PHYSIOLOGICAL ASPECTS OF STEROIDS
IN POLLEN OF Pinus ellioti Engelm.

by

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The concentrations of sterols and some physiological aspects of steroids were studied during germination of pollen of slash pine. Cholesterol, campesterol, stigmasterol, and β -sitosterol were identified by gas-liquid chromatography (GLC) and combined GLC-mass spectrometry.

On a gram fresh weight basis, the pollen contained approximately 2500 μ g total (non-water soluble) sterols; 75% was sitosterol. The total sterol content was comprised of approximately 2200 μ g free sterols and 300 μ g steryl glycosides. The concentration of free sterols peaked after 12 and 48 h, the lowest concentration was reached after 24 h. This change in concentration was primarily caused by changing sitosterol levels. Changing concentrations of free sterols paralleled opposite changes in the concentration of steryl glycosides. No steryl esters were detected during a 48 h germination period. The

aqueous media contained approximately 240 μg total sterols per gram pollen. Cholesterol accounted for approximately 75% in this fraction, contrasting sharply with 5% in the pollen (non-water soluble) fraction.

No sterols were labeled after incubation with ^{14}C -mevalonic acid. Very little, if any, sterols were synthesized, the almost constant concentration of total sterols supported this concept.

Pollen germination was depressed by the sterol biosynthesis inhibitor SKF-7997A₃, however the inhibition was probably caused by breakdown products of this compound. Pollen germination was strongly inhibited by abscisic acid (ABA), SKF-7997A₃, and cordycepin (3' desoxy-adenosine), all three compounds increased the efflux of sterols into the media. However, cholesterol efflux was less than in the controls.

Pollen germination was strongly dependent on pollen concentration, optimum germination occurred with 10 to 30 mg pollen per ml. With 2 mg $\cdot$ mg⁻¹ only 51% germinated relative to optimum pollen germination. Dimethylsulphoxide (10^{-4} M) increased the relative germination to 82%, further addition of pregnenolone, estradiol, estriol or corticosterone (each 10^{-5} M) restored germination to almost optimum levels. Animal steroid hormones increased the conductance of the pollen media; however, there was no discernible relationship between increased germination and increased conductance.

INTRODUCTION

Since the discovery of sterols and other steroids in plants, a wealth of information on the occurrence of these compounds has been accumulated. The structures are known and much progress has been made in unraveling their metabolism (Grunwald, 1975). Most animal steroids occur in plants (Knights, 1973) with the possible exception of the C_{24} bile acids. However, the isolation of 5 β -cholanolic acid from jequirity beans (Abrus precatorius) has recently been reported (Mandava, 1974). Also, most if not all plant steroids are found in certain invertebrate animals (Bergmann, 1953).

Sterols have similar functions in animals and plants (Heftmann, 1973); nevertheless, very little is known about their physiological functions in plants. Essentially two theories cover most of the proposed functions: sterols act as hormones or their precursors and sterols are structural components of membranes (Barksdale, 1969; Hendrix, 1970; Heftmann, 1975). Suggested functional roles of sterols as part of the integrity of membranes are: they prevent leakage of cells under stress conditions (Gottlieb and Shaw, 1970; Mudd and Kleinschmidt, 1970) and influence uptake of ions (Hendrix and Higinbotham, 1973; Grunwald, 1974; Archer and Gale, 1975; Carbonero et al., 1975; Karst and Jund, 1976). Steroids have been shown to

inhibit seed germination (Nord and Van Atta, 1960) and stimulate growth rates of pea embryos (Bonner and Axtman, 1937; Kögl and Haagen-Smit, 1936), corn roots (Fiedler, 1936) and onion roots (Weyland, 1948). Hylmö (1940) demonstrated that steroidal sex hormones influence sex expression in spinach. Various other physiological responses have been reported, but the type of response was largely dependent on the type of steroid applied, the particular plant or plant part involved and the time of application.

Inhibitors of sterol biosynthesis have a profound effect on plants. When Tris-(2-diethyl-aminoethyl)-phosphate-hydrochloride (SKF-7997A₃) is applied to Pharbitis nil, flower induction is inhibited (Bonner et al. 1963; Sachs, 1966). Common plant growth retardants inhibit sterol biosynthesis as well as shoot growth (Douglas and Paleg, 1974) in tobacco seedlings. Pretreatment with a steroid biosynthesis inhibitor increased susceptibility of bean leaves to ozone (Spotts et al., 1975).

The demonstrated physiological effects, but very limited understanding of the roles of steroids in plants, warrants further study of this subject.

Pollen cytoplasm, although lacking chloroplasts, contains all subcellular units common to other living cells, i.e. plastids, mitochondria, golgi bodies, endoplasmic reticulum, vesicles and ribosomes (Larsen and Lewis, 1962). Also, very rapid extension growth of pollen tubes, high metabolic rates and high sensitivity to toxic compounds (Stanley and Linskens, 1974) renders this material well-suited for a study such as this. Although collection of large

quantities of pollen is rather cumbersome, slash pine pollen is readily available and long-lived. Thus, a large well mixed sample, properly stored, provides a homogenous source for many experiments.

This study utilizes pollen of slash pine (Pinus elliottii Engelm.) to investigate steroid metabolism as related to germination. Pollen is a convenient material for biochemical and physiological studies, for when grown under proper conditions, the metabolic processes common to most living cells occur (Stanley and Linskens, 1974).

Although a number of studies have been made on the chemical composition of various pollens, very few deal with lipid metabolism (Hoeberichts and Linskens, 1968), or sterols (Kvanta, 1968). Yet, pollen contains relatively high levels of sterols (Anderson and Moore, 1923) and sometimes unusual types (Knights, 1968).

This study of slash pine pollen has the following objectives:

1. Isolate, identify and quantify the major sterols and interactions during germination, with attention to de novo synthesis and to the various categories of sterols i.e. free sterols, steryl esters, steryl glycosides and water soluble sterols.
2. Test the effects of abscisic acid, cordycepin and SKF-7997A₃ on the composition of total sterols and pollen germination.
3. A determination of the effects of pollen concentration, various temperature regimes and added sterols, including animal steroid hormones, on pollen germination.
4. Test the influence of added animal steroid hormones, sterols and abscisic acid (ABA) on cell leakage.

LITERATURE REVIEW

Historical Perspectives

Studies on sterols probably began with the investigation of dissolution products of gallstones (Vallisneri, 1733). Poulletier de La Salle isolated their main constituents circa 1769 (Smeaton, 1962; Fourcroy, 1806). Chevreuil (1816) demonstrated that the main constituent was non-saponifiable and introduced the name cholestérine (chole-bile and stereos-solid), replacing the earlier name cholestéarine (stear-fat). The alcoholic nature of cholesterol was established by Berthelot (1859), the presence of the double bond by Wislicenus (1868), the correct formula $C_{27}H_{44}O$ by Reinitzer (1888) and the molecular weight $(C_{27}H_{44}O)_1$ by Abel (1890). Braconnot (1811) isolated a fatty substance from mushrooms, which Vauquelin (1813) named agaricine, and was termed ergostérine (ergosterol) by Tanret (1889).

Beneke (1862) isolated a sterol from peas; a similar compound was obtained from Calabar beans (Physostigma venenosum) and Pisum sativum (Hesse, 1878). Hesse observed that it was not identical with animal cholesterol and named it phytosterol. Búrian (1897) discovered sitosterol and an isomer which he called para-sitosterin. It was discovered that sitosterol occurs in several isomeric forms (Anderson and Shriner, 1926; Anderson et al., 1926) and it became apparent that phytosterols are ubiquitous in the plant kingdom, occurring in all plant parts (Ellis, 1918 a,b,c; Anderson and Moore, 1923; Koessler,

1918). The sterol obtained from Calabar beans was shown to be a mixture of sitosterol and stigmasterol (Windaus and Hauth, 1906); campesterol was isolated from the oils of rape, soybean and wheat by Fernholz and MacPhyllumy (1941).

Systematic analysis of cholesterol and bile acids began around 1929 (Windaus, 1932). The discoveries during the 1920's of the hormonal functions of steroids in animals were responsible for continued extensive research. Studies of plant steroids were initiated some three decades after the discovery of cholesterol in animals, but interest in these compounds never attained the level exhibited for animal steroids. Comprehensive texts on steroids appeared in 1937, 1949 and 1959 (Fieser and Fieser, 1937, 1949, 1959), these much needed works were a welcome aid for scholars interested in most aspects of steroid research. Research on plant steroids received another strong stimulus during the early 1950's with the discovery that cortisone was effective in treatment of arthritis and other inflammatory ailments (Bergman, 1953). Soon plant steroids were used to synthesize cortisone and its derivatives. Within a decade, probably over a hundred steroids were synthesized from starting materials such as stigmasterol, cholesterol, diosgenin, and hecogenin (Appelzweig, 1959). Some five years later thousands of biologically active steroids were known (Appelzweig, 1964). Proposals have been advanced for the cultivation and breeding of plants containing steroidal sapogenins (Blunden et al., 1975) to satisfy the steroid market. It was estimated that world consumption of diosgenin would be over 1000 tons per annum by 1973 (Hardman, 1969). At present a wealth of information is available on

the occurrence of sterols in the plant kingdom. The structures are known and much progress has been made in unraveling their biosynthetic pathways (Grunwald, 1975). The recent interest in phytosterols probably began when Beerthuis and Recourt (1960) announced that these compounds can be identified by using gas-liquid chromatography. Mass spectrometry and the availability of stereo-specifically labeled sterol precursors greatly aided collection of this information (Grunwald, 1975). Although a great number of sterols have been isolated, the major sterols in higher plants were discovered before the middle of the 20th century (Bergmann, 1953).

Sterols and Other Steroids

Nomenclature

Perhydropentanophenanthrene can be thought of as the parent hydrocarbon ring system of steroids, the rings are designated A, B, C and D (Fieser and Fieser, 1959). This name however does not imply any stereochemistry; the term gonane (Formula 1 and 2) takes stereochemistry into consideration

Formula 1.
Structure of 5 α -gonane.

Formula 2.
Structure of 5 β -gonane.

This manuscript deals primarily with steroids that have a methyl-group at C-10 and C-13. The parent compounds for these series are 5 α - and 5 β -androsterane (Formula 3 and 4) (IUPAC-IUB, 1971).

All the rings are fused trans as in trans-decalin. The steroids

Formula 3.
Structure of 5 α -androstane.

Formula 4.
Structure of 5 β -androstane.

dealt with in this paper have the basic androstane structure, in addition a double bond($\Delta^{5(6)}$), a side chain at C-17 (β -position), and a hydroxyl group at C-3 (β -position). The term sterol refers only to those steroids whose structures show close resemblance to cholesterol (Formula 5, R=I). They differ from cholesterol only in the number and position of cyclic and acyclic double bonds and in the structure of the alkyl side chain. Such sterols are widely distributed in the plant kingdom (Bergmann, 1953). The major sterols of higher plants are cholesterol, campesterol (Formula 5, R=II), stigmasterol (Formula 5, R=III), and sitosterol (Formula 5, R=IV). The side chains are β -oriented at C-17.

Formula 5. Structure of cholesterol (R=I), campesterol (R=II), stigmasterol (R=III) and β -sitosterol (R=IV).

Although the known sterols possess from 8 to 10 asymmetric centers, depending on the degree of unsaturation, no isomers have as yet been found which are epimeric at positions other than C-24.

However, a few triterpene alcohols (dimethyl sterols) with α -configurations at C-17, C-18 or C-20 have been reported (Heilbron et al., 1934; Irvine et al., 1956; Lewis, 1959; Barbour et al., 1951; Dupont et al., 1951; Newbold and Spring, 1944; Haines and Warren, 1949). The alkyl groups at C-24 usually have the α -configuration in higher plants (Goad and Goodwin, 1972), while algae (Patterson, 1971) and fungi (Rubinstein et al., 1976) generally produce sterols with the 24 β -configuration. Trivial names for important steroid derivatives have been retained, these being mostly natural compounds of significant biological activity (IUPAC-IUC, 1971). Thus the trivial name campesterol is still used along with the official denotation (24R)-24-methylcholest-5-en-3 β -ol; however, the trivial names imply a specific stereochemistry.

Forms of Occurrence of Sterols

It was recognized early during steroid research that sterols often occur not only in the free form, but also are covalently bound or form various associations or conjugates. Also, sterols and some derivatives can form stable colloids (Remesov and Sosi, 1936; Remesov and Sepalowa, 1936). Gobley (1846), discovered lecithin (phosphatidyl choline) and noted that it was frequently associated with cholesterol. Cholesterol esters were discovered in wool grease (Schulze, 1873), and in blood (Hürthle, 1896). Page and Rudy (1930) summarized and corrected the voluminous literature on fatty acid esters of cholesterol.

Compounds such as citrullol, ipuranol, cluytianol and several others, formerly considered to be higher alcohols were identified as steryl-D-glucosides, primarily sitosteryl-D-glucoside (Power and Salway, 1913).

In 1964 esterified forms of steryl glucoside (fatty acids esterified at C-6 of the sugar portion) were isolated and identified (Lepage, 1964) and since discovered throughout the plant kingdom.

Adams and Parks (1967) discovered a water-soluble and physiologically active form of ergosterol and cholesterol in a commercial yeast extract. In a subsequent publication (Adams and Parks, 1968) they reported on the polysaccharide nature of the sterol binding substance. The polysaccharide was later identified as cell wall mannan (Thompson et al., 1973). Water soluble sterol complexes were also isolated from the protozoan Euglena gracilis (Brandt et al., 1969), up to 40% of the sterols occurred in the water soluble form. Subsequently, similar complexes were isolated from Kalanchoe blossfeldiana (Price, 1970) and corn (Rohmer et al., 1972). The latter researchers found water soluble complexes of sterols, sterol esters and their precursors (4 α -methyl sterols). The principal sterol binding polysaccharide of Saccharomyces carlsbergensis was identified as a mannan and that of the filamentous fungi Rhizopus arrhizus and Penicillium roquefortii as glucan(s) (Pillai and Weete, 1975). Adler and Kasprzyk (1975) isolated thirteen sterols from Calendula officinalis seedlings, about half of the sterols occurred at low concentrations in the water soluble fraction. However, Willuhn and Liebau (1976) found water soluble sterols at relatively high concentrations in the leaves of Solanum dulcamara.

It has been suggested that the water soluble sterol fraction occurs only at low concentrations, bound to enzyme systems (Rohmer et al., 1972; Adler and Kasprzyk, 1975). However, it seems almost

certain that these complexes have to be regarded as a fifth class of sterols and must be included in all studies on phytosterols (Anding et al., 1972).

Thus sterols are found in plants as free alcohols, steryl esters, steryl glycosides, acylated steryl glycosides (Lepage, 1964; Kiribuchi et al., 1966; Eichenberger and Grob, 1968) and water soluble complexes. The general occurrence and the amounts in which sterol derivatives are present (up to 29% of the total lipid fraction in apple peel [Galliard, 1968]) points to a definite function of these compounds in plants (Eichenberger and Grob, 1970).

Occurrence of Sterols in Plants in Relation to Phylogeny Sterols in Higher Plants

Isolated studies of plant sterols were presented by Dalmer (1932) and Schwab (1941). Bean (1973) compiled data on sterol contents of species in all plant phyla; however, it is still too early to generalize on sterol distributions in the plant kingdom. The listings of sterols in higher plants are very incomplete because the analyses involved: (1) few species of any family, (2) usually only part of the plants of any given species, (3) only a point in time during the plants' life cycle. In addition, reports before the mid 1960's do not contain sterols occurring in low concentrations due to insensitivity of detection methods. Nevertheless, some generalizations can be made. β -Sitosterol, stigmasterol, and campesterol have been detected in most higher plants. Cholesterol, although usually in low concentrations is equally ubiquitous. Screening of forty vegetables revealed that β -sitosterol occurred in the highest concentrations in

all plants except spinach, where α -spinasterol was highest (Oka et al., 1973). β -Sitosterol was the predominant sterol in all but two of twenty plant oils. Most oils also contained campesterol, stigmasterol, Δ -5 avenasterol, Δ -7 avenasterol, and at least traces of cholesterol and brassicasterol (Jeong et al., 1974). Methyl sterol fractions contained the 4-monomethyl sterols obtusifolliol, gramisterol and citrosadienol abundantly, cycloeucalenol to a lesser extent, and another five monomethyl sterols were tentatively identified. In addition some dimethyl sterols occurred in most oils (Jeong et al., 1975). New sterols are continually being discovered (Kusano et al., 1975; Sharma et al., 1974).

Sterols in Fungi

Knowledge of sterol composition in fungi is limited; however, a number of species of most fungal classes have been assayed.

Myxomycetes. Physarium polycephalum contains cholesterol and five other sterols commonly found in higher plants (Lenfant et al., 1970). Schyzophyllum commune contains primarily ergosterol and traces of three others (Mokady and Koltin, 1971).

Eumycetes. The fungal classes of this phylum contain a great number of different sterols. Imperfect fungi contain ergosterol primarily (Bean, 1973). Phycomycetes contain cholesterol, campesterol, stigmasterol, β -sitosterol and some other sterols (Bean, 1973), these sterols are common to higher plants. Phycomyces blakesleanus contains ergosterol and derivatives, cholesterol, lanosterol and 24-methylenelanost-8-en-3 β -ol (Mercer and Bartlett, 1974).

Goulston et al., (1975) identified 24-methylene-24,25 dihydrolanosterol and possible other ergosterol precursors in the

same species. Sterols in the Saprolegniales and Leptomitales, having cellulose cell walls, were compared with other Phycomycetes with chitin cell walls. The species with cellulose walls contain desmosterol, 24-methylene cholesterol and fucosterol, while twelve species with chitin walls contain primarily ergosterol (McCorkindale et al., 1969). Ascomycetes contain primarily ergosterol (Bean, 1973). The sterols of yeast (Saccharomyces cerevisiae) have been studied extensively, the major ones are ergosterol, zymosterol and $\Delta^{5,7,22,24}$ ergostatetraene-3 β -ol (Longley et al., 1968; Hunter and Rose, 1972). The budding yeast Kluyveromyces fragilis contains only ergosterol during its entire lifecycle (Penman and Duffus, 1974; 1976).

Basidiomycetes. Most species contain ergosterol or related C-28 sterols such as 5-dihydroergosterol, 22-dihydroergosterol and Δ^7 ergostenol (fungisterol), but some species may also contain C-29 sterols such as stigmasta-5,7-dienol and stigmasta-7,24(28)-dienol (Bean, 1973).

Sterols in Algae

Sterol composition in algae has been examined extensively during the last several years. Sterols may be used to gain insight into phylogenetic relationships between the algal classes (Patterson et al., 1974) and may provide a tool to distinguish closely related species (Seckback and Ikan, 1972). Their position at the lower end of the food chain and the sterol requirement of some organisms feeding on algae (Patterson, 1974) renders sterol research valuable to systems ecologists. Interpretation of the available data should be done with caution since it is uncertain whether sterols are synthesized by

algae or absorbed from their media (Patterson, 1971). The Rhodophyte Porphridium cruentum lacked sterols when cultured on an artificial medium (Aaronson and Baker, 1961), however, this may have been due to lack of sensitive analytical techniques. Beastall et al. (1971) found a complex mixture of sterols in this organism, the major one being 22-dehydro-cholesterol. Information on blue-green algae is scarce; cholesterol and β -sitosterol were isolated from Anacystus indulans and Fremyella diplosiphon (Reitz and Hamilton, 1968), while 24-ethyl- Δ^7 cholesterol and some minor constituents were isolated from Phormidium luridum (De Souza and Nes, 1968). The thermophilic unicellular alga Cyanidium caldarum is not fixed to any specific taxonomic group, but evidence was given that this species is a blue-green colored rhodophyte; it contains campesterol and some cholesterol (Seckback and Ikan, 1972). Reports on sterol content of Rhodophyta have been controversial (Patterson, 1971); C-28 sterols were reportedly dominant (Alcaide et al., 1969). Subsequent analysis of seven orders showed cholesterol to be the major sterol in most species, but some contained primarily desmosterol. One species contained 22-dehydro-cholesterol, and a few others contained traces of C-28 and C-29 sterols (Patterson, 1971). Similar findings were reported for 17 red algae (Fattarusso et al., 1975).

Possibly the Rhodophyta can be classified into three groups: one group containing predominantly cholesterol and a variety of C26-C29 sterols, another containing primarily 5, 6 dihydrosterols (stanols) in which cholestanol is the major one, and the third group containing 22-dehydrocholesterol primarily (Chardon-Loriaux et al., 1976).

Chlorophyta have a sterol composition in complexity similar to higher plants. For example, the genus Chlorella can be divided into three groups containing Δ^5 , Δ^7 or $\Delta^{5,7}$ sterols. Eight sterols were found in a single species (Chlorella ellipsoidea), with two being isolated for the first time (Patterson, 1974). The green algae probably have to be considered according to their taxonomic orders. The major sterols for the various orders have been reported, but a classification based on sterol contents cannot be given at the present. Some of the major sterols of the Chlorophyta are chondrasterol, ergosterol, poriferasterol, spinasterol, 28-isofucosterol, cholesterol, and 24-methylene cholesterol (Tsuda and Sakai, 1960; Patterson, 1967; Patterson and Kraus, 1965; Gibbons and Goad, 1968; Ikewawa et al., 1968; Patterson, 1969). Information on the sterol contents of other algal classes is very inadequate, and no generalization can be made at present.

Sterols in Bacteria

The early belief that sterols are absent from bacteria (Anderson et al., 1935) and certain primitive asexual algae (Carter et al., 1939) has been proven erroneous. Escherichia coli contains cholesterol, campesterol, stigmasterol and β -sitosterol (Schubert et al., 1964), Streptomyces divaceus cholesterol only and Azoto bacter Δ^7 -ergosterol, traces of three lanosterol derivatives, ergosterol and Δ^7 ,²² ergostadiene-3 β -ol. Failure to detect sterols probably was due to insensitive techniques (Bean, 1973). No trend in sterol composition can be indicated due to lack of information.

Intracellular Distribution of Sterols

Analyses of various plant cells revealed that all subcellular fractions contained sterols (Kemp and Mercer, 1968; Grunwald, 1970; Dupéron et al., 1972; Brand and Benveniste, 1972; Dupéron and Dupéron, 1973; Katayama and Katoh, 1974 b), and all fractions contained the four common categories (free, esters, glycosides and acylated glycosides) of sterols (Grunwald, 1970; Dupéron et al., 1972). Total sterols were highest in particulate fractions; microsomes were richest followed by mitochondria and chloroplasts (Kemp and Mercer, 1968; Brandt and Beneveniste, 1972; Dupéron et al., 1972; Dupéron and Dupéron, 1973; Dupéron et al., 1975). Free sterols were highest in the 500 x g fraction of tobacco leaf homogenates (Grunwald, 1970), in the starch fraction of potato tubers (Dupéron et al., 1972) and in the nuclear fraction of maize shoots (Kemp and Mercer, 1968).

Steryl esters showed the highest concentration in the supernatant of sprouting or non-sprouting potato tuber fractions (Dupéron et al., 1972; Dupéron and Dupéron, 1973) and in the supernatant fraction of maturing soybean seeds (Katayama and Katoh, 1974 a; 1974 b). Esters were also highest in the nuclear fraction of maize shoot homogenates (Kemp and Mercer, 1968).

Steryl glycosides and acylated steryl glycosides were represented primarily in the microsomes of sprouting potato tubers (Dupéron and Dupéron, 1973) and in the microsomes and mitochondria of non-sprouting potato tubers (Dupéron et al., 1972). Glycosides were almost exclusively concentrated in the microsomes of lettuce leaves (Eichenberger and Grob, 1970).

Cholesterol was a component of the chloroplasts of eight species of higher plants, the content of other sterols was typical of the species analyzed (Knights, 1971). Radio labelled mevalonic acid (MVA) administered to chloroplast preparations was incorporated into carotenoids. Very low incorporation into squalene may have been due to limited ability of chloroplasts to carry out this synthesis or slight contamination (Charlton et al., 1966). Cut stems of bean seedlings incorporated MVA initially into the supernatant, after 24 hours all label was recovered from the supernatant and the microsomes (Knapp et al., 1969).

Sterols in Pollen

Pollen often contains relatively large amounts of sterols and sometimes unusual ones (Anderson and Moore, 1923; Knights, 1968). However, absolute as well as relative amounts vary widely with species (Opote, 1975). The major sterols are β -sitosterol, 24-methylene cholesterol, stigmasterol, campesterol, pollinastanol, 28-isofucosterol (Δ 5-avenasterol) and cholesterol. No taxonomic relationships seem to exist between the sterols in pollen and the plant families (Standifer et al., 1968; Devys and Barbier, 1965; Hgel et al., 1964). Cholesterol, normally occurring at very low concentrations in plant material may constitute the major part of the total sterol fraction in pollen (Table 1).

Table 1. Cholesterol in Pollen of Some Plant Species.
Amount expressed as percent of total sterol content.

Plant species	Cholesterol as % of total sterols	Authors
<u>Hypochoeris radicata</u>	90	Devys and Barbier, 1965 Barbier, 1966 Standifer, 1966
<u>Petunia</u> sp.	100	Hoerberichts and Linskens, 1968
<u>Aster Hovic-Belgii</u>	51	Battaglini et al., 1970
<u>Helminthia echioides</u>	70	Battaglini et al., 1970

Itoh (1972) presented a review on cholesterol in plants.

Usually the more common sterols of higher plants are also the main ones in pollen, except for 24-methylene cholesterol which probably is more abundant in pollen (Barbier et al., 1960; Hügel et al., 1960; Barbier, 1966). A combined sample of nine pollen species, (gymnosperm, monocot and dicot species), showed a more or less equal distribution of mono-unsaturated C-27 and mono- and di-unsaturated C-28 and C-29 sterols (Standifer et al., 1968). Total sterol content on a dry weight basis ranged from 0.1 to 1.1 percent in bee collected pollen of ten species (Standifer, 1966). An example of the large variation of sterol composition is shown by total analysis of sterols in pollen of oil palm (Elaeis guineensis) (Richert, 1971) and giant English rape (Brassica napus L. f. annua) (Knights, 1968).

Table 2. Relative percentages of total sterol content in pollen of Brassica napus and Elaeis guineensis

Sterol	<u>B. napus</u>	<u>E. guineensis</u>
cholesterol	2.8	6.5
pollinastanol	2.4	-
24-methylene cholesterol ¹	56.0	10.5
β -sitosterol	4.2	23.5
Δ^5 -avenasterol	31.6	-
stigmasterol + fucosterol		
+ isofucosterol ²	-	38.5
campesterol	-	21.0
unknown	3.0	-

¹may contain brassicasterol²probably mostly isofucosterol (Opute, 1975)Animal Steroid Hormones in PlantsGeneral

There are some animal steroid hormones which have been found in plants. Animal steroids have been researched extensively; however, knowledge of the biosynthesis, metabolism and physiological roles of similar compounds in plants is still limited. Sterols are the biosynthetic precursors of all steroids, and it has been proposed that pathways leading to steroidal hormones are similar in plants and animals (Heftmann, 1974). Cholesterol plays a central role as precursor; sapogenins, insect moulting hormones, progesterone (Figure 1, Formula 7) and its important metabolites are derived from it (Bean, 1973). However in certain cases other plant sterols are metabolized to these compounds (Heftmann et al., 1974). Pregnenolone (Figure 1, Formula 6) is the precursor of progesterone, of androgens such as testosterone (Figure 1, Formula 15), of estrogens such as estrone (Figure 1, Formula 17), and of corticosteroids such as corticosterone

(Figure 1, Formula 10) (Heftmann, 1970). These and intermediary compounds so potent in animals have been isolated from plants. Skarziński (1933) isolated an estrogenic principle from female willow flowers, and in the same year, α -follicle hormone (α -estrone), was found in date palm pollen (Butenandt and Jacobi, 1933). It was isolated again from the same source (Hassan and Wafa, 1947) and from palm seeds, and its identity verified (Bennet et al., 1966). The hormone 5 α -androstane-3 β -16 α -17 α -triol (Figure 1, Formula 14) was identified in Rayless goldenrod, Haplopappus heterophyllus (Zalkow et al., 1964), and estrone and estradiol (Figure 1, Formula 18) from seeds of Prunus americana (Awad, 1974). Dean and coworkers (1971) quantitatively determined estrone in pomegranate using the extremely sensitive technique of competitive protein binding. They found estrone throughout the plant including the seeds, at concentrations ranging from 2.5 to 8.7 μ g per kg. 21-hydroxyprogesterone (Figure 1, Formula 9) was isolated from the husk of rice (Bahadur and Srivastava, 1970).

Metabolism of sterols to sex hormones such as androstenedione (Figure 1, Formula 11) (Sih et al., 1967; Wix et al., 1968; Nagasawa et al., 1969, 1970; Sallam et al., 1971; Marsheck et al., 1972), testosterone (Sallam et al., 1971) estrone (Sih and Wang, 19-5), and estradiol (Goddard and Hill, 1972) has been observed in many microorganisms. Testosterone was metabolized to various androgens in the free form, glycosides, and esters in tobacco tissue cultures (Hirotsu and Furuya, 1974). Pregnenolone and progesterone were elaborated from 20 α -hydroxycholesterol in leaf homogenates of Cheirantus cheiri

(Stohs and El-Olemy, 1971). The ability to synthesize sex hormones is apparently widespread in the plant kingdom (Heftmann, 1974).

Steroid hormones in pollen

It is commonly claimed in Egypt that pollen grains of the date palm (Phoenix dactilifera L.) are effective in the treatment of both female and male infertility (Soliman and Soliman, 1957). An estrogenic principle was extracted of date palm pollen (Hassan and Wafa, 1947), it showed spermatogenic as well as follicular activity in immature rats (Soliman and Soliman, 1957). Testosterone, epitestosterone (Figure 1, Formula 16) and androstenedione were identified in the pollen of Scotch pine (Pinus sylvestris) (Šaden-Krehula et al., 1971). Estrone was also found in the pollen and kernels of Hyphaene thebaica (Amin and Paleologou, 1973). Investigations of some steroid hormones and their conjugates in pollen of Pinus nigra showed the presence of testosterone, epitestosterone, androstenedione, progesterone and 17 α -hydroxyprogesterone (Figure 1, Formula 8), primarily in the free form (Šaden-Krehula et al., 1976). Very limited research has been performed on the content of animal hormones in pollen; rather large quantities of pollen are required and isolation procedures are tedious.

Biochemical Aspects of Sterols in Higher Plants

Introduction

Cholesterol biosynthesis in animals has been studied extensively for three decades (Sih and Whitlock, 1968), but only within the last decade have plant sterols received considerable attention (Goodwin, 1971). Gradually it has become clear that the more than one hundred different known sterols are not synthesized along a common route

(Goad and Goodwin, 1972). Also it is possible that water soluble sterols are key substances in the metabolism of non-structural sterols (Anding et al., 1972).

The C-30 hydrocarbon squalene is a common precursor for all steroids. It is anaerobically elaborated from the C-6 compound mevalonic acid (Cornforth, 1959), and its synthesis follows the same pathway in all organisms examined (Nes, 1971). Biochemical steps and enzymes involved were described by various researchers (Tchen, 1958; Lynen et al., 1958, 1959; Agranoff et al., 1959, 1960; Levy and Popják, 1960; Goodman and Popják, 1960). A simple picture of squalene biosynthesis is presented in Figure 2. Squalene synthesis has been shown in various plants (Capstack et al., 1962; Suga and Shishibora, 1975; Malhotra and Ness, 1972; Campbell and Weete, 1975). In some plants free geraniol and farnesol have been found rather than the pyrophosphates (Chesterton and Keckwick, 1968; Ogura et al., 1971). An intermediate compound (presqualene alcohol), previously obtained from rat liver and yeast, was identified in bramble tissue (Heintz et al., 1972).

Cyclization of Squalene

All-trans-squalene is probably the precursor of all natural cyclic triterpenes (Heftmann, 1970; Cattel et al., 1976). Anaerobic cyclizations of squalene lead to triterpenes such as tetrahymanol (Formula 20, Mallory et al., 1963) and fern-9-ene (Formula 21, Barton et al., 1969, 1971).

FIGURE 1. Structural formulas of some steroid hormones found in plant material.

Formula 20. Structure of tetrahymanol

Formula 21. Structure of fern-9-ene

For aerobic cyclization, squalene is first converted to squalene-2, 3-oxide in the presence of oxygen and NADPH (Corey et al., 1966, 1967; van Tamelen et al., 1966, 1967). Incorporation of acetate into squalene-2, 3-oxide in plant tissue has been shown by various researchers (Benveniste and Massy-Westrop, 1967; Reid, 1968; Heintz and Benveniste, 1970). Squalene-2,3-epoxide is subsequently cyclized via complex mechanisms (Goad and Goodwin, 1970; Goodwin, 1971). Two major routes exist, one leading to lanosterol in animals (Eschenmoser et al., 1955; Tchen and Bloch, 1957) and most fungi (Schwenk and Alexander, 1958; Weete, 1973), the other leading to cycloartenol in higher plants (Rees et al., 1968, 1969; Eppenberger et al., 1969), (see Figure 2).

Sterol Biosynthesis via Cycloartenol

Synthesis of sterols via cycloartenol requires an enzyme, cyclosterol isomerase, which converts a 9 β , 19 cyclosteroid to the isomeric 10-methyl- Δ^8 -steroid (Figure 3, Bu'lock and Osage, 1976). Organisms lacking cyclosterol isomerase can only follow the "animal" pathway via lanosterol. Cycloartenol has been demonstrated in several algal phyla (Goad and Goodwin, 1969; Rees et al., 1969; Doyle et al., 1972; Dickenson and Patterson, 1972; Chan et al., 1974).

Fungi present an interesting problem, for within this group of organisms the dichotomy of sterol biosynthesis occurs.. Direct cyclization to lanosterol has been shown in yeast and the zygomycete

Phycomyces (Gibbons et al., 1971) whereas the mastigomycete Saprolegnia follows the cycloartenol pathway (Bu'lock and Osage, 1976). Although some species of Chlorella synthesize ergosterol, the typical sterol of fungi, they elaborate it via the cycloartenol pathway (Doyle et al., 1972; Chan and Patterson, 1973). Lanosterol also occurs in higher plants, the latex of Euphorbia for example (Ponsinet and Ourisson, 1968), but the cycloartenol pathway is probably followed.

Biosynthesis of Common Sterols in Higher Plants

The biosynthetic pathways of the most common sterols will be considered here, these being campesterol, stigmasterol, β -sitosterol and cholesterol. Although cholesterol does not occur in concentrations comparable to the other sterols, it may occur in all higher plants, plays an important role in biological membranes, and is considered the precursor for steroid metabolism. The pathway from cycloartenol to the common sterols includes introduction of an alkyl-group at C-24 (except for cholesterol), demethylations at C-4 and C-14, opening of the 9 β , 19-cyclopropane ring, and introduction of the Δ^5 bond (Grunwald, 1975). Alkylation at C-24 is accomplished through a trans-methylation reaction with S-adenosylmethionine as the methyl donor (Alexander and Schwenk, 1957, 1958; Knights and Laurie, 1967). Two consecutive methylations result in the ethyl group of stigmasterol and sitosterol (Castle et al., 1963). Before introduction of the first methyl unit a $\Delta^{24(25)}$ bond is required (Russell et al., 1967; Tsai and Patterson, 1974). A scheme of the probable sequence of reactions leading to the ethyl group at C-24 was presented by van Aller et al. (1969) and Mercer and Russel (1975). The stereo-chemistry of the side chain alkylations has taxonomic significance (Knights, 1973).

With the exception of brassicasterol (Jacobsohn and Frey, 1967), the sterols with a methyl or ethyl group at C-24, such as campesterol, stigmasterol and sitosterol, have the α -configuration at this position (McCorkindale et al., 1969; Baisted, 1969; Bae and Mercer, 1970). Sterols with the β -configuration at C-24 such as ergosterol, poriferasterol, and clionasterol (Knights, 1968, 1971) occur in green algae and fungi (Patterson, 1971; McCorkindale et al., 1969). Oxidative demethylation reactions at C-4 and C-14 were described by Mulheirn and Ramm (1972); stoichiometric amounts of CO₂ are released (Olson et al., 1957; Gautschi and Bloch, 1957). Demethylation at C-14 is hindered by the 9 β , 19-cyclopropane ring (Grunwald, 1975); enzyme mediated opening of this ring was observed by Heintz et al., (1972). In addition to the biochemical pathways described, the pathways leading to common sterols involve stereospecific dehydrogenations, reductions and migrations of double bonds leading to the various unsaturated bonds in sterols. The exact pathways and mechanisms in higher plants are still ill-defined; however, several schemes have been presented (Grunwald, 1975; Mulheirn and Ramm, 1972; Goad and Goodwin, 1972; Anding et al., 1972; Barton et al., 1973). Figure 4 presents a scheme of proposed biosynthetical pathways leading to the major plant sterols (after Nes, 1971; Anding et al., 1972; Grunwald, 1975; Geuns, 1975).

Physiological Aspects of Sterols

Functions in Membranes

Studies in artificial membranes, erythrocyte ghosts and in vivo studies on animal and plant membranes have contributed to the

FIGURE 2. Biosynthesis of squalene.

FIGURE 3. Conversion of 19 β , 19 cyclosteroid to the isomeric 10-methyl- Δ^8 -steroid.

emerging integrated picture of the important functions of sterols. Incorporation of cholesterol into a phospholipid mono- or bilayer causes condensation and thickening of these artificial membranes (Chapman and Wallach, 1968; Lecuyer and Dervichian, 1969). Cholesterol induced stability is accompanied by a general decrease in permeability and electrical conductivity, while electrical capacity is increased (Papahadjopoulos, 1974). Models of phospholipid-cholesterol were presented by Finean (1953) and Vandenheuvel (1963). The effects of sterols on membrane properties are dependent on structure and composition of membrane phospholipids as well as concentration and chemical structure of the sterols (van Deenen, 1972; Oldfield and Chapman, 1972; Shah and Schulman, 1968). Studies of membrane incorporated sterols, relating chemical structure to function, revealed that cholesterol is the most effective membrane stabilizer. The ability of sterols to enter liposomes (artificial membranes) and erythrocyte membranes is in decreasing order: cholesterol, campesterol and sitosterol. This behavior is the same as absorption into the intestine (Edwards and Green, 1972).

Alcohol induced leakage of β -cyanin in beet root (Grunwald, 1968) and of electrolytes in barley shoots (Grunwald, 1971) was counteracted by cholesterol. Other common sterols were less effective.

The sterol composition in membranes directly influenced enzyme activities (Thompson and Parks, 1974; Papahadjopoulos et al., 1973).

Polyene antibiotics alter the permeability of plasma membranes of eukaryotic cells (Archer and Gale, 1975) through formation of hydrophobic complexes (DeKruijff and Demel, 1974). Increased permeability and associated leakage of electrolytes, caused by such

antibiotics as filipin and nystatin, were reduced by addition of sterols (Gottlieb et al., 1960; Karst and Jund, 1976). In addition to physiological functions so far described, host-pathogen relationships (Hoppe and Heitefuss, 1975; Olson 1973; Vandenheuvel, 1963) cold adaptation (Thompson and Parks, 1974), and possibly ozone damage (Spotts et al., 1975) are related to the composition of sterols in membranes.

Flowering

It has been suggested that steroid biosynthesis is involved in flower initiation (Bennet et al., 1967; Kolli, 1969). Extracts of flowering Chrysanthemum plants induced floral bud initiation in both Chrysanthemum and Xanthium (Biswas et al., 1966). Judging from extraction methods and TLC-Rf comparisons, it is likely that steroids were involved. Steroid biosynthesis inhibitors applied to Xanthium and Pharbitis inhibited floral induction (Bonner et al., 1963). Flower initiation was inhibited when the steroid biosynthesis inhibitor SKF 7997A₃ (Tris[diethylamino-ethyl]-phosphate hydrochloride) was applied to Lolium temulentum (Evans, 1964), and Pharbitis nil (Sachs, 1966). Seedling age was important to effect this response in the latter case. No systematic search for steroids, possibly connected with flower initiation has been reported.

Sterol Levels During Seed Maturation, Germination and Plant Growth

The capacity for sterol biosynthesis was greatest in the least mature fruit and decreased during development (Baistedt, 1971). Accumulation of total sterols on a dry weight basis, preceded accumulations of dry matter in soy bean (Katayama and Katoh, 1973), while

the opposite occurred in maturing corn seed (Davis and Poneleit, 1974). Dry, mature seeds of 21 species in 16 families of higher plants were analyzed for their sterol contents (Méance and Dupéron, 1973). The storage organs as well as the embryonic axis of all seeds contained the four classes of sterols, i.e. free sterols, steryl esters, steryl glycosides and acylated glycosides, in decreasing order of concentrations, except in corn where steryl esters had a higher concentration than free sterols. Steryl esters were also highest in mature seeds of longleaf pine (Pinus palustris, Vu, 1976). Total sterols as well as all categories of sterol forms varied greatly among the seeds. Also great differences were observed between the axial parts and the cotyledons. In general total sterol levels were highest in the embryonic axis, free sterols highest in the storage organs, and no particular pattern was observed for glycosidic and acylated glycosidic sterols (Méance and Dupéron, 1973).

During the early stages of germination sterol synthesis was low but increased with increasing dry matter production (Kemp et al., 1967, Baistedt, 1971; Dupéron, 1971; Bush and Grunwald, 1972). On a dry weight basis, total sterol levels dropped gradually in germinating Digitalis purpurea (Cowley and Evans, 1972), beans (Phaseolus vulgaris) and radish (Raphanus sativus) (Dupéron, 1971), but increased in tobacco (Bush and Grunwald, 1972) and in long leaf pine (Vu, 1976). No general pattern regarding concentrations of the various sterol classes was observable during germination. During germination of beans in light or dark, the tremendous loss of dry material and protein nitrogen from the cotyledons was not accompanied by a loss of free sterols or steryl esters, the same pattern was observed in

radish in darkness. When radish seeds were germinated in light in a nutrient medium, dry weight of the cotyledons increased dramatically, but protein nitrogen and free sterols increased only slightly (Dupéron, 1971). In general, sterol production parallels dry matter accumulation, the relative rate of increase may be lower or higher than the dry matter increase. Production of new organelle and plasma membranes is probably responsible for this increase (Baistedt, 1971; Kemp et al., 1967).

Sterol composition may vary significantly during a plant's life cycle. During germination of Calendula officinalis seeds, no sterol synthesis but intensive hydrolysis of sterol esters occurred. During its vegetation ester content decreased and glycoside content increased in the root, free sterols remained constant. All fractions in the shoot remained constant as well. During the flowering period, qualitative as well as quantitative changes occurred. All fractions in the root, shoot and inflorescence reached maxima, and all fractions decreased during seed maturation (Kasprzyk et al., 1968). All sterol fractions in Digitalis purpurea seedlings fluctuated throughout the year; the changes were at the same time of the year, irregardless of when the seeds were sown. The phase of maximum sterol content in the seedlings was followed by a period of no germination of the seeds (Jacobsohn and Jacobsohn, 1976). Total sterols, on a dry weight basis, increased slightly during maturation of tobacco leaves. This was attributed to an increase in free sterols (Grunwald, 1975). Free and esterified sterol contents of bean and radish paralleled increase of protein. Senescent organs and those undergoing cytological differentiation showed increased ester contents (Dupéron, 1973). Yeast

cultures maintained a constant level of steryl esters during exponential growth; however, a sharp increase of esterification occurred during the stationary phase (Bailey and Parks, 1975).

In general it can be concluded that sterol production during active growth more or less parallels dry weight production and shifts among the various categories of sterols are dependent on species, organ and physiological maturity.

Environmental Effects on Sterol Production

Temperature

Ergosterol or cholesterol administered exogenously to fungi permits growth at higher (Sietsma and Haskins, 1967; Starr and Parks, 1962) and lower temperatures (Haskins, 1965) than normal. A temperature change from 10 C to 1 C resulted in a decrease of total sitosterol, stigmasterol and campesterol in wheat shoots and roots. However, the sterols in the roots recovered to above normal concentrations after 7 to 14 days (Davis and Finkner, 1972). Rye leaves grown at 32 C contained more than double the amount of total sterols than leaves grown at 22 C (Rademacher and Feierabend, 1976). All fractions and individual sterols were higher at 32 C, the glycosides showed the largest increase. A few observations show that temperature has a drastic effect on sterol levels in plants.

Light

Reports on interactions between light and sterol production vary greatly and seem contradictory. Sterol synthesis was not stimulated when dark-grown maize seedlings were grown in light (Goodwin and Mercer, 1963); similar findings were reported for rye leaves (Rademacher and Feierabend, 1976). However, Hirayama and Suzuki (1968)

found that etiolated corn seedlings contained more than three times as much free sterols as light-grown seedlings. In etiolated barley shoots, sterol contents were somewhat higher than in light-grown shoots, which was accounted for by an increased production of free sterols (Bush et al., 1971). The levels of bound sterols were approximately the same under both conditions, except there was almost five times as much campesterol ester in etiolated shoots. Cultures of the diatom Chaetoceros simplex calcitrans showed a 106 percent increase in primary production when grown under a 12h/24h photophase as compared to continuous light. Sterol production paralleled this increase (100 percent). When UV radiation was added during the 12h/24h photophase, primary production decreased by 56 percent, but sterols increased by 110 percent (Boutry et al., 1976). Thus light apparently influences sterol production. No generalization can be made of the light effects at the present time.

Interaction of Sterols with Plant Growth Regulators

Natural or artificial growth regulators influence concentration and composition of steroids in plants. Naphthalene acetic acid (NAA) caused a large increase in sterol content of mung bean (Phaseolus aureus) hypocotyls. A stimulated turnover of cycloartenol into sterols was shown (Geuns and Vendrig, 1974; Geuns, 1975). Gibberellic acid (GA) induced an increased uptake of labelled mevalonic acid (MVA) into cotyledons and increased incorporation into free and esterified sterols in the embryonic axis of germinating Corylus avellana seeds (Shewry and Stobart, 1974). Absciscic acid (ABA) did not inhibit incorporation of MVA into sterols, but suppressed production of chlorophylls, carotenoids and chloroplastidic isoprenoid quinones (Mercer and Pughe,

1969). Auxins and kinetin, single or in combination, caused considerable variations in concentrations of free and bound sterols and steroidal sapogenins in tissue cultures of Trigonella foenumgraecum (Brain and Lockwood, 1976; Lockwood and Brain, 1976). Auxins produced a large increase of diosgenin in tissue cultures of Solanum xanthocarpum (Heble et al., 1971), and Dioscorea deltoidea (Marshall and Staba, 1976); also diosgenin and yamogenin production was increased in seeds and fruit walls of Balanites orbicularis (Hardman and Wood, 1971). Seeds of Trigonella foenumgraecum and tuber tissue of Dioscorea deltoidea increased sapogenin yield up to 35 percent when supplied with synthetic or natural growth regulators (Hardman and Brain, 1971). Kimura et al., (1975) made the interesting observation that stigmasteryl β - D glucoside, isolated from peanut (Arachis hypogaea) plants, significantly enhanced elongation of Avena coleoptile segments in the presence of IAA. This was the first report of a sterol glycoside acting synergistically with a plant growth regulator.

Physiological Aspects of Steroid Hormones

The functions of steroid hormones are virtually unknown. Antheridiol (Figure 1, Formula 19) produced by Achlya species induced production of male hyphae (Machlis, 1972). This compound is probably the only steroid that is considered a plant sex hormone, however, testosterone and estradiol were found to have sex hormone activity in yeast (Takao et al., 1970). The role of steroids in growth and reproduction of fungi was reviewed by Hendrix (1970). Androgens and progestational hormones accelerated the growth of Staphylococcus aureus and Cornebacterium pyogenes (Moursi, 1966). Deoxycorticosterone and progesterone strongly inhibited cell growth and division in

Tetrahymena (Hamana and Iwai, 1971). Deoxycorticosterone inhibited H^+ release, blocked uptake of K^+ , decreased the resting membrane potential and caused rapid cytoplasmic clumping in Neurospora crassa. These phenomena were explained by assuming that dexamethasone blocked the electrogenic efflux pump for H^+ , that is associated with membrane ATP-ase (Slayman and Van Etten, 1974).

Various interesting physiological responses were observed when hormones were applied to higher plants. Estrone increased the germination rate of isolated pea embryos (Kögl and Haagen-Smit, 1936), also estrone and estradiol significantly increased the germination of Pinus sylvestris seeds in the dark (Kopcewicz, 1970 a). In vitro germination of Chrysanthemum pollen was greatly increased with addition of testosterone, androstenedione, androstenolone (Figure 1, Formula 12) and androsterone (Figure 1, Formula 13). Of these, testosterone was the more potent promoter at a concentration of 100 ppm (Matsubara, 1969). Growth of higher plants is influenced by steroid applications; estrone, estradiol and β -sitosterol stimulated growth of dwarf pea seedlings, whereas cholesterol and testosterone had no effect (Kopcewicz, 1969 a). Steroid hormones also influence flowering; estrone and estradiol induced flowering in the cold-requiring long day plant Cichorium intybus under non-inductive conditions (Kopcewicz, 1970 c). Endogenous estrogens in developing bean plants appeared at the period of flower formation, reaching a maximum at flower bud and pod formation, then decreased again. Estrogens were absent from ripe seeds (Kopcewicz, 1971). Treatment of monoecious cucumber plants with estradiol or testosterone significantly increased the number of female

flowers (Gawienowski et al., 1971). Applications of estrogens influenced the endogenous levels of growth regulators; pea and pine seedlings treated with estrogens showed large increases of auxin (Kopcewicz, 1970 b), estrogens caused large increases of gibberellin in Cichorium intybus (Kopcewicz, 1970 b). Applications of steroid biosynthesis inhibitors caused suppression of flowering in short day (Bonner et al., 1963) and long day plants (Evans, 1964).

Thus it appears that animal steroid hormones cause striking physiological responses in plants. Thus it seems possible, that at the molecular level interactions between sex hormones and certain cellular components are the same in animals and plants (Heftmann, 1974).

MATERIALS AND METHODS

Chemicals

Digitonin, testosterone, androstenedione, estrone, estradiol, estriol, progesterone, pregnenolone, corticosterone, hydrocortisone, cholesterol, stigmasterol, α -cholestane, nystatin, and chloroamphenicol were purchased from Sigma Chemical Company. Campesterol, β -sitosterol, and gas chromatography column material (5% OV-101 on gaschrom Q, 80-100 mesh) was obtained from Applied Sciences Laboratories, Inc. Tris-(2-diethylaminoethyl)-phosphate trihydrochloride (SKF-7997A₃) was purchased from Smith, Kline and French Laboratories and cordycepin from Calbiochem. Absciscic acid was obtained from the Department of Fruit Crops, University of Florida. Cholesterol-¹⁴C, cholesterol-³H, cholesteryl palmitate-¹⁴C, and 2-¹⁴C mevalonic acid was purchased from New England Nuclear Company. All solvents were analytical grade.

Pollen Sources

Pollen was collected in the University of Florida slash pine seed orchard. Male strobili were enveloped with plastic sausage casings in late January, 1975. When the strobili started to shed in February, they were cut off and hung to dry in a humidity controlled room. After collection, the pollen was sifted through a 0.15 mm wire screen to remove extraneous matter. Subsequently, the pollen was put in a desiccator in the refrigerator for two days. Approximately 500 g of pollen obtained from various trees was mixed, split up in small lots in airtight vials, and stored in a freezer until used.

Germination of Pollen

Pollen was germinated in aqueous media at 30 C in a shaking water bath. The basic media always contained deionized water, 15 $\mu\text{g. ml}^{-1}$ of the fungicide nystatin and 15 $\mu\text{g. ml}^{-1}$ of the bacteriostat chloramphenicol. For sterol determinations, 250 mg of pollen was added to 25 ml of the basic media in a 250 ml erlenmeyer. At various intervals an aliquot was withdrawn and germination determined microscopically. Pollen was considered to have germinated when the emerging tube had attained a length equal to the diameter of the pollen grain. At least 300, but usually 500, pollen grains were counted per sample. Batches determined to be contaminated by bacterial or fungal growth were discarded. Germination was approximately 65% after 48 h of incubation in the basic medium.

Extraction and Fractionation of Sterols

Procedures used for sterol extraction and fractionation were based on methods described by Stedman and Rusaniswkyi (1959); Keller et al., (1969); Grunwald (1970); and Price (1970).

Germinating pollen was filtered and put into a glass bottle with 15 ml acetone and 30 g glass beads (0.45-0.52 mm). The bottle was mechanically shaken in a Braun (Model MSK) homogenizing mill for 1.5 min., while being cooled with an evaporating liquid CO₂ stream. The homogenate was transferred to a Soxhlet apparatus and acetone was added to a total volume of 50 ml. After 24 h of extraction, the acetone extract was divided into two equal aliquots and evaporated to dryness under reduced pressure.

For total sterol determinations, 50 ml 95% ethanol, 0.15 ml concentrated H_2SO_4 and approximately 0.01 μCi ^3H -C-cholesterol was added. Samples were taken in triplicate for determination of total activity. Subsequently the mixture was refluxed for 15 h to cleave the steryl glycosides. Then 15 ml 10% KOH in 95% ethanol was added and the mixture was refluxed for 30 min. to hydrolyze the steryl esters. The hydrolysate was neutralized with 5N H_2SO_4 in ethanol. The resulting mixture was partitioned three times against 30 ml hexane. The combined hexane fractions (hexane fraction 1) were extracted three times with 30 ml 90% methanol. The methanol fractions were combined and back extracted twice with 30 ml hexane (hexane fraction 2). Hexane fractions 1 and 2 were combined, evaporated to dryness under reduced pressure, and redissolved in 20 ml boiling absolute ethanol. The sterols were precipitated by addition of 10 ml hot digitonin solution (2% w/v digitonin in 80% v/v ethanol). The mixture was allowed to cool and stand for 24 h at room temperature, followed by 24 h of storage in a refrigerator. The sterol digitonides were washed three times with 5 ml 80% methanol followed by three washings with diethylether, using a clinical centrifuge operated at top speed (approx. 5000 x g). After drying at room temperature the precipitate was hydrolyzed with 2 ml pyridine at 70 C for 2 h and was left at room temperature for 12 h.

The digitonin was precipitated with 12 ml diethylether and centrifuged. The ether layer was removed to recover the total free sterols. The ether was evaporated and the sterols were redissolved in double distilled and dried (anhydrous Na_2SO_4) ethylacetate containing

α -cholestane as internal standard. The sterols were then ready for injection into the GLC.

The second aliquot of the acetone extract was used to determine the free sterols, steryl esters and steryl glycosides. The extract was evaporated to dryness under reduced pressure, taken up in 50 ml 95% ethanol, and partitioned three times against hexane. The ethanol fraction contained the glycosides and the hexane fraction contained the free sterols and steryl esters. Approximately $2\mu\text{Ci}^3\text{H}$ -cholesterol and $0.01\mu\text{Ci}^{14}\text{C}$ -cholesterol palmitate were added to the hexane fraction, and samples were taken in triplicate to determine ^3H and ^{14}C activities.

A 1.8×7 cm column was packed with 6 g dry silica gel (100-200 mesh), and the fraction containing the free sterols and steryl esters was added as a slurry. Serial elution was as follows: 50 ml hexane (discarded), 100 ml 10% benzene in hexane (discarded), 100 ml 40% benzene in hexane (steryl esters), 50 ml benzene (discarded) and 150 ml chloroform (free sterols). The free sterol, steryl ester, and steryl glycoside fractions were all dried under reduced pressure. The free sterols were redissolved in 30 ml warm 95% methanol and partitioned three times against 30 ml hexane. The hexane fractions were combined and evaporated to dryness. The free sterols were then precipitated with digitonin as previously described.

Steryl esters were refluxed in 30 ml 5% KOH in 95% methanol for 30 min. After cooling and neutralizing with 5% H_2SO_4 in ethanol, the hydrolysate was partitioned three times against 30 ml hexane. The combined hexane fractions were evaporated to dryness and the sterols were precipitated with digitonin. Subsequent procedures were the same as previously described.

FIGURE 5. Flow chart for procedures used in the extraction and fractionation of sterols in pollen of Pinus elliotii.

The steryl glycosides were refluxed for 12 h in 30 ml 95% methanol containing 0.15 ml concentrated H_2SO_4 . At this point 0.01 $\mu\text{Ci}^{14}\text{C}$ -cholesterol was added and samples were taken in triplicate for later calculations of recovery. After hydrolysis and neutralization with 10% KOH in methanol, the hydrolysate was partitioned three times against 30 ml hexane. The combined hexane fractions were evaporated to dryness and the sterols were precipitated with digitonin.

Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer, model B 2450. The radioactive samples were dissolved in 5 ml toluene containing 4 g l^{-1} BBOT (2, 5-bis-2-[5-tert.-butyl-benzoxazolyl]-thiophene).

A flow chart demonstrating in brief form the main procedures of separation for the sterol categories is shown in Figure 5.

Sterol Identification and Quantification

Sterols were identified using combined GLC-mass spectrometry and co-chromatography with authentic sterols on a gas-liquid chromatograph. GLC-mass spectrometry was executed with a double beam, double focusing AEI MS-30 Mass Spectrometer coupled with a silicone membrane separator to a Pye Gas Chromatograph. The ion source was operated at 220° C and the electron beam at an energy of 70 eV. Data from the combined system were recorded and analyzed by an AEI DS-30 digital computer.

Quantification of sterols was effected with a Packard Becker Gas Chromatograph Model 421. A 180-cm long glass column with 4-mm internal diameter was used. The column was packed with 5% OV-101 (dimethyl silicone) on Gaschrome Q, 80-100 mesh. Operating temperatures were as follows: injection ports 270° C, column 245° C, and

the detectors 270° C. Gas flows were: Helium 100 ml min⁻¹, air 350 ml min⁻¹, and H₂ 90 ml min⁻¹. All samples contained α-cholestane as an internal standard. Relative retention times and relative areas of the peaks were measured in relation to α-cholestane. Areas were determined by cutting out the peaks and weighing.

Sterol concentrations were calculated using the following formula:

$$\text{Amount of sterol } (\mu\text{g}) = (\text{STD RA})^{-1}(\text{C}_{\alpha\text{-chol}})(V_{\text{GLC}})(\text{Rec})^{-1}(\text{STE RA})$$

STD RA = standard relative area = sterol peak area divided by the peak area of an equal amount of the internal standard, α-cholestane.

C_{α-cho}l = concentration of α-cholestane expressed in μg·μl⁻¹

V_{GLC} = volume of solvent containing α-cholestane and added to dry GLC sample.

STE RA = sterol relative area = area of sterol peak divided by area of α-cholestane peak (GLC record of sample).

Rec. = recovery = total activity of GLC sample divided by total activity in the original sterol extract.

When ³H and ¹⁴C labels were mixed, the contribution of activity of each isotope was calculated by using formulas published by Bransome (1970).

$$C = N_1 - N_2(h_1h_2^{-1})$$

$$H = N_2 - N_1(C_2C_1^{-1})$$

where C = ¹⁴C dpm in sample

H = ³H dpm in sample

C₁ = ¹⁴C efficiency in Channel 1 (100% = 1.00)

C₂ = ¹⁴C efficiency in Channel 2

h_2 = ^3H efficiency in Channel 1

h_2 = ^3H efficiency in Channel 2

N_1 = net total observed counts in Channel 1

N_2 = net total observed counts in Channel 2

Since operating conditions were such that ^3H did not contribute to the channel which was more efficient for ^{14}C the two formulas reduce to:

$$C = N_1 C_1^{-1}$$

$$H = (N_2 - C_{C_2}) h_2^{-1}$$

^{14}C -Mevalonic Acid Incorporation

DL- 2^{14}C mevalonic acid DBED salt, $\text{MVA}-(\text{C}_6\text{H}_5\text{CH}_2\text{NHCH}_2\text{CH}_2\text{NHCH}_2\text{C}_6\text{H}_5)_{1/2}$ was checked for purity using paper chromatography (Bloch et al., 1959). MVA was applied to strips of 4 x 50 cm Whatmann 1 and developed with tert-butanol:formic acid:water, 40:10:16. Descending chromatography was at room temperature for 8 h. The chromatograms were cut into 5 mm strips and put into vials. Five milliliters aquasol was added and the activity was determined with a liquid scintillation spectrometer. A major peak with a relative retention time of 0.80-0.85, within the range reported by Bloch et al., (1959), contained approximately 98% of the administered activity. The remaining 2% were located in a small zone tailing the major peak. The MVA was considered adequately pure for incorporation into pollen.

Two batches of 250 mg pollen were germinated for 36 h. The basic medium contained in addition MVA (10^{-6} M) and ^{14}C -MVA with a final specific activity of $46 \text{ mCi} \cdot \text{mmole}^{-1}$. Subsequently the pollen was filtered from the medium and thoroughly rinsed with water. Procedures previously described were used to isolate the sterols. The

GLC was equipped with a streamsplitter; 90% of the effluent was diverted into a gas proportional counter to record the activity of the various sterol peaks. A Packard gas proportional counter was operated under the following conditions: operating potential 1700 V, time constant 50 sec., range 200 cpm, helium carrier gas flow 100 ml min⁻¹, and propane quench gas flow 5 ml min⁻¹.

¹⁴C-Cholesterol Administration

4-¹⁴C-cholesterol with a final specific activity of approximately 40 mCi·mmole⁻¹ and a final concentration of 10⁻⁵M was added to two batches of pollen (250 mg pollen in 25 ml basic medium) and allowed to germinate for 36 h under standard conditions. The pollen was filtered and rinsed thoroughly with 10⁻⁵ M cholesterol in water. The cholesterol in the rinse solution was originally dissolved in ethanol, the final concentration of ethanol was 0.2% v/v. Subsequently, standard procedures were used to isolate and quantify the sterols. Radioactivity was determined with a proportional counter as already described for ¹⁴C-MVA incorporation.

Effects of Inhibitors on Sterol Composition and Pollen Germination

The following inhibitors were tested: abscisic acid (ABA), tris-(2-diethylaminoethyl)-phosphate trihydrochloride (SKF-7997A₃) and 3'-deoxyadenosine (cordycepin). The inhibitors were added to batches of pollen (10 mg·ml⁻¹). The pollen was allowed to germinate at 30 C, and the percentage germination was determined at regular intervals. The following conditions prevailed: ABA was dissolved in a saturated calcium-phosphate buffer (pH = 6.0). The ABA concentration was 1 x 10⁻³M. SKF-7997A₃ was dissolved in a saturated

calcium phosphate buffer (pH = 6.0). Concentrations were 5×10^{-6} , 1×10^{-5} , 2×10^{-5} , and 5×10^{-5} M. Cordycepin was dissolved in the basic medium at concentrations of 2×10^{-5} , 3×10^{-5} , 5×10^{-5} , and 1×10^{-4} M.

For determination of total sterols, duplicate batches of 250 mg pollen in 25 ml water were allowed to germinate for 36 h. Inhibitor concentrations were: ABA - 10^{-3} M, SKF - 7997A₃ - 1×10^{-5} M, and cordycepin - 3×10^{-5} M. Total sterol composition was determined according to the methods already described. The media of the ABA experiment were also assayed for total sterol composition.

Effect of Pollen Concentration on Germination

Pollen was suspended in 5 ml basic medium and allowed to germinate for 36 h at 30 C in a shaking water bath. Pollen concentrations were 2, 4, 5, 8, 10, 15, 20, 30, 50 and $100 \text{ mg} \cdot \text{ml}^{-1}$. The experiment was executed in duplicate.

Two batches of pollen with a concentration of $8 \text{ mg} \cdot \text{ml}^{-1}$ were incubated for 20 min and a similar set was incubated for 12 h. Following the incubation times, the pollen was filtered from the media and the media added to batches of fresh pollen. The final pollen concentrations were $2 \text{ mg} \cdot \text{ml}^{-1}$. The pollen was incubated for 36 h under standard conditions after which the germination was determined. The experiment was performed twice in duplicate.

Effects of Selected Steroid Hormones on Pollen Germination

Batches of pollen ($2 \text{ mg} \cdot \text{ml}^{-1}$) were incubated for 36 h in basic medium plus dimethylsulphoxide (concentration DMSO = 10^{-4} M). The following hormones were tested: Progesterone, pregnenolone,

corticosterone, hydrocortisone, estradiol, and estriol. The final concentration of each hormone was 10^{-5}M . They were dissolved in 100% ethanol and added to the media by forcing them through a fine hypodermic needle while the media were being stirred vigorously. No crystals could be observed in the light microscope. The final concentration of ethanol was 0.4% (v/v). The experiment was conducted twice in triplicate.

Effects of Sterols on Germination Under Various Temperature Regimes

Batches of pollen ($10\text{ mg}\cdot\text{ml}^{-1}$) were incubated for 36 h at 22, 24, 26, 28, 32, 34, 36, 38 and 40 C. The basic media contained in addition 10^{-4}M DMSO. The following comparisons were made: control (no ethanol); 0.4% ethanol; 0.4% ethanol plus 10^{-5}M cholesterol; and 0.4% ethanol plus 10^{-5}M sitosterol. The experiment was performed twice in duplicate.

Effect of Some Steroids on the Conductivity of the Germination Medium

Batches of pollen ($2\text{ mg}\cdot\text{ml}^{-1}$) were incubated under standard conditions. The media contained 10^{-4}M DMSO and 0.4% (v/v) ethanol. The following steroids were tested: cholesterol, sitosterol, progesterone, pregnenolone, corticosterone, hydrocortisone, estradiol, and estriol. Also 10^{-3}M ABA in basic medium was tested; the pollen concentration was $10\text{ mg}\cdot\text{ml}^{-1}$. The final concentrations of the steroids were 10^{-5}M . After 30 min. the pollen was filtered off and the specific conductance of the media were measured (Lab-Line mho-meter, Lab-Line Instruments, Inc.). The experiment was performed in triplicate.

RESULTS

Identification of Sterols Isolated from Pollen of *Pinus elliotii*

A representative GLC retention pattern of total sterols isolated from pollen that had germinated for 36 h is presented in Figure 6 and Table 3. The peaks correspond to the internal standard α -cholestane (peak 1), and the pollen sterols cholesterol (peak 2), campesterol (peak 3), stigmasterol (peak 4), and β -sitosterol (peak 5). Minor peaks that occasionally appeared on the gas-liquid chromatograms were scanned by the mass-spectrometer. These effluents did not correspond to any sterols but were probably hydrocarbons. Co-chromatography of authentic pure sterols mixed with pollen sterols showed perfect overlap. The mass spectrum data are presented in Tables 4, 5, 6 and 7, corresponding to cholesterol, campesterol, stigmasterol and β -sitosterol, respectively.

The fragmentation patterns and relative intensities of the various ions show that the sterols isolated from pine pollen correspond with standards of authentic sterols. The intensities of fragments obtained from stigmasterol deviated to some degree from the standard, most intensities were lower in the sample. However the main ions with $m/e = 412$ (molecular ion), $m/e = 273$ (M-side chain), and $m/e = 229$, (M-[side chain + 17]) agreed well with the standard. Masses with $m/e = 231$, (M-[side chain = 42]), and $m/e = 229$ (M-[side chain + 27 + 17]) were considerably higher than the standard due to

Figure 6. Gas-liquid chromatogram of sterols extracted from *Pinus eliottii*.

contributions of campesterol. Campesterol and stigmasterol were poorly separated by the GLC system coupled to the MS, as was shown by the chromatogram. However, the GLC system used for quantitative determinations of sterols effected a good separation of these sterols, as is shown in Figure 6. A table of typical retention times of authentic and native pollen sterols is presented in Table 3. It would seem reasonable to suggest that pollen of Pinus ellioti contains cholesterol, campesterol, stigmasterol and β -sitosterol.

Total Sterol Levels During Germination of Pine Pollen

Total sterol levels (excluding sterols in the media) were corrected for losses using ^{14}C recovery values. Sterol contents are shown in Figure 7. Total sterol levels were almost constant throughout the germination period, except for β -sitosterol which showed an increase after 6 h but subsequently dropped to previous levels. Total sterol levels remained around $2500 \mu\text{g} \cdot \text{g}^{-1}$ (0.25% F.W.). Cholesterol, normally occurring at very low concentrations in plant tissue, also had the lowest concentrations in pine pollen. β -sitosterol had the highest concentration ($1840 \mu\text{g} \cdot \text{g}^{-1}$; 72% of total sterols). The temporary elevation of β -sitosterol levels is not readily explained. No de novo synthesis from MVA occurred (see page 57). The possibility exists that it is elaborated from isoprenoid phosphate precursors, squalene or cycloartenol. However, the latter two compounds have never been shown to accumulate in plant tissues. Steroidal precursors such as dimethyl or mono-methyl sterols are unlikely since they did not occur at detectable levels. Sterols in the free or various bound forms seem to be the end products of steroid metabolism in higher plants. Although various reports indicate that these forms

Table 3. Relative retention times¹ with respect to α -cholestane, of pure sterols and those extracted from pine pollen that had germinated for 36 hours.

Compound	Peak Number	Relative Retention Times	
		Standard	Pollen Sterols
α -cholestane	1	1.00	
Cholesterol	2	1.79	1.79
Campesterol	3	2.31	2.31
Stigmasterol	4	2.49	2.50
β -sitosterol	5	2.86	2.86

¹Operational characteristics: Column length; 180 cm, internal diameter 4 mm; packing OV-101 on gaschrom Q (80-100 mesh); helium carrier gas flow 100 ml·min⁻¹. Operating temperatures; Column 250 C, isothermal; flame ionization detector and injection ports 275 C.

Table 4. Mass spectral data of cholesterol extracted from slash pine pollen.

Fragmentation	m/e	Relative Intensity	
		Standard	Pollen Sterol
Molecular ion M^+	386	76	67
M-15 ($CH_3 = 15$)	371	42	56
M-18 ($HOH = 18$)	368	66	60
M-[15 + 18]	353	57	60
M-[67 + 18] ($C_5H_7 = 67$)	301	50	50
M-[93 + 18] ($C_7H_9 = 93$)	275	100	100
M-[121 + 18] ($C_9H_{13} = 121$)	247	42	42
M-[108 + 18] ($C_8H_{12} = 108$)	260	15	15
M-side chain	273	28	40
M-[side chain + 18]	255	57	50
M-[side chain + 42]	231	35	52
M-[side chain + 42 + 18]	213	67	92
M-[side chain + 27]	246	15	21
M-[side chain + 27 + 17] ($OH = 17$)	229	28	35

Table 5. Mass spectral data of campesterol extracted from slash pine pollen.

Fragmentation	m/e	Relative Intensity	
		Standard	Pollen Sterol
Molecular ions M^+	400	95	84
M-15	385	57	59
M-18	382	95	79
M-[15 + 18]	367	64	68
M-[15 + 67]	315	58	54
M-[18 + 93]	289	93	101
M-[18 + 121]	261	45	36
M-[18 + 108]	274	31	18
M-[side chain]	273	42	39
M-[side chain + 18]	255	83	67
M-[side chain + 42]	231	52	51
M-[side chain + 42 + 18]	213	100	100
M-[side chain + 27]	246	18	10
M-[side chain + 27 + 17]	229	51	38

Table 6. Mass spectral data of stigmasterol extracted from slash pine pollen.

Fragmentation	m/e	Relative Intensity	
		Standard	Pollen Sterol
Molecular ion M^+	412	53	31
M-15	397	11	0
M-18	394	26	16
M-[15 + 18]	379	12	0
M-[18 + 67]	327	6	0
M-[18 + 93]	301	18	0
M-[18 + 121]	273	23	26
M-[18 + 108]	286	5	0
M-[side chain]	273	23	26
M-[side chain + 18]	255	100	100
M-[side chain + 42]	231	18	41
M-[side chain + 42 + 18]	213	49	76
M-[side chain + 27]	246	5	0
M-[side chain + 27 + 17]	229	24	29

Table 7. Mass spectral data of β -sitosterol extracted from slash pine pollen.

Fragmentation	m/e	Relative Intensity	
		Standard	Pollen Sterol
Molecular ion M^+	414	97	82
M-15	399	55	52
M-18	396	93	67
M-[15 + 18]	381	59	58
M-[18 + 67]	329	54	53
M-[18 + 93]	303	93	89
M-[18 + 121]	275	39	25
M-[18 + 108]	288	15	7
M-[side chain]	273	42	37
M-[side chain + 42]	231	54	51
M-[side chain + 18]	255	79	65
M-[side chain + 42 + 18]	213	100	100
M-[side chain + 27]	246	21	13
M-[side chain + 27 + 17]	229	47	38

are interconverted no reports exist that extensive metabolism of sterols to animal steroid hormones or bile acids occur in higher plants. Possible other metabolites such as steroidal sapogenins or alkaloids have never been detected in pines. Another possible explanation for the temporary increase in β -sitosterol is reabsorption from the aqueous media. Fresh pollen (time = 0) had been wetted with water and filtered. Wetting was necessary to achieve grinding of the pollen in acetone. Thus during the short contact with water, soluble sterols may have entered the liquid phase. Reabsorption during the first 6 h would explain the temporary elevation. The total levels of sterols in the pollen at 6 h is approximately the same as the total combined levels of sterols in the pollen and media after 36 h. Thus it is probable that little, if any, sterol biosynthesis occurred during the entire germination period.

Levels of Total Sterols in Aqueous Media

Since pollen has been germinated in aqueous media, testing for water soluble sterols leaked into the media seemed appropriate. Analysis for total sterols after 36 h incubation time revealed that approximately 12% of the total sterols (combined pollen and media pool) was recovered from the media. Table 8 compares the total sterol composition in the pollen and media. Approximately 10% of the total campesterol, stigmasterol and β -sitosterol pool (pollen and media combined) was recovered from the aqueous fraction. Interestingly 75% of the total cholesterol content was recovered from the media. Figure 7 shows that the total sterol concentration in the pollen after 24 h is approximately 10% lower than the concentration

at time zero. Thus the drop in sterol levels in the pollen during germination could be explained by gradual leakage of sterols into the aqueous media during the first 24 h. It is possible that a relatively large part of the water soluble sterols were derived from non-viable pollen. The experiments described here do not allow to distinguish between the two possible sources.

DL-2 ^{14}C : Mevalonic Acid Incorporation

Since a temporary increase in total β -sitosterol levels during pollen germination was observed, labelled MVA was administered to the incubation media. Total sterols were extracted after 36 h. Only 6.4% of the radioactivity was recovered from the pollen acetone extract. GLC data showed that total sterol levels and composition were normal. However, the graph obtained from the gas proportional counter revealed that none of the four sterols under consideration were labelled. Although the setting of the proportional counter was very sensitive (200 cpm full scale), the graph did not show any activity levels significantly above the background (40 cpm). Since there is no reason to believe that MVA was not taken up by the pollen, it must be concluded that no de novo synthesis of sterols from MVA, a precursor in all systems analyzed, had occurred.

^{14}C -Cholesterol Administration

^{14}C -labelled cholesterol (10^{-5}M) was added to batches of pollen ($10\text{ mg} \cdot \text{ml}^{-1}$), and the pollen was allowed to germinate for 36 h under standard conditions. Total sterols were assayed by GLC coupled to a gas-proportional counter. Sterol levels were normal however, and none of the sterols, including cholesterol, were labelled. It was concluded that no uptake of cholesterol had occurred.

Determination of Free Sterols, Steryl Esters
and Steryl Glycosides

Separation of the various forms of occurrence of sterols was described previously. Separation of steryl esters and free sterols by column chromatography on silica gel was first performed with the hexane fraction of fresh (non-incubated) pollen. A mixture of ^3H -cholesterol and ^{14}C -cholesteryl palmitate was added to the hexane fraction. The final concentrations of the added cholesterol and cholesteryl palmitate were 10^{-5} M. The experiment, performed in duplicate, showed that good separation was accomplished (Figure 8). The graph shows that the labeled cholesteryl palmitate contained some free cholesterol and the labeled cholesterol contained some esterified form. These contaminations were so small, however, that they were considered insignificant. Unfortunately no labeled steryl glycoside was available and attempts to prepare them according to methods described by Meystre and Miescher (1944) gave poor results.

Free sterols made up the bulk of the total sterol content in pollen during the entire germination period (Figure 9). The average total content of free sterols was approximately $2200 \mu\text{g} \cdot \text{g}^{-1}$ pollen. The levels were fairly constant except for sitosterol that showed fluctuations, with peaks after 12 h and 48 h and the lowest concentration after 24 h. Within the limits of error, the content of free cholesterol was constant. Free cholesterol represented almost the total cholesterol pool, since cholesteryl glycosides contributed very little.

Steryl glycoside levels were approximately constant for stigmasterol and cholesterol. Campesteryl and sitosteryl glycoside levels

increased after 6 h, peaked around 25 h and returned to almost original levels after 36 h (Figure 10). Average total steryl glycoside levels were approximately $470 \mu\text{g} \cdot \text{g}^{-1}$ pollen. Excluding water soluble sterols, the glycoside fraction comprises about 16% of the total sterol content. The increased content of β -sitosteryl glycoside coincides with a decrease in free β -sitosterol. These changes occur at the start of germination, which occurs after 10-12 h incubation time. After 36 h, which is about the end of the germination period, but not tube elongation, steryl glycoside levels had decreased to original levels and were still the same after 48 h.

Steryl esters were not detected during the entire observation period of 48 h. Although some labeled ester was eluted off the silica gel columns, no significant peaks could be detected on the GLC records. The absence of steryl esters in this pollen species was also supported by the fact that the sum of the free sterols and steryl glycosides equals, within the limits of error, the total sterol content.

Effects of Absciscic Acid, SKF-7997A₃ and Cordycepin on
Composition of Total Sterols and on Pollen Germination

The effect of 10^{-3} M ABA on pollen germination is shown in Figure 11. This concentration caused approximately 44% inhibition of germination after 36 h. The pollen as well as the media were assayed for total sterol contents. Table 9 compares total sterol levels in pollen and media of controls (basic media plus phosphate buffer) and ABA treatments. The media with ABA and the control experiments contained a saturated calcium phosphate buffer with pH = 6.0. ABA caused a considerable efflux of sterols into the media (Table 9).

Table 8. Total sterol content of pollen and media after 36 hours incubation time ($\mu\text{g}\cdot\text{g}^{-1}$ pollen).

	Cholesterol	campesterol	stigmasterol	β -sitosterol
pollen	46	455	246	1722
media	128	43	21	151

Table 9. Levels of total sterols ($\mu\text{g}\cdot\text{g}^{-1}$ fresh pollen) in pollen and media after 36 hours incubation time in basic media containing 10^{-3}M ABA.

Sterol	Control			10^{-3}M ABA		
	pollen	medium	total	pollen	medium	total
cholesterol	46	128	174	127	47	174
campesterol	445	43	488	227	229	456
stigmasterol	246	21	267	186	80	266
β -sitosterol	1722	151	1873	659	971	1630
Total	2459	343	2802	1199	1327	2526
% Total	88	12	100	47	53	100

Table 10. Total sterol composition ($\mu\text{g}\cdot\text{g}^{-1}$ fresh pollen) in pollen germinated in basic media (control) and media containing $1 \times 10^{-5}\text{M}$ SKF-7997A₃, or $3 \times 10^{-5}\text{M}$ cordycepin. The incubation periods were 36 h.

Sterol	Control	SKF-7997A ₃	Cordycepin
cholesterol	46	143	102
campesterol	445	414	337
stigmasterol	246	278	187
β -sitosterol	1722	1311	1351
Total	2459	2146	1977

FIGURE 7. Total sterol content and composition during germination of slash pine pollen.

SAMPLE NUMBER (10 ml effluent per sample)

Figure 8. Separation on silica gel column of ^3H -cholesterol and ^{14}C -cholesteryl palmitate added to the hexane fraction of slash pine pollen extract.

FIGURE 9. Total free sterol content and composition during germination of slash pine pollen.

FIGURE 10. Total sterol glycoside content and composition during germination of slash pine pollen.

Whereas only 12% of the total sterol content was recovered from the control media, ABA treatment resulted in 53% being in the media. However, the behavior of cholesterol was quite the opposite; the control media contained 75% of the total cholesterol pool and the media with ABA contained only 27%. Since the bulk of the cholesterol effluxes into the medium under standard incubation conditions, ABA seems to prevent this efflux selectively.

SKF-7997A₃ was administered to the pollen incubation medium at concentrations of 5×10^{-6} , 1×10^{-5} , 2×10^{-5} , and 5×10^{-5} M. The effects on pollen germination are shown in Figure 12. Approximately 44% inhibition of germination was obtained with 1×10^{-5} M SKF-7997A₃ after 36 h. This concentration was used in pollen media for determination of total sterol contents. Table 10 compares the composition of total sterols in the controls and SKF treated pollen. Again cholesterol levels in treated pollen were high. Campesterol and stigmasterol were not significantly different from the control, however, the sitosterol content was lower.

The effects of cordycepin (3' deoxyadenosine) on the rate of pollen germination was tested at concentrations of 2×10^{-5} , 3×10^{-5} , 5×10^{-5} and 1×10^{-4} M. The effects of cordycepin on pollen germination are shown in Figure 13. Approximately 49% inhibition of pollen germination was obtained with 3×10^{-5} M cordycepin in the basic medium after 36 h. This concentration was used in the media of pollen batches that were incubated for 36 h and assayed for total sterols. The results are shown in Table 10. Total sterol levels were approximately 33% lower in the cordycepin treated pollen, however

FIGURE 11. Effect of 10^{-3} M ABA on germination of slash pine pollen.

FIGURE 12. Effect of SKF-7996A₃ on germination of slash pine pollen.

FIGURE 13. Effect of cordycepin on germination of slash pine pollen.

cholesterol levels were more than double. Again, as in the ABA treatment, cholesterol behaved differently, although less pronounced here.

Effects of Pollen Concentration on Germination

Aliquots of slash pine pollen were germinated in basic media for 36 h. Pollen concentrations ranged from $2 \text{ mg} \cdot \text{ml}^{-1}$ to $100 \text{ mg} \cdot \text{ml}^{-1}$. This experiment provides the reference for studies in which steroids are tested for their possible influence on the effects of pollen concentration on germination. As demonstrated in Figure 14, optimal pollen concentrations ranged from 10 to $30 \text{ mg} \cdot \text{ml}^{-1}$. Concentrations higher than $30 \text{ mg} \cdot \text{ml}^{-1}$ caused a slight decrease of germination; below $10 \text{ mg} \cdot \text{ml}^{-1}$ germination decreased rapidly showing an almost linear dose response.

For further tests, aliquots of pollen of optimum concentration ($10 \text{ mg} \cdot \text{ml}^{-1}$) were incubated in the standard medium and the amount of germination was determined over a 48 h period. Figure 15 demonstrates the typical sigmoid curve for pollen germination.

In further tests aliquots of pollen ($8 \text{ mg} \cdot \text{ml}^{-1}$) were incubated in water for 20 min and 12 h. After incubation the media were filtered from each treatment and added to 2 mg batches of fresh pollen. These fresh aliquots were allowed to germinate for 36 h and the germination was assayed. Table 11 shows that addition of pollen incubation media to small quantities of pollen restores the germination to maximum values.

FIGURE 14. Effect of pollen concentration on germination of slash pine pollen.

FIGURE 15. Percent germination of slash pine pollen during 48 hours incubation in basic medium.

Table 11. Average relative percent germination of slash pine pollen after 36 hours incubation in different media*.

	Relative percent germination
Control 1	100
Control 2	51
Medium 1	107
Medium 2	99

*Control 1 10 mg pollen ml⁻¹ in basic medium
 Control 2 2 mg pollen ml⁻¹ in basic medium.
 Medium 1 2 mg pollen ml⁻¹ in medium obtained from pollen
 (8 mg · ml⁻¹) incubated for 20 min in basic medium.
 Medium 2 2 mg pollen ml⁻¹ in medium obtained from pollen
 (8 mg · ml⁻¹) incubated for 12 h.

Effects of Some Steroid Hormones and DMSO on Pollen Germination

Animal steroid hormones do occur in pollen, for example in pollen of Pinus nigra (Šaden-Krehula et al., 1976) and Pinus sylvestris (Šaden-Krehula et al., 1971). Although nothing is known about the function of native animal steroids in pollen, they greatly increased the germination of Chrysanthemum pollen in vitro (Matsubara, 1969).

Preliminary experiments with slash pine pollen indicated that the animal steroid hormones had no effect on germination when optimum pollen concentrations ($10 \text{ mg} \cdot \text{ml}^{-1}$) were used. Germination is affected, however, when low ($2 \text{ mg} \cdot \text{ml}^{-1}$) pollen concentrations were used, as shown in Table 12. Surprisingly DMSO, the carrier used in all experiments, caused the largest increase in germination. All hormones, except hydrocortisone, caused an additional increase. The combination of 10^{-4} M DMSO and 5×10^{-4} M hormones in the media restored germination to almost optimum levels. DMSO alone had little effect when the pollen concentration was optimal, and there was no difference between 10^{-4} and 10^{-3} M DMSO.

Effects of Some Sterols, Animal Steroid Hormones and ABA on the Conductivity of the Germination Media

Conductivity of media in which pollen has been incubated for a short time has often been used as a measure of germinability. An inverse relationship between these parameters exists (Dillon and Zobel, 1957; Ching and Ching, 1976). Increased conductivity of the pollen media is attributed to a larger amount of dead pollen.

Preceding experiments indicated that pollen has to efflux a certain quantity of compounds into the medium in order to achieve

Table 12. Germination of slash pine pollen in media containing DMSO and animal steroid hormones.*

Pollen (mg·ml ⁻¹)	Hormone (10 ⁻⁵ M)	DMSO (molarity)	Relative average % germ. ($\bar{x}$)	Standard error ($s_{\bar{x}}$)
2	---	0	53	4.1
2	---	10 ⁻⁴	82	3.5
2	progesterone	10 ⁻⁴	87	4.9
2	pregnenolone	10 ⁻⁴	96	3.6
2	estradiol	10 ⁻⁴	99	6.3
2	estriol	10 ⁻⁴	96	6.9
2	corticosterone	10 ⁻⁴	96	5.0
2	hydrocortisone	10 ⁻⁴	81	3.4

*Results of two identical experiments both done in triplicate. There was no significant difference between the two experiments (factorial, completely random design), thus six values were obtained per treatment.

maximum germination. Steroids can partially remedy the adverse effect of low pollen concentration on germination. These observations suggested an experiment to test steroids for their possible influence on the conductivity of the media. ABA was also included since it caused a considerable change in the concentrations of sterols in the incubation media; also ABA increases membrane permeability as was described previously.

Batches of pollen ($2 \text{ mg} \cdot \text{ml}^{-1}$) were incubated for 30 min in media containing DMSO (10^{-4} M), ethanol (0.4% v/v) and steroids (10^{-4} M). Also 10^{-3} M ABA in a saturated calcium phosphate buffer ($\text{pH} = 6.0$) was tested. After incubation the pollen was removed by filtering and the conductance of the media was measured (Table 13). The media obtained from the high pollen concentration ($10 \text{ mg} \cdot \text{ml}^{-1}$) had the highest conductance, while the media of the low pollen concentration had a conductance proportionately lower. Addition of cholesterol or sitosterol did not significantly change the conductance of the media. Preliminary experiments where cholesterol or sitosterol was added to media containing pollen with a low concentration showed that there was no effect on germination. Progesterone, pregnenolone and corticosterone caused significant increases in conductance of the media, these steroids also caused increased germination of low concentration pollen (see Table 12). However, estradiol and estriol caused no increased conductance, yet increased the germination of low concentration pollen. Hydrocortisone had no effect on conductance nor germination. Thus the observed pattern of conductance of the media and germination of low concentration pollen was as follows: three steroids did not change conductivity or germination, three steroids

Table 13. Effect of some sterols, steroid hormones and ABA on the conductivity of pollen incubation media.*
(Concentrations: DMSO 10^{-2} M, ethanol 0.4% v/v, steroids 10^{-4} M, ABA 10^{-4} M).

Treat- ment	Additions to basic media	Pollen conc. (mg·ml ⁻¹)	Average conduct. ($\bar{x}$) (μ Mho's)	Δ Cond. (μ Mho's)	Standard error($s_{\bar{x}}$)
1		10	6.69		.085
2	Dimethylsulfoxide	0	0.12		.006
3	DMSO	2	1.48		.080
4	DMSO + alcohol	2	1.50		.023
5	DMSO + alc. + chol.	2	1.54	0.04	.020
6	DMSO + alc. + sit.	2	1.56	0.06	.020
7	DMSO + alc. + andr.	2	1.81	0.31	.025
8	DMSO + alc. + prog.	2	1.79	0.29	.042
9	DMSO + alc. + pregn.	2	1.70	0.30	.026
10	DMSO + alc. + estr.d.	2	1.51	0.01	.036
11	DMSO + alc. + estr.	2	1.49	-0.01	.021
12	DMSO + alc. + cortic.	2	1.80	0.30	.046
13	DMSO + alc. + H-cortis.	2	1.50	0.00	.051
14	P. buffer	2	2.86		.007
15	P. buffer + ABA	2	4.97	2.11	.332

*Treatment 4 served as control for treatment 5-13, and treatment 14 served as control for treatment 15.

Abbreviations: chol = cholesterol, sit. = sitosterol, andr. = androstenedione, prog. = progesterone, pregn. = pregnenolone, estr.d. = estradiol, estr. = estriol, cortic. = corticosterone, H-cortis. = hydrocortisone, P. = phosphate, ABA = abscisic acid.

did increase media conductivity as well as increase germination, and the two applied estrogens caused no change in conductivity but did increase germination. ABA, previously shown to inhibit pollen germination considerably, caused an increase in the conductance of the media of over 40%.

Effect of Sterols on Germination under Various Temperature Regimes

Indications that sterols may play a role in cold adaptation were the bases of the following experiments.

Cholesterol and β -sitosterol solutions were added to the incubation media at concentrations of 10^{-5} M. The media contained in addition 10^{-4} M DMSO and 0.4% (v/v) ethanol. The pollen ($10 \text{ mg} \cdot \text{ml}^{-1}$) was incubated for 36 h at various temperatures. Figure 16 and Table 14 show the results. Ethanol depressed germination over the entire temperature range. This effect was alleviated by cholesterol over the temperature range of 22 to 30 C, between 24 and 26 C recovery was nearly complete. β -Sitosterol had no effect over the entire temperature range. At 40 C no pollen remained viable over the test period. The membrane stabilizer cholesterol was effective in reducing, or even preventing, decreased germination at lower temperature caused by the addition of ethanol. Unfortunately the results do not allow one to speculate on the effects of cholesterol on cold acclimation per sé, since ethanol was present in these experiments. Thus cholesterol did counteract reduced germination due to ethanol, in the lower temperature range, and never was the amount of germination in the cholesterol treatment above the control.

FIGURE 16. Effect of cholesterol and β -sitosterol on germination of slash pine pollen under various temperature regimes

Table 14. Percent stimulation of germination of slash pine pollen by 10^{-5} M cholesterol and β -sitosterol at various temperatures. ^{1/}

Temp (C)	22	24	26	28	32	34	36	38	40
cholesterol	27.4 ^{2/}	50.4 ^{3/}	13.1 ^{2/}	8.9	0.7	-7.4	2.6	-2.8	-
β -sitosterol	1.7	-2.9	0.7	-0.4	-4.9	-1.8	0.4	-10.6	-

^{1/}Control: 10^{-4} M DMSO and 0.4% (v/v) ethanol in water.

^{2/}Significantly different from control $\alpha = 0.05$

^{3/}Significantly different from control $\alpha = 0.01$

DISCUSSION

Cholesterol, campesterol, stigmasterol and β -sitosterol were identified in pine pollen by isolation, gas-liquid chromatography (GLC) and combined GLC-mass spectrometry. The glycosidic saponin digitonin was used in the isolation procedures. Digitonin forms stable and sparingly soluble compounds, sterol digitonides, exclusively with sterols that contain a free β -hydroxyl group (Windaus, 1909). GLC co-chromatography of authentic pure sterols mixed with pollen sterols gave perfect overlap. Mass spectra showed fragmentation patterns and relative intensities of the major ions closely corresponding to the behavior of authentic sterols. It would seem reasonable to suggest that pollen of Pinus elliotii contains the four sterols mentioned above.

Total sterol levels were almost constant throughout the germination period except for sitosterol. Concentrations remained around $2500 \mu\text{g} \cdot \text{g}^{-1}$ (0.25% F.W.); this value is within the range reported for various pollen species (Standifer, 1966). Cholesterol, normally occurring at very low levels in plant material (Itoh, 1972), also had the lowest concentrations in pine pollen. Sitosterol had the highest concentrations. It is also the major sterol in seedlings of Pinus elliotii (Laseter et al., 1973). The temporary changes of sitosterol levels are possibly due to exchange with the sterol pool in the medium. Labelled MVA, the common precursor for steroids, was not incorporated in any of the four sterols. Little if any sterol

synthesis occurs during the entire germination period. This seems reasonable, considering that this pollen species does not require any carbon or energy source in the incubation media.

Water soluble sterols were first discovered in 1967 (Adams and Parks, 1967) and have since been reported by various authors (Adams and Parks, 1968; Brandt et al., 1969; Price, 1970; Rohmer et al., 1972; Pillai and Weete, 1975). Up to 40% of the sterols in Euglena gracilis were water soluble (Brandt et al., 1969). Pine pollen also contained a considerable amount of water soluble sterols (approximately 12%). Interestingly, 75% of the total cholesterol pool was recovered from the aqueous media. The observation that cholesterol levels are constant during the entire germination period suggests that a large amount is immediately released when pollen comes into contact with water. Cholesterol has been shown to be the primary membrane stabilizer (Edwards and Green, 1972; Oldfield and Chapman, 1972). Cholesterol induced stability is accompanied by a general decrease in permeability of the membrane (Papahadjopoulos, 1974). The behavior of pollen seems to support this observation; when pollen is suspended in water, the bulk of the cholesterol pool is lost to the medium and the conductance of the medium increases from 0.01 to $6.69 \mu\text{Mhos} \cdot \text{cm}^{-1}$. The possibility exists that the efflux of cholesterol is necessary to allow for leakage of compounds which prevent or delay pollen germination. This subject will be discussed further with regard to effects of pollen concentration, effects of steroid hormones and effects of DMSO on pollen germination. The ^{14}C cholesterol experiment indicated that no cholesterol was taken

up by the pollen. This seems unusual and the possibility exists that the cholesterol added to the medium is complexed and becomes unavailable.

Free sterols made up the bulk of the total sterol content during the entire germination period. The average content of $2200 \mu\text{g} \cdot \text{g}^{-1}$ represents 88% of the total sterols in pollen, excluding the water soluble fraction. The fluctuating concentrations of sitosterol are only explainable by assuming that exchange with sitosterol in the medium occurred, provided the hypothesis that no sterol synthesis occurs, is correct. The content of free cholesterol remained around $60 \mu\text{g} \cdot \text{g}^{-1}$ fresh pollen. This may indicate that no new membrane material is synthesized. During tube extension, vesicles derived from the endoplasmic reticulum, or the Golgi apparatus (Stanley and Linskens, 1974; Van der Woude and Morre, 1968), are incorporated into the tip of the growing pollen tube by a process of inverse pinocytosis (Rosen, 1968). Thus the possibility exists that the membrane material needed for pollen tube growth during the initial 48 h is pre-existing.

Average steryl glycoside contents were $470 \mu\text{g} \cdot \text{g}^{-1}$ or 16% of total sterol content excluding the water soluble fraction. Siteryl glycoside concentrations increased at the beginning of germination and decreased to previous levels at the end of the germination period. The increasing concentration of sitosteryl glycoside coincided with a decrease of free sitosterol. Some reports indicate that sitosteryl glucosides give physiological responses resembling growth regulators. They had a synergistic effect with auxin in the avena coleoptile test, (Kimura et al., 1975; Smith and Van Staden, 1978) showing a biphasic dose response (Tietz et al., 1977), increased

the growth rate of cress seedling roots and shoots, and stimulated (like IAA) the H^+ -efflux of protoplast suspensions of Vicia faba (Tietz et al., 1977). It is speculated that steryl glycosides are connected with pollen germination, possibly promoting germination or pollen tube extension.

No steryl esters were detected during the entire observation period. The absence of steryl esters in this pollen species is supported by the fact that the sum of the free sterols and steryl glycosides equals, within the limits of error, the total sterol content. The significance of this observation is obscure, no definite roles for steryl esters have been reported previously.

ABA caused a considerable inhibition of pollen germination and was also responsible for a large efflux of sterols into the medium. However cholesterol showed opposite behavior, whereas 74% of the total cholesterol pool was recovered from the control medium, the medium with ABA contained only 27% of the total pool. Various short and long term effects of ABA related to plant responses have been reported (Milborrow, 1974). In relation to experiments described here, some of the short term effects of ABA are mentioned. Shoots of intact tomato plants treated with ABA showed an increased rate of root exudation (Tal and Imber, 1970). ABA applied to carrot root discs increased the flux of water either into or out of root cells (Glinka and Reinhold, 1971, 1972). Increased root exudation was attributed to increased permeability of the cells (Glinka, 1973). K^+ transport into the exudate of excised corn and barley roots was inhibited by ABA (Cram and Pitman, 1972); ion secretion into xylem tissue was also influenced (Pitman et al., 1974). Tanada (1968) discovered that red

light changed the electrical charge on cell surfaces causing them to attach to glass charged with phosphates. This effect was quickly enhanced by addition of extremely small amounts of ABA to the solution.

These reports and the observation that ABA caused increased losses of campesterol, stigmasterol and sitosterol to the medium, but drastically decreased loss of cholesterol -- the major membrane stabilizer -- points strongly to direct effects of ABA on membrane properties. It would be interesting to investigate the possibility that ABA conjugates with the cholesterol in biological membranes.

SKF-7997A₃ caused a considerable depression of pollen germination (1×10^{-5} M gave 47% inhibition), this effect was unexpected since little if any sterol synthesis occurs in germinating pollen. Biosynthesis is probably blocked between lanosterol and zymosterol (Holmes and DiTullio, 1962). However, this compound also inhibits steroid biosynthesis in plants not following this biosynthetic pathway and has other physiological effects (Bonner, et al., 1963). The compound is labile and the breakdown products are also physiologically inhibitory (Evans, 1964). Therefore there is a good possibility that breakdown products caused inhibition of pollen germination not related to sterol biosynthesis. In particular, the greatly decreased efflux of cholesterol, but not other sterols, suggest that secondary effects are involved. Any specific comments on the mode of action in the system under consideration seems therefore purely speculative.

Cordycepin (3' deoxyadenosine) strongly inhibited pollen germination, 3×10^{-5} M resulted in approximately 55% inhibition. Cordycepin inhibits RNA synthesis; 3' deoxyadenosine 5' triphosphate

competed with ATP in various RNA polymerization reactions (Shigeura and Gordon, 1965). It was shown that m-RNA was inhibited (Penman et al., 1968), in particular, RNA-polymerase II was most sensitive to cordycepin (Horowitz et al., 1976). RNA synthesis does take place in germinating pollen (Mascarenhas, 1971). There is strong evidence that dormant or masked forms of m-RNA and t-RNA are present prior to germination and are quickly activated upon germination (Linskens, 1971; Mascarenhas, 1971). The general theory, concerning formation of m-RNA, maintains that slow germinating pollen (like slash pine pollen) requires synthesis of m-RNA (Stanley and Linskens, 1974; Nygaard, 1972, 1973).

The level of cordycepin used in the pollen germination test was somewhat lower (7.5 g ml^{-1}) than levels used in experiments performed by the authors just mentioned ($10\text{-}25 \text{ g ml}^{-1}$). Auxin induced elongation of Avena coleoptile segments was greatly inhibited by $25\text{-}100 \text{ mg}$ cordycepin ml^{-1} (Cline et al., 1974). Thus, slash pine pollen is quite sensitive to cordycepin. The selective effects of cordycepin on sterol concentrations in this pollen is not easy to explain. The shape of the curves relating cordycepin concentration to germination indicate that no recovery occurs for a certain amount of pollen, therefore it seems reasonable that increased losses of sterols are due to loss from dead pollen. However cholesterol does not follow this pattern, its level in treated pollen was twice as high as in the control experiment.

Pollen germination was much affected by the concentration of pollen in the media. Maximum germination was obtained between 10 and $30 \text{ mg pollen ml}^{-1}$, and only half as much pollen germinated

when the concentration was $2 \text{ mg} \cdot \text{ml}^{-1}$. The inhibitory effect observed at low pollen concentrations was fully alleviated if media were used in which pollen was incubated prior to addition of fresh batches of pollen. The mutual stimulation of pollen on germination is a general phenomenon. Holubinsky (1945) succeeded in germinating 15 pollen species belonging to 11 families. All species showed the concentration effect strongly.

DMSO (10^{-4} M) added to pollen ($2 \text{ mg} \cdot \text{ml}^{-1}$) restored germination to 82% of the optimum value, further addition of pregnenolone, estradiol, estriol or corticosterone (each $5 \times 10^{-4} \text{ M}$) restored germination very close to optimum values. Hydrocortisone and progesterone had no significant effects. DMSO had little effect when pollen concentrations were optimal ($10 \text{ mg} \cdot \text{ml}^{-1}$), and there was no difference between 10^{-4} and 10^{-3} M DMSO. The effects of the steroid hormones seem general and non-hormonal since the concentrations were relatively high, and also the hormones vary so widely in structure. The use of animal steroid hormones was suggested by a report (Matsubara, 1969) that animal hormones greatly stimulated germination of Chrysanthemum pollen. The amount of stimulation was dependent on the particular hormone added. The effect of DMSO was quite unexpected. It has been reported that soluble components in pollen are responsible for mutual stimulation of germination. In particular, in pollen of Petunia hybrida heat labile proteins, with a molecular weight between 5×10^4 and 10^5 recovered from the pollen eluent were able to restore germination. For an extensive treatment on this subject see Kirby (1977). DMSO is often used as a passive general carrier in uptake experiments. The effect of DMSO on pollen germination indicates that the action is

probably related to a change in membrane properties. Efflux of certain compounds alone does not result in normal germination. Replacement of the pollen media (after short incubation) with water does not restore germination. Simple osmotic effects do not explain this behavior either. Germination of pine pollen in a dilute calcium phosphate buffer is decreased. Also there is no difference between 10^{-4} and 10^{-3} M DMSO on germination of pine pollen. Complete media analysis may solve this question; also, a study of the effect of DMSO on membranes may prove fruitful.

Conductivity of pollen media has often been used as a measurement of pollen germinability (Dillon and Zobel, 1957; Ching and Ching, 1976). Since DMSO and animal steroids caused significant increases of germination when pollen concentrations were low, measurement of the conductivity of the media was suggested. ABA was also included in the experiment since it affected pollen germination and sterol contents in the media. No obvious relationships exist between conductivity increases due to steroids and increased germination of low concentration pollen. However, of six steroids tested for their influence on germination, four had a significant positive effect and three of these increased conductivity as well. Of the two that did not increase germination, one did not increase conductivity. Cholesterol and sitosterol did not cause a change in conductivity. ABA caused a drastic increase of conductivity in the medium, this is in agreement with observations that ABA causes leakage of cell membranes in other systems.

The effect of added cholesterol and sitosterol on pollen germination was tested over the temperature range of 22 to 40 C. Since

the sterols were added as an ethanolic solution, all media contained ethanol (0.4% v/v). Ethanol caused a decrease in germination over the entire temperature range. This decrease was counteracted by cholesterol over the temperature range of 22 to 30 C and between 24 and 26 C recovery was complete. Sitosterol had no effect over the entire temperature range. Cholesterol has been shown to be the primary membrane stabilizer by various researchers (Chapman and Wallach, 1968; Lecuyer and Dervichian, 1969; Papahadjopoulos, 1974). There is evidence that membranes contain heterogenous (gel and liquid-crystalline) lipid domains. Studies on crystal-liquid to crystal phase transitions have revealed that the transition temperature (T_c : onset temperature of transition) is dependent on various factors including the phospholipid-sterol ratio and the chemical structure of the sterol (van Deenen, 1972; Oldfield and Chapman, 1972; Shah and Schulman, 1968). The transition temperature decreases with more unsaturation, shorter chains, and cis rather than trans unsaturation. Within a certain range of cholesterol concentration a condition of intermediary fluidity is produced. Below T_c in the presence of cholesterol the hydrocarbon chains in the membrane lipids are more mobile than without cholesterol, above T_c less mobile. Above T_c the steroid nucleus decreases flexing of the hydrocarbon chains, below T_c it prevents them from crystallizing into the rigid crystalline condition (Oldfield and Chapman, 1972). Thus, cholesterol present within a certain concentration range imparts the "correct fluidity" to the membrane over a relatively wide temperature range. Studies of membrane incorporated sterols, relating chemical structure to function, have revealed that cholesterol is the most effective membrane

stabilizer. The ability of sterols to enter liposomes (artificial membranes) and erythrocyte membranes is in decreasing order; cholesterol, campesterol and sitosterol. This behavior is the same as absorption into the intestine (Edwards and Green, 1972). The chemical composition of membranes directly influenced enzyme activities. Changes in ergosterol levels in yeast produced concomitant changes in the phase transitional temperature pattern of mitochondrial ATP-ase, an inverse relationship between sterol content and transition temperature was established (Thompson and Parks, 1974). Many studies have been reported concerning the roles of sterols in membrane integrity, flux of water and ions, action of solvents with low polarity and effect of polyene antibiotics (Papahadjopoulos et al., 1973; De Kruijff and Demel, 1974; Carbonero et al., 1960; Hendrix and Higinbotham, 1973; Gotlieb et al., 1960; Karst and Jund, 1976; Olson, 1973). These studies all support the theory that sterols, in particular cholesterol, counteract various stresses placed upon biological membranes.

During cold acclimation of some tissues a significant shift in the overall lipid composition occurs (Kuiper, 1970). Yeast sterol mutants showed that different sterol compositions caused different temperature characteristics of two mitochondrial enzymes (Thompson and Parks, 1974).

The results obtained with cholesterol and alcohol addition to the media support the described observations. Unfortunately, these experiments do not allow the separation of the effects of decreased temperature and alcohol. Nevertheless, cholesterol counteracts the

combined effects of ethanol and low temperature on the germination of pine pollen.

Results of the various experiments allow some suggestions about the roles of sterols in pollen of Pinus elliotii. Some additional experiments are suggested to substantiate these speculations.

Sterols are essential components of biological membranes. Results of some experiments indicated that no de novo sterol biosynthesis occurs during germination. This makes pine pollen desirable experimental material. Quantitative measurements of the various free and bound sterols in the cytoplasts, mitochondria, vesicles and supernatant would show sterol distribution. Sterol analysis over time would show a shift from sterol contents in the vesicles and/or supernatant to the growing membranes of the pollen tubes. Tube growth results from addition of vesicles to the tube tips by inverse pinocytosis. Distributional changes over time may also reveal what sterols are particularly used as storage or transport forms.

Steryl glycosides, in particular sitosteryl glycosides may influence germination. At the beginning of germination concentrations increased rapidly, reached a peak during the middle of the germination period and declined to pregermination concentrations at the end. Sitosteryl glycosides have auxin-like properties. External application of this compound may reveal its role.

Cholesterol is the major membrane stabilizer, it behaved quite different from other sterols. Whereas more sterol material leaked into the medium when pollen was placed under stress (metabolic inhibitors, ethanol) cholesterol was actually taken up from the medium. This behavior was particularly evident when ABA was applied.

ABA also caused increased leakage of polar compounds as shown by a large increase of the conductivity of the pollen media. Direct analysis of isolated membranes, ion and water flux measurements as well as shifts in UV spectra of cholesterol and ABA mixtures, may shed light on this question.

It was shown that pollen germination was very dependent on the concentration of pollen in the aqueous media. Addition of 10^{-4} M DMSO greatly increased germination at low pollen concentrations. It is not clear how DMSO operates in this system, or if sterols are involved. However, DMSO is considered an effective passive carrier. Fractionation and analysis of pollen and media extracts, before and after application of DMSO may indicate what compounds are involved with pollen concentration and germination interactions.

It has to be kept in mind that approximately 35 percent of the pollen was non-viable, this probably causes certain shifts in sterol distribution. Analysis of pollen with zero viability may allow some data corrections.

The small effects of animal steroid hormones on pollen germination (pollen at low concentrations) as well as conductivity of the germination media seems non-specific and non-hormonal. No definite conclusions can be drawn from these experiments.

Cholesterol counteracted decreased germination due to the combined effects of lower temperatures and addition of alcohol. This supports observations that cholesterol counteracts stresses placed on membranes as well as the involvement of cholesterol in cold-adaptation.

SUMMARY

This study concerned the composition and concentrations of sterols in pollen of Pinus elliotii, as well as some physiological aspects of sterols, animal steroid hormones, and inhibitors added to the incubation media. Cholesterol, stigmasterol, campesterol and sitosterol were identified by gas-liquid chromatography (GLC) and combined GLC-mass spectrometry (GLC-MS). Total sterol levels, excluding sterols released into the aqueous media, were approximately $2500 \mu\text{g} \cdot \text{g}^{-1}$ fresh pollen (0.25% F.W.) during the 48 hour germination period. Total sitosterol levels increased during the first 6 hours and returned to original levels after 12 hours; sitosterol accounted for almost 75% of the total non-water soluble sterol content. The aqueous media, analyzed after 36 hours, contained approximately $240 \mu\text{g}$ sterols g^{-1} pollen (12% of total combined sterol pool). However, 75% of the total cholesterol pool was water soluble. Free sterols ($2200 \mu\text{g} \cdot \text{g}^{-1}$ fresh pollen) made up the bulk of the total non-water soluble sterols. The free sitosterol content reached peaks after 12 hours and 48 hours, and the lowest concentration after 24 hours. Total steryl glycoside levels increased after 6 hours, reaching a peak after 24 hours, and then returned to almost original levels after 36 hours. The increasing sitosterol glycoside content almost equalled the decreasing content of free sitosterol. Steryl esters were not detected during the entire germination period; this was supported by the fact that total sterol levels approximately equalled the total combined levels of free sterols

and steryl glycosides. Incorporation of ^{14}C -mevalonic acid indicated that very little, if any, sterols were synthesized from this universal precursor of terpenoids. The almost constant levels of total sterols supported this concept. Depressed germination caused by the sterol biosynthesis inhibitor SKF-7997A₃ was probably caused by breakdown products of this compound. Inhibitory effects other than on sterol biosynthesis is supported in the literature. Absciscic acid (ABA), SKF-7997A₃, and cordycepin (3' deoxy-adenosine) all inhibited pollen germination extensively, and all three inhibitors increased the efflux of sterols into the aqueous media. However, cholesterol efflux was less than in the controls. In particular, whereas 74% of the total cholesterol pool was recovered from the control medium, the medium with ABA contained only 27% of the total pool.

Pollen germination was strongly affected by the concentration of pollen in the media. Optimum germination was attained between 10 and 30 mg pollen ml⁻¹. At 2 mg · ml⁻¹ germination was only 51% of that at optimum levels of pollen in the media. Addition of 10⁻⁴ M dimethylsulphoxide (DMSO) to pollen (2 mg · ml⁻¹) increased relative germination to 82%. Addition of pregnenolone, estradiol, estriol or corticosterone (each 10⁻⁵ M) along with DMSO restored germination to almost that of controls with optimum levels of pollen. Animal steroid hormones increased the conductance of the pollen media, however there was no relationship between increased germination and increased conductance. Decreased germination, due to the combined effects of sub-optimal temperatures and ethanol, was counteracted by addition of cholesterol to the medium.

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BIOGRAPHICAL SKETCH

Josephus Karl Peter was born September 9, 1940, in Aalten, The Netherlands. He attended the High School "Marianum" in Groenlo, The Netherlands, from 1952 until 1956. He was then employed as a laboratory technologist at the Royal Dutch Salt Industries (Hengelo, The Netherlands) from 1956 until 1958. He was subsequently employed on passenger ships with the Rotterdam Lloyd and the Holland America Line from 1959 until 1960. During 1961 and 1962 he attended the "School for Laboratory Technologists" (Diploma) in Arnhem, The Netherlands. He was employed by the University of Nijmegen (The Netherlands) in the Department of Chemical Cytology as a laboratory technologist from 1963 until 1965. After extensive travelling he settled in Gainesville, Florida, where he was employed by the University of Florida, Department of Fruit Crops, as a laboratory technologist from 1967 until 1968. Meanwhile he entered college at the University of Florida where he received his B.S. in chemistry with a minor in languages (August 1971); Master of Agriculture (forest physiology) with a certificate in Latin American Studies (August 1973) and Ph.D. in agronomy (plant physiology) in June 1979. He started employment with the U. S. Forest Service, Forestry Sciences Laboratory in Starkville, Mississippi, in June 1978.

Josephus Karl Peter is a member of Xi Sigma Pi (Honorary Forestry Society) and Sigma Xi (Scientific Research Society of North America). He is married to Rita Engels and has a five-year-old son, Yuri.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Ray E. Goddard, Chairman
Professor in Agronomy

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Robert H. Biggs
Professor, Fruit Crops

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Charles A. Hollis III
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