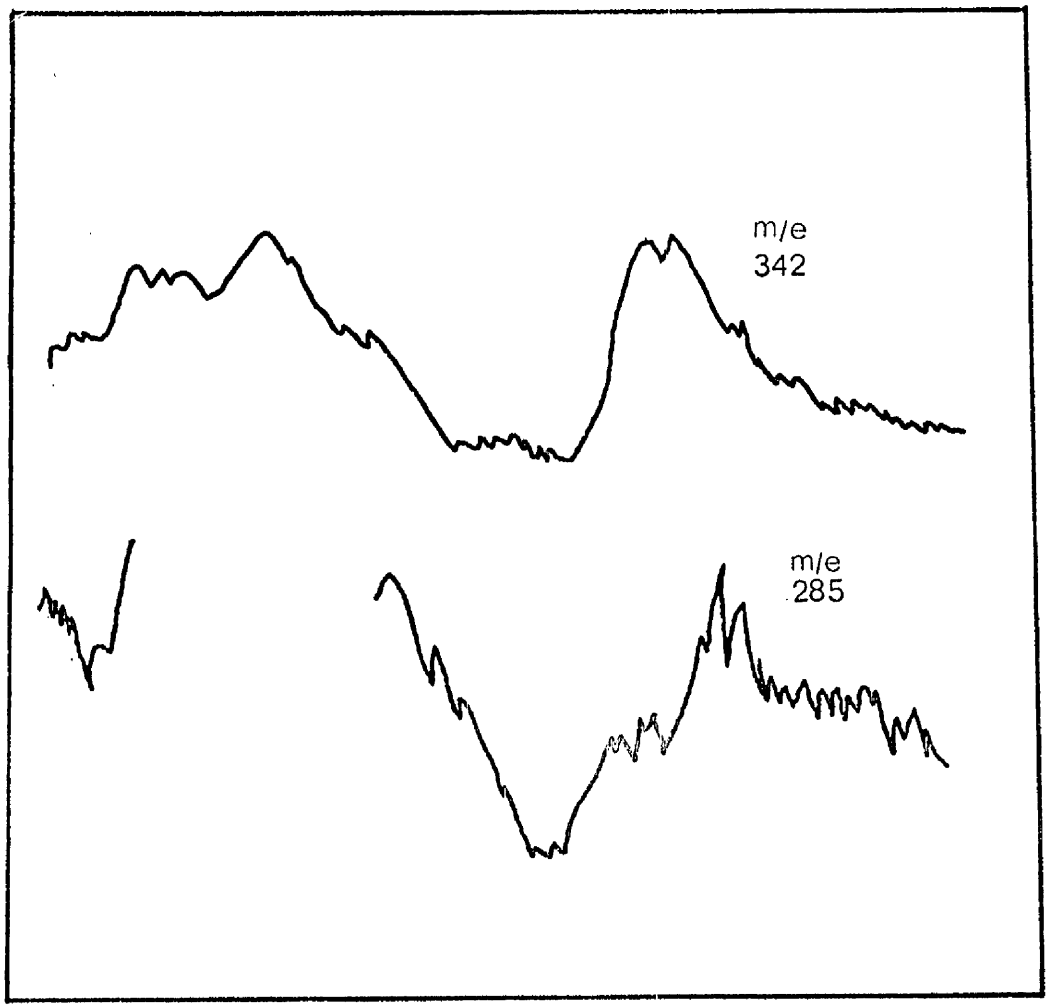

m/e
342
m/e
285

Figure 1:5 Ion current from the MPM analysis of the presumptive E_1 fraction from Lipidex.

(Flowering *P. vulgaris*)

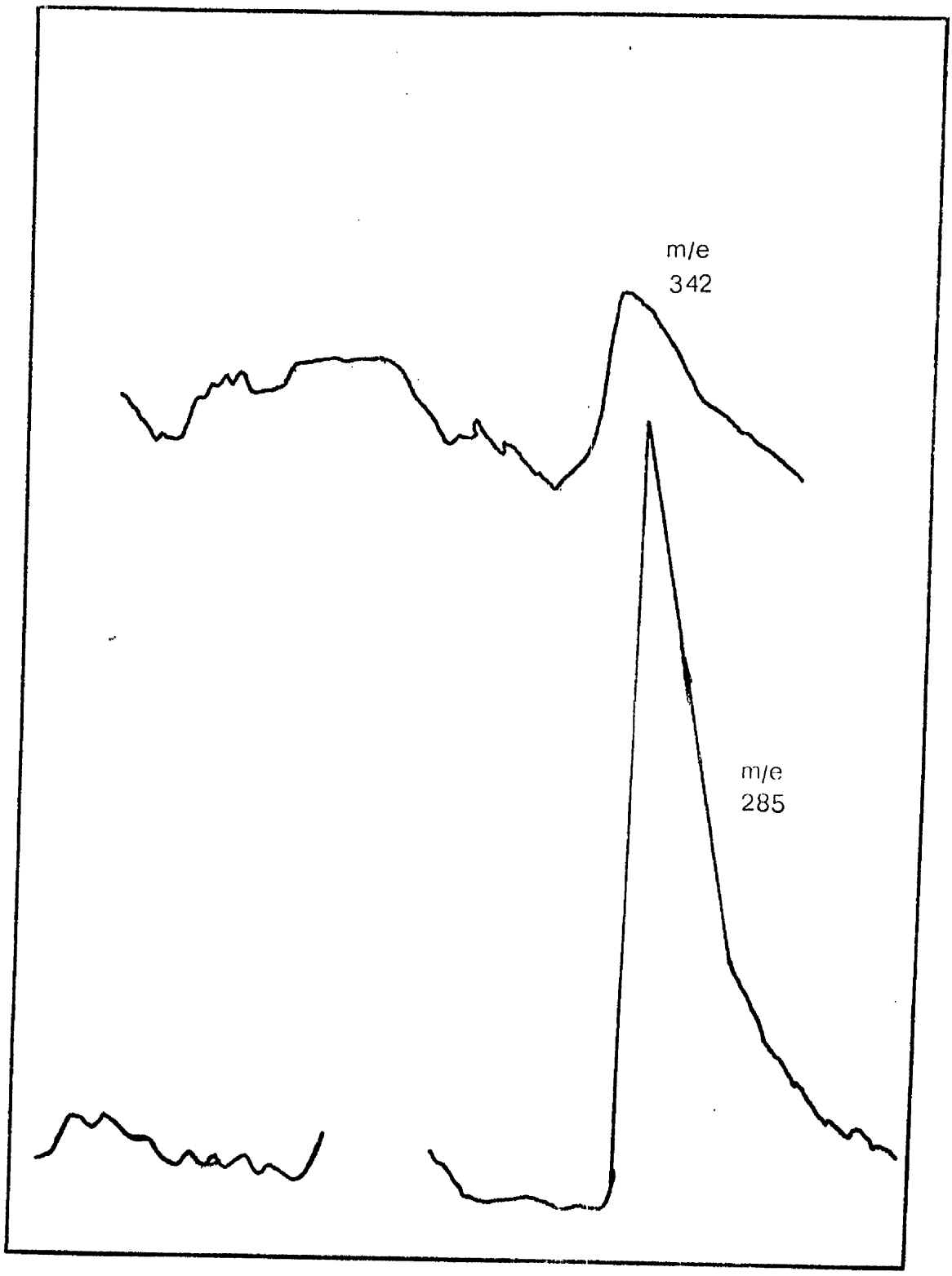

m/e
342
m/e
285

Figure 1:6 MPM analysis of 4 ng E_2 TMSi and 10 ng E_2 TMSi at m/e 416, 342 and 285.

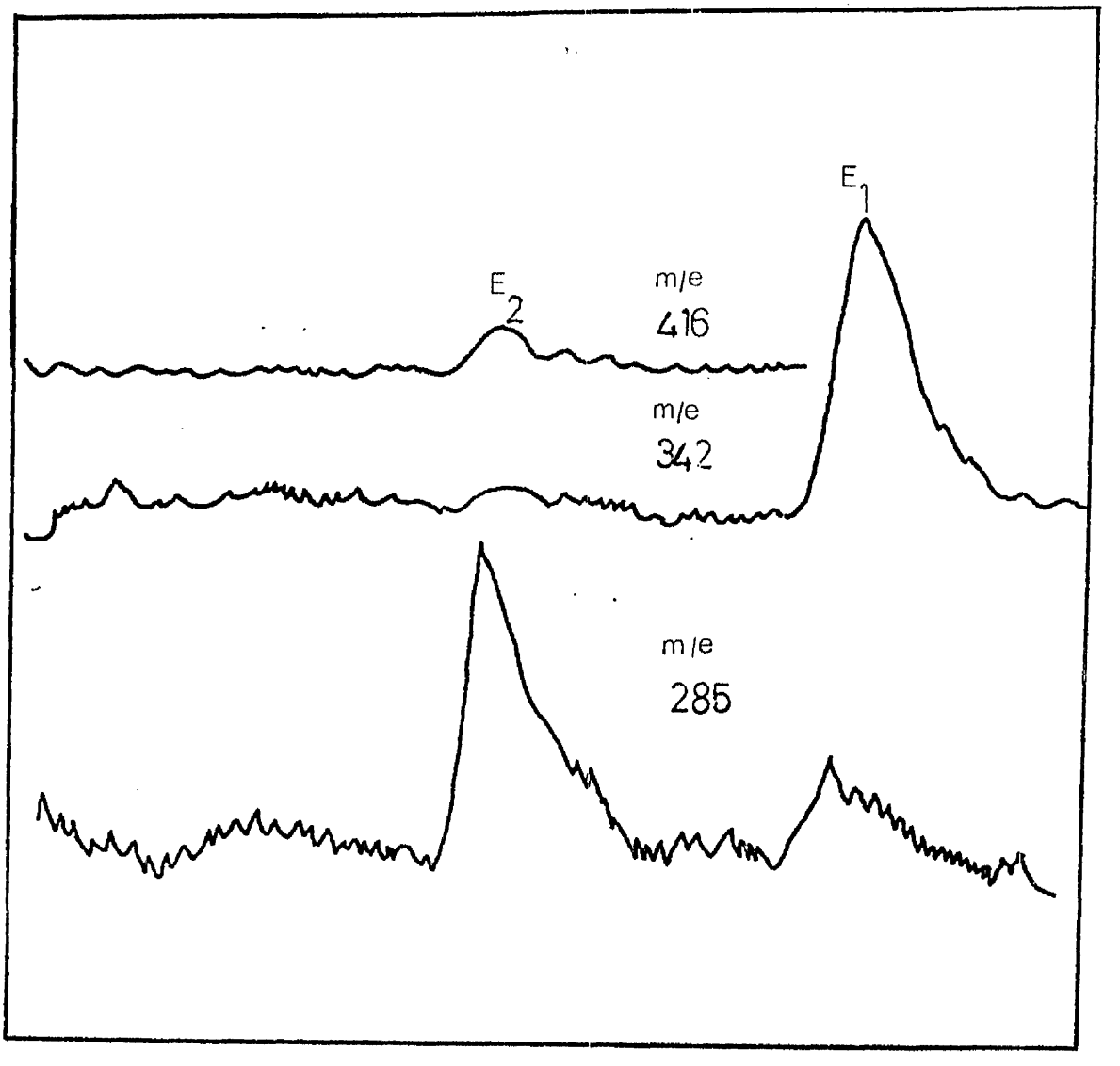

E1
E2
m/e
416
m/e
342
m/e
285

Table I : 5 Estradiol equivalent detected in different stages of development of the dwarf French bean

Summary of Results

Plant Material		cpm	Weight (pg)	Total E_2 equiv. in sample (ng)	E_2 equiv. Kg^{-1} (ng)
Seeds	I	2686	165	33	528
	II	3552	92	36.8	589
Vegetative	I	2693	165	33	528
	II	3875	82	32.8	525
Flowering	I	2791	150	30	480
	II	3758	825	33	525

Sample II is a 50% dilution of sample I.

These samples were aliquots removed at the time of the SIM analyses described in Table I : 3 replicate I.

No further RIA studies were carried out until MPM assays were undertaken. The second MPM investigation was performed in tandem with RIA and the initial results obtained from the RIA are summarised in Table I : 6.

Figure 1:7 A typical standard curve obtained from the radioimmunoassay of 10 - 200 pg estradiol

T : 13,217 cpm

B_o : 8,317 cpm

N : 199 cpm

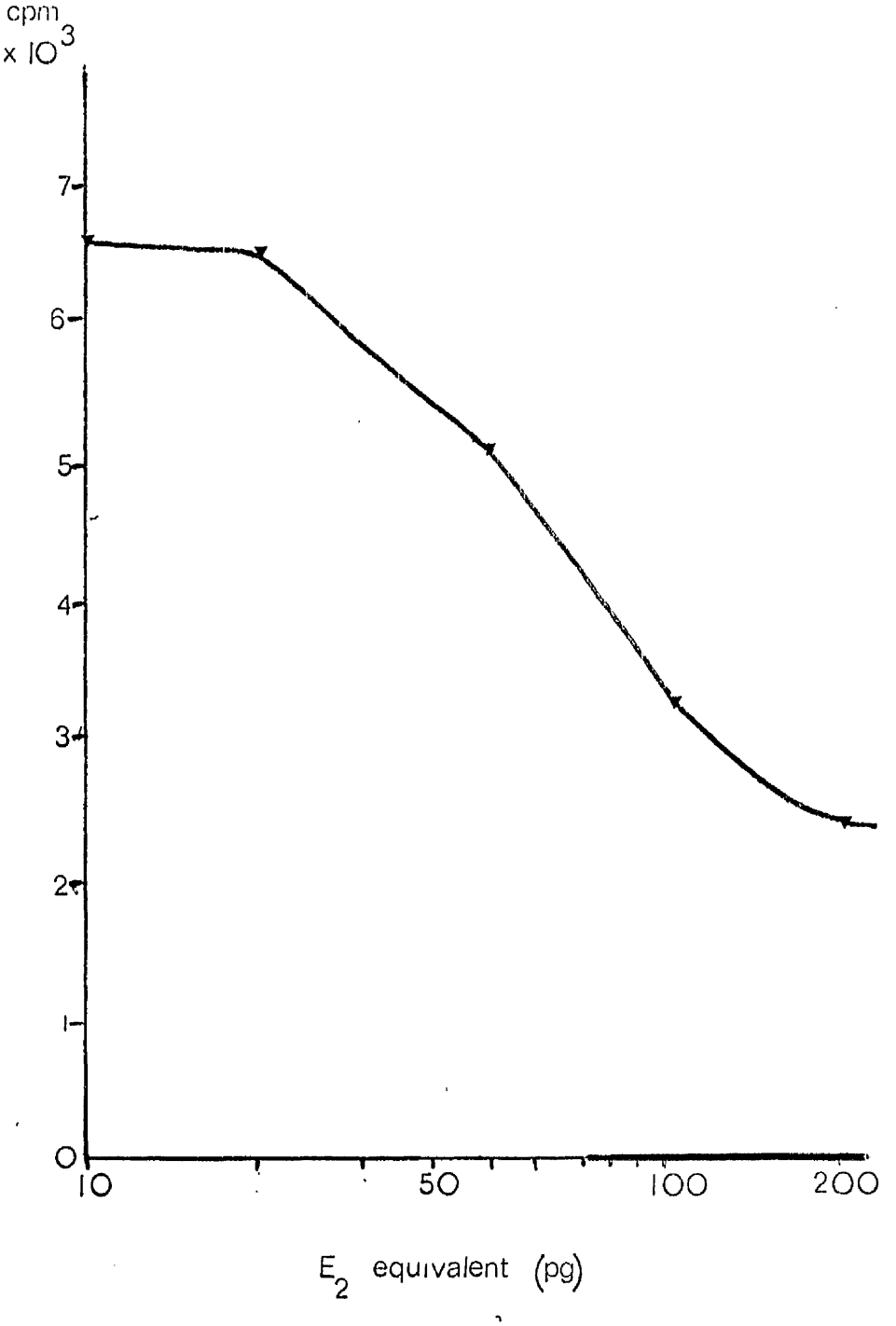

cpm
x 10^3
7
6
5
4
3
2
1
0
10
50
100
200
E2 equivalent (pg)

Table I : 6 RIA II of the putative E_2 fractions from Dwarf French Bean

Developmental Stage	Amount (µl)	cpm	E_2 equivalent (pg)
*Seeds	10	1810	>> 200
	20	1098	>> 200
	50	446	>> 200
*Seeds Hyd.	10	898	>> 200
	20	450	>> 200
	50	184	>> 200
Vegetative	50	1513	>> 200
Vegetative Hyd.	50	4000	92
Flowering	50	4374	72
Flowering Hyd.	50	2221	200

All samples dissolved in 1 ml solvent (except * 2 ml).

Positive responses were found in all samples especially in seeds; vegetative and hydrolysed flowering fraction. The response was so large, in excess of 200 pg, as to be outside the range of the calibration curve.

Samples were then further diluted until the response was on the scale. (Table I : 7).

Table I : 7 Dilutions of RIA II

Sample	Amount (µl)	E_2 equivalent (pg)	Total Sample (ng Kg^{-1})
Seeds	10	80	264
	20	190	314
Seeds Hydrolysed	10	35	1155
	20	68	1122
Vegetative	20	29	43.5
	50	62	37.2
	100	95	28.5
Vegetative Hyd.	20	42	21
	50	90	18
	100	140	14

All samples uncorrected for sample loss.

The results from this assay demonstrated that there was more E_2-like compound in the seed fraction (264 ng Kg^{-1}) than in the others (e.g. 37.2 ng Kg^{-1} in vegetative). Although the growing plant extracts had an internal standard present, whereas this particular seed fraction did not, it was deemed necessary to assay seed fractions with internal standards. As a further check the fraction from seeds was chromatographed on thin layer in solvent system (c), divided into ten equal zones and each tested for response to binding.

Results from one such study indicated three major zones of activity, the largest of which was at the origin and thus not likely to be E_2. The other zones of activity had similar R_f values to E_2 (0.2 - 0.3) and E_1 (0.5 - 0.6) with the E_2 zone being the highest. Moreover, each zone gave a response to the assay.

Further assays elicited positive responses to E_2 in seeds and other fractions although none as high as in Table I : 7. These data are listed in Table I : 8.

Table I : 8 E_2 equivalent from 125 g of seeds of P. vulgaris

Sample	Amount µl	E_2 equiv. (pg)	Extraction effic. (%)	Sample total (ng)	Weight per Kg of Tissue (ng)
Seeds	20	62	22.65	13.6	108.8
Seeds Hyd.	20	50	32.27	7.75	62.0
Seeds II	25	85	39.85	8.5	68
Seeds II Hyd.	25	58	10.73	21.62	172.9
Blank	50	40	40.1	1.99	-

Although the amount of tissue assayed was only 125 g, (cf 250 g in other studies) the amounts of E_2 detected (108.8 ng Kg^{-1}) were much

smaller than would be expected from previous studies (e.g. Table I : 6 and 7), on diluting these samples by half, the amount of E_2 equivalent detected was also halved, which indicates that the substance binding had the same binding characteristics as E_2.

Thus it is on a qualitative basis only that the RIA results substantiate those of the GC-MS experiments. Regardless, however, of the quantitative constraints of the respective systems both RIA and GC-MS support the notion that steroidal estrogens exist in extracts of Phaseolus tissues.

Having demonstrated that estrogens were present in extracts of P. vulgaris and that there is considerable evidence that they also exist in the post-embryonic plant itself, it is important to establish that the presence of these hormones, which exist in man, are not due to contaminants of the plant in the first place or of glassware and chemicals during analysis. One method already described in part I of the Results, is the assay of solvent blanks; the other method is to show that the plant is indeed capable of synthesising such hormones. Thus the remaining parts of the Results presented in this thesis are aportioned under four main headings:

II Metabolism and uptake of [^{14}C]-estrone in the dwarf French bean.

III Metabolism of [6,7-^{3}H]-estrone sulphate in dwarf French bean.

IV Metabolism of uptake of [^{14}C]-E_2 by P. vulgaris.

V Biosynthesis of estrogens from dl-[2-^{14}C]-MVA DBED.

II

Metabolism and uptake of [^{14}C]-estrone in the dwarf French bean

(a) It is well known that estrone and estradiol are readily interconvertible in the presence of NADPH in animal cells (see Grant, 1969). Consequently, it was decided to attempt this interconversion in plants. In the first series of experiments 20 μCi [^{14}C]-E_1 were administered to 40 dwarf French bean plants (M and M, 9). Vegetative plants were harvested 17 days after potting up, and the phenolic fraction obtained in the usual way. Analysis of an aliquot by TLC (solvent system (a)) revealed three major peaks of radioactivity. A relatively large zone of radiocarbon (46.4%) was detected at the origin, and although most of the remainder (44.2%) was located at R_f zone 0.38 - 0.6 there was a conspicuous area of activity (9.4%) at R_f zone 0.15 - 0.23. The larger of these two peaks might be reasoned as being [^{14}C]-E_1 although not entirely co-chromatographing with the radioactive markers (Figure II : 1). A further aliquot was eluted on Lipidex and the fractions collected, assayed by scintillation spectrometry and the results obtained shown in Figure II : 2. The first 40 ml eluted contained 42% of the total radiocarbon and is thought to be the radioactivity coming through with the solvent front. The second major peak was located within the elution volume of [^{14}C]-E_1 (50-65 ml). A small amount of radioactivity was detected between 100-130 ml corresponding to 3.47% of the total radiocarbon assessed. This peak fell within the elution volume of standard [^{14}C]-E_2.

Thirty-three days after potting up, the flowering plants were harvested and the phenolic fraction surveyed for the presence of [^{14}C]. Figure II : 3 reveals the elution profile obtained from an aliquot of the phenols chromatographed on Lipidex. The peak at the solvent front was not as evident in this fraction as that from the vegetative plants, and it only accounted for 19.95% of the total radioactivity, from 25-50 ml. Radiocarbon (52.60%)

Figure II : 1 TLC radiochromatogram of -

(a) [^{14}C]-estrone and estradiol

(b) Phenolic fraction from *P. vulgaris* (vegetative)

(Solvent system a)

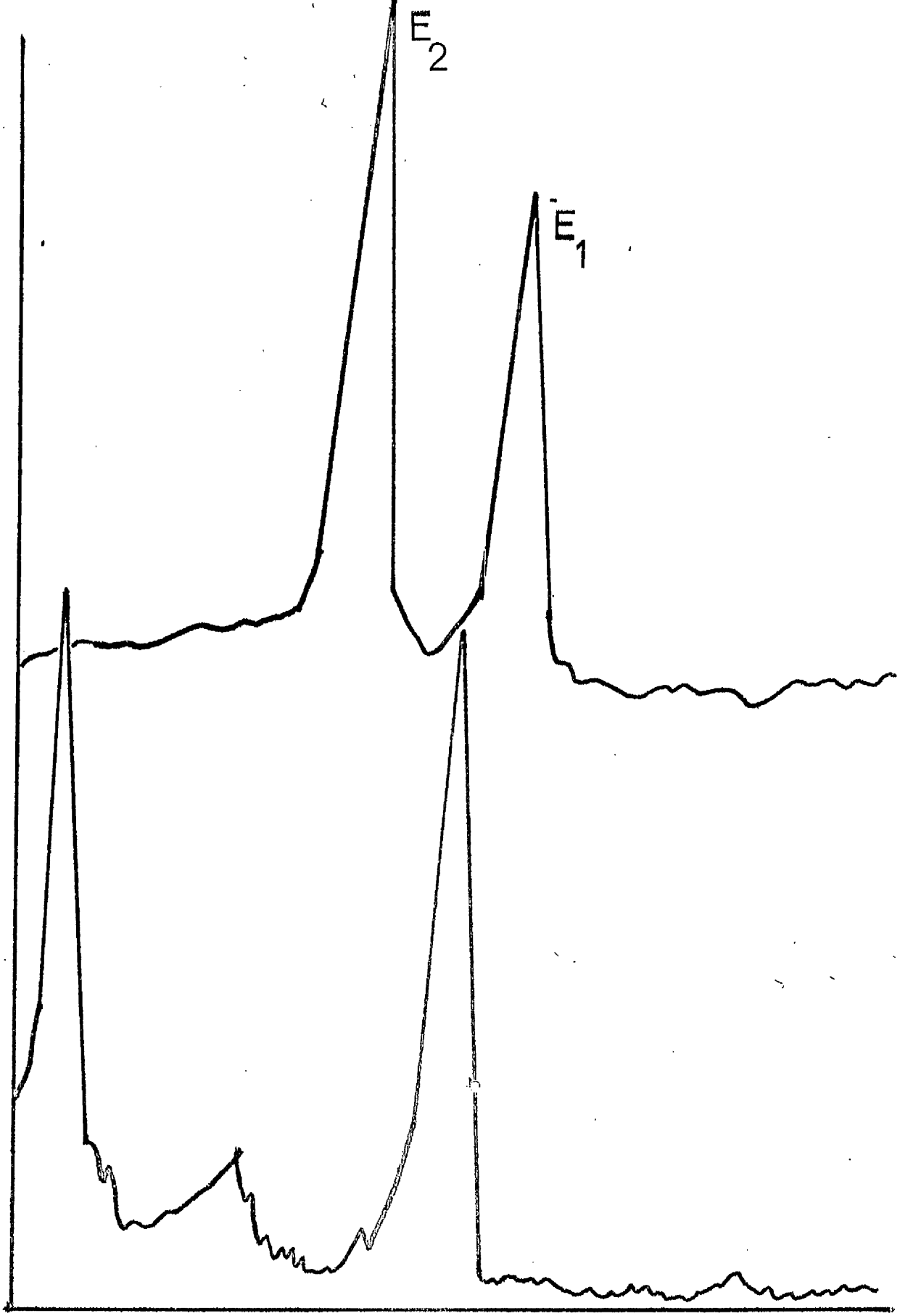

E2
E1

Figure II : 2 Elution profile from Lipidex column: phenolic fraction from vegetative *P. vulgaris*.

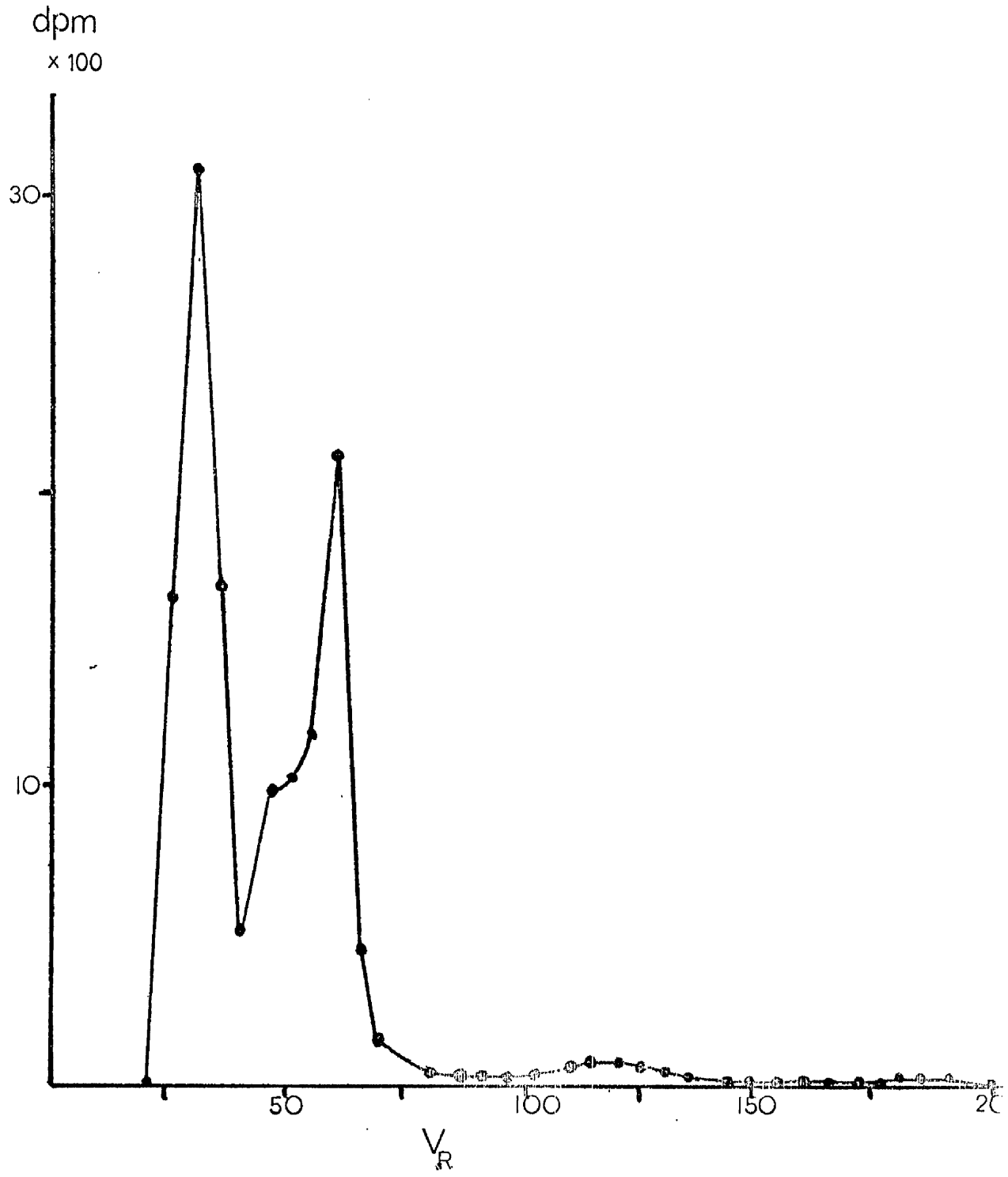
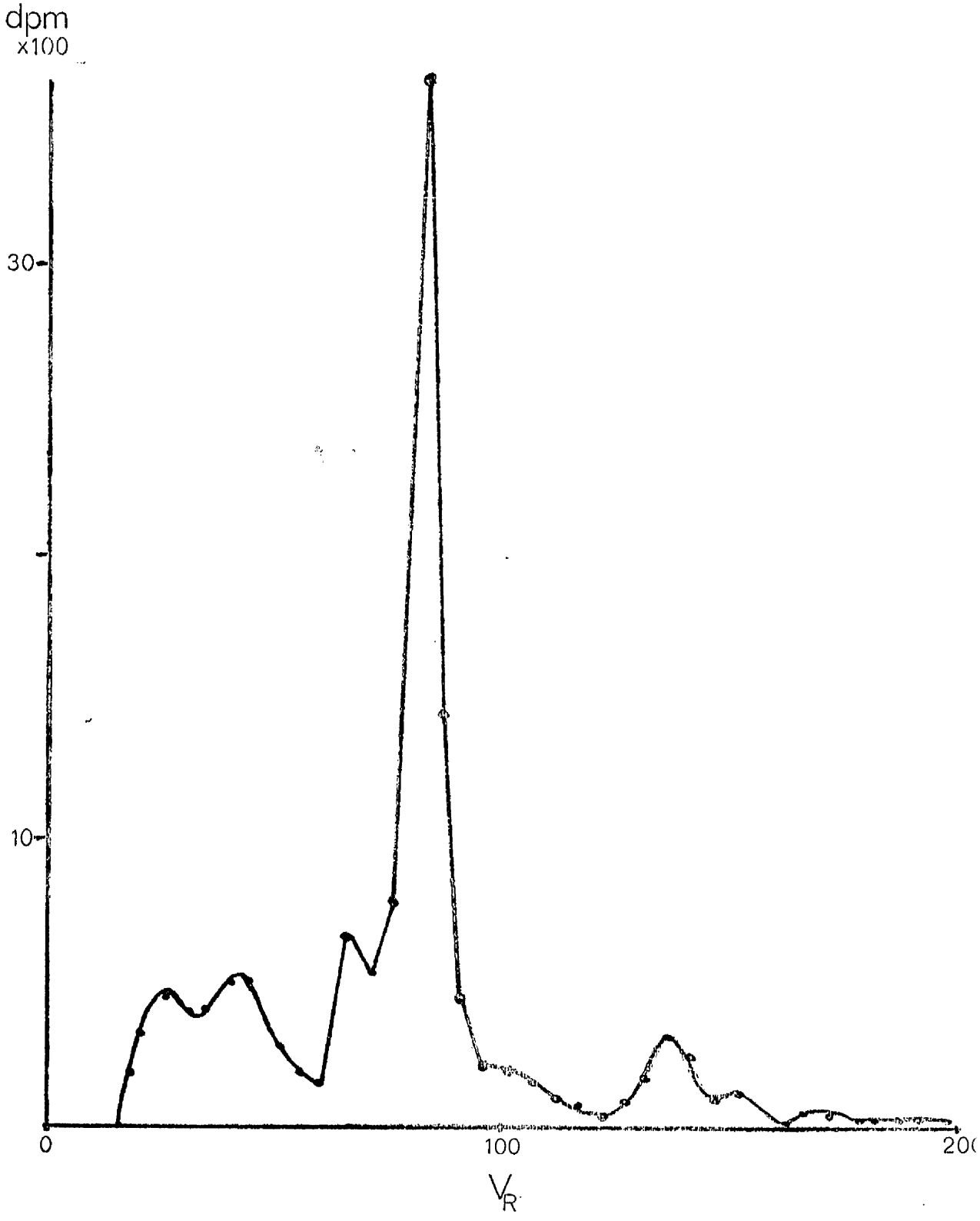

dpm
x 100
30
10
50
100
150
V_R

Figure II : 3 Elution profile from Lipidex column: phenolic fraction from flowering *P. vulgaris*.


dpm
x100
30
10
0
100
200
V_R

was eluted from 75-100 ml (the elution volume of standard [^{14}C]-E_1) and a further 9.14% of the [^{14}C] was eluted between 125-150 ml and this was within the elution volume of E_2. Chromatographing another aliquot on TLC (solvent system (a)) and assaying by the TLC radiochromatogram scanner gave the trace shown in Figure II:4. Three zones of radiocarbon can be observed; one at the origin (47.4%); one at R_f zone 0.36 - 0.49 (40.8%) and one at R_f 0.13 - 0.25 (11.7%). This latter zone co-chromatographed with [^{14}C]-E_2.

A further aliquot was eluted on Lipidex and the zone corresponding to E_2 was collected, reduced to dryness and crystallised to constant specific activity. Table II : 1 shows constant specific activity after three experiments.

Table II : 1 Radioactivity (dpm) found on recrystallising putative [^{14}C]-E_2 with non-radioactive E_2

Replicate	Weight of sample (mg)	dpm (above bg)	dpm mg^{-1}
I	2.8	169	60
II	2.07	139	66
III	4.61	292	63

The remaining phenolic fraction was derivatised with BSA and analysed by radio-GLC. A peak with the same retention time as that of standard [^{14}C]-E_2 was obtained.

The [^{14}C]-E_1 radiochemical was chromatographed in solvent system (b) to check the purity of the solution. The resultant trace is shown in Figure II : 5, and it is evident that there is no significant zone of radioactivity corresponding to E_2.

(b) Metabolism of [^{14}C]-E_1 in various zones of P. vulgaris

In this series of experiments the plants which had been fed with the

Figure II : 4 TLC radiochromatogram of -

(a) Standard [^{14}C]-E_1 and E_2

(b) Phenolic fraction flowering plants.

(Solvent system a)

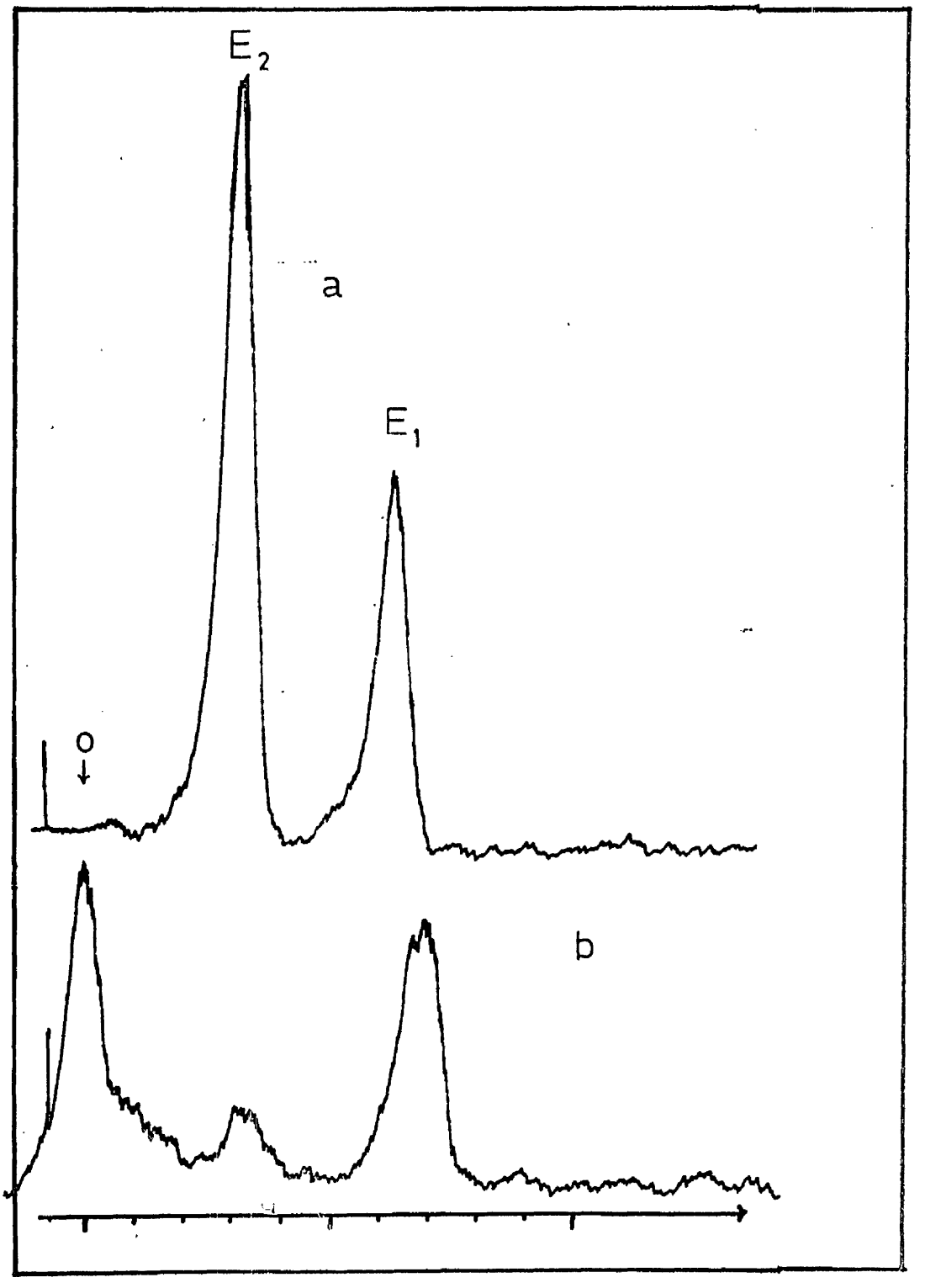
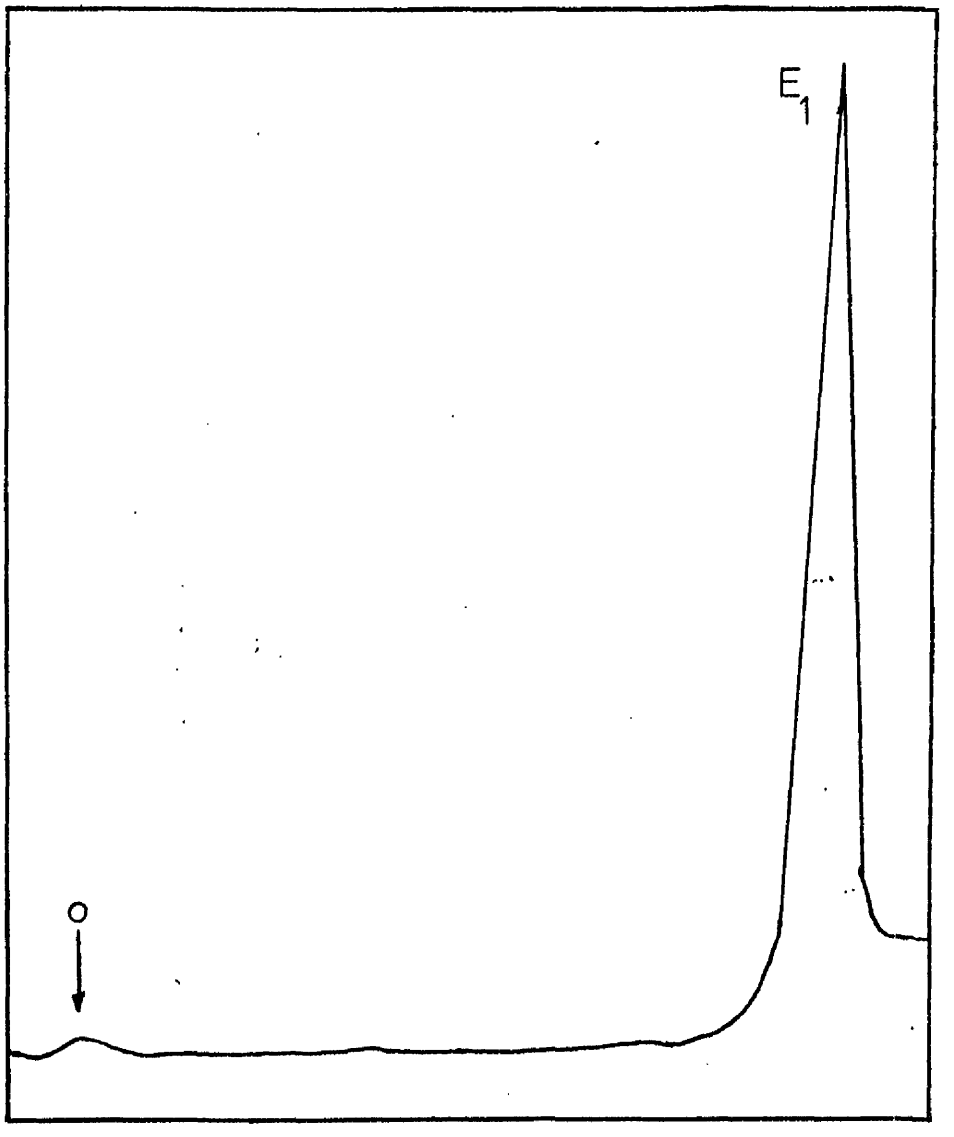

E2
a
E1
O
b

Figure II : 5 TLC radiochromatogram of [^{14}C]-E_1 in solvent system (b).


E1
O

radiocarbon were sectioned into four or five different zones and examined for [^{14}C] in the phenolic fractions.

Twenty seedlings were fed 40 μCi [^{14}C]-E_1 and allowed to grow as before.

(i) Vegetative plants

Eleven days after potting up, nine plants were analysed for the biotransformation of [^{14}C]-E_1. At this stage the plants were almost identical in development to the previous vegetative assays. After dividing the plants into the various fractions viz: roots, shoots, primary leaves and trifoliate leaves, the fresh weight of the tissue was determined before solvent extraction. The weight of the residue was obtained and known aliquots removed for assay in the Oxymat sample oxidiser. On purification, radiocarbon present in the phenolic fraction was assessed (Table II : 2).

Table II : 2 Radiocarbon in various zones of dwarf French bean

Plant Organ	Tissue weight (g) Before	After	[^{14}C] in phenolics	% incorp	Total [^{14}C] (%)	[^{14}C] in tissue residue	Total (%)
Roots	16.2	0.99	40,072	0.10		300,292	
Shoots	9.2	1.08	409,675	1.03	516,313	10,690,926	11,766,058
Primary Leaves	12.1	2.04	64,699	0.16	(1.30)	746,291	(29.71)
Trifoliate Leaves	5.0	0.69	1,867	0.005		28,549	

The incorporation percentages were calculated on the assumption that the nine plants contained 39.6 x 10^6 dpm, the total donated to the plants.

The bulk of the radioactivity was found in the phenolic fraction of

the stems. Indeed it represented 79.3% of the total [^{14}C] recovered from the phenolic fractions of the various zones, albeit only 1.03% of the total administered. Just over 0.26% incorporation was isolated from the other three fractions.

The cotyledons, which normally abscinded at or prior to potting, were also extracted but not purified to phenols before analysis. Combustion of the residue revealed that 317,151 dpm remained after extraction (0.4% of total applied).

Aliquots of the phenolic fractions were chromatographed in solvent system (d) (Figure II : 6). In the roots, three zones of activity can be observed with over 66% being located at the origin. A further 23.1% of the radiocarbon was observed at the R_f zone 0.23-0.32 which corresponded to standard estrone. The remaining 10.38% of the radioactivity was found at R_f 0.13 - 0.21 which was similar to that of standard estradiol.

In the stem, a similar pattern was seen, with three major areas of radiocarbon. The ratio of the peaks, however, was slightly different with only 30.2% of the radioactivity present at the origin and 50.5% at R_f 0.27 - 0.35 ([^{14}C]-E_1). R_f 0.12 - 0.23 corresponds to E_2.

Analysis of the cotyledon fraction revealed that most of the label was located at the origin and in the estrone region.

TLC of the phenolic fractions from the primary leaves, although there being more overall radioactivity than that of the roots, demonstrated the presence of only two major zones. The origin accounted for approximately 34.8% of the radiocarbon compared with 65.2% in the E_1 zone (R_f 0.19 - 0.32). There was very little evidence for the presence of [^{14}C]-E_2.

The remainder of the phenolic fractions were loaded onto thin-layers and developed in solvent system (b), (Figure II : 7). The peak observed at the origin in Figure II : 6 was present in all fractions except in the

Figure II : 6 TLC radiochromatograms from the phenolic fractions of different regions of the plant. (Solvent system d).

(a) Primary leaves

(b) Stem *

(c) Roots

(d) Cotyledons

* 100 cps full scale deflection and time constant 30 sec.

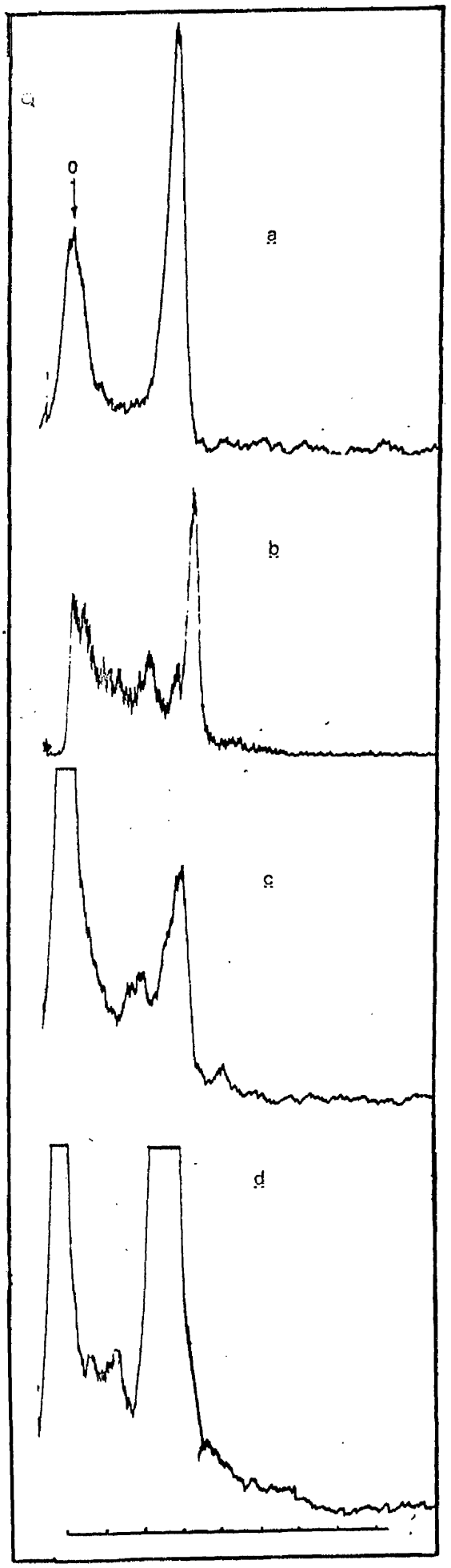

a
b
c
d

Figure II : 7 TLC radiochromatograms from phenolics of regions of *P. vulgaris*.

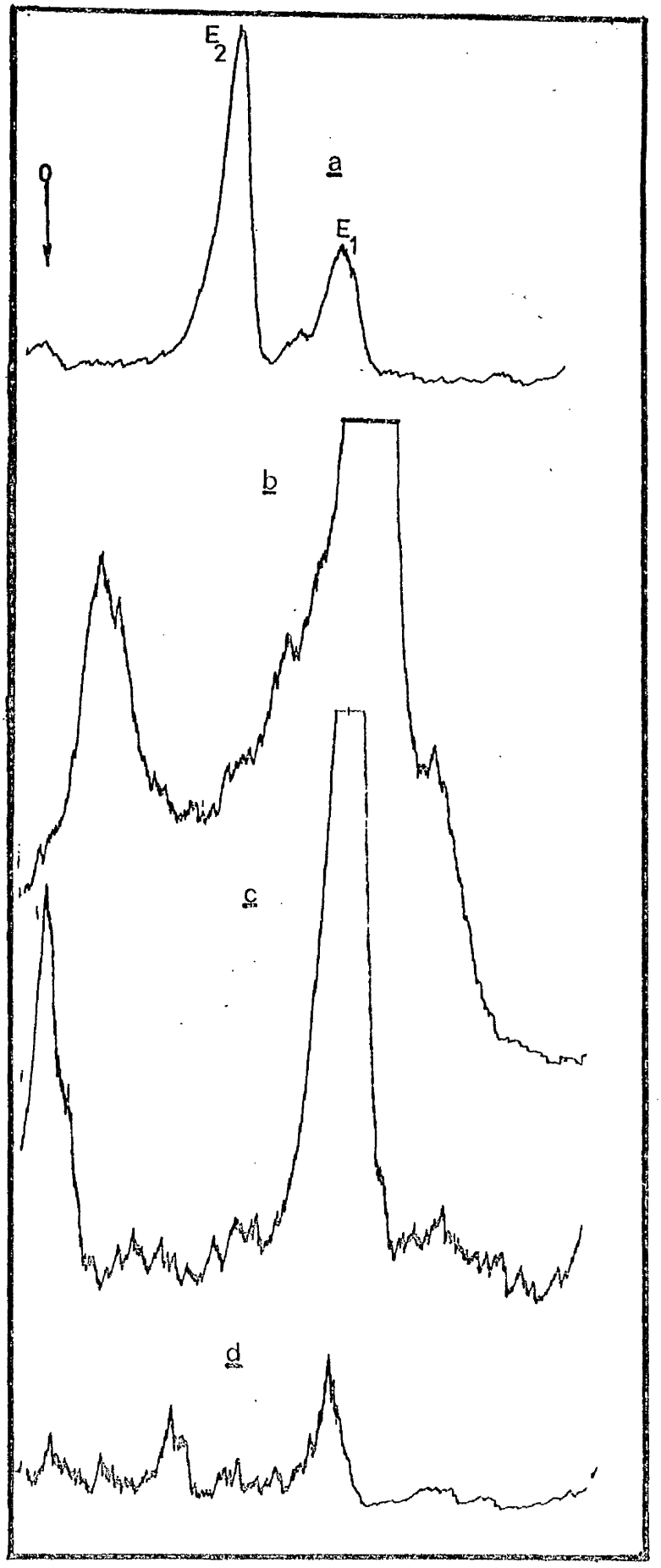
(Solvent system b).

(a) [^{14}C] standard E_1 and E_2

(b) Stem

(c) Cotyledons

(d) Primary leaves


E2
a
E1
b
c
d

primary leaves. There was also little evidence for any radioactivity associated with the zone corresponding to estradiol.

Zones of interest were scraped from the plates and eluted in small chromatographic columns with either 10% EtOH in benzene or MeOH and aliquots removed for radioassay. (Table II : 3).

Table II : 3 R_f and radioactivity of two zones from TLC plates developed in solvent system b.

Plant Organ	Zone 1	[^{14}C]	Zone 2	[^{14}C]
Root	origin	3,958	0.27 - 0.47	924
Stem	0 - 0.27	34,783	0.27 - 0.67	29,801
Cotyledons	0 - 0.07	9,024	0.27 - 0.47	1,867
Primary Leaves	-	-	0.27 - 0.4	2,988
Trifoliate Leaves	-	-	0.27 - 0.4	927

Zones 2 were chromatographed on thin layers in solvent system (e) (Figure II : 8). Only in the stem was there any evidence of estradiol with most of the radioactivity being located at the origin and at the R_f agreeing with E_1.

The origins were chromatographed in a more polar solvent system (f) with E_1 and E_1SO_4 as markers and the radioactivity estimated by TLC radiochromatography (Figure II : 9). Three zones of activity were resolved in extracts of the stem, cotyledon and to a lesser extent in that of roots. One of these zones was placed at a similar R_f to the estrogens, and the bulk of the remainder at the origin. In the cotyledon fraction there was a zone of radioactivity with a similar mobility to E_1 sulphate. The TLC plate was also assessed by autoradiography on X-ray film (Figure II:10).

Figure II : 8 TLC radiochromatograms from phenolic fractions of zones of P. vulgaris (Solvent system e).

(a) Standard [^{14}C]-E_1 and E_2; [^{3}H]-E_1SO_4

(b) Stem *

(c) Root

(d) Primary leaves

* 30 cps full scale deflection; time constant 30 sec.

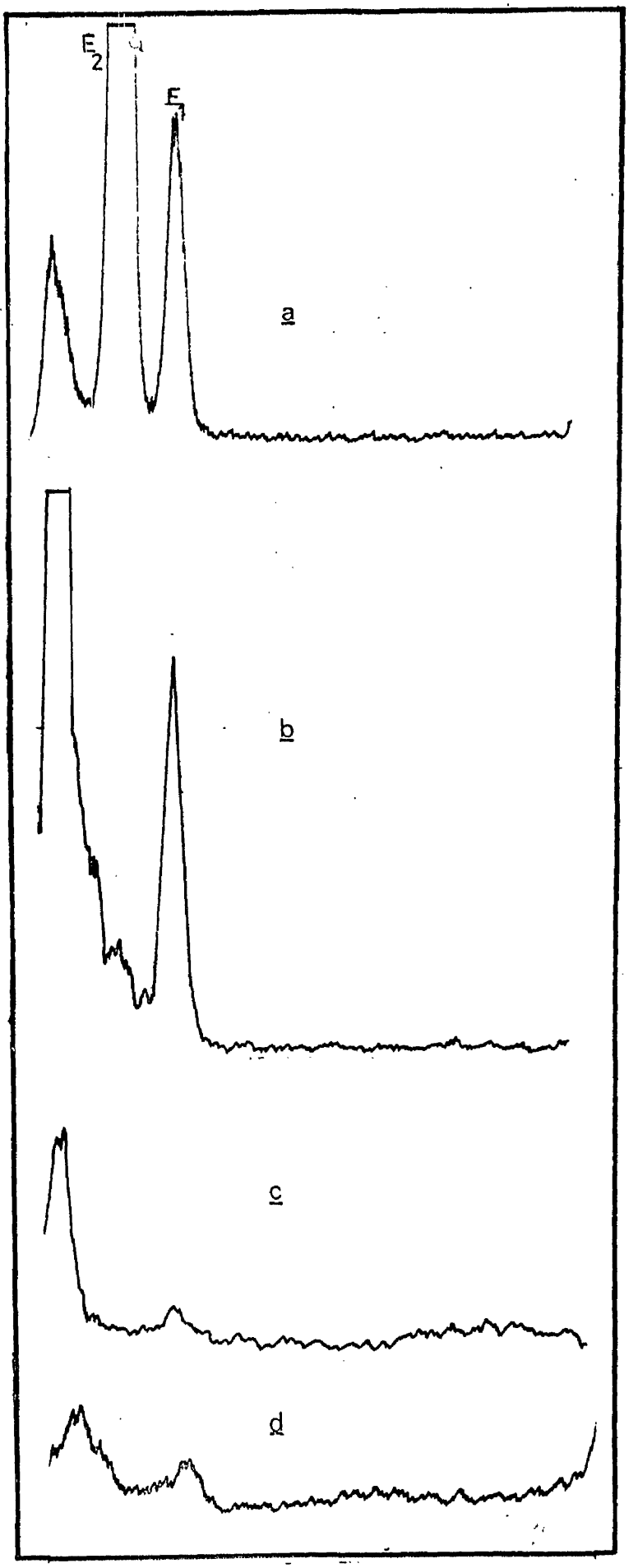

E2
E1
a
b
c
d

Figure II : 9 TLC radiochromatograms of origins from the phenolic fractions. (Solvent system f).

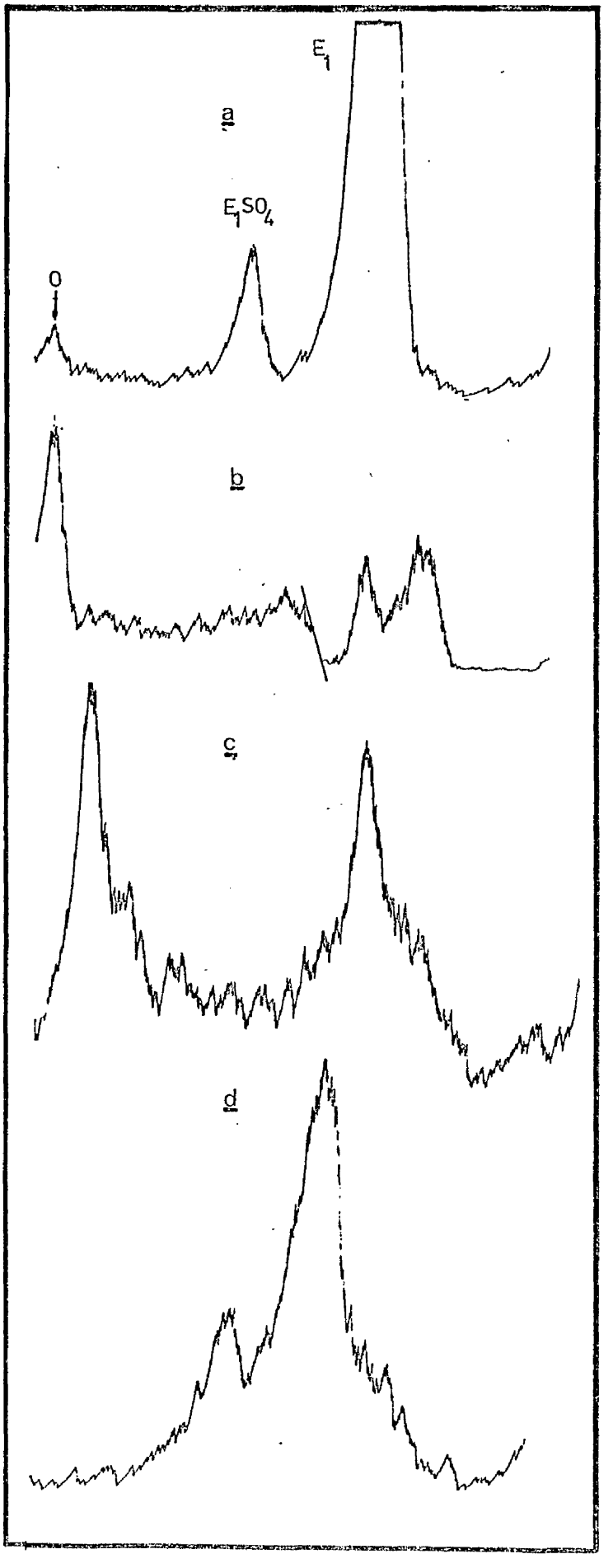
(a) $[^{14}C]$-E_1 and $[^{3}H]$-E_1SO_4

(b) Stem *

(c) Root

(d) Cotyledon

* Scan started with attenuation of 10 cps f.s.d. changed to 3 cps at marker.


E1
a
E1SO4
O
b
c
d

Close examination of this plate revealed six zones of activity in the cotyledons, and three in the stems. The stem chromatogram was overloaded and as a result there was poor resolution. Four zones, some very slight indeed, were observed in the root. There was no peak corresponding to the [^{3}H]-E_1 sulphate in the markers due to the fact that this technique is inappropriate for this isotope.

As some measure of control the contents remaining in the vials in which the seedlings were incubated were also analysed (Figure II:11). There was evidence for more than one zone of activity in this plate, with a zone at the origin and one at a similar R_f to E_2.

(ii) Flowering dwarf French bean

Nine flowering plants (31 days after potting) were divided into their various portions as before, with the addition of flowers extracted in the normal fashion and the phenolic fractions isolated. Aliquots were removed from the tissue residue and the radiocarbon remaining was assessed by means of the sample oxidiser and scintillation spectrometry (Table II : 4).

Table II : 4 [^{14}C] in phenolic fraction and remaining in tissue after extraction

Plant Organ	Tissue weight Before	After	[^{14}C] in phenolics	% incorp	Total [^{14}C] %	[^{14}C] in tissue residue	Total %
Root	46.8	5.813	14,677	0.04		172,930	
Stem	16.1	3.37	354,300	0.89	386,762	1,255,789	2,620,571
Primary Leaves	15.5	2.20	12,048	0.03	(0.98)	1,153,294	(6.62)
Trifoliate Leaves	26.1	4.33	4,217	0.01		36,105	
Flowers	2.2	0.16	1,520	0.003		2,453	

Of the [^{14}C] extracted from the tissues 91.6% was secured from the

Figure II : 10 Photograph taken from autoradiography on X-ray film.

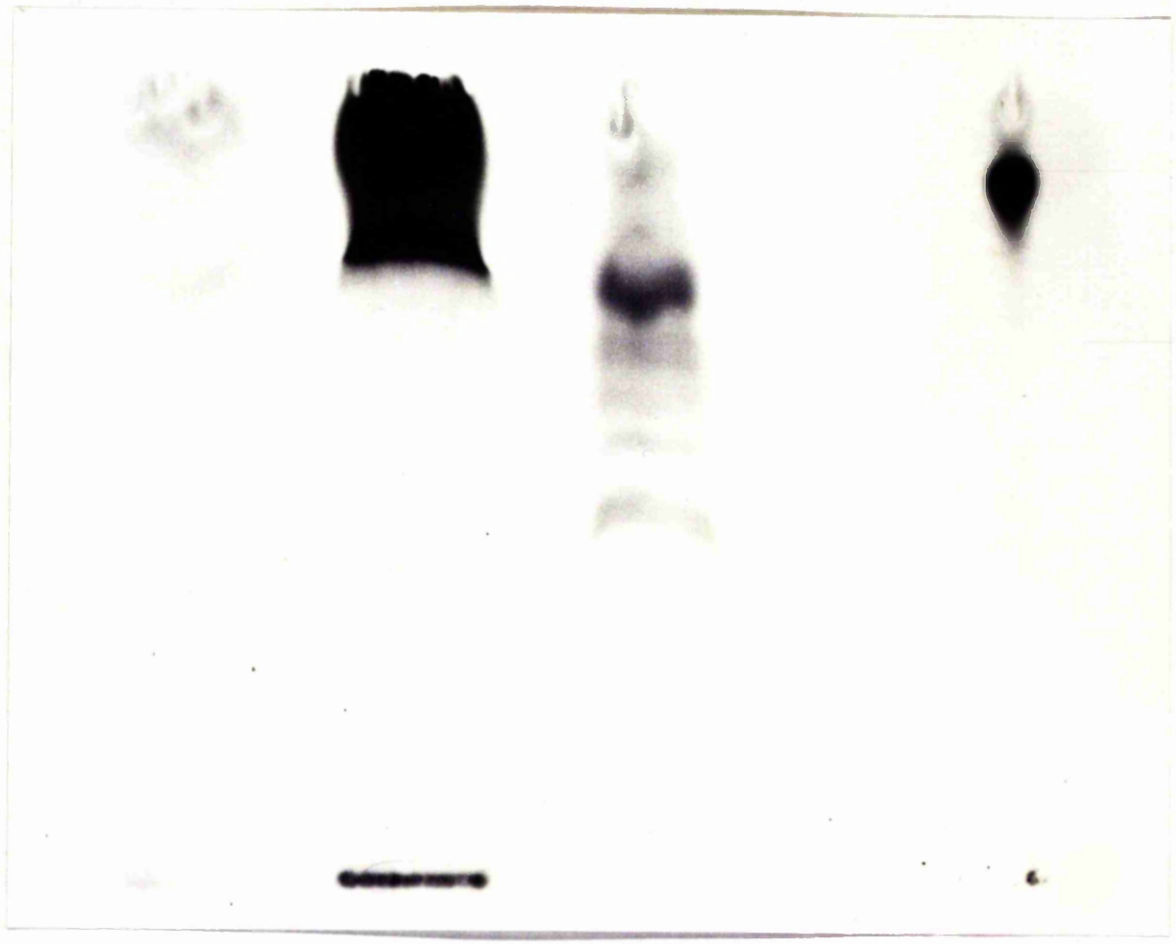
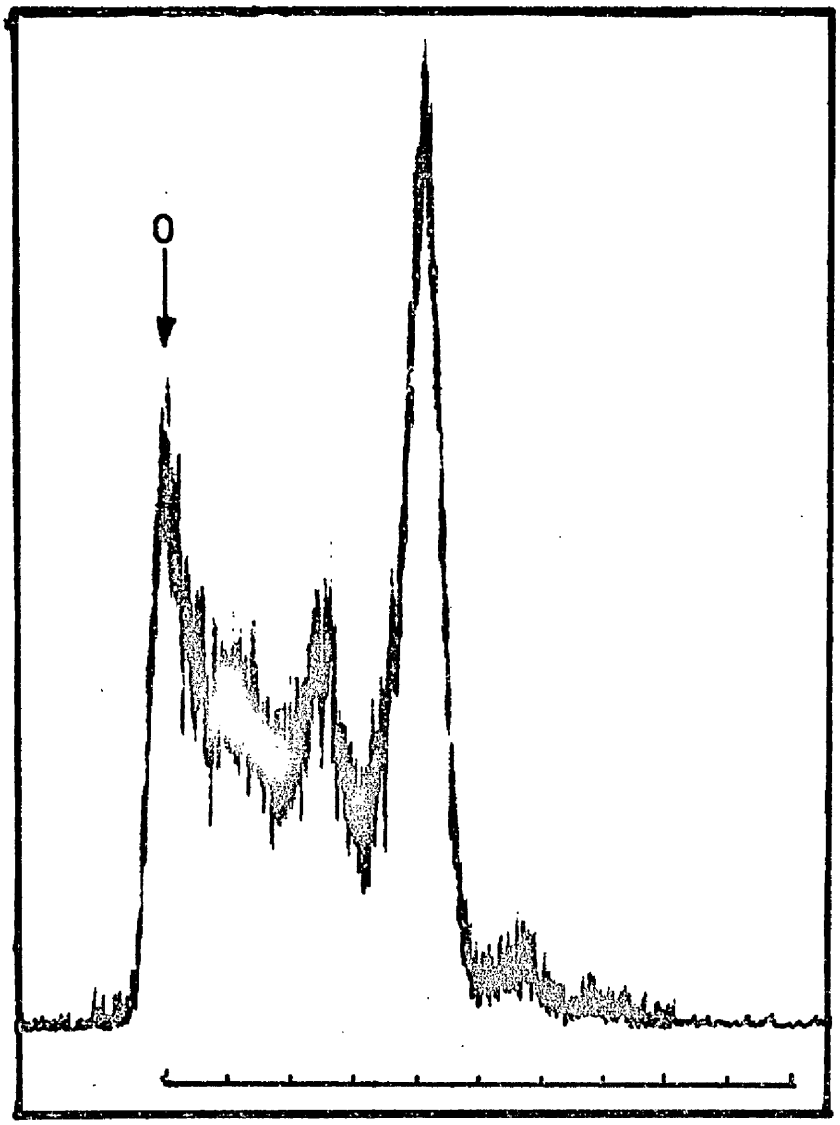


Figure II : 11 TLC radiochromatogram of [^{14}C] remaining in incubating medium (Solvent system b).


O

stem fraction although this only accounted for 0.89% of the total radiocarbon fed to the plant. Smaller amounts were found in the roots, primary leaves, trifoliate leaves and flowers. Analysis of the residue revealed that 47.92% of the [^{14}C] was situated in the stem with a further 44.01% in the primary leaf residue.

The phenolic fractions were then loaded onto 250 μm TLC plates and chromatographed in solvent system (c). Reference to Figure II : 12 revealed that in the shoot and root tissue there were three major zones of activity while in the primary leaves there were only two. The peak at the origin accounted for ca 25% of the radioactivity whereas that in the primary leaves represented 35.1% and that in the root 51.6%. Small peaks corresponding to 15.4%, 15.8% and 6.5% of radioactivity present in shoot, root and primary leaves respectively were located at a similar R_f to E_2.

Very little radioactivity was detected in the phenolic fraction of either the trifoliate leaves or the flowers. Consequently the TLC adsorbent was cut up into 30 sections each placed in a scintillation vial and the [^{14}C] assessed by scintillation spectrometry. The results obtained are shown in Figure II : 13 with large peaks at the origin and corresponding to E_1. This was further confirmed by placing the remainder of the adsorbent on the plate in contact with X-ray film for five weeks, when two zones corresponding to those above were detected.

The zones corresponding to E_2 were removed from the shoot and root plates and eluted. Although 57,759 dpm were located in shoot, only 1,976 dpm was detectedin the same zone in roots. These were chromatographed in solvent system (e) (Figure II : 14). Three zones of radioactivity were detected with one corresponding to E_2.

A peak at the origin alone was observed in roots. The zone corresponding to estradiol from the shoot fraction was obtained in the con-

Table II : 12 TLC radiochromatograms of the phenolic fractions of zones of flowering P. vulgaris Solvent system (c).

(a) $[^{14}C]$-E_1 and E_2

(b) Stem*

(c) Roots

(d) Primary leaves

* 30 cps full scale deflection and 30 sec time constant

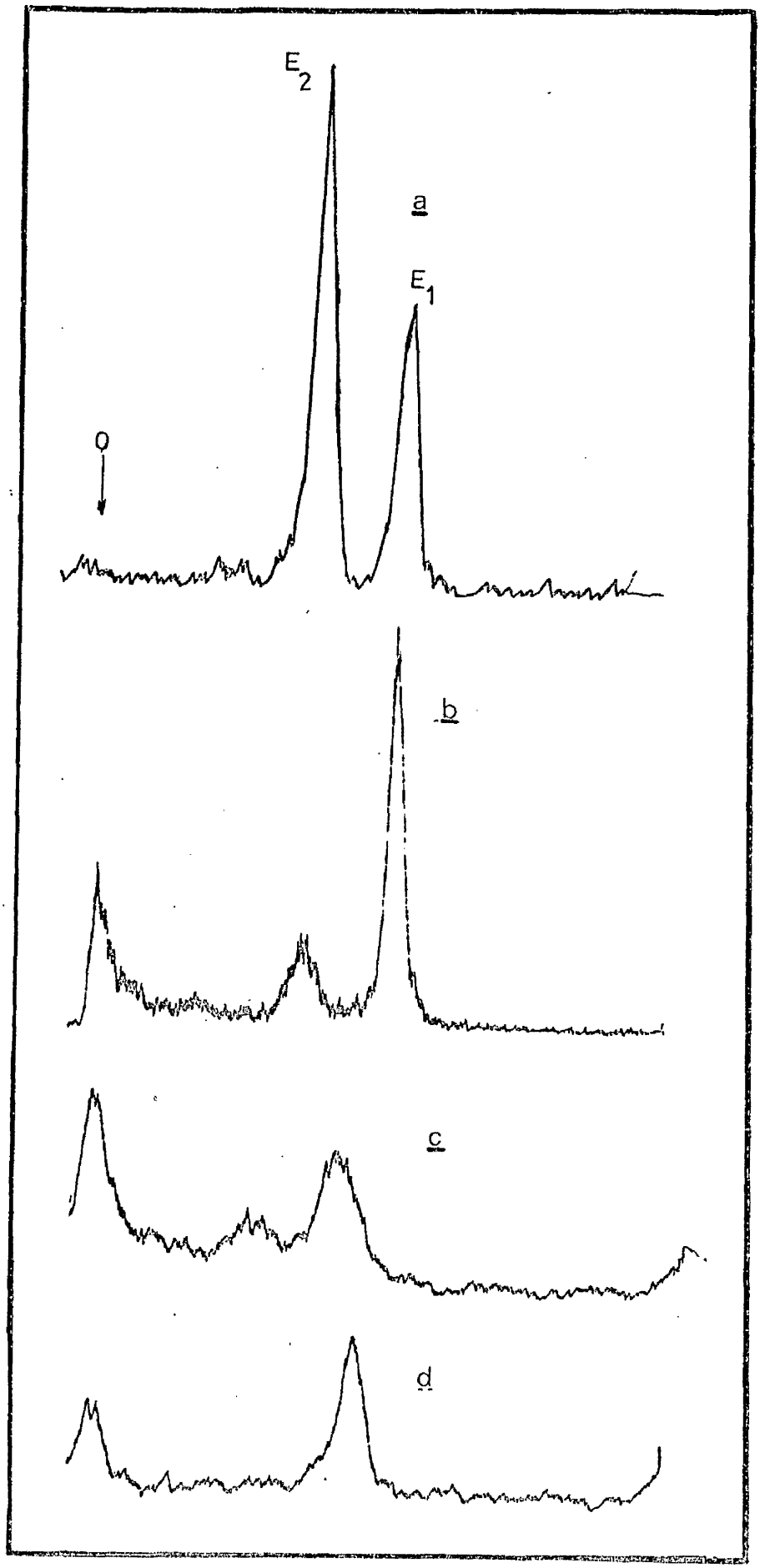

E2
a
E1
0
b
c
d

Figure II : 13 Histograms of radiocarbon from TLC assayed by scintillation spectrometry.

(Solvent system c)

(a) Flowers

(b) Trifoliate leaves

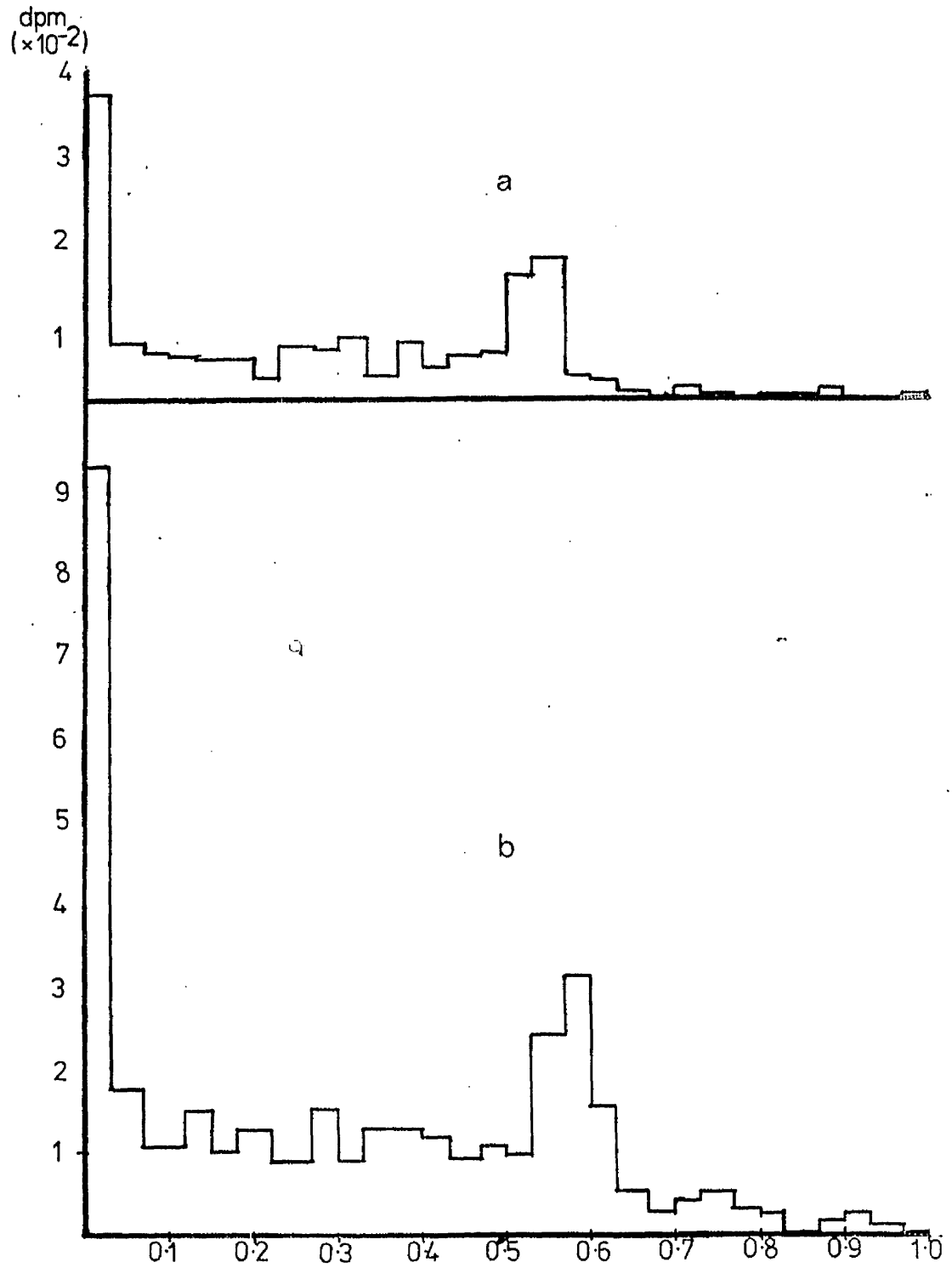

dpm
(×10⁻²)
4
3
2
1
a
9
8
7
6
5
4
3
2
1
b
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0

Figure II : 14 TLC radiochromatograms of putative E_2 zones. (Solvent system e).

(a) Standards $[^{14}C]$-E_1 and E_2 and $[^{3}H]$-E_1 sulphate.

(b) Stem

(c) Root

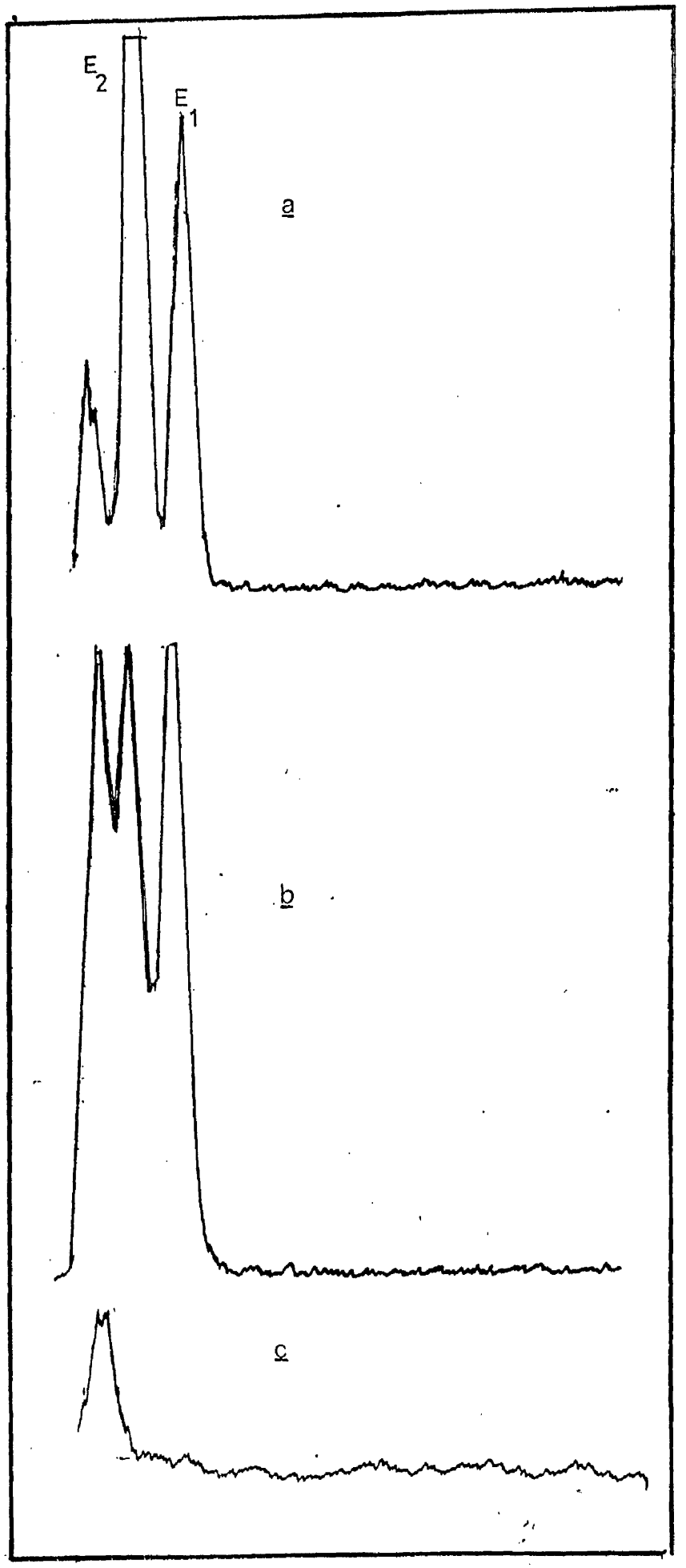
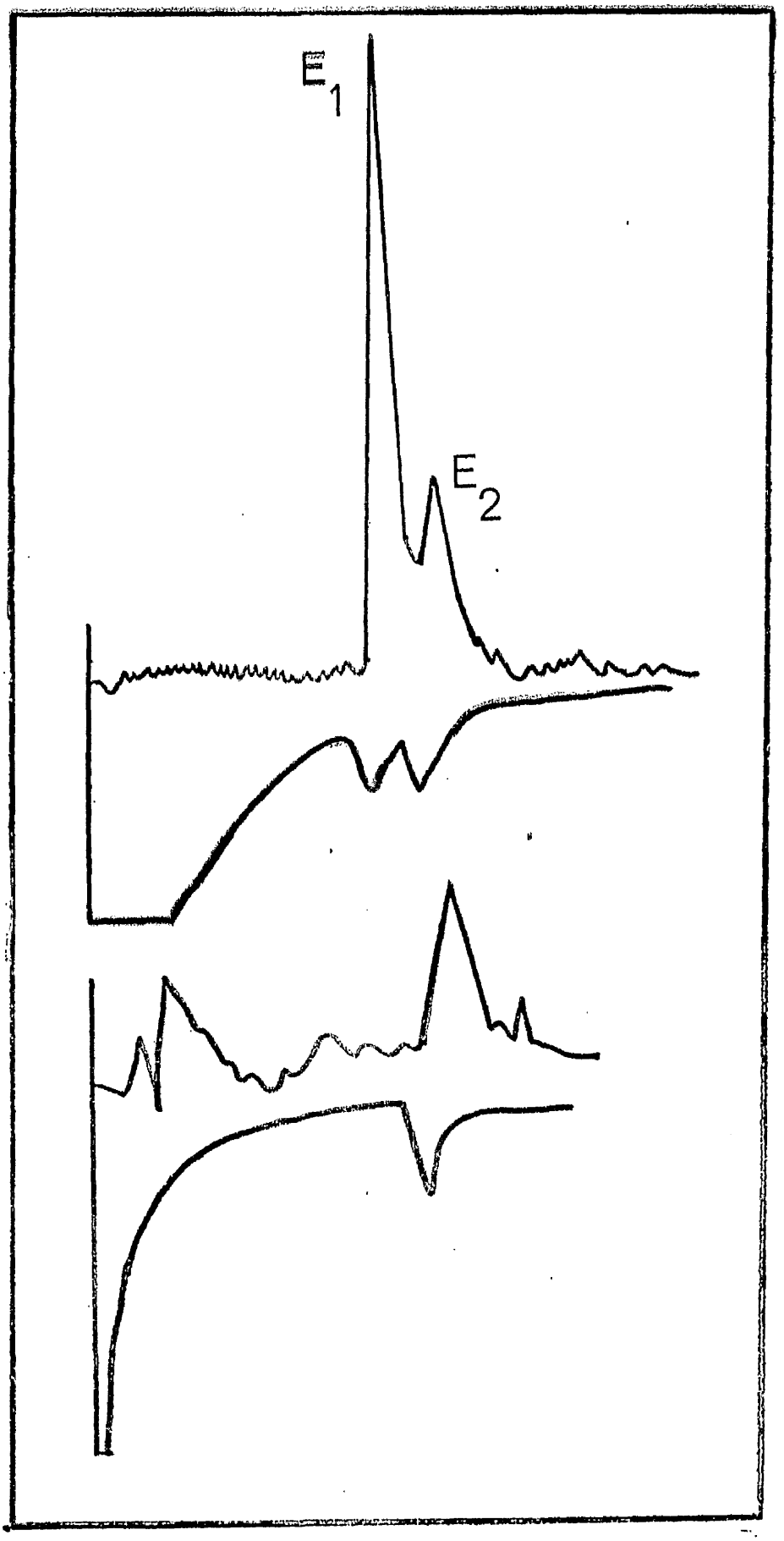

E2
E1
a
b
c

Figure II : 15 Radiochromatographs from 5 ft. 5% OV 101 column.

(a) Standard [^{14}C]-E_1 and E_2

(b) Putative E_2 from the stem phenolic fraction.


E1
E2

ventional manner, reacted with BSA and chromatographed on radio GLC (Figure II : 15). One peak with the same retention time as authentic [^{14}C]-E_2 TMSi was obtained.

(c) Distribution of [^{14}C] in dwarf French bean after the application of [^{14}C]-E_1

Concurrently with the metabolism study an assessment was carried out of the uptake of the radiocarbon by the seedlings. This also involved evaluating the most useful method for estimating the label within the various sections.

Table II : 5 summarises the data obtained from the first series of experiments where 0.5 μCi of [^{14}C]-E_1 was fed to the plants.

Table II : 5 Distribution I of [^{14}C] in seedlings

	Prior to potting up		Vegetative			
	*[^{14}C] (dpm)	%	**[^{14}C] (dpm)	%	***[^{14}C] (dpm)	%
Apex	1,942	0.96	0	0	51	0.005
3rd Internode	-	-	0	0	44	0.004
2nd Trifoliate	-	-	37	0.02	219	0.02
2nd Internode	-	-	40	0.02	75	0.007
1st Trifoliate	-	-	309	0.16	1,922	0.18
1st Internode	-	-	452	0.23	278	0.02
Primary Leaves	34,786	17.2	5,472	2.88	47,472	4.43
Epicotyl	12,732	6.3	4,632	2.44	5,316	0.50
Hypocotyl	152,827	75.5	115,813	61.0	1,009,784	94.34
Roots	-	-	63,055	33.22	5,097	0.48
Total	202,287	99.96	189,810	99.97	1,070,258	99.99

* Plant material extracted with scintillation fluid (3 replicates)

** Plant material extracted with acetone before addition of scintillation fluid.

*** Analysis by sample oxidiser.

Table II : 6 Distribution of [^{14}C] in flowering plants fed with [^{14}C]-E_1 from experiment I

	[^{14}C] dpm	%
Flowers	27	0.004
5th Trifoliate	0	0
4th Trifoliate	282	0.04
4th Internode	39	0.005
3rd Trifoliate	382	0.05
3rd Internode	35	0.005
2nd Trifoliate	397	0.05
2nd Internode	64	0.009
1st Trifoliate	639	0.09
1st Internode	339	0.05
Primary leaves	9,781	1.36
Epicotyl	9,038	1.26
Hypocotyl	669,534	93.28
Roots	27,154	3.71
Total	717,711	99.93

From the three plants analysed before potting some radiocarbon was observed in the apex of two of the plants, however, the majority of the label remained in the hypocotyl (over 76.5% with a range of 53.9 - 99% in the replicates) with only some 3.6% of the label reaching the primary leaves. Nevertheless, the 202,287 extracted from the plant tissue only accounts for 18.39% of the label added to the plants. In the vegetative plants the same sort of labelling pattern was observed with between 2.88 and 4.43% of the total extractable [^{14}C] in the leaves but again most of the label in the hypocotyl region 61% in the case of the acetone-extracted plants and 94.34% in the combusted material.

Radiocarbon was detected as far as the 2nd trifoliate leaf with the more significant amount being found in the combusted material (219 dpm). Although this only represented 0.02% of the total label administered it was over five times the background (40 cpm for this assay) and must therefore be regarded as real.

The most significant fact, however, is that only 189,810 dpm was located in the tissue extracted by acetone whereas over 1,070,000 dpm were secured by combustion of the tissue.

In the flowering plants a similar pattern was observed (see Table II : 6), small amounts of radioactivity being detected in the trifoliate leaves, although nothing significant in the flowers (Figure II : 16).

In the second series of experiments involving metabolism in different regions of the plants, one plant was removed at the vegetative stage and one at the flowering (Table II : 7 and Figure II : 17).

Figure II : 16 % distribution of [^{14}C] in the various plant regions. (Plants supplied with [^{14}C]-E_1.)

(a) Seedlings after incubation

(b) Vegetative

(c) Flowering

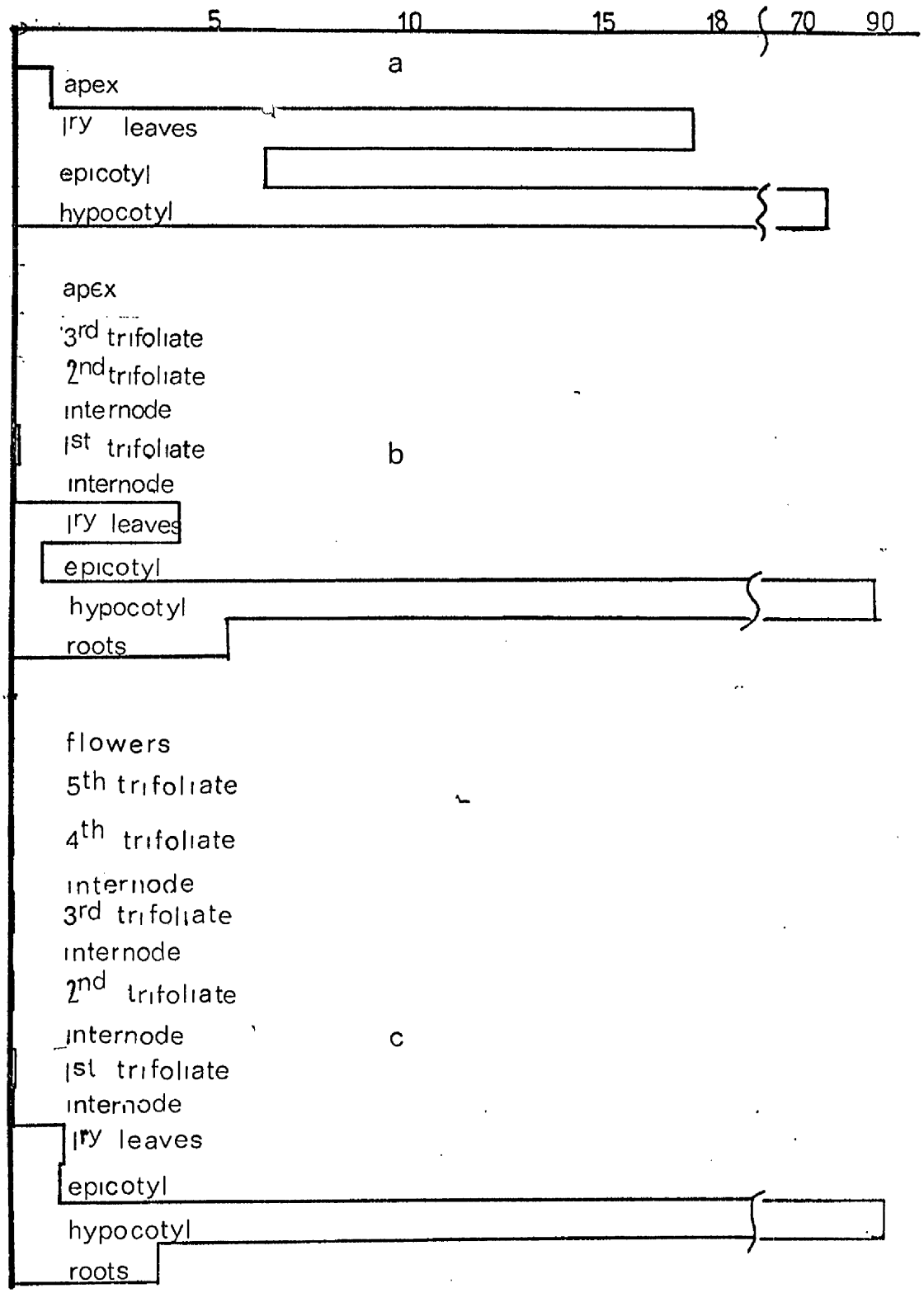

5
10
15
18
70
90
a
apex
Iry leaves
epicotyl
hypocotyl
apex
3rd trifoliate
2nd trifoliate
internode
Ist trifoliate
internode
Iry leaves
epicotyl
hypocotyl
roots
b
flowers
5th trifoliate
4th trifoliate
internode
3rd trifoliate
internode
2nd trifoliate
internode
Ist trifoliate
internode
Iry leaves
epicotyl
hypocotyl
roots
c

Figure II : 17 % distribution (II) of [^{14}C] in plants regions (as fed with [^{14}C]-E_1).

(a) Vegetative

(b) Flowering

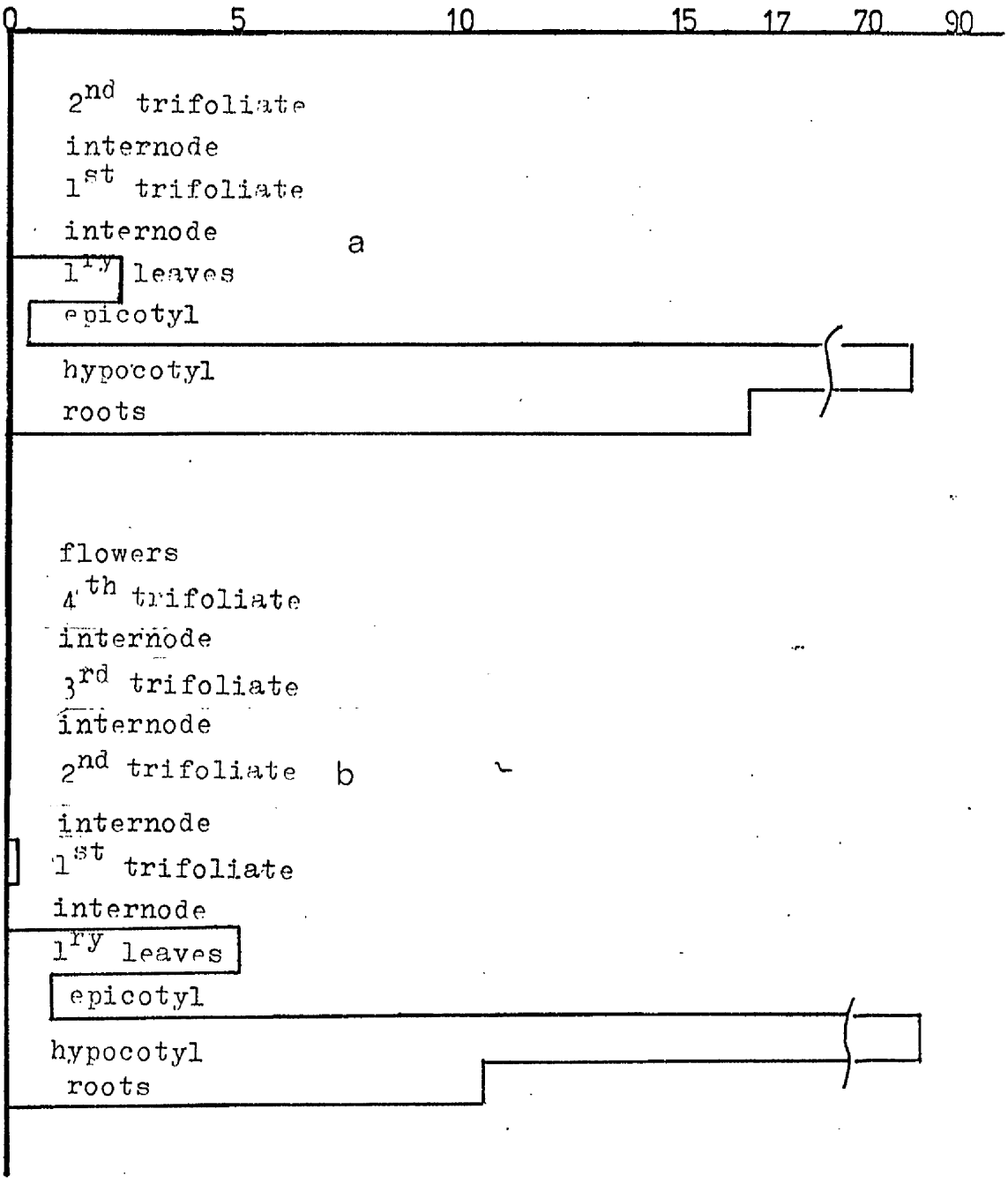

0
5
10
15
17
70
90
2nd trifoliate
internode
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots
a
flowers
4th trifoliate
internode
3rd trifoliate
internode
2nd trifoliate
internode
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots
b

Table II : 7 Distribution of [^{14}C] after feeding with [^{14}C]-estrone (II)

	Vegetative		Flowering	
	[^{14}C] dpm	%	[^{14}C] dpm	%
Flowers	-	-	511	0.02
4th Trifoliate	-	-	98	0.004
4th Internode	-	-	477	0.02
3rd Trifoliate	-	-	709	0.03
3rd Internode	-	-	514	0.02
2nd Trifoliate	107	0.005	1,174	0.05
2nd Internode	-	-	255	0.01
1st Trifoliate	647	0.03	6,207	0.24
1st Internode	208	0.01	580	0.02
Primary leaves	49,245	2.52	128,905	5.1
Epicotyl	9,561	0.49	25,178	1.0
Hypocotyl	1,576,597	80.5	2,080,974	82.9
Roots	321,123	16.4	265,427	10.6
Total	1,957,488	99.9	2,511,009	100.0

The overall pattern was very similar to that obtained in the earlier plants with 511 dpm extracted from the flowers - again only 0.02% of total extracted but equivalent to twenty times background (25 cpm for this assay), with similar amounts detected in the internodes and upper trifoliate leaves. As usual over 84% of label was situated in the hypocotyl.

Thus there is evidence by TLC, column chromatography, crystallisation to constant specific activity and radio-GLC that P. vulgaris can

transform E_1 to E_2, and this appears to be synthesised in the stem. In this investigation it was established that the most efficient method of ascertaining any radiochemical in plant tissues is assaying by the Oxymat sample oxidiser. The study has also demonstrated that less than 25% of the radiocarbon moves above the hypocotyl which confirms the results obtained from the study of E_1 conversion to E_2 in different zones of the plant.

III

Metabolism of [6,7-^{3}H]-estrone sulphate in whole plants

(a) Because of the trouble encountered with the uptake of estrone a suitable alternative was sought. Estrone sulphate, a water-soluble, ether-insoluble steroid conjugate was therefore employed as a precursor.

Twenty-one seedlings were each treated with 5 µCi of potassium [6,7-^{3}H]-estrone monosulphate (M and M, 10). Five days later, at potting up, the cotyledons were removed and assayed for radioactivity. The cotyledons afforded 1,361,990 dpm when extracted. An aliquot was chromatographed in solvent system (f) with [^{3}H]-E_1 sulphate and [^{14}C]-E_2 as markers (E_1 and E_2 are not separated in this system.) Figure III : 1a shows that there were two zones of radioactivity. Tritium was detected at the R_f corresponding to E_1 sulphate (18.2%). The remainder of the label was situated at R_f 0.66 - 0.81 which corresponded to the free estrogens. Further aliquots were chromatographed in solvent systems (d) and (e) and assayed by TLC radiochromatography (Figure III : 1b and c). There was 25.1% of the radioactivity present at R_f zone 0.25 - 0.39 and a further 27.2% located at 0.36 - 0.47 which corresponded to E_2 and E_1 respectively (Figure III : 1b). A similar pattern was observed in Figure III : 1c where three zones of activity were evident, 23.3% at the origin, 38.3% at R_f zones 0.22 - 0.29 and 0.31 - 0.41 which co-chromatographed with E_2 and E_1 respectively.

The remaining crude extract was reacted with BSA and assayed by radio-GLC. Two peaks with retention times closely resembling those of standard [^{14}C]-E_1 and E_2 standards were obtained.

Figure III : 2 shows the TLC scan of the feeding solution (137,000 dpm) remaining after incubation and although there was breakdown (probably to E_1) there was no significant amount of radioactivity co-chromatographing with E_2.

Figure III : 1 TLC radiochromatograms of the cotyledon extracts in different solvent systems Plants fed with $[^3H]$-E_1SO_4.

(a) Solvent system (f)
(b) Solvent system (d)
(c) Solvent system (e)

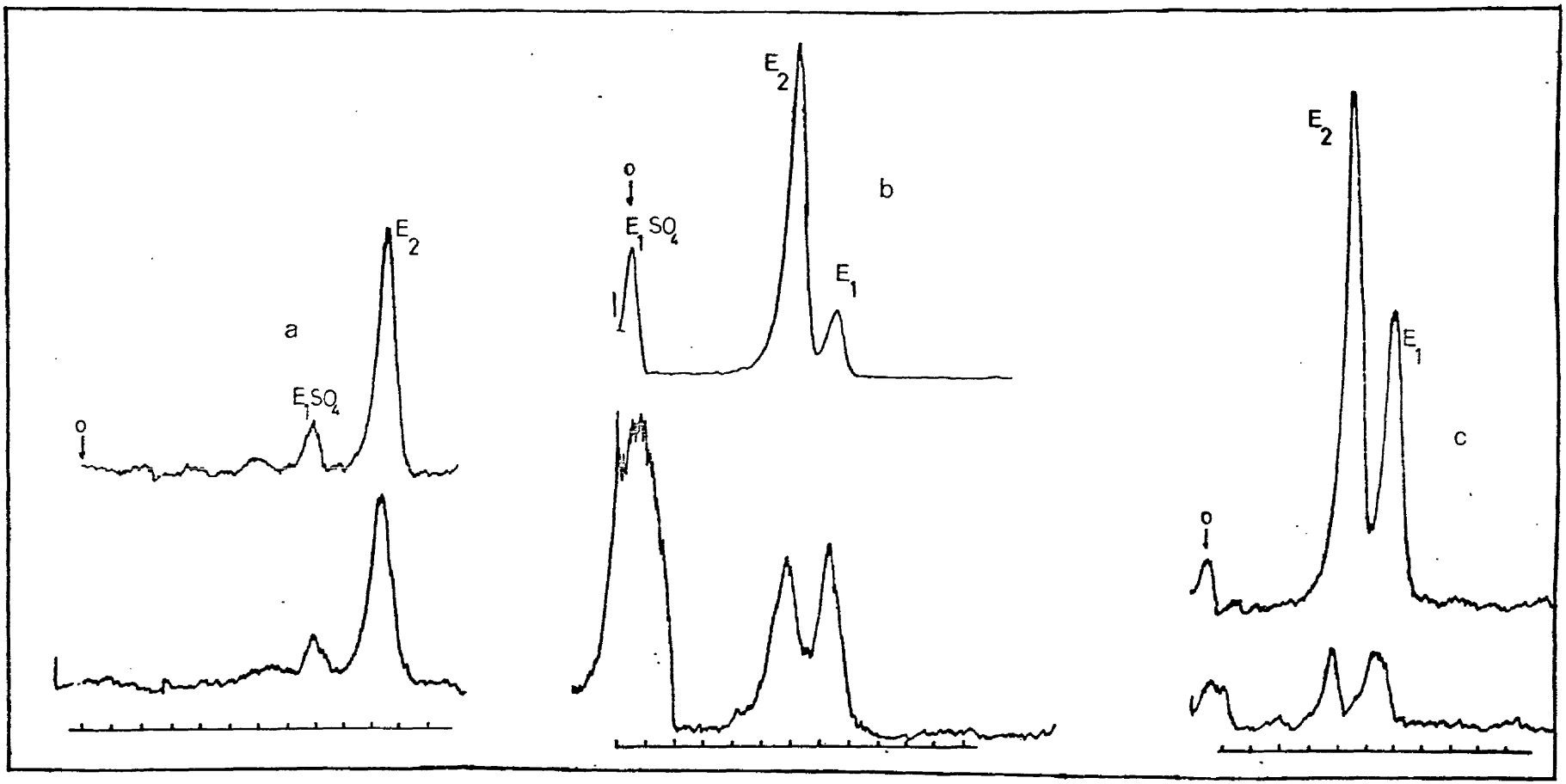
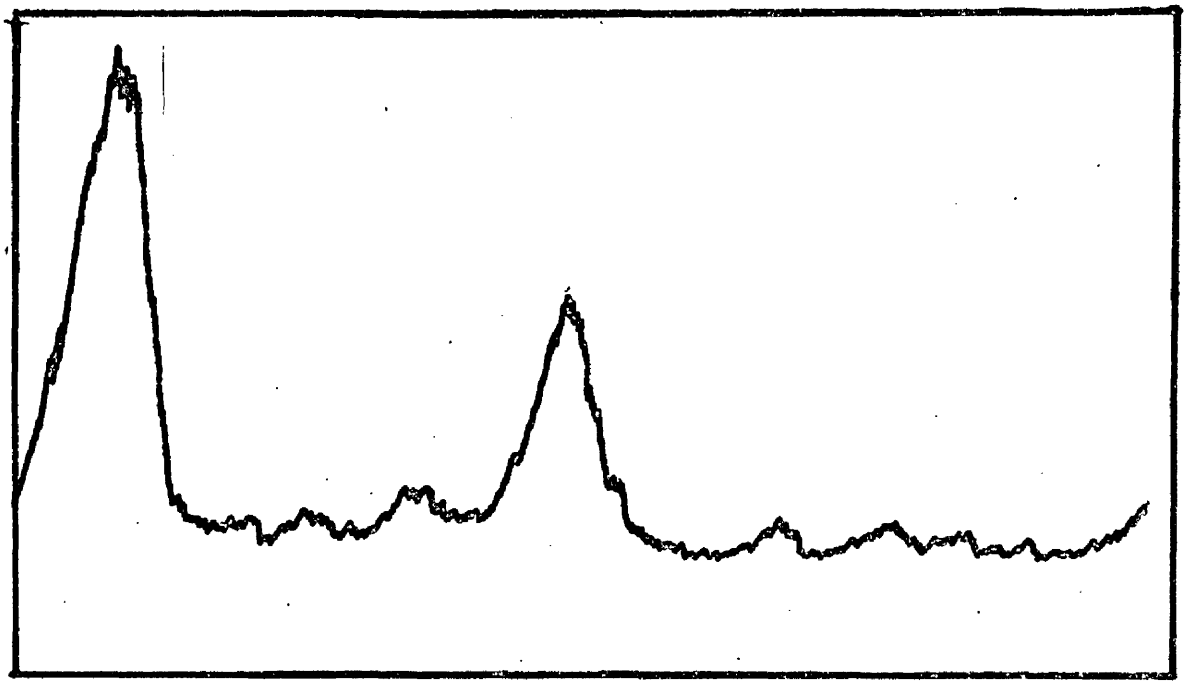

a
E2
E1SO4
o
b
E2
o
E1SO4
E1
c
E2
E1
o

Figure III : 2 TLC radiochromatogram of [^{3}H] remaining in the incubating medium.
(Solvent system b).



Twenty-seven days after potting up, nine plants (65 g) were harvested and processed as previously described. At this stage of development the plants were producing flower buds.

The phenolic fraction obtained from these plants was chromatographed in solvent systems (b), (d) and (e) and results exhibited in Figure III : 3. In all but the C_6H_6 : Pr-2-OH chromatogram there were two major zones of radioactivity which corresponded to E_1 and E_2 respectively. On chromatographing along with $[^{14}C]$-E_1 and E_2 as TLC standard no differences were detected between standard and sample. Although chromatographed on GLC, however, little evidence was obtained for the presence of either peak, as any peaks of radioactivity were too close to background noise to be unequivocal.

Flowering plants were harvested after 34 days. The phenolic fractions (337,500 dpm) were chromatographed in solvent systems (b), (d) and (e) (Figure III : 4). The now familiar pattern was observed with peaks of radioactivity with similar chromatographic mobilities to E_1 and E_2 but the putative E_2 was greater than E_1 and although the ratio does not differ between solvent systems 2.2:1(b), 4.75:1(d) and 5.4:1(e). Radiocompounds with the same retention time as E_1 and E_2 TMSi ethers were found on analysing this fraction by radio GLC.

(b) Metabolism of $[^3H]$-estrone sulphate in various plant organs

(i) Vegetative

Having established that E_1 sulphate can be metabolised to estrone and estradiol, the theme was continued. In this instance, however, only 2 μCi were applied to each plant. Nine days after potting up, the plants were harvested and sectioned into the appropriate tissues. Extraction and purification determined the presence of tritium in the phenolic fraction (Table III : 1).

Figure III : 3 TLC radiochromatograms of phenolic fractions of budding dwarf French bean supplied with $[^3H]$-E_1SO_4.

Solvent systems:-

(a) - $CHCl_3$:MeOH (95:5)

(b) - C_6H_6LPr-2-OH (95:5)

(c) - Light petrol:EtOAc (75:25)

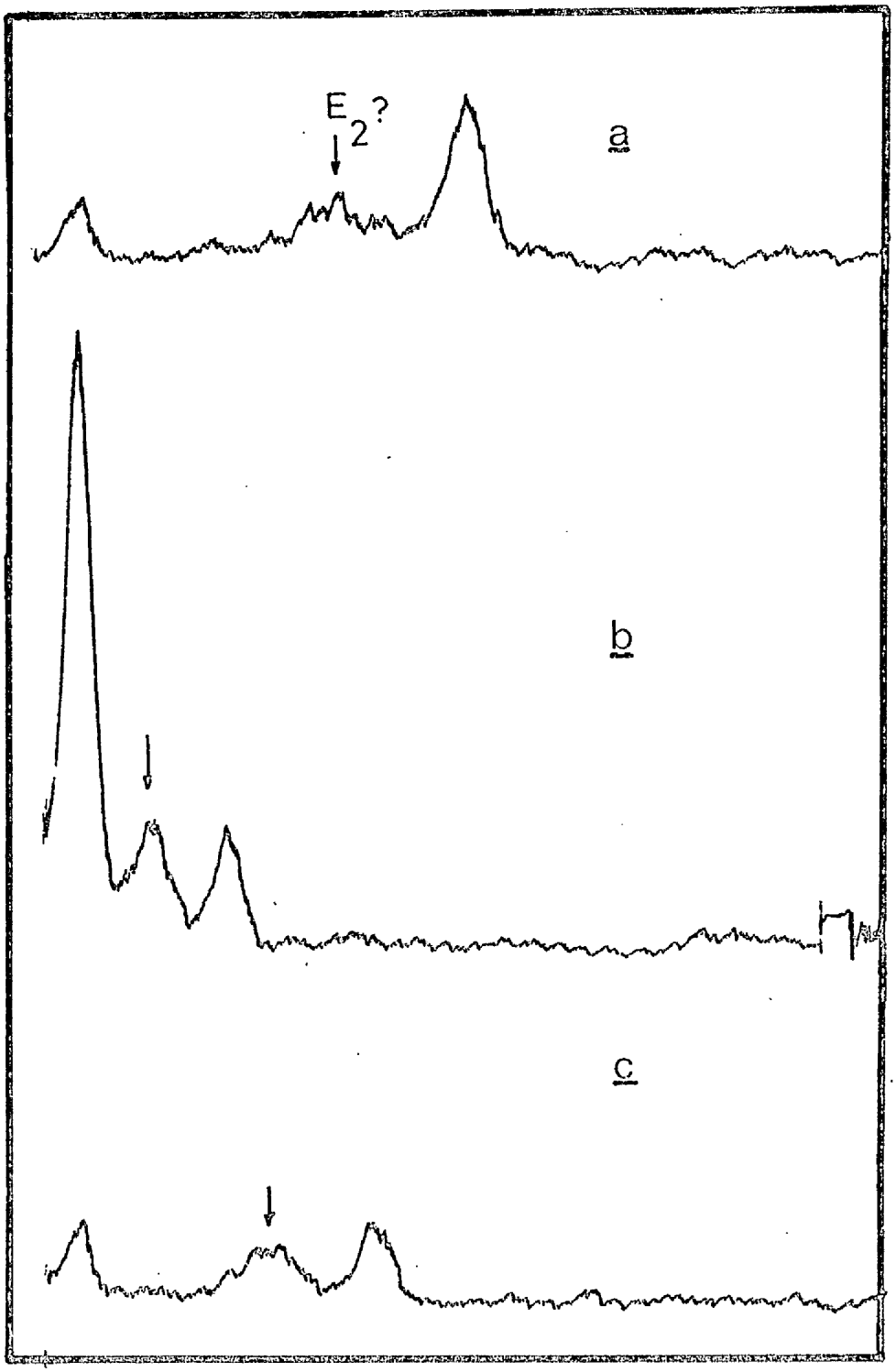

E2?
a
b
c

Figure III : 4 TLC radiochromatograms of phenolic fractions from flowering dwarf French bean.

(a) $CHCl_3$:MeOH (95:5)

(b) C_6H_6:Pr-2-OH (95:5)

(c) Light petrol:EtOAc (75:25)

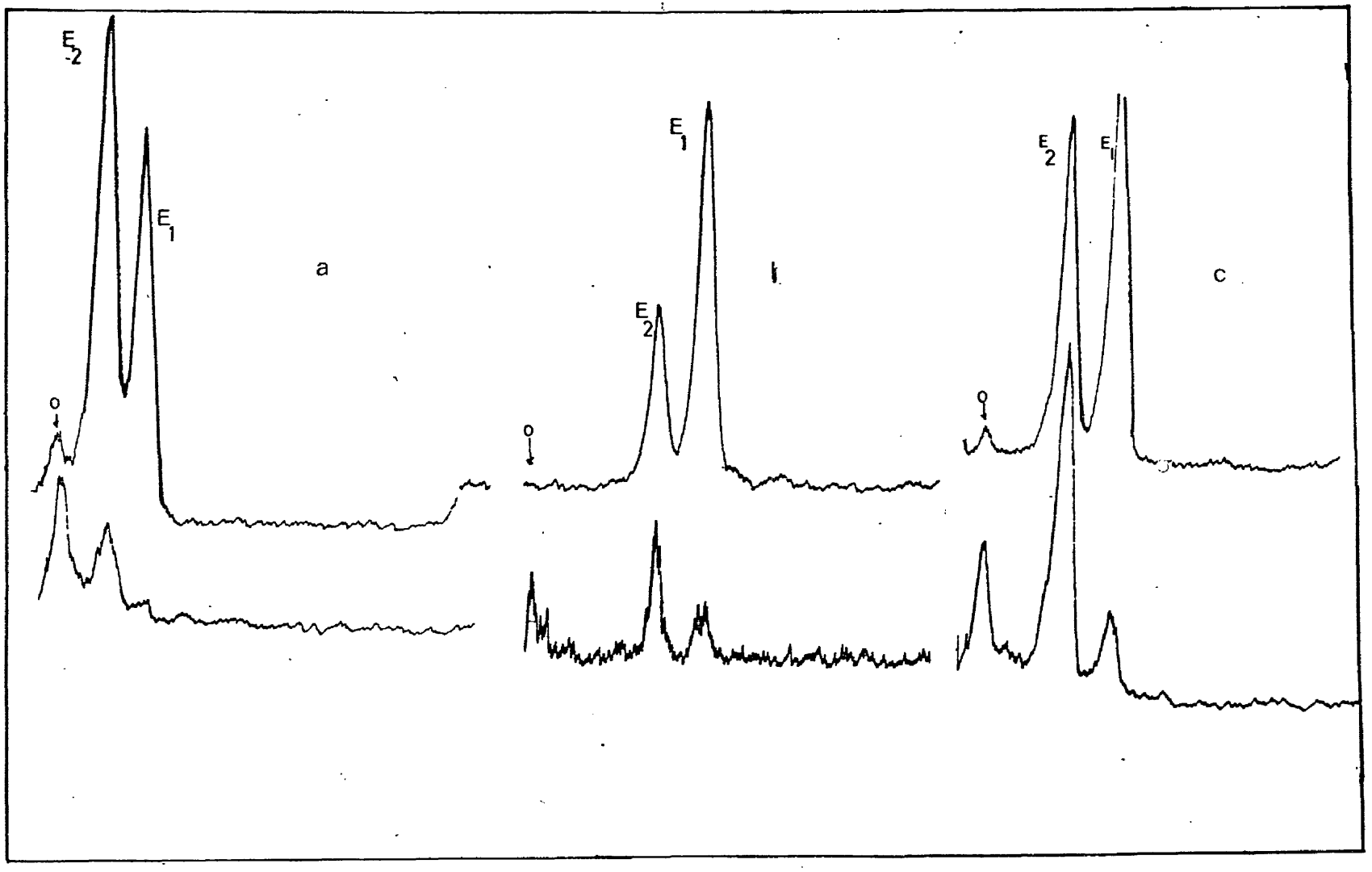

E2
E1
a
o
E1
E2
b
o
E2
E1
c
o

Table III : 1 Tritium in plants after extraction

Tissue	Weight (g) Before	After	dpm in phenolics	%	Total (%)	dpm in tissue	%	Total (%)
Root	16.0	1.4	14,717	0.012		533,881	1.35	
Shoot	10.0	0.87	24,083	0.06	54,000	7,029,612	17.75	12,296,166
Primary Leaves	16.0	1.29	23,800	0.06	(0.14)	5,216,849	13.17	(32.6)
Trifoliate Leaves	6.0	0.59	1,400	0.03		145,824	0.37	

42,144 dpm (0.05%) was extracted from cotyledons from 20 plants with 3,278,612 (4.14%) remaining after extraction (assay by Oxymat).

The phenolic fractions were chromatographed on thin-layers which were scanned for radioactivity. Little tritium was detected on the plate.

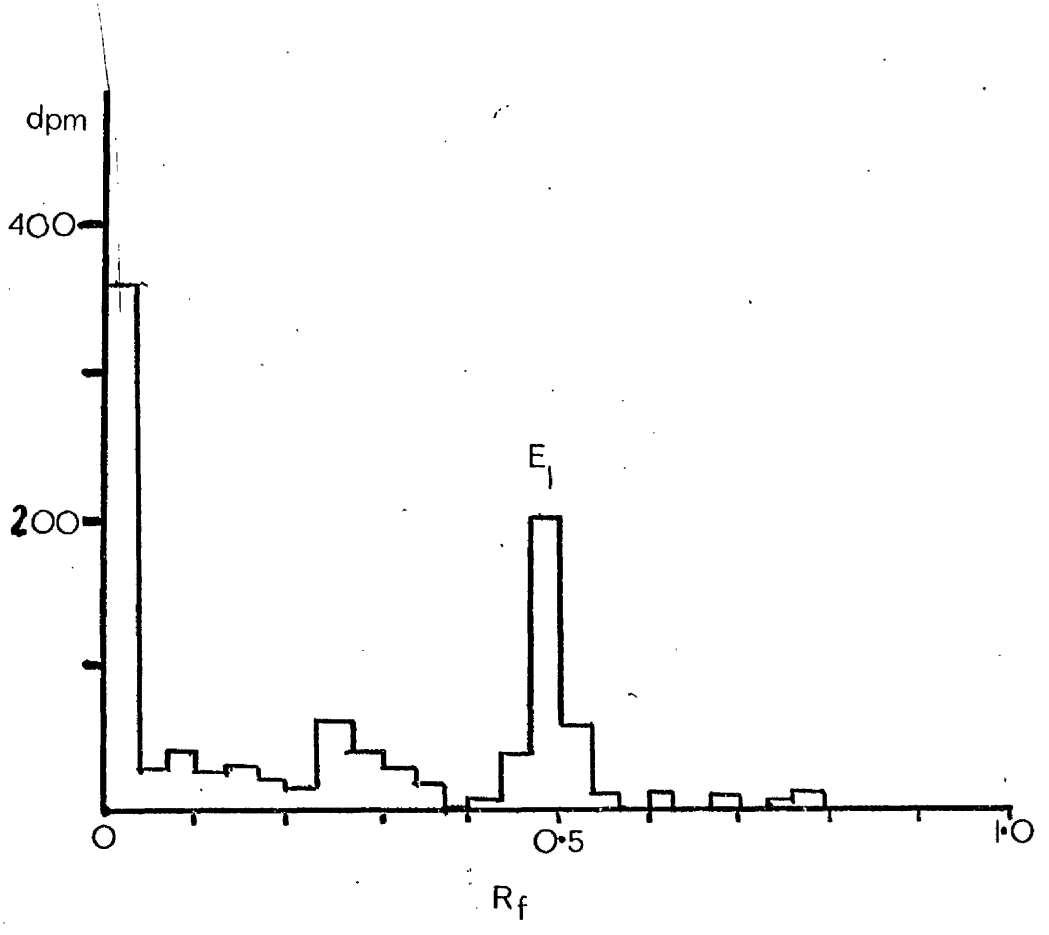
The chromatograms were then divided into 30, 0.5 cm strips and the tritium assessed by liquid scintillation spectrometry. As a result of low levels of tritium present in the root and trifoliate leaves little radioactivity was detected by this method, apart from some located at the origin.

TLC of the phenolic fraction of stem in solvent system (b) (Figure III : 5) revealed two major peaks - one at the origin and one with a similar R_f to E_1. On the other hand, there was also a zone of radioactivity corresponding to E_2. A similar pattern also occurred in the primary leaves.

Elution of this zone on Lipidex and analysis by scintillation spectrometry revealed little or no radioactivity at the relevant retention volume (100 - 120 ml).

Figure III : 5 Scintillation spectrometric analysis of $[^3H]$ on TLC of vegetative shoot fraction.

(Solvent system b)


dpm
400
200
E1
0
0·5
1·0
Rf

(ii) Flowering

Flowering plants (30 days after potting up) were taken for assay. The weights before and after extraction and tritium in the fractions are given in Table III : 2.

Table III : 2 [^{3}H] located in the phenolic fractions and plant tissues

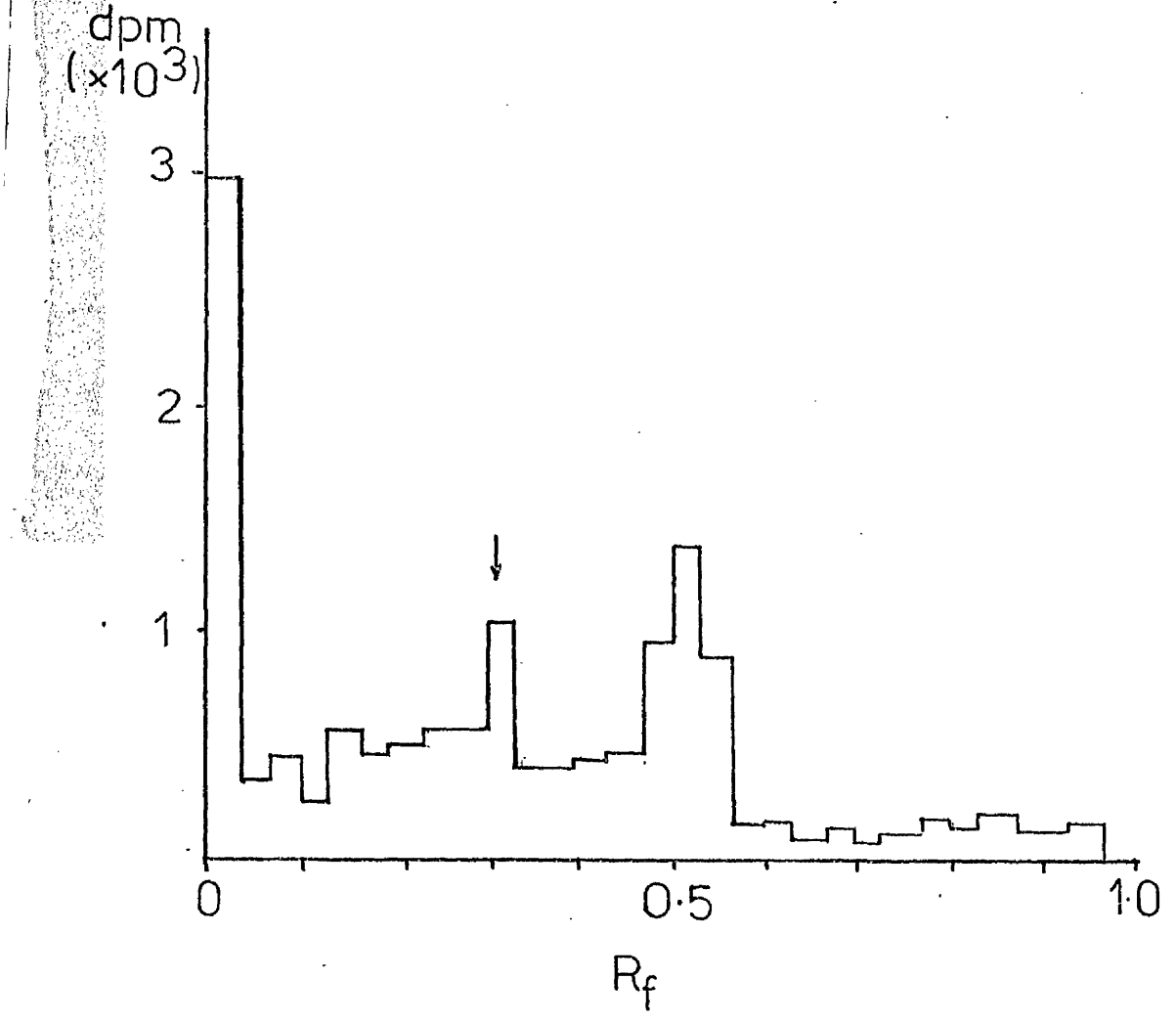
Zone	Weight (g) Before	After	^{3}H in phenolics	% incorp	Total (%)	^{3}H in tissues	Total (%)
Flowers	4.9	0.29	8,640	0.02		22,274	
Trifoliate Leaves	22.7	2.66	158,940	0.40		103,099	
Primary Leaves	11.7	1.035	160,349	0.40	724,429 (1.83)	3,765,938	18,514,255
Shoot	12.1	1.3	382,350	0.96		8,173,256	(46.76)
Root	43.7	1.108	14,150	0.04		6,449,688	

As in the previous section, the phenolic fractions were chromatographed in solvent system (b) and the chromatogram divided into 30 zones. Aliquots were removed from each and assayed by scintillation spectrometry. Little tritium was observed in roots and flowers save a little at the origin. Although there was a considerable amount of radioactivity at a similar R_f to estrone there was no radioactivity at an R_f similar to E_2.

In the shoot and primary leaves there was evidence for radioactivity at the same R_f as E_2. Figure III : 6 shows the scintillation spectrometric data from the shoot in histogram form. The zone at R_f 0.27 - 0.33 (which co-chromatographed with E_2) accounted for 12.7% of the radioactivity.

The zones corresponding to estradiol in the primary leaves (3,000 dpm) and stems (40,577) were eluted on Lipidex. Most of the radioactivity

Figure III : 6 Scintillation spectrometric analysis of $[^3H]$ in the phenolic fraction from stems (Solvent system b).


dpm
(×10³)
3
2
1
0
0·5
1·0
Rf

recovered was at the origin with little at a similar retention volume as E_2 (less than three times background).

(c) Distribution of $[^3H]$ after the application of $[^3H]$-estrone sulphate

As in part II of the Results, the distribution of tritium in plants incubated with $[^3H]$-E_1 sulphate was studied. Table III : 3 summarises the data obtained from the first of these experiments.

Table III : 3 Distribution of label in experiment I

	Before potting		Budding		Flowers	
	$[^3H]$ dpm	%	$[^3H]$ dpm	%	$[^3H]$ dpm	%
Apex	4,716	0.04	-	-	-	-
Flowers (or buds)	-	-	771	0.007	2,499	0.02
4th internode	-	-	-	-	394	0.003
3rd trifoliate	-	-	943	0.008	1,643	0.01
3rd internode	-	-	-	-	3,611	0.03
2nd trifoliate	-	-	53,878	0.47	67,149	0.58
2nd internode	-	-	2,442	0.02	5,835	0.05
1st trifoliate	-	-	36,531	0.32	87,498	0.76
1st internode	-	-	1,277,250	11.18	17,484	0.15
Primary leaves	659,012	5.6	4,137,321	36.24	5,162,302	45.05
Epicotyl	566,821	4.82	1,441,343	12.63	1,623,156	14.16
Cotyledons	457,494	3.89	-	-	-	-
Hypocotyl	10,070,733	85.64	4,450,541	38.99	4,143,237	36.16
Roots	-	-	14,227	0.12	344,590	3.01
Total	11,758,776	99.99	11,415,247	99.98	11,459,398	99.98

Although some 85.64% of the extractable tritium was located in the hypocotyl,

Figure III : 7 % distribution I of [^{3}H] in dwarf French bean.

(a) Plants prior to potting

(b) Budding plant

(c) Flowering plant

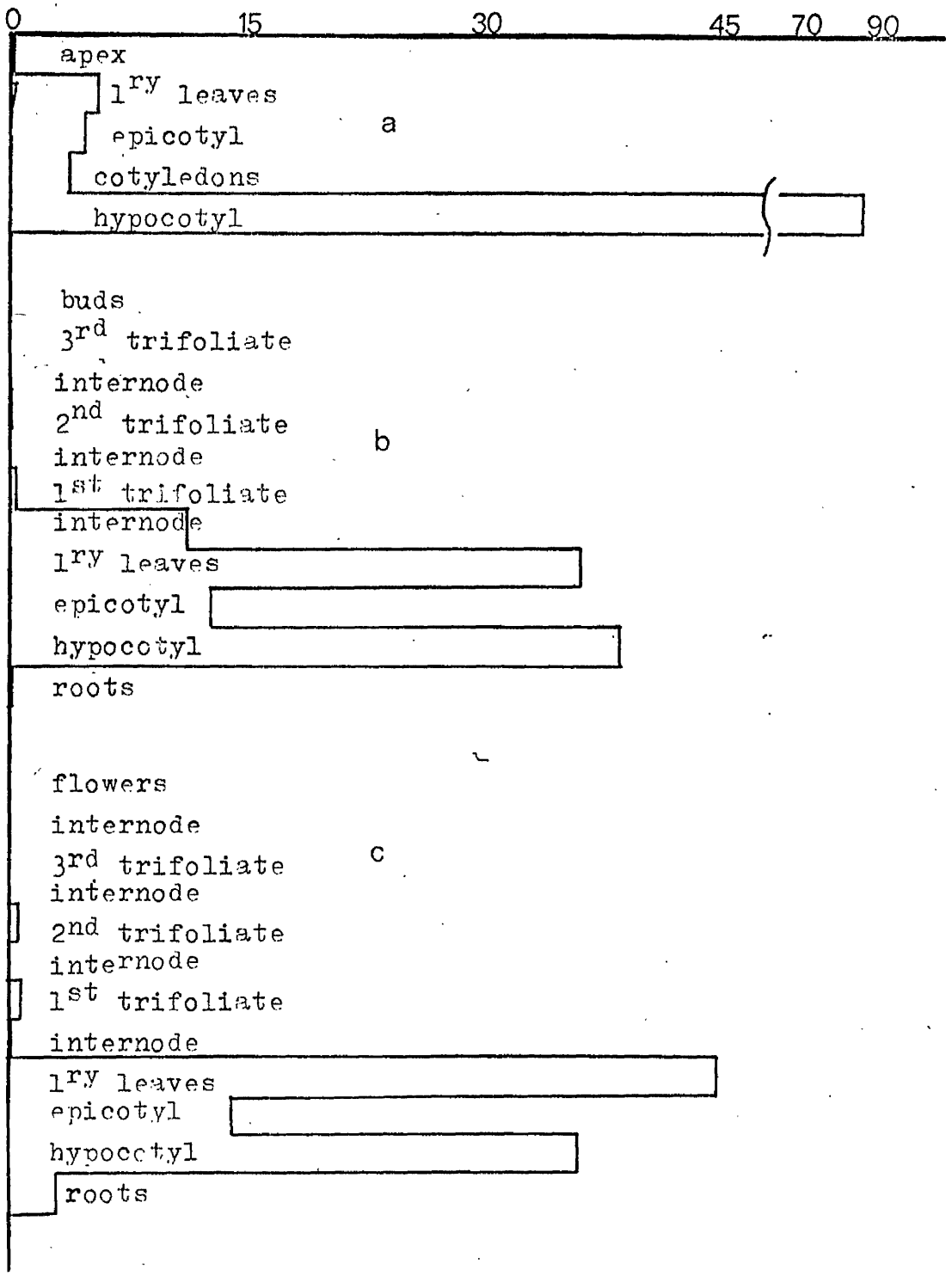

0
15
30
45
70
90
apex
1ry leaves
epicotyl
cotyledons
hypocotyl
a
buds
3rd trifoliate
internode
2nd trifoliate
internode
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots
b
flowers
internode
3rd trifoliate
internode
2nd trifoliate
internode
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots
c

Table III : 4 Distribution of [^{3}H] in dwarf French bean II

	Vegetative		Flowering	
	^{3}H dpm	%	^{3}H dpm	%
Apex	1,135	0.02	-	-
Flowers	-	-	1,194	0.03
3rd trifoliate	-	-	9,075	0.24
3rd internode	-	-	1,556	0.04
2nd trifoliate	1,600	0.03	26,367	0.71
2nd internode	1,196	0.02	2,443	0.06
1st trifoliate	15,282	0.33	44,953	1.21
1st internode	3,453	0.07	4,120	0.11
Primary leaves	1,878,260	40.19	917,723	24.77
Epicotyl	455,595	9.75	208,554	5.6
Hypocotyl	1,983,784	42.45	2,451,647	66.17
Roots	332,968	7.12	37,343	1.01
Total	4,673,273	99.98	3,704,975	99.95

the remaining label was evenly distributed throughout the remainder of the seedling with slightly more in the primary leaves compared to the epicotyl and the cotyledons. The total radioactivity extracted by combustion was equal to 99.9% of the total added.

Whereas in the estrone-fed plants the label in the hypocotyl prior to potting up tended to remain there throughout the development of the plant, both budding and flowering plants revealed that the tritium in the hypocotyl accounted for only 38.99% and 36.16% of the total in the budding and flowering plants respectively, and the primary leaves 36.24% and 45.05% respectively. Also, the label was detected further up the plant than was normally detected in estrone-fed plants with 0.47% (budding) and 0.58% (flowering) in the second trifoliate leaf (Figure III : 7).

In the second experiment, although only 2 μCi were donated to each plant a similar distribution was observed in the vegetative plant (Table III : 4, Figure III : 8). Although the trifoliate leaves, 1.21% (first trifoliate) and 0.71% (second trifoliate) and hypocotyl (over 66%) in the flowering plant contained proportionately more compared to the previous experiment less was detected in the primary leaves (24.77%). These results, however, are preliminary, representing the distribution in only one plant for each phase.

It appears, therefore, that these experiments have produced evidence from TLC, column chromatography and radio GLC that estrone sulphate was converted to estrone and estradiol in flowering plants. There is also evidence for the conversion of E_2 in budding plants by TLC and elution on Lipidex.

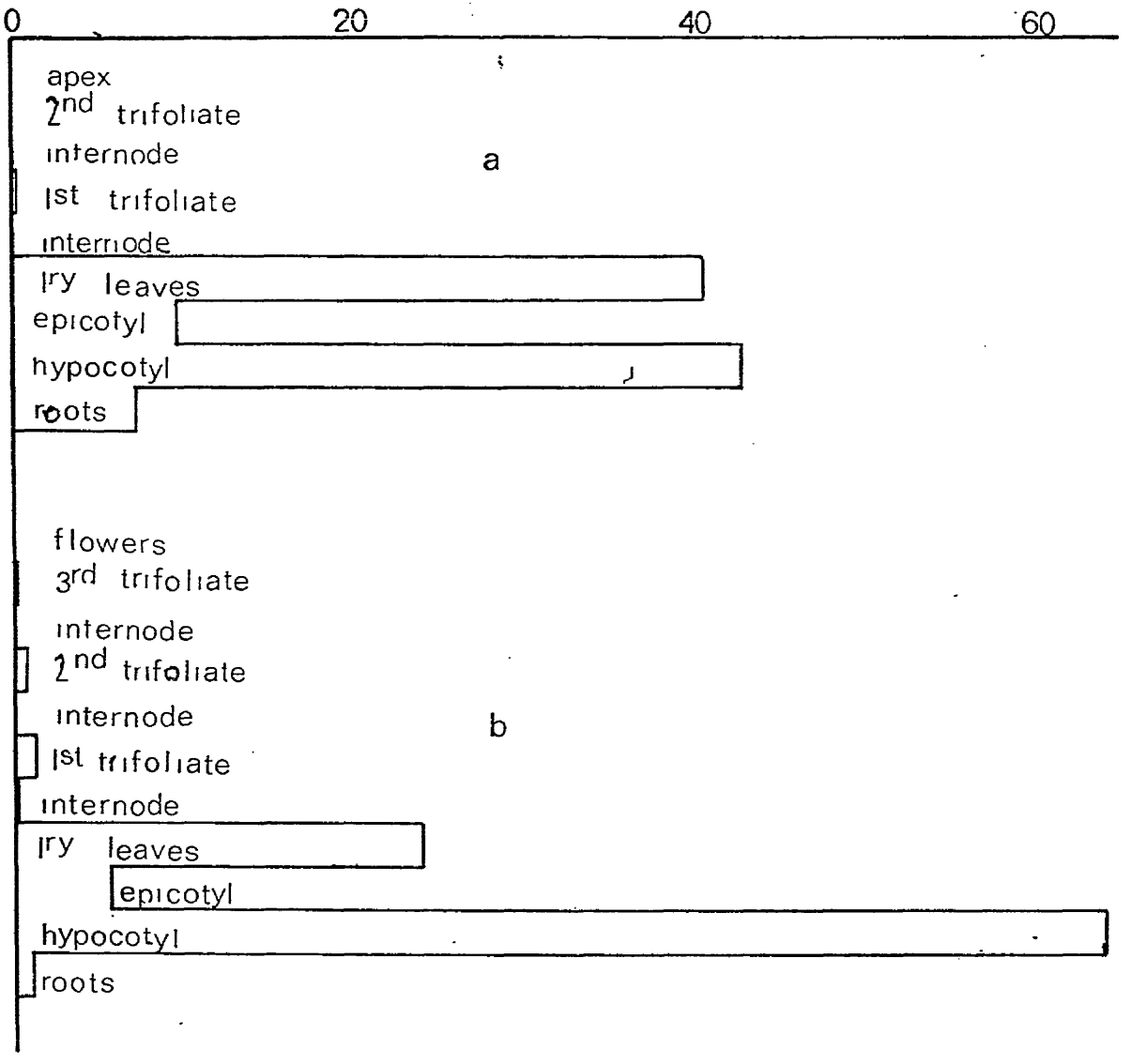
Although the cotyledons have been demonstrated to convert E_1SO_4 to both E_1 and E_2 by all the above methods only TLC evidence is available for the other plant regions.

Estrone sulphate can be distributed throughout the plant with over 24% of the radioactivity reaching the primary leaves and between 36-60% left in the hypocotyl.

Figure III : 8 % distribution II of [^{3}H] in P. vulgaris

(a) Vegetative

(b) Flowering


0
20
40
60
apex
2nd trifoliate
internode
1st trifoliate
internode
dry leaves
epicotyl
hypocotyl
roots
a
flowers
3rd trifoliate
internode
2nd trifoliate
internode
1st trifoliate
internode
dry leaves
epicotyl
hypocotyl
roots
b

IV

The metabolism and uptake of $[^{14}C]$-E_2 by the dwarf French bean

(a) The previous parts have described the evidence obtained for the conversion of estrone to estradiol and therefore it was deemed logical the examine the reverse process.

Initially, 5 μCi of $[^{14}C]$ estradiol were administered to the cut stems of 11 plants: after collecting the cotyledons and potting up, the cuttings were left for five weeks until flowering at which time they were analysed for metabolism of the supplied radiochemical.

Figure IV : 1 provides details of the $[^{14}C]$ detected in a $CHCl_3$:MeOH extract from the cotyledons (15,210 dpm), chromatographed on TLC in solvent system (b) and developed for 10 cm. There was a small peak, less polar than the estradiol, at a relative retention factor of 1.48 - estrone has a retention factor of 1.45.

Figure IV : 2 shows the traces obtained from the chromatograms in three different solvent systems. Estrone had relative retention values of 1.2, 1.44 and 1.67 in solvent systems (b), (d) and (e) respectively. The phenolic fraction had peaks with relative retentions of 1.28, 1.48 and 1.67 in these solvent systems.

Having obtained some chromatographic evidence for the conversion of estradiol to estrone, the experiment was modified to include the analysis of vegetative plants also.

Fourteen plants were fed with 26 μCi $[^{14}C]$-E_2, and incubated in the usual manner. Extraction of the cotyledons isolated 17,529 dpm which was chromatographed in solvent system (c) (Figure IV : 3). Two major zones of activity were seen on this plate the larger of which was at the origin, and the other with the same R_f values as estradiol. There was a small area of activity with relative retention factor of 1.54 (1.00 for E_2

Figure IV : 1 TLC radiochromatograms of –

(a) Standard [^{14}C]-E_1 and E_2

(b) Cotyledon extract I
($CHCl_3$:MeOH 95:5)

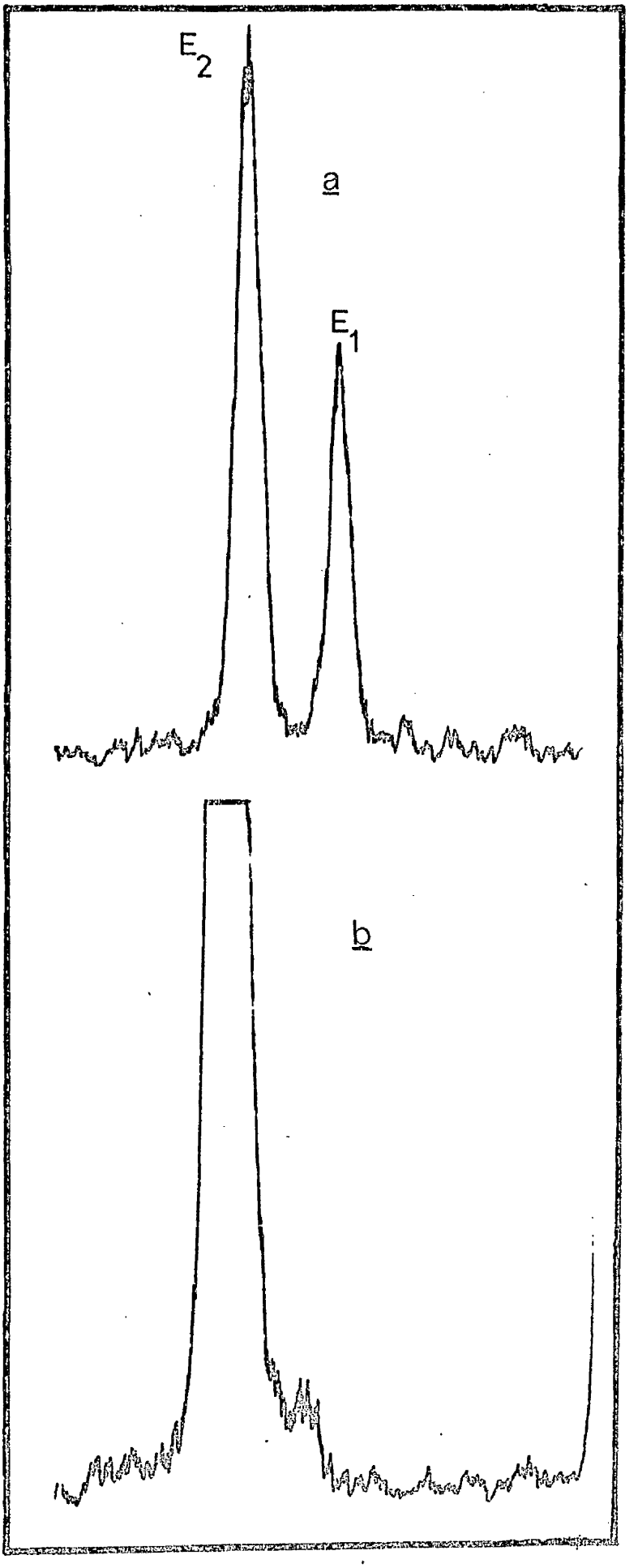

E2
a
E1
b

Figure IV : 2 TLC radiochromatogram of phenolic fractions from flowering dwarf French bean.

Solvent systems:-

(a) $CHCl_3$:MeOH (95:5)

(b) Light petrol:Ethylacetate (75:25)

(c) Benzene:isopropanol (75:25)

(Standard [^{14}]-E_1 and E_2 the uppermost peaks)

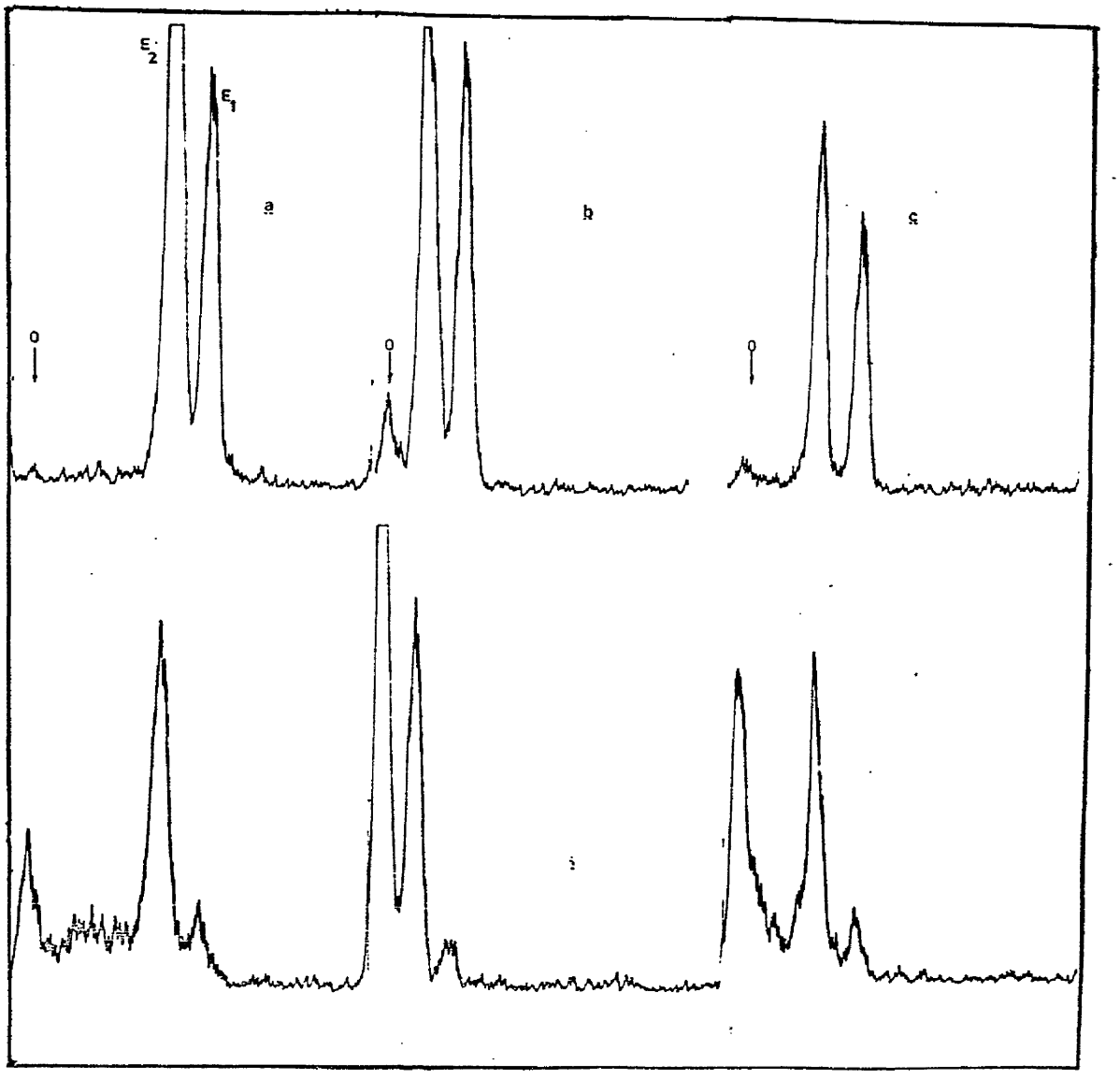
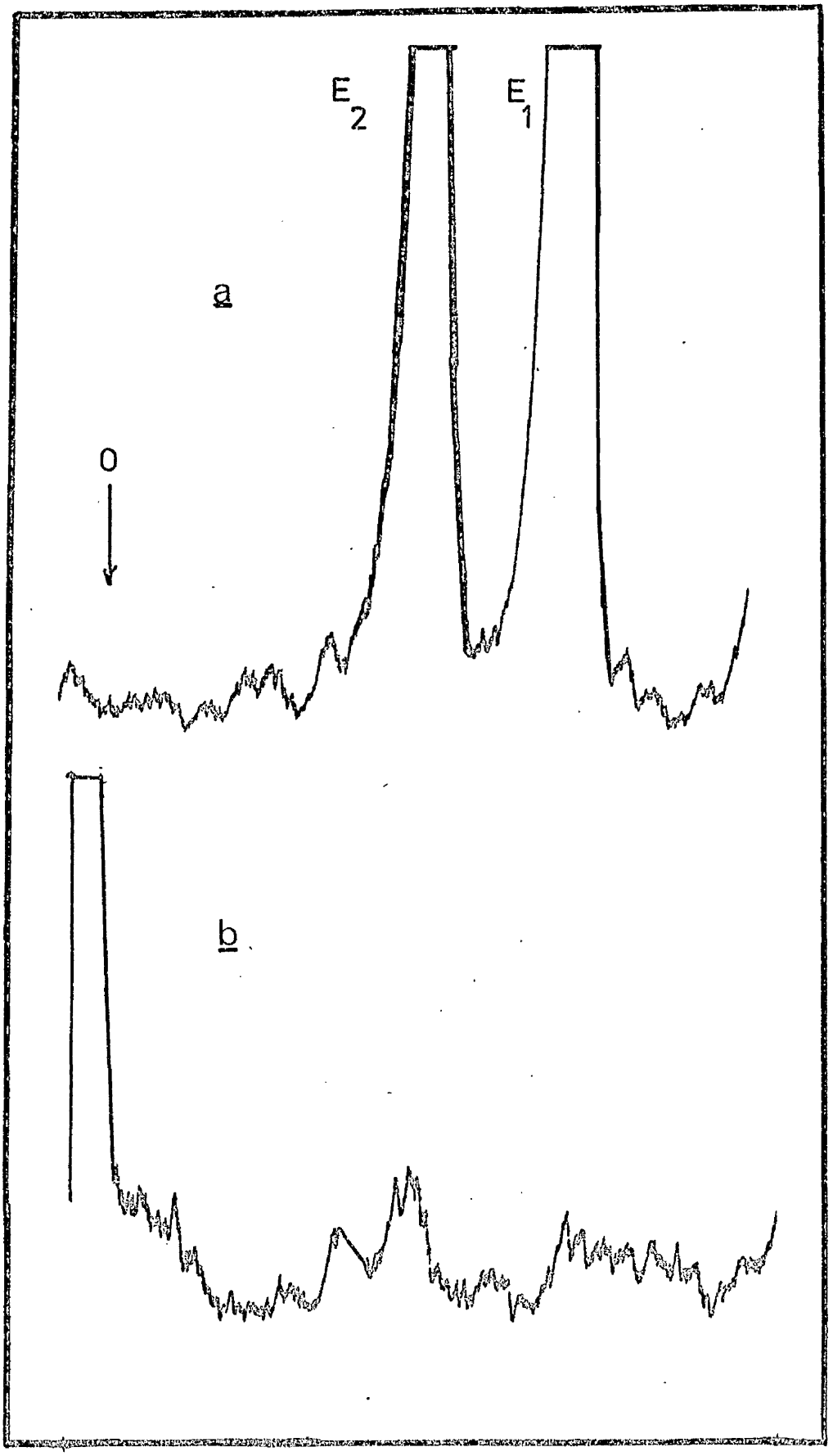

E2
E1
a
b
c
0
0
0

Figure IV : 3 TLC radiochromatogram of –

(a) Standard [^{14}C]-E_1 and E_2

(b) Cotyledon extract II
($CHCl_3$:acetate 95:5)


E2
E1
a
0
b

and 1.38 for E_1). On extraction of the cotyledons by the Oxymat a further 52,630 dpm were detected.

Figure IV : 4a shows the trace obtained from chromatographing the feeding solution residue after the incubation period. A number of zones of radioactivity can be seen, the largest of which is at the origin. There is also a peak at R_f 0.64 which could possibly be [^{14}C] estrone. Chromatographing in solvent system (d) gave the pattern seen in Figure IV : 4b with a peak at the same R_f as E_1.

Twelve days after potting up, six plants were harvested and the phenolic fraction analysed for [^{14}C]. The phenolic fraction was isolated (40,000 dpm) and these chromatographed on TLC developed in solvent system (c). Figure IV : 5 shows the trace obtained from assaying the plate by TLC scanner. Three major zones of activity are observed; one at the origin, one at a similar R_f to estradiol and one other major zone with a similar R_f to estrone. Due to the likelihood of there being more than one peak, here the TLC plate was divided into 30 zones and small aliquots of each assayed for [^{14}C] by scintillation spectrometry (Figure IV : 6). A much clearer picture of the radiocarbon present in the plate was obtained. Apart from the zone corresponding to estradiol there were six peaks, one of which had the same R_f as estrone. This particular zone was removed from the TLC plate, eluted in a small chromatography column with benzene:ethanol (9:1) and the 13,964 dpm obtained, eluted on Lipidex in the usual fashion. Aliquots (20%) were assayed by scintillation spectrometry (Figure IV : 7).

Although a number of peaks were seen the largest was that eluted between 90 - 100 ml, which is the elution volume of estrone. This fraction was collected, found to contain 2,440 dpm and analysed by radio GLC but no peaks were obtained.

Analysis of the residue revealed that 19,715,518 dpm (80.42%) remained.

Figure IV : 4 TLC radiochromatograms of the incubating medium prior to potting ([^{14}C]-E_1)

(a) $CHCl_3$:Acetic Acid (95:5)

(b) Benzene:iospropanol (95:5)

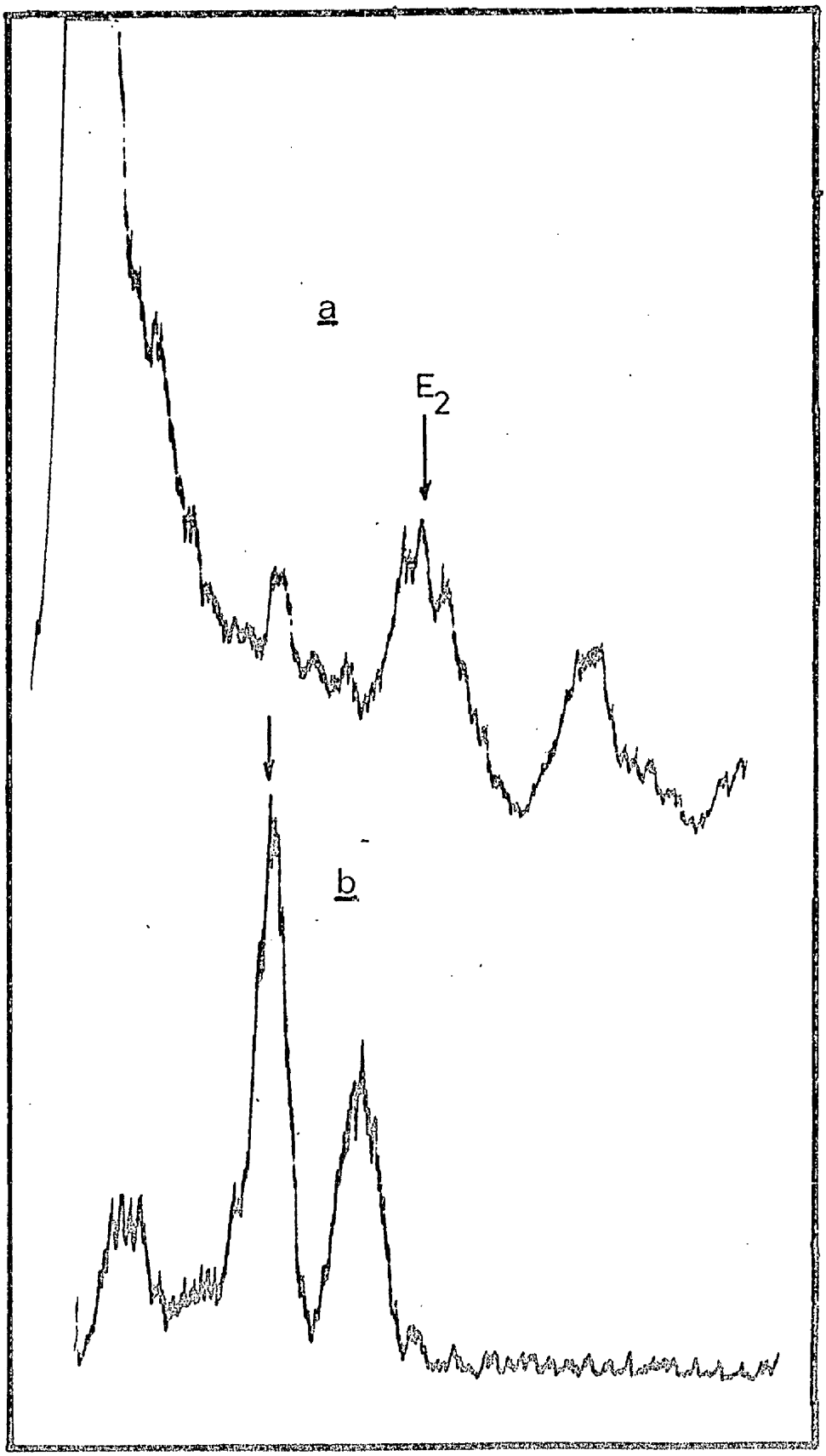

a
E2
b

Figure IV : 5 TLC radiochromatogram of the phenolic fraction from vegetative dwarf French bean.
(Solvent system c).

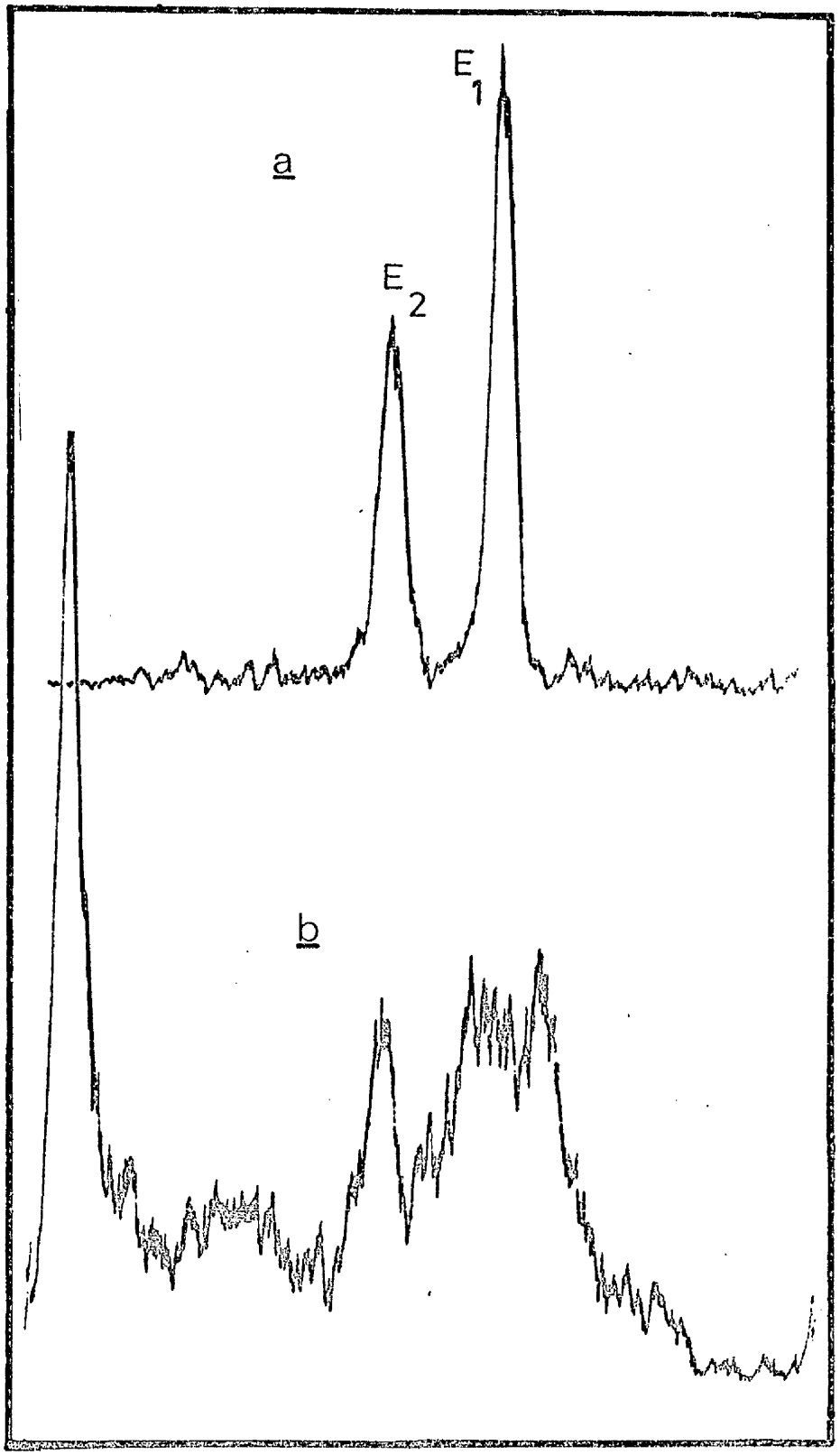

E1
a
E2
b

Figure IV : 6 TLC plate assayed by scintillation spectrometry

(See Figure IV : 5)

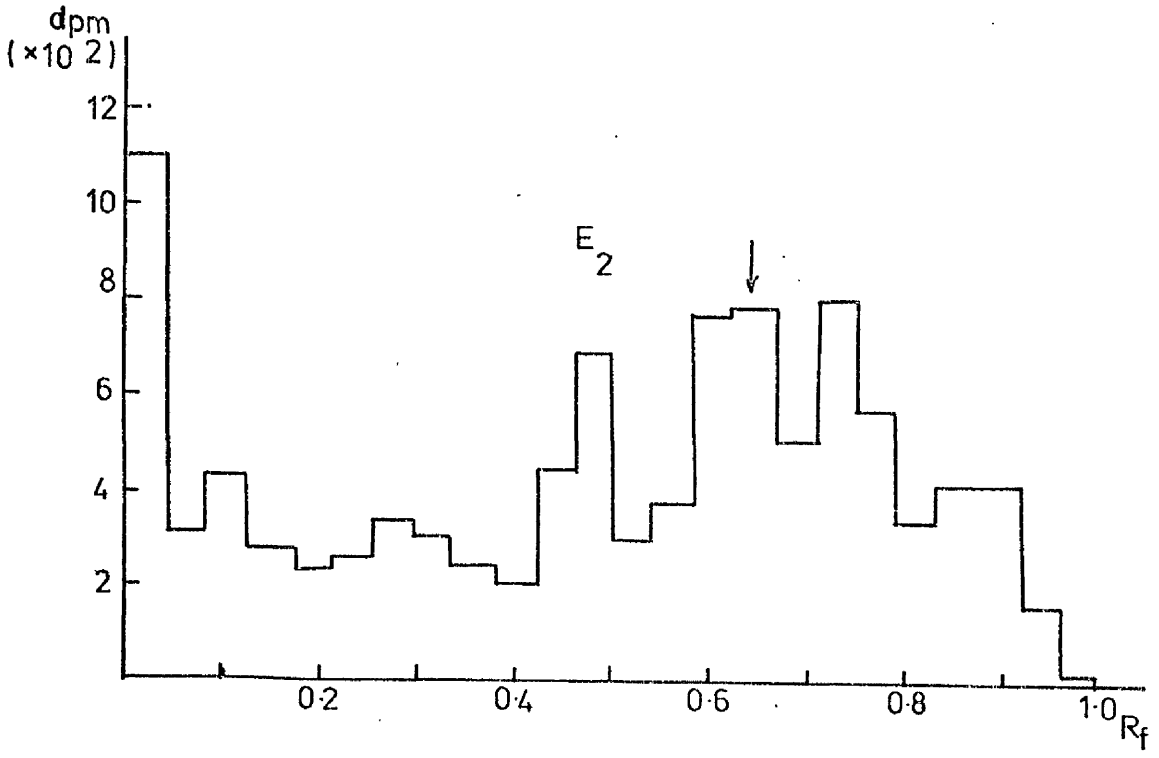
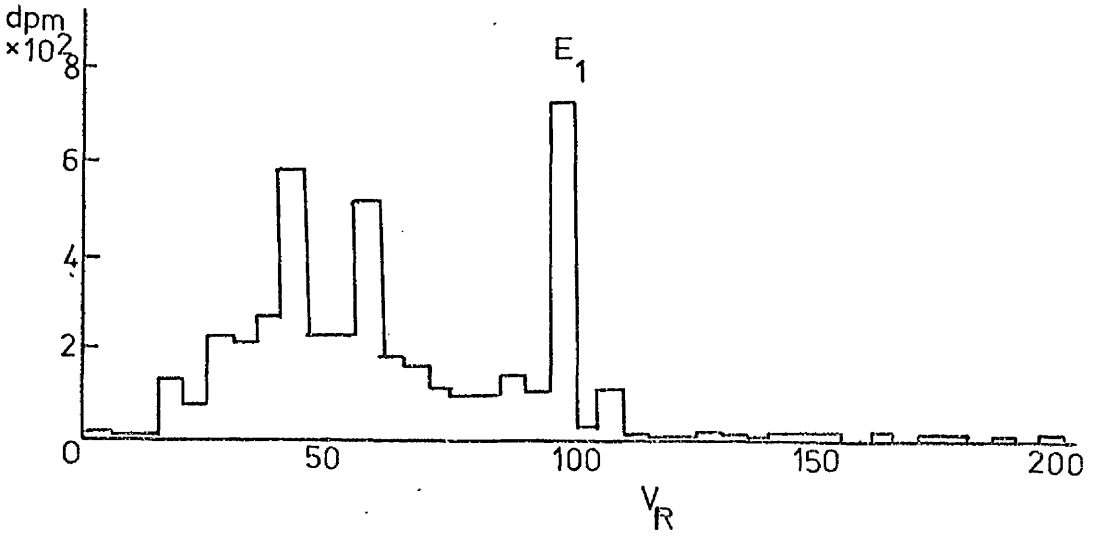

dpm
(×10 2)
12
10
8
6
4
2
E2
0·2
0·4
0·6
0·8
1·0
Rf

Figure IV : 7 Elution profile from Lipidex 5,000 of the putative [^{14}C]-E_1 zone from TLC (vegetative).


dpm
×10²
8
6
4
2
0
50
100
150
200
E1
VR

Thirty-five days after potting up, the remaining plants, which were flowering profusely, were harvested and processed. The phenolic fraction was isolated (367,274 dpm) and chromatographed in solvent system (c) (Figure IV : 8).

Four major zones of activity were seen, with the largest at the origin. The peak at R_f 0.53 had a similar R_f to standard estrone. This zone was removed and loaded onto a 650 mm column of Lipidex. Aliquots (10%) were analysed for [^{14}C] (Figure IV : 9). There was some evidence of [^{14}C] from 50 - 70 ml (9,542 dpm) but this was not confirmed by radio GLC.

(b) Distribution of [^{14}C] in plants supplied with [^{14}C]-estrone

The results from this study are summarised in Table IV : 1 and 2, Figures IV : 10 and 11, where over 89% of the radiocarbon remained in the hypocotyl whereas between 1.9 and 6% reached the primary leaves. The pattern observed is similar to that seen in plants fed with [^{14}C]-E_1.

The conversion of estradiol to estrone has, therefore, been demonstrated by TLC on three different solvent systems and by column chromatography on Lipidex.

Figure IV : 8 TLC radiochromatogram of phenolic fraction of flowering French bean ($CHCl_3$:Acetic acid 95:5)

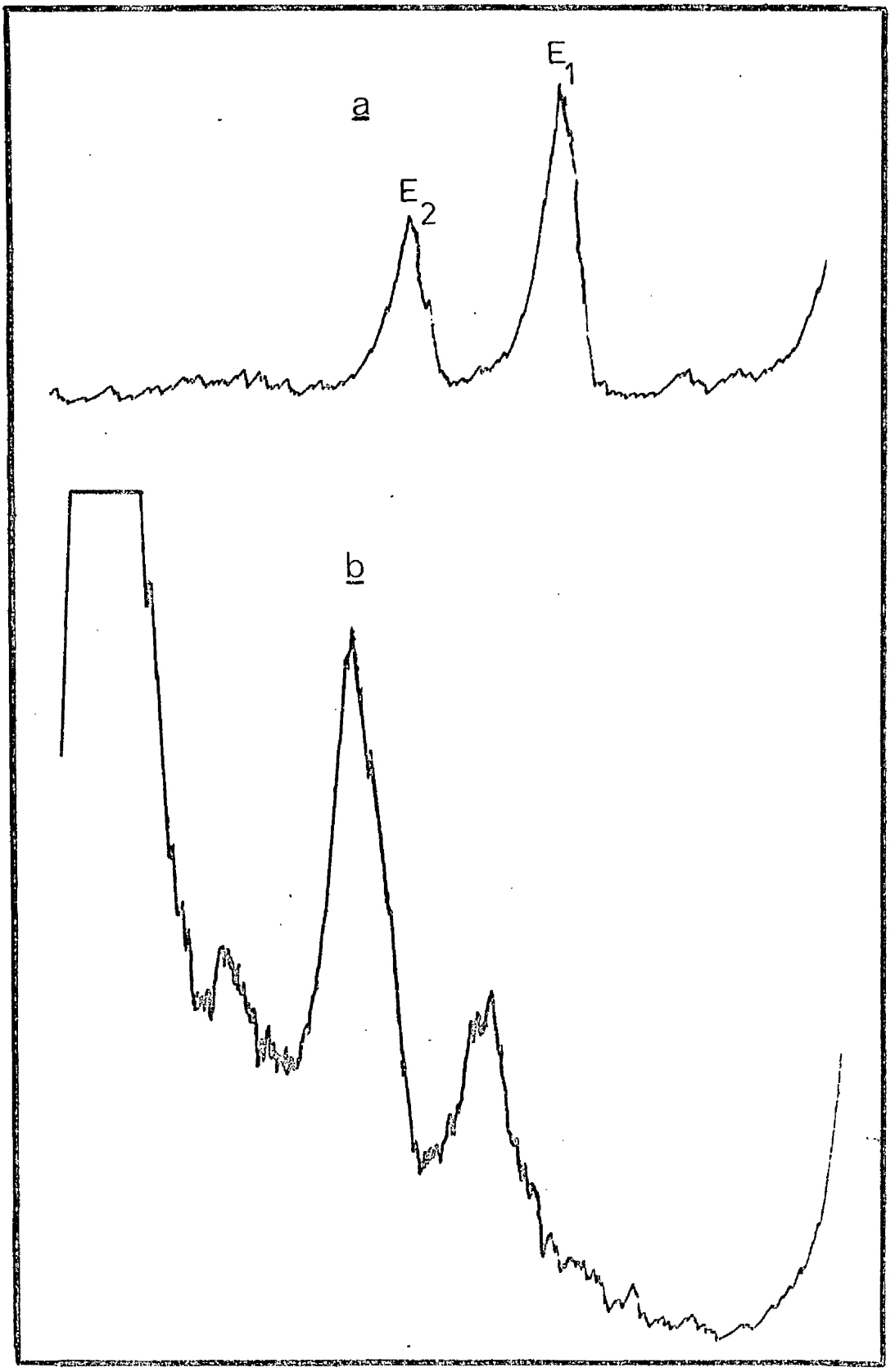

E1
a
E2
b

Figure IV : 9 Elution profile from Lipidex 5,000 of -

(a) Standard [^{14}C]-E_1 and E_2

(b) Putative E_1 zone from thin layer (Flowering French bean)

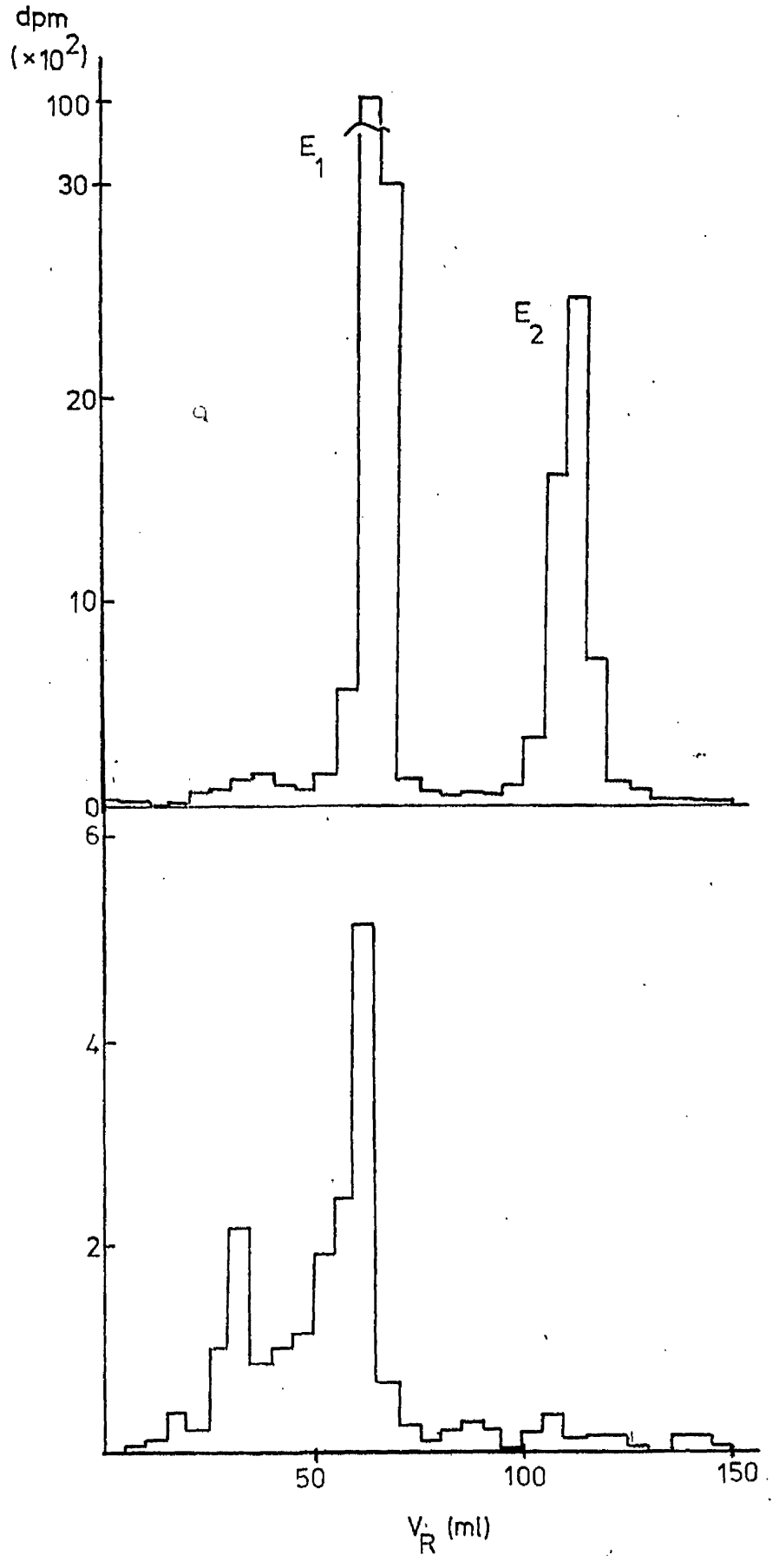

dpm
(×10²)
100
30
20
10
0
E1
E2
6
4
2
50
100
150
VR (ml)

Table IV : 1 Distribution of [^{14}C] in plants fed with [^{14}C]-E_2

	Prior to potting		Flowering	
	[^{14}C] (dpm)	%	[^{14}C] (dpm)	%
Flowers	-	-	-	-
Apex	98	0.008	14	0.001
4th Internode	-	-	9	-
3rd Trifoliate	-	-	38	0.002
3rd Internode	-	-	32	0.002
3rd Trifoliate	-	-	150	0.016
2nd Internode	-	-	162	0.02
1st Trifoliate	-	-	437	0.04
1st Internode	-	-	161	0.02
Primary leaves	44,845	3.6	18,116	1.93
Epicotyl	33,198	2.7	8,723	0.93
Hypocotyl	1,145,275	92.1	902,891	96.75
Roots	19,592	1.6	2,776	0.28
Totals	1,243,008	100.08	933,505	99.99

Table IV : 2 Distribution II of [^{14}C] in plants fed with [^{14}C]-E_2

Organ	Vegetative		Flowering	
	[^{14}C]	% incorp	[^{14}C]	% incorp
Flowers or Apex	387	0.008	457	0.01
3rd Trifoliate	-	-	382	0.009
3rd Internode	-	-	403	0.01
2nd Trifoliate	403	0.008	1,292	0.03
2nd Internode	530	0.01	236	0.006
1st Trifoliate	2,355	0.046	3,047	0.075
1st Internode	1,094	0.02	895	0.02
Primary leaves	326,019	6.45	254,165	6.29
Epicotyl	148,756	2.92	73,702	1.82
Hypocotyl	4,560,666	89.42	3,668,770	90.76
Roots	56,597	1.11	37,956	0.93
Total	5,100,087	99.99	4,041,872	99.96

Figure IV : 10 % distribution I of [^{14}C] in P. vulgaris

(Plants treated with [^{14}C]-E_2.)

(a) After incubation

(b) Flowering

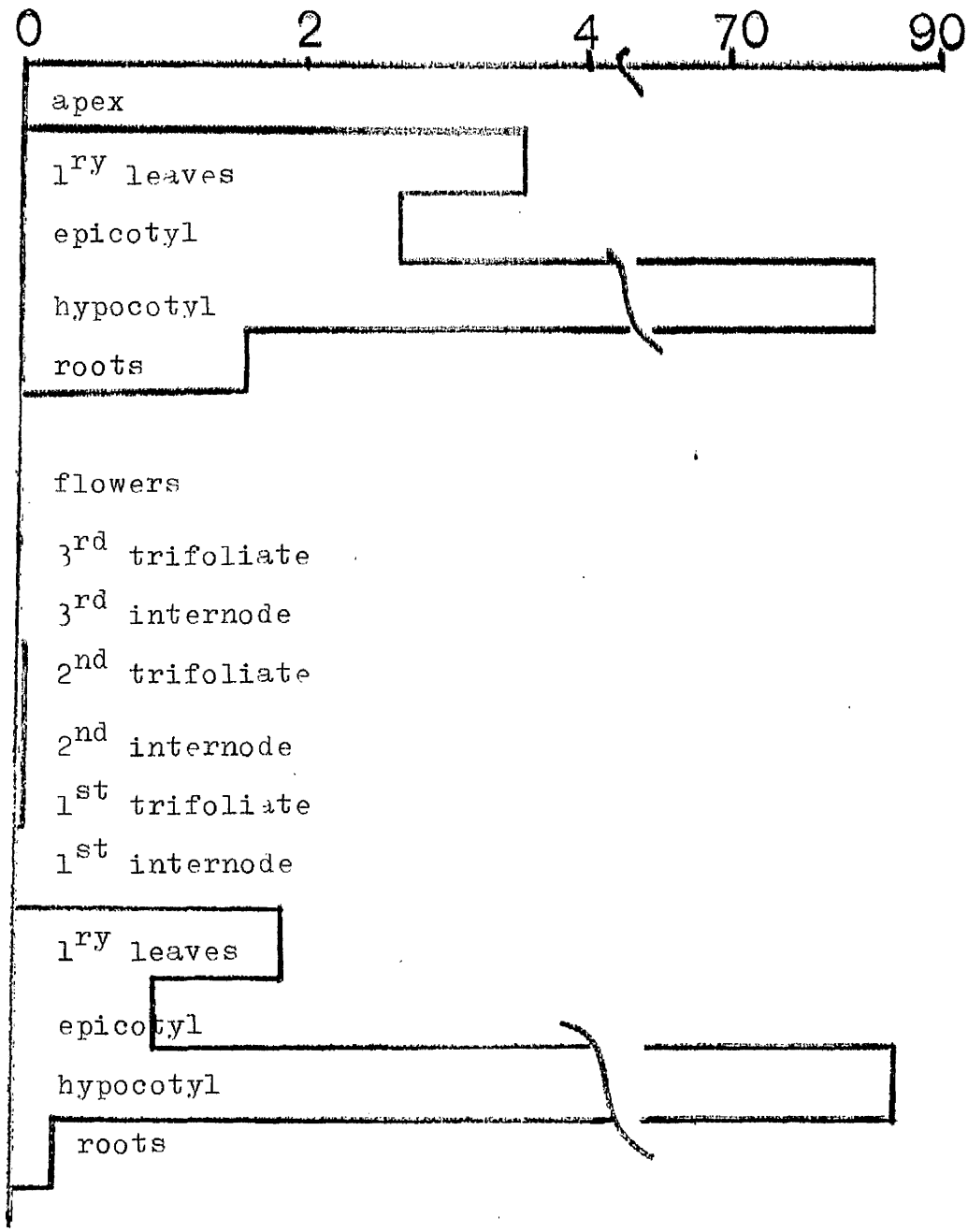
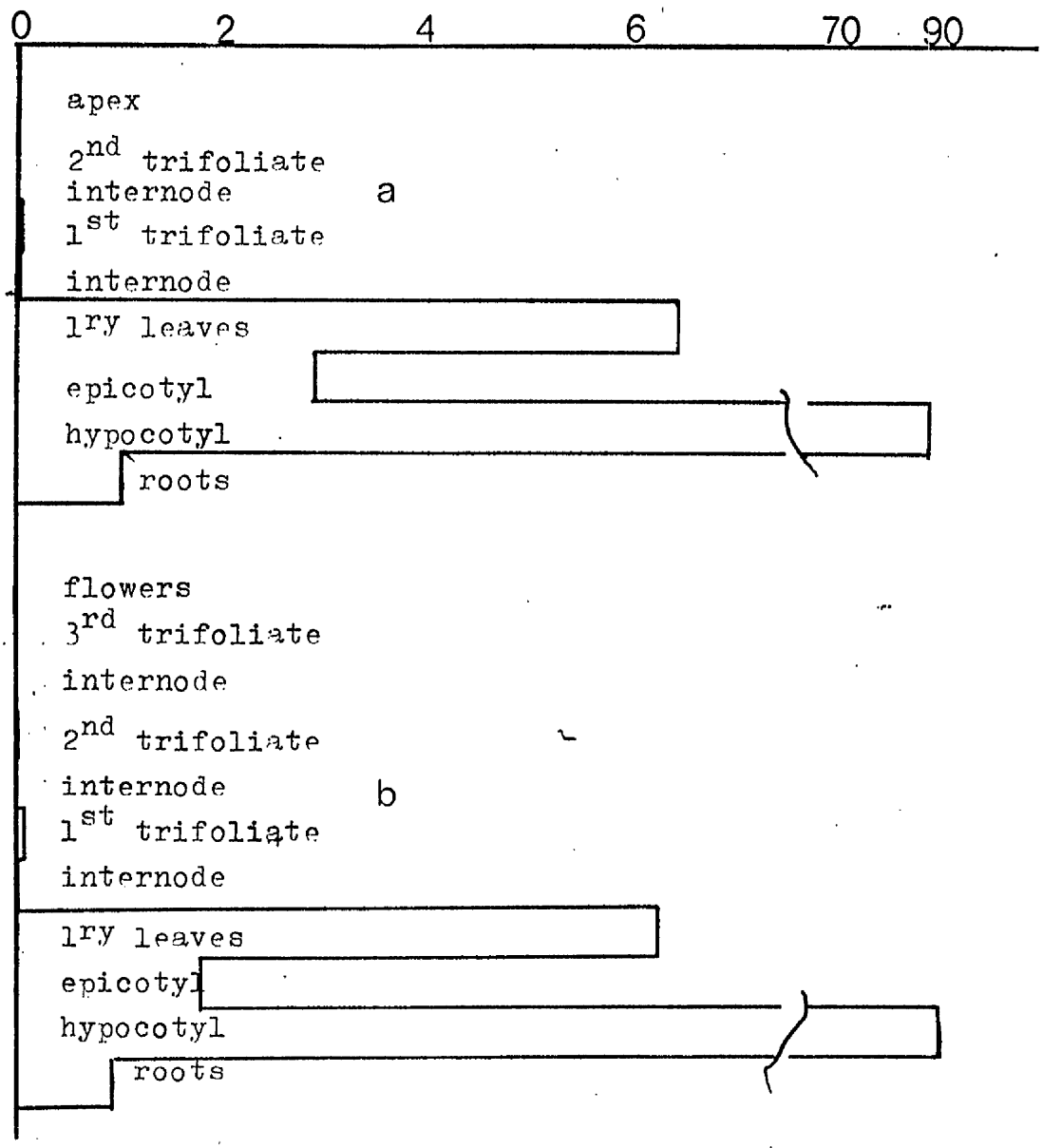

0
2
4
70
90
apex
1ry leaves
epicotyl
hypocotyl
roots
flowers
3rd trifoliate
3rd internode
2nd trifoliate
2nd internode
1st trifoliate
1st internode
1ry leaves
epicotyl
hypocotyl
roots

Figure IV : 11 % distribution II of [^{14}C] in P. vulgaris

(Plants treated with [^{14}C]-E_2)

(a) Vegetative

(b) Flowering


0
2
4
6
70
90
apex
2nd trifoliate
internode
a
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots
flowers
3rd trifoliate
internode
2nd trifoliate
internode
b
1st trifoliate
internode
1ry leaves
epicotyl
hypocotyl
roots

V

Biosynthesis of estrogens from dl-[2-^{14}C] mevalonic DBED

(a) That estrone and estradiol are interconvertable does not necessarily imply that the plant can synthesise these compounds, as there may be, for example, a metabolic block earlier in their biosynthetic pathway. The biosynthesis of estrogens from [^{14}C] MVA (the triterpenoid precursor) was, therefore, investigated and as preliminary studies during the course of this work suggested that flowering was required for estrogen biosynthesis the initial investigation was carried out on flowering plants.

Ten seedlings were each supplied with 2 μCi of the DBED salt of dl-[2-^{14}C] mevalonic acid (M and M, 11) and incubated for 5 days. As this experiment was performed in the months of November and December, 38 days elapsed before the plants attained the appropriate flowering condition.

The plant tissue was extracted in 4N NaOH (M and M, 11) and it was found that the phenolic fraction contained 245,140 dpm which was equivalent to 0.56% of the radioactivity administered to the plant but, as only 50% of the racemic mixture is available to the plant, this represents 1.1% incorporation into the phenolic fraction.

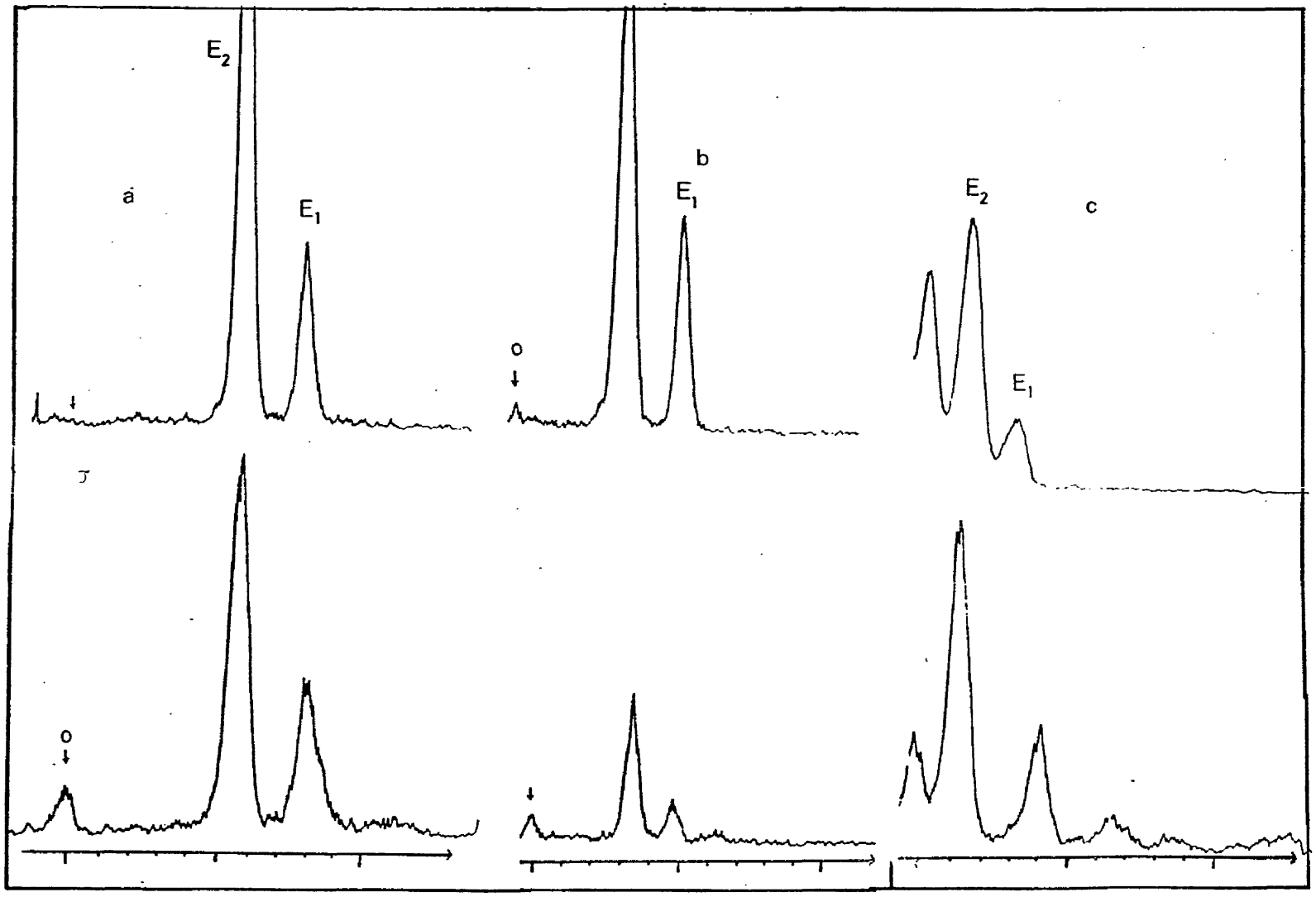
This was chromatographed in solvent systems (b), (d) and (e) and reference to the TLC radiochromatogram traces (Figure V : 1a, b, c) demonstrated that the majority of the radioactivity was concentrated in two zones with a further small amount located at the origin. R_f zones 9.29 - 0.45 and 0.51 - 0.61 (Figure V : 1a) accounted for 65 and 26.5% respectively of the radiocarbon on the plate and these corresponded to standard E_2 and E_1 respectively. The ratio of peak heights (Figure V : 1b) were very similar to those in Figure V : 1a with the peak at R_f values 0.19 - 0.27 possessing over 67% of the label detectable on the plate; this peak also co-chromatographed with E_2. The other zone of radioactivity (R_f values 0.28 - 0.35) was found not to co-chromatograph with E_1 (0.35 - 0.45) in this solvent system. A similar pattern was observed in the TLC radio-

Figure V : 1 TLC radiochromatogram scan of the phenolic fractions of [^{14}C] MVA fed plants in various solvent systems.

(a) Solvent system b.

(b) Solvent system d.

(c) Solvent system e.


E2
E1
a
b
E2
E1
c
E1
o
o

chromatogram trace from solvent system (e) (Figure V : 1c) with the relative proportions of the zones of radioactivity being closely similar to those found in the other TLC radiochromatogram traces (Figure V : 1b and c). R_f zone 0.05 - 0.15 accounted for 59% of the radioactivity and co-chromatographs with E_2. The other area of radioactivity did not have the same mobility as estrone. Comparison of R_f zone 0.23 - 0.33 with that of E_1 (0.13 - 0.25) implied that this compound was not estrone.

The putative estradiol zone on the chromatogram developed in solvent system (a) was removed and eluted before crystallising to constant specific activity with non-radioactive carrier (Table V : 1).

Table V : 1 Radioactivity found when crystallising with non-radioactive E_2.

Replicate	Weight of sample (mg)	dpm	dpm mg^{-1}
I	2.19	1,682	768
II	1.76	1,207	686
III	1.29	924	716

The remaining phenolic fraction was derivatised with BSA and analysed by radio GLC. The traces obtained are shown in Figure V : 2 with the solvent front of the FID trace taken as a reference point. Both Figure V : 2 b and c had one major peak with the same retention time as standard estradiol; Figure V : 2c represented the putative E_2 co-injected with ca. 700 dpm [^{14}C]-E_2 and the attenuation had to be doubled to keep the peak on scale.

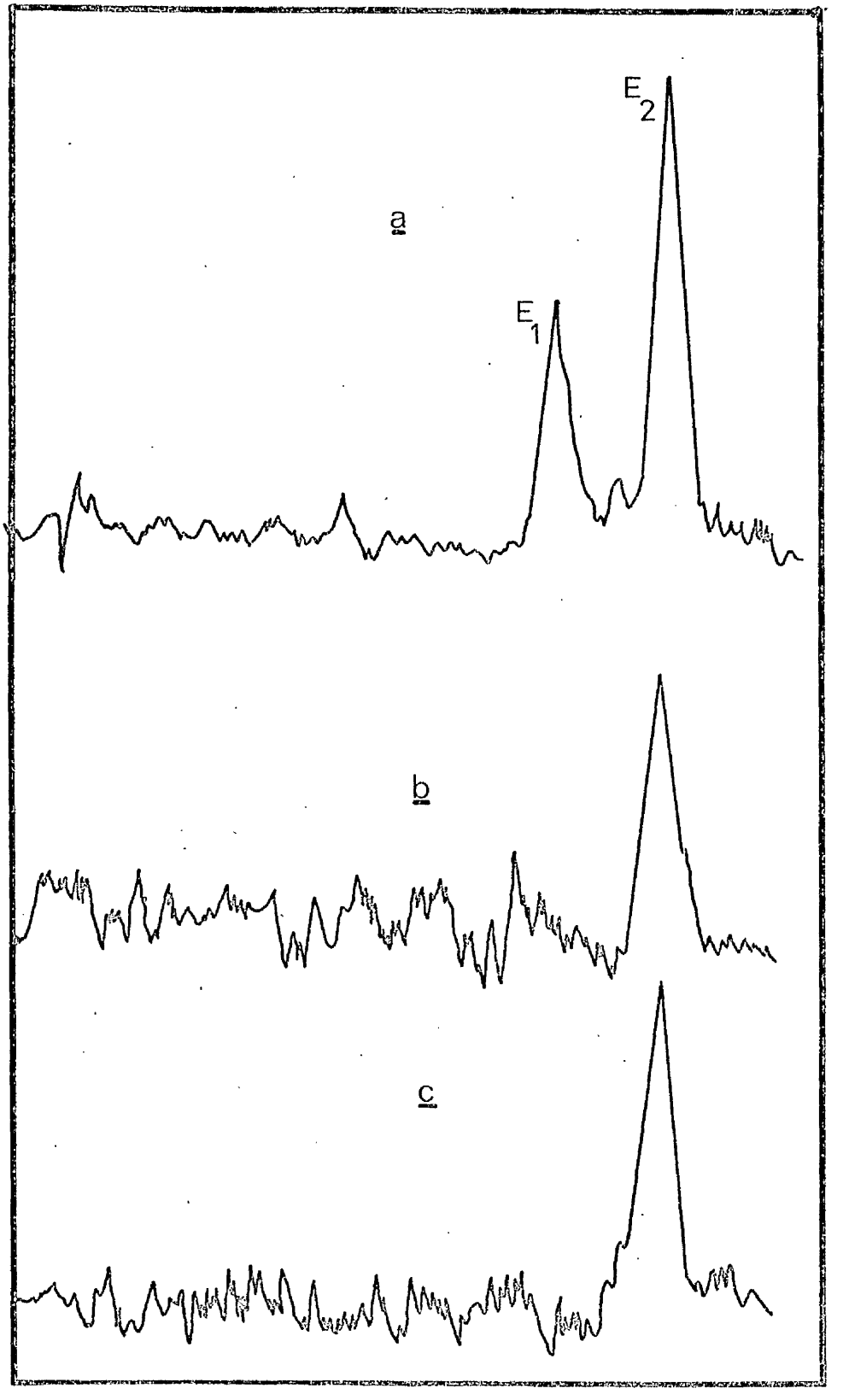
A small peak at the same retention time as estrone can be seen in Figure V : 2b but it is too close to background to be unequivocal.

Figure V : 2 Radio GLC chromatogram of the phenolics of [^{14}C] MVA fed plants.

(a) Standard [^{14}C]-E_1 and E_2.

(b) Phenolic fraction at 500 cpm FSD.

(c) Phenolic fraction + 700 dpm [^{14}C]-E_2 at 1,000 cpm FSD. 5 ft. 5% OV 101 240°C 40 ml min^{-1}.


E2
a
E1
b
c

(b) Metabolism of [^{14}C] MVA in different zones of dwarf French bean

(i) Vegetative analysis

Ten plants were harvested twelve days after potting up and divided into four regions. The phenolic fractions were obtained in the usual way and aliquots removed for radioassay as were aliquots from the extracted tissue (Table V : 2).

Table V : 2 Weight of tissue before and after extraction and radiocarbon in phenolic fraction and tissue residue

Plant Organ	Weight (g) Before	after	[^{14}C] in phenolics	%	Total (%)	[^{14}C] in tissue	Total %
Trifoliate Leaves	13.2	1.34	10,265	0.02		164,138	
Primary Leaves	13.2	2.08	61,747	0.14	140,587	5,111,716	17,037,059
Stem	10.3	1.74	57,265	0.13	(0.31)	11,126,846	(38.7)
Root	16.2	1.09	10,950	0.02		634,359	

Cotyledons had 597,417 (1.36%) dpm remaining after extraction

The phenolics were chromatographed on thin-layers in solvent system (b) and assayed by radiochromatography (Figure V : 3).

The traces of shoot and primary leaves (Figure V : 3b and c) exhibited a number of peaks. It is noteworthy that the zone of activity at R_f 0.32 - 0.55 was present in all fractions and was the largest peak. It seems likely, however, that this merely represents more than one zone of radioactivity. In the shoot there was a peak, although relatively small, at R_f 0.15 - 0.24 which corresponds to E_2. These zones of activity were removed, eluted and chromatographed in solvent system (d) (Figure V : 4). The zone removed from trifoliate leaves showed no significant zones of

Figure V : 3 TLC radiochromatogram of phenolic fractions of plant regions in solvent system (b). (Vegetative).

(a) Trifoliate leaves

(b) Primary leaves

(c) Shoot

(d) Root

(e) Cotyledon extract

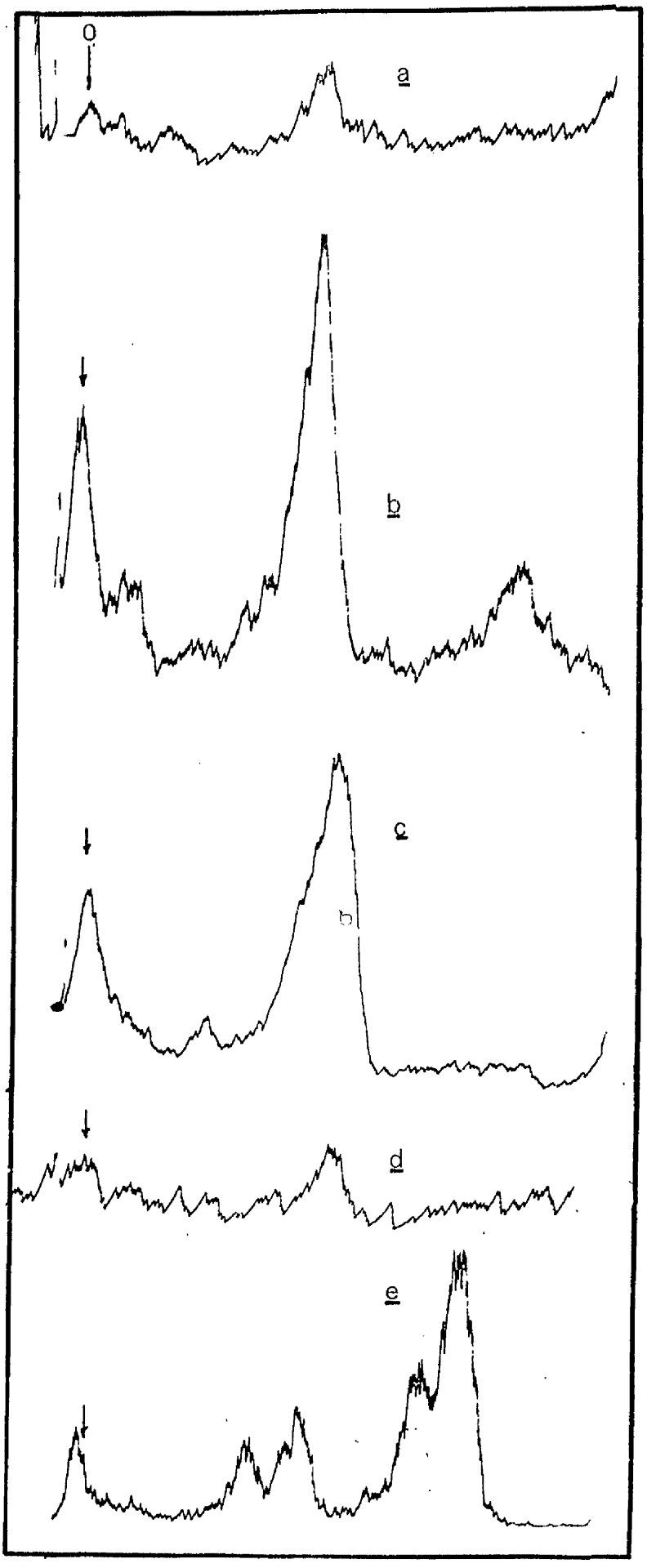

0
a
b
c
d
e

Figure V : 4 TLC radiochromatogram of zones of interest from previous TLC (solvent system d).

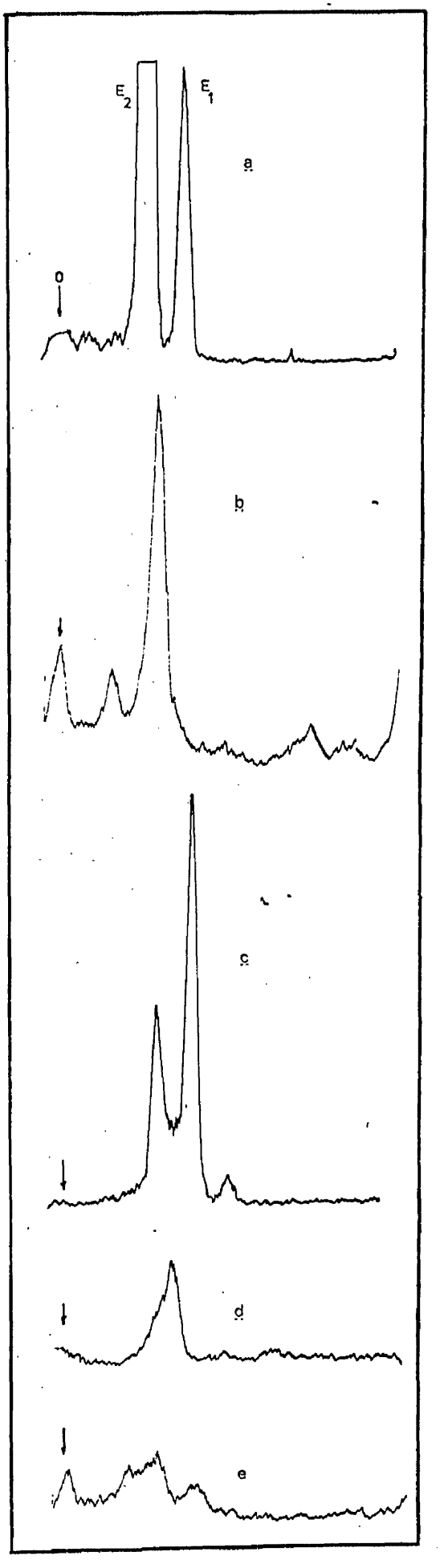
(a) Standard [^{14}C]-E_1 and E_2

(b) Putative E_1 zone primary leaves

(c) Putative E_1 zone stem

(d) Putative E_1 zone root

(e) Putative E_2 zone stem


E2
E1
a
0
b
c
d
e

radioactivity, but the primary leaves and stem fractions resolved into three zones each. In particular, the largest of the peaks in the stem fraction co-chromatographed with E_1 and the other large peak with E_2. The presumptive E_2 fraction from stem, on chromatography, revealed a zone of activity at a similar R_f to E_2. The zones thought to be E_2 and E_1 were assayed by radio GLC but no peak was obtained from those thought to be E_2 (probably due to the small amount of $[^{14}C]$ in the sample). Those zones thought to be E_1 on chromatographing gave large amounts of radioactivity at the solvent front but none elsewhere, implying that this zone was not in fact estrone.

(The cotyledon fraction (Figure V : 3e) showed peaks corresponding to E_1 and E_2 but these were not confirmed on rechromatography).

(ii) Flowering plants

Thirty-two days after potting up, the flowering plants were harvested and the $[^{14}C]$ in the phenolic fractions assayed (Table V : 3).

Table V : 3 Weight of tissue before and after extraction and the radiocarbon in the phenolic fractions and tissue residue

Plant Organ	Weight (g) Before	Weight (g) After	$[^{14}C]$ in phenolics	%	Total dpm (%)	$[^{14}C]$ in residue	Total %
Flowers	5.6	0.48	2,217	0.005		4,828	
Trifoliate Leaves	30.75	5.05	1,000	0.002		13,037	
Primary Leaves	16.75	2.28	79,181	0.18	249,459	264,129	10,621,395
Stem	17.5	3.69	125,663	0.29	(0.56)	9,286,111	(24.14)
Root	52.7	8.42	41,398	0.094		1,053,290	

The phenolic fractions were chromatographed on thin-layers in solvent

system (c) (Figure V : 5). There were very obvious differences between the primary leaves and stem fractions. In the stem phenolic fraction there were two peaks not present in the primary leaves, one at R_f 0.36 (4) and the other at R_f 0.74 (7). Peak 4 co-chromatographed with $[^{14}C]$-E_2. Eluting this zone from the stem fractions gave 7,253 dpm and the equivalent zone from primary leaves provided only 2,000 dpm. These zones were reacted with BSA and chromatographed by radio GLC (Figure V : 6). The sole peak obtained co-chromatographed with standard $[^{14}C]$-E_2. Analysis of the primary leaves gave no indication of $[^{14}C]$-E_2. The zone at R_f 0.4 - 0.58 (5) in stem afforded 17,446 dpm and from primary leaves (0.4 - 0.63) 24,699 dpm. These zones were thought, initially, to be estrone but analysis by radio GLC produced zones of radioactivity close to the solvent front and certainly did not co-chromatograph with estrone.

This part of the results has provided evidence - TLC (in three different solvent systems); radio GLC and crystallising to constant specific activity - that estradiol can be synthesised from MVA by P. vulgaris tissues and investigation of various parts of the plant implies that this synthesis can occur in the stem.

Figure V : 5 TLC radiochromatogram of the phenolic fraction of various zones of [^{14}C] MVA plants in solvent system c. (Flowering).

(a) Standard [^{14}C]-E_1 and E_2

(b) Primary leaves

(c) Stem

(d) Root

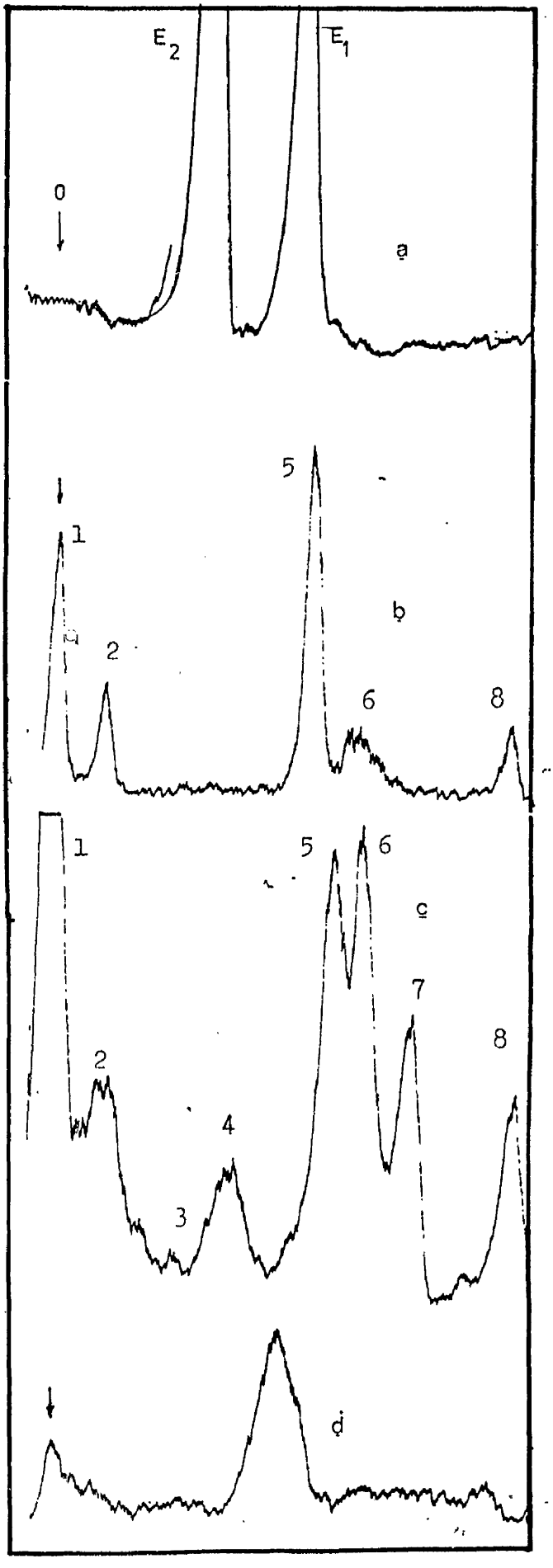
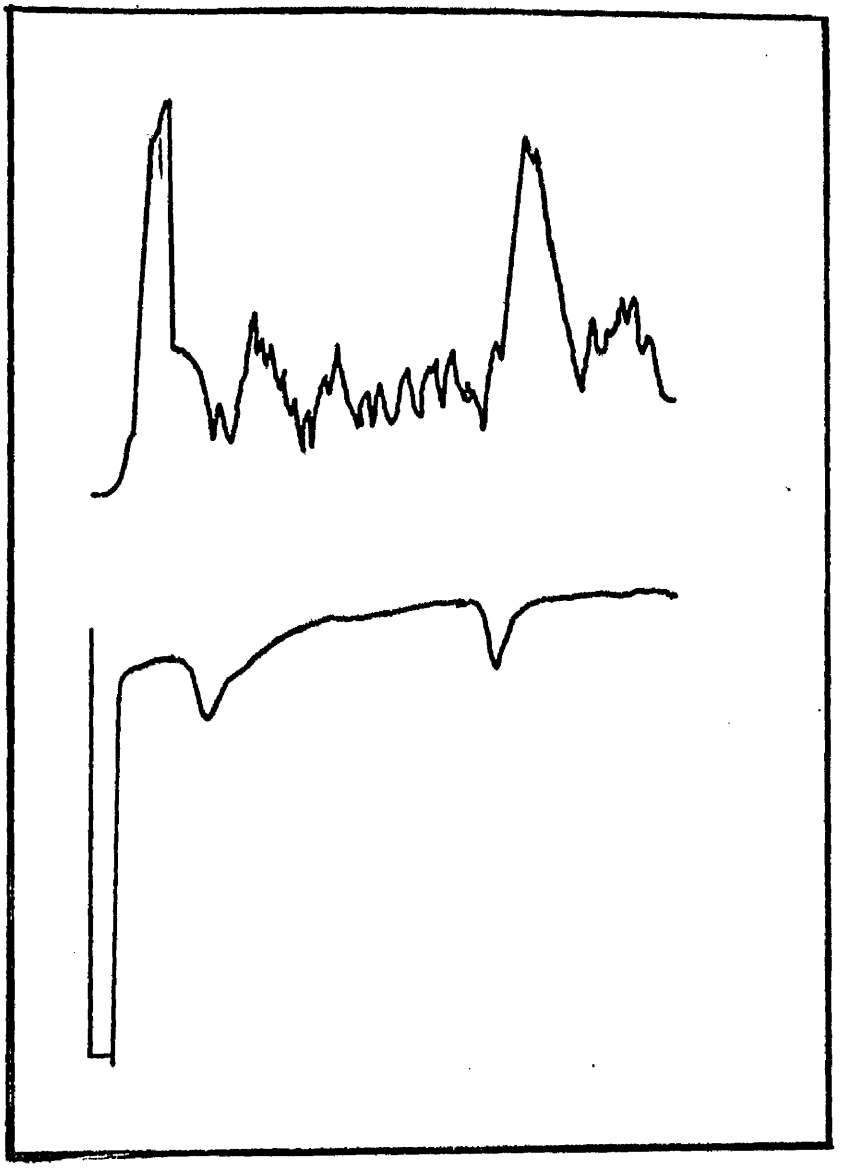

E2
E1
0
a
1
2
5
6
8
b
1
2
3
4
5
6
7
8
c
d

Figure V : 6 Radio GLC chromatogram of putative E_2 zone from stem.

5 ft. 5% OV 101 250°C 50 ml min^{-1}



DISCUSSION

I Endogenous Estrogen Analysis

(a) Extraction and purification methods

In order to demonstrate the presence of endogenous steroidal estrogens in extracts of the dwarf French bean it is essential to review and develop published analytical methods. As far as could be ascertained there has been no rigorous approach on this particular theme.

The oft-quoted studies of Kopcewicz (1971, 1972a, b) do not make mention of internal standards. It must be stressed, however, that such internal labelling can only assess the efficiency of the various purification stages. There would not appear to be an adequate arbiter of extraction efficiency although the development of suitable techniques is clearly called for, perhaps utilising the extraction of labelled endogenous components, thus enabling monitoring to be carried out.

It is apparent from the studies on the efficiency of partition procedures that there is a decrease in efficiency with increase in concentration of NaOH except in method two which has an efficiency close to that of 2N NaOH in Method 1. A possible reason for this observation may relate to the fact that the 2N and 4N NaOH in Method 1 (M and M, 7) and the NaOH in Method 2 produced large quantities of emulsion with the organic phase during partitioning and the backwashing may have removed this emulsion leading to subsequent loss of radioactivity from the aqueous fraction.

After the phenolic fraction was taken up in either ether or dichloromethane it was noted that when the aqueous fraction became colourless the radioactivity had been almost entirely removed. This would imply that in the absence of internal standard thorough partition had occurred. A further check was made on the efficiency by counting the radioactivity remaining after the alkali washing in the ethereal fraction (the neutral fraction). Owing to the very high colour quench of such samples, no meaningful results

could be obtained as the higher the quench the steeper is the slope of the calibration curve (especially below AES ratio 0.1) and the more difficult it is to make accurate measurements.

In those experiments designed to test the efficiency of purification techniques a considerable amount of development was necessary to devise methods of counting the radioactivity in the samples and in the course of these studies "Unisolve" (Koch-Light) was found to be the most suitable, and certainly the toluene:PPO scintillation cocktail is inappropriate.

With this Unisolve scintillation cocktail, water was added (4 ml) to the organic fractions to maintain constant quench in all samples, and it was found that the differences in counting efficiencies with either water, 1N, 2N or 4N NaOH were negligible.

From these studies it was possible to decide on the method of purification, particularly with regard to the normality of the alkali. In addition, Method 1 employed less toxic organic solvents, an important consideration in routine experimentation.

Critical appraisal of the results shows that under ideal conditions, the maximum purification efficiency does not exceed 62.9%. A profitable line of future research which would be of great merit would be the development of a purification method of high efficiency, brevity and simplicity. High pressure liquid chromatography (e.g. Reeve and Crozier, 1976) appears to offer a promising alternative.

Clearly then, the purification procedure in this work of partitioning, TLC and column chromatography, have been of some value but capable of great improvement.

(b) Endogenous estrogens

With the knowledge that the purification procedures would lead to probably 50% recovery of endogenous estrogens, initial studies were concerned with identifying estrogens on a purely qualitative basis. Much time was spent assaying these compounds by conventional GLC techniques

but neither TLC nor elution on Lipidex columns afforded samples pure enough to allow accurate assessment by such means. Even temperature programmed analysis could not resolve the estrogens from other phenolic compounds. Occasionally, it was possible to obtain peaks with similar retention times to estradiol TMSi but these were normally situated on the shoulder of much larger peaks.

Because of these problems and the fact that identification of an unknown compound, on the basis of retention time alone, is open to question, a more definitive approach was attempted by employing GLC combined with mass-spectrometry. The early analyses were by single-ion-monitoring where it is possible to detect compounds in the sub-nanogram level if the ion is sufficiently intense (see Palmer et al, 1975). Mirocha et al (1973) (cited in VandenHeuvel and Zacchei, 1976) have used this approach for the identification of diethylstilbestrol in swine foodstuff. The extract was so impure that it was impossible to detect it as its TMSi ether on a FID chromatogram. Nevertheless, on monitoring the molecular ion of the derivative (m/e 412) signals were observed for both the cis- and trans-isomers.

Such is the case when putative E_2 fractions (as their TMSi ethers) were analysed by SIM. The peaks shown in Figures I : 1 and 2 show positive peaks fully resolved from any others but the total-ion-traces from the FID revealed that such peaks co-chromatographing with E_2 TMSi were on the shoulders of larger peaks as in conventional GLC studies. The identification of E_2 TMSi from seeds was demonstrated twice on a 1% SE 30 column and once on a 3 ft. OV 210 + OV 17. The SE 30 identifications were of the molecular ion (m/e 416), but due to problems experienced with the mass-spectrometer on the occasion when the second column was employed, the voltage had to be increased to 70 eV, which reduces the number of ions at m/e 416 for identification because of fragmentation of the molecule.

Nevertheless, this does increase the abundance of the base ion (m/e 285). This ion with the same retention time as that from standard estradiol (Table I : 3) was detected in the phenolic fraction of seeds. Although useful as substantiating evidence this ion is also present in E_1 TMSi, albeit with a different retention time. It was felt that identification was more positive with the molecular ion in SIM analyses.

The identification of estradiol was demonstrated by analysis on two columns with different properties. SE 30 operates as a molecular sieve, i.e. compounds are eluted by increasing molecular weight, whereas OV 210 and OV 17 have ketone retaining properties thus reversing the positions of E_1 and E_2 with respect to elution on SE 30. E_2 was shown to be present in vegetative fractions on two occasions and on two different columns but E_2 has only been shown once by SIM in flowering plants.

The second method (MPM) has been reviewed by Palmer and Holmstedt (1975) and VandenHeuvel (1976). The method was first used in 1968 by Hammer et al. who continuously and simultaneously recorded up to three mass numbers characteristic of the substance being analysed. The drug chlorpromazine and its metabolites were identified by the retention times of the ions and their relative intensities.

This technique has several advantages: it is one of the most sensitive gas chromatographic detection systems known and can only be matched by the less selective electron capture detection system for certain compounds; it is anything from 100 - 1,000 times more sensitive than flame-ionisation detection or GC-MS. Indeed, mass fragmentograph studies of estrogens in urine samples have detected 5 pg E_2 which was six to seven times greater than background signal noise (Adlercreutz and Hunneman, 1975). It has also been demonstrated for certain estrogens that the detector response is directly related to the amount of sample injected in the range of 50 - 1,000 pg (Brooks and Middleditch, 1971).

As samples of the same order of magnitude as those employed in the SIM assays could be detected by such a system and having the ability to monitor several ions concurrently (2 ions per compound) this method was employed as a further, more definitive, identification procedure and in order to attempt the quantification of the estrogens.

It was found that seeds gave the most E_2 with evidence for some in the vegetative fractions. In the first of these analyses $[^{14}C]$-E_2 was employed as internal standard (see Table I : 4) and due to its low specific activity, nanogram quantities of E_2 should have been detected. In all cases excepting seeds and vegetative plants, the amount of E_2 detected could be accounted for by the amount of internal standard remaining in the sample. What is more, the solvent blank sample and the hydrolysed seed fraction appeared to have less than was expected from the amount of radiochemical remaining. To overcome this, an internal standard of high-specific activity was employed in further studies viz: $[2,4,6,7\text{-}^{3}H]$-E_2 which had a specific activity of 334 mCi mg^{-1}. Thus if 100,000 dpm of radiochemical were added to the extract this would be equivalent to an addition of only 135 pg.

Problems were encountered with the mass-spectrometer at this juncture and it was not possible to obtain a detector response which was directly related to the amount of sample injected. Therefore, attempts to quantify the levels of estradiol by this method had to be abandoned. This did not diminish the fact that E_2 was observed in fractions of seeds with perhaps some evidence in vegetative plants.

In some of the mass fragmentography assays especially those in which E_2 was the internal standard a very large peak was obtained at m/e 285 with the same retention time as E_1 (Figure I : 5). It was larger than the molecular ion (m/e 342) and hence it could not have originated from E_1. Repeating the analysis on another mass-spectrometer (courtesy of Dr R Anderson Forensic Science Department, Glasgow University) which was equipped with

a jet separator (compared with the membrane separator of the AEI mass-spectrometer used in the majority of our assays) found that the peak at m/e 285 was the height expected for E_1. These results infer that artefacts are produced from the membrane which interfere with analyses. It is possible that in this case E_2 TMSi was adsorbed on to the membrane and not removed until the E_1 TMSi was eluted from the column some time later. At this time some of the fractions eluted on Lipidex at the same retention volume as E_1 were found to contain compounds with retention times closely similar to that of estrone. Fractions obtained from seeds and flowers are described in Figures I : 4 and 5; these are the results from only one experiment as a further two assays could not confirm their presence. The compound, if E_1, was certainly real as no peak of a similar nature was found in the solvent blank.

With all the difficulties encountered in GC-MS studies it was decided early on in these studies to attempt to employ RIA in tandem with GC-MS analyses. Initial assays were encouraging with E_2 equivalent detected in seeds, vegetative and flowering plants. These samples were from the same fractions as were analysed by SIM (Table I : 3 replicate I), and thus the presence of a substance which has properties similar to E_2 in RIA and GC-MS has been demonstrated.

One of the difficulties with RIA is that it can be influenced by compounds which although binding to the antiserum are not estradiol. Estrone is known to have 10% of the affinity of E_2 for the antiserum and other compounds from plant tissue (the so-called phytoestrogens) are also known to bind to E_2-antiserum. One method of eliminating the possibility of contamination by other substances is to assay a series of dilutions of the unknown and, if it exhibits "parallelism", i.e. its binding curve has a similar shape to the calibration curve, the unknown is likely to be the same as the standard.

The dilution assays which were performed were shorter than the ideal, usually having only two or three aliquots assayed, but in the majority parallelism was established to a certain extent. The fact that some were not entirely parallel, although the possibility of experimental variation cannot be ruled out, implies that there may be some binding of other compounds. This, then, make the quantification of plant samples more difficult to perform. The problem was highlighted when the E_2 fraction from seeds (Table I : 6 and 7) was chromatographed on thin-layers and divided into ten "R_f zones". Binding was found at the R_f zone corresponding to E_2 as well as two other places. The component at the origin had a great deal of affinity for the antiserum and this probably accounts for the large amounts estimated in the total sample. The amount of binding at the R_f zone similar to E_2 is similar to that found in samples assayed since this time.

The samples which were acid-hydrolysed before purification cause some concern as in many instances they appear to have less E_2 than their non-hydrolysed counterparts. The reason for this is not at all clear. It is possible that the hydrolysis step destroys a component in the free fraction which can bind with the antiserum. This, however, would not explain similar results obtained from GC-MS. Furthermore, there are some samples in which the hydrolysed fraction contains more E_2 than the non-hydrolysed fraction (see Tables I : 6, 7 and 8).

Problems of this nature make the interpretation of results troublesome, and consequently, it was decided that these data would be used only on a qualitative basis.

Thus it appears that the tissues of P. vulgaris contain a substance which behaves in a similar fashion to E_2 both chromatographically (TLC, column and GC-MS) and in RIA, and consequently it can be concluded that there is a high possibility that this substance is indeed E_2.

Although multiple peak monitoring has been employed in the analysis of drugs (VandenHeuvel, 1976) and steroids in other fields (Adlercreutz and Hunneman, 1973, Adlercreutz et al., 1974) it has rarely been used in the analyses of plant extracts. Noteable exceptions are Rivier and Pilet, (1974) who identified and quantified indole-3-acetic acid (IAA) in maize by this method as well as Hillman et al., (1977) and MacDougall and Hillman (in press) in the quantification of IAA in P. vulgaris.

As far as is known this is the first reported identification of estrogens in plants by GC-MS using either SIM or MPM. Furthermore, it is only the second identification of estrogens in plants by RIA. Bennett et al., (1966) based their identification on a number of criteria. The substance was found to co-chromatograph on TLC in three separate solvent systems. On isolating and reducing it with $LiAlH_4$ it was found to co-chromatograph with E_2. It was also found to have an infra-red spectrum closely similar to authentic estrone. The identification of estrone from pomegranate seeds was similar using specific and non-specific colour reactions, of the substance and its acetate. Reducing as above gave a compound with similar chromatographic behaviour to E_2. Derivatising to its acetate gave the chromatographic mobility expected for E_2 acetate.

In the absence of more sophisticated instrumentation, these are highly commendable procedures for the identification of estrone. Nevertheless, one would doubt their validity in plant extracts for the quantitation of compounds. Due to the very nature of plant extracts there are likely to be contaminants which will interfere with the colour reaction. This is particularly true for the Kober reaction in which the colour reaction is influenced by artefacts from acid hydrolysis (Adlercreutz et al., cited in Grant, 1967). It is therefore unfortunate that Kopcewicz (1971, 1972a, b) should employ this method of identification and without the rigorous TLC analyses performed by Bennett et al., (1966) and Heftmann et al., (1966) and this must therefore question the accuracy of his identification.

Gawienowski and Gibbs (1969) succeeded in obtaining GC evidence for E_1 as well as that from TLC and quantified the peaks thus obtained by measuring peak heights and referring to a standard curve. It is unfortunate that a GLC standard had not been incorporated in the analysis as this would have made any quantification more precise.

Dean et al. (1971) employed RIA as a means of both quantifying and identifying estrone from pomegranate. While it is undoubtedly interesting that their estimate of the amounts in the seed are some 4,000 times less than that of Heftmann, it is unfortunate that, if this is the main means of identification, a more rigorous approach was not employed. There is no mention in the paper of the carrying out of dilution assays to ascertain whether parallelism could be established, however, known aliquots of estrone were added to the plant extracts and were observed to add to the response within 2% of that expected.

Further qualitative identification of estrogens has been demonstrated in moghat root and pollen from Egyptian palm by TLC and colour reactions combined with evidence from melting point and mixed melting point determinations, (E_1) and in peach seeds by colour reactions and infra-red spectra (E_1 and E_2).

It is noteworthy that in all of these so-called quantitative studies no allowance was made for losses during sample purification. Radioactively labelled substances should be added as early as possible in the process and assessed at the final stage. Not only does this account for any losses during processing but also from replicate to replicate.

Taking account of these limitations it is certain that all quantitations previously performed are equivocal and, on this basis can only be considered as qualitative. On the other hand, there is neither mention of the reproducibility of the results nor of solvent blanks and as such how accurate

are these early studies?

One of the most confusing and time-consuming areas of plant physiological work revolves around disputes concerning the presence or absence of metabolically important compounds. This work is no exception. Negative results, especially with GC-MS studies have provided frustrating delays in the early stages yet it has been possible with experience to demonstrate repeatedly the presence of steroidal estrogens in Phaseolus extracts both by GC-MS and RIA that any doubt must now be considered as negligible. It was necessary, however, to detail the negative results that have been encountered using the described analytical procedure: any research worker continuing these studies must be aware of the difficulties in gaining the requisite experience.

One further aspect demands brief mention. There are many known estrogens and it would be unwise to think that the list would not be extended. In the future it would be desirable to assay for all of these compounds with the preferred aim of establishing a "metabolic profile". Such information would be invaluable in establishing metabolic interrelationships inorganisms during various stages of growth and differentiation as well as disease and vigour. Obviously the procedures employed in this thesis work, but must be regarded as prime candidates for development. The plethora of analytical procedures which are available or becoming available should enable more rigid and efficient extraction and purification to be accomplished. It would appear that the detection and identification by gas chromatography and mass spectrometry are relatively well established and development in this area seems to be of a technical rather than a fundamental nature, e.g. capillary columns, separator styles and data handling. On the other hand, the extraction and purification stages would readily lend themselves to innovations such as high pressure liquid-chromatography, modern methods of liquid-chromatography and automatic handling methods.

II Metabolism and uptake of labelled compounds

In order to establish that the steroidal estrogens in extracts indeed represent the endogenous compounds as opposed to contaminants, the study of their possible biosynthesis was undertaken.

A method was sought that would allow the uptake of sufficient label. The procedure was modified from that employed by Atallah et al. (1975) which was originally used by Krotkov (1948). Despite the fact that seedlings had to undergo some surgery to allow uptake to take place, the labelled substances were at least taken up in aqueous solution.

Analysis of the phenolic fraction of the vegetative plants by thin-layer chromatograpny and elution on Lipidex gave little evidence of conversion of estrone to estradiol. The most convincing evidence came from the Lipidex elution profile in Figure II : 2 where there was a zone of radioactivity with a similar retention volume to $[^{14}C]$-E_2, however, it was too close to background radiation to be unequivocal. This was further confirmed when the putative E_2 fraction was chromatographed on radio GLC with no radioactivity observed at the same retention time as estradiol. If evidence for conversion of estrone to estradiol in vegetative plants was slight, the analysis of the estrogen fraction in flowering plants produced a clearer indication. Elution of an aliquot of the phenols on Lipidex (Figure II : 3), thin-layer chromatography in three solvent systems and radio GLC analysis all exhibited zones of radioactivity which corresponded to standard $[^{14}C]$-E_2. Further corroboratory evidence was obtained from crystallising to constant specific activity with non-radioactive estradiol a zone corresponding to $[^{14}C]$-E_2. (Table II : 1).

Thus having demonstrated by four different methods that estrone is converted to estradiol in whole plants, the second study attempted to show where the estradiol was being synthesised.

On harvesting, the vegetative plants were divided into various organs

including the seed leaves - the cotyledons. All plant parts with the exception of the stem gave no evidence of conversion of estrone to estradiol. In the stem, however, on chromatographing in benzene:propan-2-ol a zone of radioactivity corresponding to estradiol was detected and also appeared to be present in $CHCl_3$:MeOH. Subsequent analysis on further TLC and radio GLC failed to confirm the presence of estradiol in stems.

Analysis of flowering plants gave a similar pattern to the vegetative with the only evidence for estradiol being in the stem, with perhaps some conversion in roots. The conversion in the stem was corroborated thereafter by radio GLC and TLC in solvent system (e). Reference to Table II : 5 reveals that 91.6% of the [^{14}C] detected in the various tissues was extracted from the stem fraction, and it seems likely that the estradiol converted in the stem was due to there being a good deal more label in the stem than elsewhere.

Thus it has been shown on two occasions that estrone can be converted to estradiol. It should be mentioned that estradiol was not the only conversion that occurred as can be seen from the amount of radioactivity which was present at the origin in those TLC systems that separate the estrogens. Chromatography with a more polar solvent system separates a number of components (Figure II : 9). The identification of these compounds is beyond the scope of this thesis, but the crude cotyledon fraction is likely to contain C-3 esters and even glycosides, which due to conjugation at C-3 would not normally be isolated in the phenolic fractions (see Fieser and Fieser, 1959).

As a measure of uptake, plants were taken at the time of harvesting and monitored throughout for the presence of [^{14}C]. This also involved the assessment of various extraction media. It is evident from the results that only *ca.* 5% of the [^{14}C] added was extracted from the tissue extracted with either the toluene scintillation fluid or acetone, whereas almost 100%

has been extracted from the tissue assayed by the sample oxidiser. This latter procedure was found to be time consuming on the Oxymat sample oxidiser with normally three to four minutes required for each sample as it has to be loaded manually. This becomes a more lengthy process when assaying samples with varying levelsoof radioactivity as there is at least 1% carry over between each analysis. Thus wash and priming cycles are required between high and low activity samples. This problem was normally alleviated by assaying samples in what was thought to be an ascending order of radioactivity. Set against these disadvantages are the facts that 100% recovery can usually be obtained leading to samples with relatively constant quench.

In all stages of development in the plant, the highest levels of radioactivity remain in the hypocotyl with between 61 and 94% of the total extracted radiocarbon located here. Those samples with somewhat less in the hypocotyl tended to have more in the roots. The primary leaves accounted for most of the remaining [^{14}C] although varying amounts were detected throughout the plant including flowers. In the second set of samples it is of interest that the recovery is only of the order of 50% and this can only be attributed to losses in the Oxymat itself in which some problems with the catalyst were experienced.

These data also reaffirm those results obtained in the metabolism section that the bulk of radioactivity is located in the hypocotyl. This was thought to be due to the relative insolubility of estrone in water. A suitable alternative was thus sought which may be transported more readily throughout the plant. One such compound would seem to be estrone sulphate, a urinary product in animals which is both water-soluble and ether-insoluble, and although there is no evidence so far for the presence of any estrogen sulphate in plants it was thought that its water-soluble properties may prove valuable in an experimental system.

This experiment involved the use of tritiated E_1SO_4 as this compound was not available as the [^{14}C] isotope. This is unfortunate as [^{3}H] is less easily detected that [^{14}C].

Analysis of the cotyledon fraction located 1.38% of the total tritium added to plants. On analysis on TLC (Figure III : 1) and radio GLC two peaks corresponding to estrone and estradiol were obtained.

Analysis of the budding plants although giving both TLC and radio GLC evidence for estrone only gave TLC evidence for the presence of estradiol as a result of the small amounts present (Figure III : 3). The flowering plants, however, although only seven days older, revealed phenolic fractions which on thin-layers possessed a compound with the same retention time as estradiol and which was present in very large amounts relative to estrone. Radio GLC of the phenolic fraction resulted in a peak with a similar retention time as estradiol but none corresponding to estrone. This appears to confirm their relative proportions.

As in the [^{14}C] feeding experiments the investigation was repeated to try to establish the site of synthesis. In this experiment, however, only 2 μCi was applied to each seedling and this caused problems in the analysis due to the low efficiency of counting of [^{3}H] samples.

The extraction solvent was changed to $CHCl_3$:MeOH (Eneroth and Nystrom, 1968) (from $CHCl_3$:MeOH:BuOH) and reference to Table III : 1 reveals that although 0.14% of the tritium is isolated in the phenolic fractions only 32.6% remains in the tissue after extraction. If it is assumed that the remaining 67% was indeed extracted either very little conversion to free estrogens has occurred or the purification methods are inefficient. This latter suggestion is unlikely to be the whole story as the purification method was the same as for the estrogens. TLC of these fractions revealed some radioactivity at a similar R_f to estrone. In the fractions from shoot and leaf there was some radioactivity at the same R_f as E_2 (Figure III : 5).

It was not possible, however, to confirm this by elution on Lipidex, probably due to the low levels of tritum in the sample. What radioactivity was present was less than three times background and thus too close to background radiation to be significant.

Extraction of the flowering plants procured almost ten times the tritium in the phenolic fraction as in the previous analysis, although more label remained in the tissue after extraction than in the vegetative analysis.

Again although there was radioactivity at a similar R_f value to estradiol in shoots and primary leaves, this was not confirmed on elution on Lipidex. It is possible, however, that radiochemical breakdown had occurred.

Although the trifoliate leaves had a similar quantity of tritium there was no evidence for estradiol, and this suggests that trifoliate leaves may not contribute to the estradiol pool in the whole plant.

Monitoring the uptake of the estrone sulphate before potting up resulted in a similar pattern to that in estrone with the bulk of the tritium remaining in the hypocotyl but by the time of harvesting the primary leaves contained between 24 and 45% of the total with most of the remainder in the epicotyl and hypocotyl.. It is also apparent that more label reaches the upper parts of the plant perhaps relating to its being a negatively charged ion which is capable of rapid and long distance transport in the xylem which has negatively charged walls.

Thus it has been demonstrated that P. vulgaris has the capability of converting [^{3}H]-estrone sulphate to estrone and both [^{3}H]-E_1 sulphate and [^{14}C]-estrone to estradiol.

As mentioned earlier, estrone and estradiol are interconvertible in animals and it was thus decided to attempt the reverse conversion. In the first of these experiments only the flowering plants were examined for metabolism. Examination of the cotyledon extract gave very little

evidence for the conversion of estrone to estradiol which was confirmed on repetition of the analysis. Nevertheless, in the flowering plants (Figure IV : 2) there is a peak with the same R_f value as estrone in three solvent systems. This preliminary experiment though, did not involve high levels of radiocarbon and consequently it was repeated with the addition that vegetative plants were also analysed.

On chromatographing the phenolic fractions on thin-layers in $CHCl_3$: CH_3COOH a peak with similar R_f to estrone was obtained. Elution of the zone corresponding to estrone and chromatographing on Lipidex over 2,000 dpm (0.005%) was detected at the same retention volume as standard estrone. Attempts to chromatograph this relatively small fraction on radio GLC proved unfruitful.

The phenolic fraction of the flowering plants afforded a higher proportion of the radiocarbon (over 350,000 dpm) and a peak corresponding to estrone (Figure IV : 7). This zone was eluted and chromatographed on Lipidex and 9,542 dpm isolated from the same retention volume as $[^{14}C]$-E_1. Radio GLC analysis proved inconclusive as no radioactivity was detected. Problems were experienced with the radio GLC at this time and it appears that the sensitivity of the instrument may have been greatly reduced.

It is noteworthy that the estrone levels appear relatively higher in the vegetative fraction than they do in the flowering plants as this fits in with the results found in the conversion of estrone to estradiol. In the vegetative plants fed with estrone there is little estradiol whereas in those fed with estradiol there is proportionately the same amount of estrone as estradiol. This suggests that the equilibrium for interconversion may lie towards estrone early on in the development of the plants and as the plant ages the equilibrium shifts in favour of estradiol. In animal tissues the equilibrium is thought to be in favour of E_1 (see Grant, 1969).

The uptake of estradiol is very similar to that of estrone with between 89 and 96% of the [^{14}C] remaining in the hypocotyl, and less than 0.1% above the primary leaves. The distribution pattern of E_1 and E_2 compared with E_1 sulphate implies that E_1 and E_2 do not so much preferentially remain in the hypocotyl as do so due to their insolubility in the aqueous medium.

Analyses of the feeding solutions after incubation have proved interesting with more than one zone of activity apparent in them all, and in a few cases peaks corresponding to those being sought in the plant. The pattern observed was different from that in the cotyledons of all extracts and normally from the rest of the plant. Notably estrone sulphate has only a peak corresponding to estrone remaining (Figure III : 2).

Plants fed with estradiol do pose a problem as relatively high levels of estrone-like peaks were obtained in the incubation medium (Figure III : 4) where the proportions are very similar to those in the vegetative plants. There is, however, very little estrone-like compound in the cotyledon fraction.

The cause of this apparent autometabolism could be attributed to a number of factors: breakdown of the compound in solution; the action of enzymes released from the cut stems; bacterial contamination in the solutions; and metabolism of the compound within the stem and leaching out again during incubation. The first of these suggestions does seem unlikely as both estrone and estradiol are very stable compounds not thought to break down significantly in solution (see Fieser and Fieser, 1959). Bacterial contamination could account for the degradation as Schubert et al. (1967) have reported the degradation of cholesterol to 4-androstene-3,17-dione by Streptomyces olivaceous. The aromatisation of ring A has been demonstrated in Escherichia coli which can convert 4-androstene-3,17-dione to estradiol (Goddard and Hill, 1972). Though this is certainly

a possibility with the incubation method employed little turbidity was observed and there was no evidence of fungal mycelia. It is possible that the action of enzymes at the cut stems may have a greater influence on the degradation. Furthermore, it is unlikely that this contamination would account for the metabolic pattern observed in the plants compared to the feeding solution.

Thus, although there has been demonstrated an interconversion of estrone and estradiol this is no proof of an ability to synthesise them from basic precursors. Apart from some of the objections already mentioned it is possible that the plant has the enzymes necessary for this simple transhydrogenation but there may be a block further down the pathway. If it could be demonstrated that the plant could synthesise estrogens from precursors such as mevalonic acid much of the preceding argument would be tenable.

Initial metabolic studies also implied that estradiol conversion was at a maximum during flowering, therefore, the first experiments involved assaying flowering plants. In the first analysis the extract was boiled in 4N NaOH to give a measure of the total estrogens. This method isolated 245,140 dpm which accounts for 0.56% of the total radioactivity applied to to the plant but as MVA is a racemic mixture only half of any applied is available to the plant, making this equivalent to 1.1% incorporation. Figure V : 1a, b and c demonstrate that there is a zone of radioactivity corresponding to estradiol which was further corroborated by radio GLC, and crystallising to constant specific activity (Table V : 1). At first it was also thought that estrone had been isolated Figure V : 1a but thin-layer in different solvent systems and gas chromatography failed to verify this.

In the second experiment MVA incorporation into the phenolic fractions of various zones of the plant was studied. The plant tissue was extracted in $CHCl_3$:MeOH (instead of 4N NaOH) and thus any estrogens isolated from

the phenolic fractions would only have come from the free pool. By this method 140,587 dpm were obtained from the phenolic fractions (Table V : 2). This was considerably less than was isolated in the previous experiment (245,140 dpm) but the plants used were younger and it is probable that this would account for the lower incorporation. More radiocarbon was isolated from the primary leaves (61,747 dpm) than the stem (57,265 dpm). The roots and trifoliate leaves afforded a similar quantity to each other (10,000 dpm). Although the primary leaves and stem had similar quantities isolated in their phenolic fractions, the stem fraction had over twice as much as the primary leaves remaining after extraction and the roots had over five times that of the trifoliate leaves. TLC of the trifoliate leaves and roots in solvent system (b) showed a similar pattern with only one major zone of radioactivity (Figure V : 3a, c) showed different patterns. The primary leaves fraction not only had the peaks at the origin and R_f zone 0.32 - 0.55 but also had a zone of activity near the solvent front. It is possible that this was a sterol fraction which had carried over from the neutral fraction. There was also a small zone of radioactivity close to the origin which was not present in the stem fraction. In the shoot fraction there was a relatively small zone of activity at R_f 0.15 - 0.24 which corresponded with E_2.

This zone and that at R_f 0.32 - 0.55 was chromatographed in solvent system (d). The putative E_2 zone on chromatography in this system (Figure V : 4e) revealed some radioactivity at the same R_f as E_2. Analysis of this zone by radio GLC gave no indication of radioactivity at the same retention time as E_2. It would appear that the zones isolated from primary leaves and stem were different as the mobilities of the zones differed. In the stem two major peaks were observed, the larger of which had a similar R_f to E_1 and the smaller one had an R_f similar to E_2. Nevertheless, this latter zone was much higher than that isolated from the $CHCl_3$:MeOH chroma-

togram, and having run with the estrone it is possible that this peak represents 17α-E_2 although subsequent analysis by TLC and GLC was not able to confirm that this was an estrogen. Radio GLC analysis of the fraction corresponding to E_1 and the large zone in primary leaves gave radioactivity at the solvent front and none with similar retention times to E_1 or E_2.

Thus there is evidence for E_2 from vegetative plant extracts in two solvent systems but little for the presence of E_1.

Analysis of the flowering plants isolated a similar amount of radiocarbon in the phenolics (249,459) as in the first experiments, but there was also less remaining in the tissue after extraction and the question must be asked whether this was due to greater incorporation, more efficient extraction or perhaps both factors were contributing.

Chromatography in solvent system (c) revealed a very similar pattern to that obtained from the vegetative fractions (Figure V : 5). There were, however, many more peaks to be seen in the stem fraction with peaks corresponding to E_2 and possibly E_1. These same peaks were not apparent in the primary leaves, and although less radiocarbon was isolated from the primary leaves than was isolated from the stems this would not account for the differing profiles. Perhaps the zone at R_f similar to E_2 was present and could not be detected but the peaks 5 and 6 were in differing proportions between the two fractions (assuming compound 6 was the same in both fractions). Peaks 3 and 7 were also not present in the leaf fraction. Peak 8 probably represented some sterols which had not been thoroughly removed in the purification procedure. Although the identification of these other compounds was not attempted it is probable that they were non-steroidal phenolics but this requires affirmation as it was not even established whether they were steroidal in nature.

Analysis of the putative estradiol fraction from stems on radio GLC proved positive but the presence of E_1 could not be confirmed by this method.

Thus it has been demonstrated on two different occasions that estradiol can be biosynthesised from MVA precursor by flowering dwarf French beans and evidence from the vegetative plants suggests that there is synthesis as early as twelve days after potting up (i.e. 17 days total incubation).

Thus not only has it been demonstrated that estrone and estrone sulphate are converted to estradiol, a hitherto unreported process, but that estradiol can be synthesised from MVA. This also suggests that the enzymes necessary for such a pathway are present in plants or can be induced by treatment with the exogenous precursor. The fact that estradiol is synthesised from MVA implies that this conversion is carried out by the plant and not by bacteria (see Grant, 1969).

Although this is the first successful incorporation of MVA into estrogens, Bennett et al. (1967) applied a single dose of [2-^{14}C] MVA to six plants of Haplopappus heterophyllus and harvested them at intervals from 3 days to 6 months. In this latter plant alone was radiocarbon isolated from the phenolic fractions. [^{14}C] with similar R_f values to estriol and estradiol were found but were too close to background radiation to be unequivocal. As was shown in the above study, there was a zone of activity with a similar R_f to estrone in one solvent system but further chromatographic analyses failed to identify it as any known estrogen.

The question must be asked why it appears that there has been difficulty in isolating [^{14}C] estrone from the MVA fed plants. This may be influenced by the position of estrone in the biosynthetic pathway and the ease with which estrone is converted to estradiol (and vice versa). If estradiol were a precursor this would explain in part at least why it appears to accumulate to a lesser degree. It is also possible that if estrone is quickly metabolised to other compounds, (e.g. conjugates such as esters, glycosides etc.) it may not be able to accumulate under our experimental conditions (see Brown and Wetter, 1972). In the E_2 feeding studies there

is the situation in which E_1 accumulation is possible with large quantities of E_2 flooding the pool and allowing sufficient E_1 to be present at any one time to be detectable. There is also the suggestion from these studies that estrone is synthesised earlier in the development of the plant and indeed if this is the case, it may be that continued study of the vegetative plants fed with [^{14}C] may establish that this is the case, and implying that estrone is before estradiol in the pathway. Any interpretation from such preliminary investigations must be treated with caution.

One of the reasons that confounds interpretation of such data is the knowledge that a large proportion of the label remains in the tissue, and in particular in the stem fractions. It is difficult, therefore, to assess percentage incorporation. It may be advantageous to extract for a longer period in $CHCl_3$:MeOH and finally to reflux the residue in ethanolic KOH (McKean and Nes, 1977) which should certainly increase the amount of label extracted from the plant. It is also likely that there are mechanical problems in extracting radiocarbon from stem sections showing secondary thickening which are difficult to homogenise.

In addition only a small percentage of the total radioactivity applied appears to be isolated in the phenolic fractions and while the limited extraction efficiency may play a large part in this, the purification method may also need reappraisal. Of course, it could be that the remaining label is to be found in the non-phenolic fractions and one obvious line of research would be the investigation of the radiocarbon in the acidic and neutral fractions in which one would also isolate those free estrogens not isolated in the alkali wash.

It would seem that the method of feeding the radio isotopes is far from ideal for while uptake approaches 100%, ca. 80% remains in the hypocotyl and in fact the majority of this is situated in the lowest 0.5 cm, except in the case of the [^{3}H] estrone sulphate. Even the MVA did not move

throughout the plant as one would expect (Knapp., 1969). Being water soluble the MVA would have been thought to be distributed throughout the plant, but this is not the case with a relatively large proportion remaining in the stem. This may, of course, be due to the preferential transport of the biologically active enantiomer which seems unlikely in view of the fact that the MVA is taken up in the transpiration stream. Distribution of labelled sitosterol was found to be quite thorough throughout the tissues of Pelargonium hortorum (Atallah et al., 1975) as shown by autoradiography. Indeed, sterol was translocated to the top leaves with 48 hours. It is unfortunate, however, that the amount of radioactivity in the fractions was not presented and consequently useful comparisons cannot be made.

There is also the problem that the use of cut stems introduces a very artificial system and another method of application should be sought. There are many methods of application of radioactive substances which have been tried - all with varying success, (Bennett et al., 1967, Tso and Cheng, 1971, Shewry and Stobart, 1974, Atallah et al., 1975).

The cuticle of any leaf constitutes a considerable barrier to permeation. One attractive alternative to the above method is the application of the steroid in a Tween-80-water solution to the leaf surface. (The surfactant aids in the dispersal of the steroid in the water). Atallah et al. (1975) claim almost 100% uptake by this method and very rapid distribution of label throughout the plant.

The use of isotopic tracers is based on the principle that the isotopic atoms, having identical electronic configurations to those of normal atoms, behave chemically in the same way. This assumption must be qualified to allow for isotopic effects which result in the slower rupture of bonds and can lead to a slower attainment of equilibrium than in those containing only normal atoms. Nevertheless, in most cases isotope effects are not thought to be of great significance in studies of biosynthesis (see Brown and Wetter, 1972).

As with any investigations of this nature, the addition of any exogenous compound must interferewith pool sizes (see Brown and Wetter, 1972). Molecular interactions involve very low amounts of compound (e.g. 10 $\mu g\ Kg^{-1}$ represents about 10 ng per plant) and the application of 2 μCi of [2-^{14}C] MVA adds 47 ng to the pool. Thus caution must be exercised when interpreting results.

It is known, for example, that the early reactions up to the formation of squalene take place under anaerobic conditions and under the influence of soluble enzymes while the remaining reactions require oxygen and particulate enzymes (see Goad and Goodwin, 1972). Thus at least two compartments are involved in steroid biosynthesis.

It cannot be assumed, therefore, that the utilisation of exogenous material is by normal pathways and it may involve a secondary pathway in another compartment which under the normal course of events is never activated as the precursor pool size never attains the critical level which triggers this alternate pathway (see Oaks and Bidwell, 1970).

This is obviously a situation which is difficult to resolve as even the use of high specific activity radioisotopes still involves the addition of some exogenous compound to the pool. Nevertheless, this does not alter the fact that the enzymes required for the synthesis of estrogens appear to be present and constitutes firm evidence that the estrogens isolated in section I could certainly have been synthesised by the plant.

The results obtained from the distribution studies were unusual in that there appeared to be little movement of label into the metabolic sinks as can be observed for other plant growth substances (Colquhoun, 1974). The observed phenomenon may be a function of the incubating method and consequently this aspect merits further examination.

Further advancements and improvements in experimental design need to be considered. Autoradiography of plant tissues to determine in which cells the radioisotope (from estrogens) is situated would be valuable in establish-

ing a site of synthesis (Atallah et al., 1975).

Also, with the knowledge that human placental mitochondria can aromatise androstenedione to E_1 and E_2 (Renwick et al., 1973) and that microsome fractions of P. vulgaris var. Tender pod can incorporate label from [^{14}] MVA into sterol (Knapp 1969) and chloroplast, mitochondria and endoplasmic reticulum of P. vulgaris have been shown to contain sterol, (Brandt and Benveniste, 1972) this lends weight to the logic of investigating sub-cellular particles for their ability to synthesise estrogens. Other studies would almost certainly involve the investigation of the kinetics of the pathway and therefore, the rate of turnover, of the estrogens in the plant.

Further feeding studies should involve the uptake of estrogens by seeds and flowers and the feeding of labelled sterols, e.g. cholesterol and sitosterol, to dwarf French bean plants in order to determine which, if either, can incorporate label into estrogenic fractions, and perhaps the other intermediates too.

Concluding Remarks

When studying the role of molecular components in biological material a three-fold approach should be employed. Firstly the presence of the compound of interest should be established and quantified unequivocally preferably on an individual tissue and cell component basis. It is then important to demonstrate the ability of the organism to metabolise and indeed biosynthesise the component. Once these two criteria have been met it is logical to examine its physiological role.

This thesis has been designed to investigate the first and second of these approaches and while much of the research was of a preliminary nature awaiting further development it is perhaps pertinent at this stage to discuss the possible role of animal sex hormones, in plants.

The function of these compounds in animals has been reviewed briefly in the Introduction and one of the most important roles of these compounds is their effect on the sexual reproduction of animals. With the isolation of sex hormones from the reproductive parts of the plant (flowers, fruits seeds etc.) perhaps the most attractive of these roles is in the regulation of flowering and thence sexual reproduction (see Heftmann, 1975).

Attractive though it may be, the evidence supporting such a hypothesis appears to be conflicting. Several authors claim to show effects of steroidal estrogens in plants (see Thimann, 1935 and Bonner, 1937; Leshem, 1967; Kopcewicz, 1970 and 1971; Gawienowski et al., 1971). This view contrasts with the results obtained by numerous other authors whose work is reviewed by Thimann (1935); Bonner (1937) and Heftmann (1963). Also, it has been noted that when the sterile flowers of brussel sprouts are treated with androsterone they develop stamens and anthers, which are not present on control plants (Dr. T. Thomas pers. communication). Unfortunately no viable pollen has been obtained from such studies.

Steroid inhibitors have been noted to retard flowering in plants

(Sachs, 1966; Douglas and Paleg, 1974), and sterols have been applied to these plants with varying effects on flowering. As far as can be ascertained, however, the authors did not apply sex hormones in the experiments.

Effects of steroid estrogens on plants have been investigated in collaboration with S. Hewitt and preliminary results suggest no effect in leaf discs of senescent P. vulgaris.

Although this similarity of physiological role between animals and plants is certainly appealing, it may be too simplistic a proposition. Such a notion implies that the steroids (as in animals) would be effective at very low concentrations in regulating the structure and function of certain tissues in the intact organism (Villee, 1961). In animals the steroids are active in "target-organs", and are normally transported to these "target-organs" from the other parts of the organism in which they are synthesised. In this respect one would expect that the apex prior to flowering could be termed as the target organ, and the sex-hormones accumulating in the sexual parts of the plant as development proceeds.

This suggests an obvious theme of research - the study of flowering development on the steroidal sex hormones (both endogenous and biosynthetic studies) and possibly the micro-injection of various sex-hormones into flower buds in an attempt to alter the sex-expression.

Such an effect must of necessity involve change at a fundamental level. In animal tissues estradiol is thought to mediate its effect on the target organs by acting on the nucleus. This is dependent on the presence of cytoplasmic receptors, because, although estradiol has been demonstrated to bind to chromatin in the nuclei of non-target organs, normally this only occurs when the cytosol receptor is present to carry the hormone to the nucleus, (see Villee, 1961 and O'Malley, 1973).

While even the existence of such receptors in plants is a matter

for conjecture, objections already come to bear. These receptors are known not to be specific for estrogens. Compounds like diethylstilbestrol (a synthetic estrogen) and isoflavones (phytoestrogens ingested in the diet) are known to bind to the estradiol receptor and indeed to the chromatin (the phytoestrogens irreversibly so) (see Grant, 1969; Shutt and Cox, 1972 and Shutt, 1976). If these phytoestrogens can affect animal receptors it is highly probable that they would interfere with any cytosol receptor in the plant. Thus it is possible that a different sex-hormone has evolved in plants. It is interesting in this respect that there have been reports of increased RNA and DNA levels in plants under the influence of both estrone and estradiol (Mitra and Sen, 1975; Martinez-Honduvilla et al., 1976).

Because the presence of estrogens has been established in plants does not of necessity imply that they have any physiological role. Sterols, which are implicated by many to have an important structural role in membranes (see Nes, 1974 and Grunwald, 1975), are known to be precursors of sex-hormones in animals (see Heftmann, 1975).

In addition the corticosteroids - cortisol and corticosterone - have been reported to influence elongation growth in Mung bean (Geuns, 1974). Thus it is not inconceivable that the steroidal estrogens in plants originate from the remnants of a primaeval enzymic system which evolved a different physiological role, i.e. the estrogens may represent the degradative by-products of membrane components. If the corticosteroid pathway is present in plants progesterone is their precursor as well as estrogens giving the link between estrogens and the corticosteroids.

This thesis presents some information on the presence, biosynthesis and metabolism of steroid estrogens in an herbaceous dicotyledonous plant. It is perhaps premature to speculate on the significance of such observations especially in terms of elucidating metabolic and economic roles.

That levels or any other parameters of a named compound can be correlated with a physiological event is by and large insufficient evidence of a regulatory function. Thus these findings must be reviewed in the context of providing a few links in a convoluted and anastomosing catenary sequence of events.

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** Review Article.