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Production of crude drugs

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The crude drug that reaches the pharmaceutical manufacturing line will have passed through various stages, all of which influence the nature and amount of active constituents present. These aspects will be considered under the headings 'Source Materials', 'Environmental Conditions', 'Cultivated and Wild Plants', 'Collection', 'Drying', 'Storage' and 'European Regulations'.

SOURCE MATERIALS

It is imperative that correct identification of the source material is made. Adulteration may be accidental, particularly if collection is made from wild plants, or it may be deliberate. Failure in this area can result in poisoning (e.g. hemlock fruits mistaken for other umbelliferous fruits) or inactive products (e.g. substitution of St John's wort with other vegetable material when demand exceeds supply). For pharmacopoeial drugs, precise macroscopic and microscopic characters are available.

For the isolation of specific constituents, the source can vary, e.g. particular steroids may be obtained from various diverse plants (q.v. Chapter 25) or hyoscine from a number of solanaceous species. Recently a potential problem concerning the production of the oral antiviral against avian flu—Tamiflu® (oseltamivir)—arose from a shortage of Chinese staranise (q.v.), the source of the starting material (shikimic acid, see Fig. 18.8) for the synthesis. However, the toxic Japanese star anise, regarded as an adulterant of the Chinese drug, also contains shikimic acid and could provide an alternative source (D. V. C. Awang and M. Blumenthal, *HerbalGram*, 2006, **70**, 58).

In the article 'Plant part substitution—a way to conserve endangered medicinal plants' S. Zschocke *et al.* (*J. Ethnopharm*., 2000, **71**, 281) explore the possibility of using the leaves of plants that are traditionally used for their barks, bulbs and roots, thus conserving the plant.

ENVIRONMENTAL CONDITIONS

Plant growth and development, and often the nature and quantity of secondary metabolites, are affected by temperature, rainfall, aspect, length of day (including the quality of light) and altitude. Such effects have been studied by growing particular plants in different climatic areas and observing variations. The findings of such research are illustrated by work on cannabis by El-Kheir *et al.* in 1986 in which seeds of cannabis, grown in England and rich in CBD and devoid of THC, when cultivated in Sudan started to produce THC in the first generation and in the second generation contained up to 3.3% THC with a further decrease (down to 0% in some plants) of CBD (see 'Cannabis' for explanation of chemistry). However, it is impossible to control all the variables in such experiments, and special laboratories (phytotrons) have been constructed in which all the factors are independently controllable. Even so, a meaningful expression of the results can often present some difficulty. For example, a particular factor may lead to the development of a small plant which, when analysed on a percentage dry weight basis, indicates a high proportion of metabolite, even though the overall yield per plant could be quite low. Conversely, certain nutrients may result in the production of large plants with a somewhat low analytical figure for constituents on a percentage dry weight basis, but yield per plant may exceed that of the control.

Temperature

Temperature is a major factor controlling the development and metabolism of plants. Although each species has become adapted to its own natural environment, plants are frequently able to exist in a considerable range of temperature. Many tropical and subtropical

plants will grow in temperate regions during summer months, but lack frost resistance to withstand the winter. In general, the highest temperatures are experienced near the Equator, but as the temperature falls about 1°C for every 200 m of elevation, it is possible in, say, Jamaica to have a tropical climate on the coast and a temperate one in the mountains. The annual variations in temperature are just as important as the temperature of the hottest month. At Singapore the annual range of temperature is as little as 1.5°C, whereas Moscow, with its hot summers and cold winters, has a range of 29.3°C. In general, the formation of volatile oils appears to be enhanced at higher temperatures, although very hot days may lead to an excess physical loss of oil. The mean optimum temperature for nicotine production in *Nicotiana rustica* is 20°C (lower at 11–12°C and at 30°C). Several authors have indicated that fixed oils produced at low temperatures contain fatty acids with a higher content of double bonds than those formed at higher temperatures.

Rainfall

The important effects of rainfall on vegetation must be considered in relation to the annual rainfall, its distribution throughout the year, its effect on humidity and its effect coupled with the water-holding properties of the soil. Variable results have been reported for the production of volatile oils under different conditions of rainfall and may in some instances be coupled with the development of glandular hairs. Continuous rain can lead to a loss of water-soluble substances from leaves and roots by leaching; this is known to apply to some plants producing alkaloids, glycosides and even volatile oils. This could account for low yields of some active constituents in wet seasons from plants whose general condition appears to be good.

With *Cassia angustifolia* (Tinnevelly senna) it has been shown that short-term drought increases the concentration of sennosides A+B but in the longer term causes loss of leaf biomass (H. Ratnayaka *et al*., *Planta Medica*, 1998, **64**, 438).

Day-length and radiation characteristics

Plants vary much in both the amount and intensity of the light which they require. In the wild state the plant will be found where its shade requirements are met, and under cultivation similar shade must be provided. In certain cases research has shown that light is a factor which helps to determine the amount of glycosides or alkaloids produced. With belladonna, stramonium and *Cinchona ledgeriana* full sunshine gives a higher content of alkaloids than does shade. At Gif-sur-Yvette experiments indicated that with *Datura stramonium* var. *tatula* long exposure to intense light brought about a sharp increase in hyoscine content at the time of flowering. An important *in vivo* reaction in the formation of the antitumour alkaloids of *Catharanthus roseus* is exemplified by the dimerization of the indole alkaloids catharanthine and vindoline leading to vinblastine; Hirata *et al.* (*Planta Med.*, 1993, **59**, 46) demonstrated that irradiation of intact plants with near ultraviolet light in the range 290–380 nm (peak 370 nm) stimulates the synthesis of dimeric alkaloids, probably by inducing catharanthine oxidation as a trigger reaction. This observation has support from *in vitro* studies.

It has been shown that under long-day conditions peppermint leaves contain menthone, menthol and traces of menthofuran; plants grown under short-day conditions contain menthofuran as a major component of the volatile oil. Furthermore a long photoperiod for young leaves activates the reduction pathway with conversion of menthone to methol. In studies on the day–night changes in the relative concentrations of volatiles from flowers of *Nicotiana sylvestris* and other species a marked increase (about tenfold) in aromatic compounds including benzyl alcohol was detected at night, whereas no increase in the volatiles (e.g. linalool, caryophyllene) originating from the mevalonic acid pathway (q.v.) was noted (J. H. Loughrin *et al*., *Phytochem*., 1990, **29**, 2473).

The daily variation in the proportion of secondary metabolites is probably light-controlled and is discussed more fully under 'Collection Times'. Many plants initiate flowers only in certain day-lengths, and where flowering is essential this factor must be carefully considered before planting in a new region. Presence or absence of light, together with wavelength range, have a marked effect on the secondary metabolite production of some plants in tissue culture.

The type of radiation which plants receive is also important. With *Ocimum basilicum*, C. B. Johnson *et al.* (*Phytochemistry*, 1999, **51**, 507), have found, in relation to herbs raised under glass and receiving no UV-B radiation, that supplementary UV-B radiation increases levels of both the phenyl-propanoids and terpenoids of the leaves. Flavonoids and anthocyanins are also known to be influenced by UV-B radiation. Depletion of the ozone layer and the consequent effect of increased radiation at the earth's surface has been a topic of much recent speculation. Concerning medicinal plants, R. Karouson *et al.* (*Phytochemistry*, 1998, **49**, 2273) raised two chemotypes of *Mentha spicata* which were subjected to increased UV-B radiation equivalent to a 15% ozone depletion over Patras, Greece. In one chemotype essential oil production was stimulated by the treatment while a similar non-significant trend was noted with the other.

Altitude

The coconut palm needs a maritime climate and the sugar cane is a lowland plant. Conversely, tea, cocoa, coffee, medicinal rhubarb, tragacanth and cinchona require elevation. In the case of *Cinchona succirubra* the plants grow well at low levels but produce practically no alkaloids. The bitter constituents of *Gentiana lutea* increase with altitude, whereas the alkaloids of *Aconitum napellus* and *Lobelia inflata* and the oil content of thyme and peppermint decrease. Other oil-producing plants may reach a maximum at certain altitudes. Pyrethrum gives the best yields of flower-heads and pyrethrins at high altitudes on, or near, the Equator. It is therefore produced in East Africa and north-west South America. However, vegetative growth is more lush under irrigated conditions at lower altitude, so the propagation farms (for the vegetative multiplication of plants) are, in Ecuador, situated at lower levels than the final commercial farms.

G. A. Statti *et al.* (*Fitoterapia*, 2004, **75**, 212–216) studied bergamot (*Citrus bergamia*) grown at different altitudes and solar exposures in Calabria, Italy; they found both chemical (linalool, linalyl acetate composition) and biological (antioxidant and antifungal activities) diversities.

The flowering heads of *Arnica montana*, grown in Austria in experimental plots at altitudes between 590 and 2250 m showed no altitude effect on the total contents of sesquiterpene lactones and flavonoids but the latter with vicinal-free hydroxy groups in ring B increased with altitude relative to the other flavonoids. Caffeic acid derivatives were 85% higher at the summit compared with the valley (R. Spitaler *et al*., *Phytochemistry*, 2006, **67**, 407). For a study of the effect of altitude on the podophyllotoxin content of the aerial parts and underground organs of *Podophyllum hexandrum* populations from the Kumaun region of the Indian Central Himalayas, see M. Nadeem *et al*., *Planta Med*., 2007, **73**, 388.

CULTIVATED AND WILD PLANTS

Certain drugs are now obtained almost exclusively from cultivated plants. These include cardamoms, Indian hemp, ginger, and peppermint and spearmint for oil production. Others include Ceylon cinnamon,

linseed, fennel, cinchona and opium. In other cases both wild and cultivated plants are used. Some plants have been cultivated from time immemorial (e.g. flax, opium poppy and coca). Others are now grown because supplies of the wild plants are insufficient to meet the demand or because, owing to sparse distribution or inaccessibility, collection is difficult. Cultivation is essential in the case of drugs such as Indian hemp and opium, which are subject to government control, and recently for those wild plants in danger of over-exploitation and which have now been given CITES (q.v.) listing. In many cases cultivation is advisable because of the improved quality of the drug which it is possible to produce. The improvement may be due to the following.

1. The power to confine collections to species, varieties or hybrids which have the desired phytochemical characters (e.g. aconite, cinnamon, fennel, Duboisia, cinchona, Labiate drugs and valerian).
2. The better development of the plants owing to improved conditions of the soil, pruning, and the control of insect pests, fungi, etc.
3. The better facilities for treatment after collection. For example, drying at a correct temperature in the cases of digitalis, colchicum, belladonna and valerian, and the peeling of cinnamon and ginger.

For success in cultivation it is necessary to study the conditions under which the plant flourishes in the wild state and reproduce these conditions or improve on them. Small changes in ecology can affect plant products; thus, satisfactory rubber trees grow wild in the Amazon basin but cleared areas converted to rubber plantations have been a failure.

Soils

Different plant species vary enormously in their soil and nutritive requirements, and this aspect has received considerable attention with medicinal plants. Three important basic characteristics of soils are their physical, chemical and microbiological properties.

Variations in particle size result in different soils ranging from clay, via sand, to gravel. Particle size is one factor influencing water-holding capacity, and some plants (e.g. *Althaea officinalis*) which produce mucilage as a water-retaining material contain less mucilage when grown on soil with a high moisture content. Although particular species have their own soil pH tolerances (*Datura stramonium* 6.0–8.2, *Majorana hortensis* 5.6–6.4), no marked influence of pH value within the tolerance range has been demonstrated for essential oils (*Mentha piperita*) and alkaloids (*D. stramonium*). All plants require calcium for their normal nutrition but plants known as caliphobous plants (e.g. *Pinus pinaster* and *Digitalis purpurea*) cannot be grown on chalky soils, probably owing to the alkalinity. In other cases different varieties of the same species may grow on different soils. For example, in Derbyshire, UK, *Valeriana officinalis* var. *sambucifolia* is common on the coal measures, but avoids the limestone, where it is replaced by *Valeriana officinalis* var. *mikanii*.

The effect of nitrogen-containing nutrients on alkaloid production has received considerable study (solanaceous drugs including *Nicotiana*, opium); generally nitrogen fertilizers increase the size of the plants and the amounts of alkaloids produced but, as indicated elsewhere, the method of expressing the results of such experiments is important. The effects of nitrogen on glycoside and essential oil contents appear variable; presumably in these cases the final result arises from the general effect of nitrogen on the plant's metabolism. Nitrogen fertilization has been shown to increase the silymarin content of the fruits of *Silybum marianum* grown on reclaimed ground. The effect of potassium on alkaloid production shows no consistent trend, but an interesting example is the increase in putrescine production in barley grown on a potassium-deficient medium, where it is possible that the organic base has been formed to act as a substitute for potassium ions. It has long been maintained that trace amounts of manganese are necessary for the successful production of *Digitalis purpurea* and more recently it was shown that a regimen of manganese and molybdenum feeding over the two years of development of *D. grandiflora* gives significant increases in glycoside yield.

Propagation from seeds

To ensure success the seeds must be collected when perfectly ripe. If not planted immediately, they should normally be stored in a cool and dry place and must not be kiln-dried. Some seeds such as cinnamon, coca and nutmegs rapidly lose their power of germination if allowed to dry or if stored for quite short periods. Long storage of all seeds usually much decreases the percentage which germinate.

Although seeds are naturally sown at the season when they ripen, it is frequently more convenient, especially in the case of the less hardy exotic species, to defer sowing until the spring. In some cases, however, immediate sowing of the fresh seed is advisable. For example, it has been shown that if the seeds of *Colchicum autumnale* are air-dried even for a few days, only about 5% germinate in 1 year and some may not germinate for 5 years; whereas if sown as soon as the capsules dehisce, 30% will germinate in the first year. In some instances, as with *Datura ferox* and foxglove, seeds may remain viable in the ground for many years before germinating. With *Erythroxylum coca* and *E. novogranatense* the seeds stored at 4°C for 24 days gave, respectively, 29% and 0% germination (E. L. Johnson, *Planta Med.*, 1989, **55**, 691). Seeds may, if slow germinating, be soaked in water or a 0.2% solution of gibberellic acid for 48 h before sowing; more drastic methods, such as soaking in sulphuric acid in the case of henbane seeds, or partial removal of the testa by means of a file or grindstone, have also been recommended. With *Ipomoea purga* (jalap) scarification of the seeds has been the secret of success in obtaining 95% germination in eight days (A. Linajes *et al.*, *Economic Botany*, 1994, **48**, 84).

Time of seed-sowing may affect the active constituents, as illustrated by *Chamomilla recutita*—for 17 cultivars investigated most gave a significantly higher yield of oil if they were spring-sown rather than autumn-sown and the oil composition also varied (O. Gasic *et al.*, *J. Ess. Oil Res.*, 1991, **3**, 295, through *Chem. Abs.*, **116**, 37955).

Propagation by vegetative means

The following examples of vegetative propagation may be mentioned.

1. By the development of *bulbs* (e.g. squill); *corms* (e.g. colchicum); *tubers* (e.g. jalap and aconite); or *rhizomes* (e.g. ginger).
2. By *division*, a term usually applied to the separation of a plant which has a number of aerial stems or buds, into separate parts each having roots and a growing point. This method may be used for althaea, rhubarb, gentian and male fern.
3. By *runners* or *offsets* (e.g. chamomile and the mints).
4. By *suckers* or *stolons* (e.g. liquorice and valerian).
5. By *cuttings* or portions of the plant severed from the plant and capable of developing roots. Success by this method has been extended to a large number of plants by the use of rooting hormones (see Chapter 12) and by the employment of mist propagation.
6. By *layers*. A layer is a branch or shoot which is induced to develop roots before it is completely severed from the parent plant. This is done by partly interrupting the food supply by means of a cut or ligature and embedding the part. Alternatively the slit portion of the branch is enclosed in moist peat, surrounded by moss, and the whole enclosed in polythene. This method has been used successfully for the propagation of cascara.

7. By *grafting* and *budding*. Grafting is an operation in which two cut surfaces, usually of different but closely related plants, are placed so as to unite and grow together. The rooted plant is called the *stock* and the portion cut off the *scion* or *graft*. In Guatemala young *Cinchona ledgeriana* scions are grafted on *Cinchona succirubra* root-stocks, eventually giving a tree which produces bark rich in the alkaloid quinidine. Grafting of female scions of *Myristica fragrans* on male stocks may be used to increase the proportion of fruit-bearing trees in the plantation. The method has been used considerably in phytochemical research to study sites of synthesis of metabolites etc. *Budding* consists of the introduction of a piece of bark bearing a bud into a suitable cavity or T-shaped slit made in the bark of the stock. Budding is largely used for *Citrus* species, selected strains of sweet orange, for example, being budded on sour stocks.
8. By *fermentation*. This process applies particularly to the production of moulds and bacteria, and is extensively used in the manufacture of antibiotics, lysergic acid derivatives and some vitamins.
9. By *inoculation*. Specific to ergot whereby the spores of the fungus are artificially cultured and injected into the rye heads by special machines,
10. By *cell culture* followed by differentiation; see Chapter 13.

Hydroponics

Plants can be cultivated without soil by the use of an artificial aqueous nutrient medium. The system is suitable for raising plants under laboratory conditions for biogenetic and other studies. It is used commercially for such crops as tomatoes and strawberries but is uneconomic for the large-scale production of common medicinal plants.

S. J. Murch *et al.* (*Planta Med.*, 2002, **68**, 1108) have obtained data showing that a greenhouse hydroponic system can be effectively used for the production of St John's wort containing the active constituents hypericin, pseudohypericin and hyperforin.

COLLECTION

Drugs may be collected from wild or cultivated plants, and the task may be undertaken by casual, unskilled native labour (e.g. ipecacuanha) or by skilled workers in a highly scientific manner (e.g. digitalis, belladonna and cinchona). In the USA the explosive demand for some herbs has led to concern over wholesale uncontrolled collection, so-called wildcrafting, resulting in the over-harvesting of such plants as *Panax quinquefolium, Polygala senega, Echinacea* spp. and *Cimicifuga racemosa* (black cohosh). Elsewhere *Prunus africana* (pygeum bark) found from Nigeria to Madagascar, *Rauwolfia serpentina* from India, and *Turnera diffusa* (damiana) from Mexico are other examples of over-exploitation.

A strategy for the sustained harvesting of *Camptotheca acuminata* (Nyssaceae), the source of the anticancer drug camptothecin, has been described by R. M. Vincent *et al.*, (*J. Nat. Prod.*, 1997, **60**, 618). The alkaloid is accumulated in young leaves and by their repeated removal axillary bud outgrowth is stimulated giving an increased harvestable amount of camptothecin in a non-destructive manner. Further studies by S. Li *et al.* (*Planta Med.*, 2002, **68**, 1010) showed that camptothecin accumulates primarily in the glandular trichomes of the leaves and stems with overall variation among *Camptotheca* species and varieties, and significantly, according to tissue ages and seasons. Details of the two best strains for cultivation are given.

With *Hypericum perforatum* it has been shown that from the first bud phase to the open flower stage the contents of dianthrones, quercetin derivatives and hyperforin increase; in the unripe fruits dianthrones and quercetin glycosides decrease whereas the hyperforin content increases (D. Tekelová *et al.*, *Planta Med.*, 2000, **66**, 778).

The season at which each drug is collected is usually a matter of considerable importance, as the amount, and sometimes the nature, of the active constituents is not constant throughout the year. This applies, for example, to the collection of podophyllum, ephedra, rhubarb, wild cherry and aconite. Rhubarb is reported to contain no anthraquinone derivatives in winter but anthranols which, on the arrival of warmer weather, are converted by oxidation into anthraquinones; also the contents of *C*-glycosides, *O*-glycosides and free anthraquinones in the developing shoots and leaves of *Rhamnus purshiana* fluctuate markedly throughout the year.

The age of the plant is also of considerable importance and governs not only the total quantity of active constituents produced but also the relative proportions of the components of the active mixture. A few examples are given in Table 11.1 but some ontogenetic variation of constituents must exist for all plants.

There is increasing evidence that the composition of a number of secondary plant metabolites varies appreciably throughout the day and night. In some cases—for example, with digitalis and the tropane alkaloid-containing plants which have been extensively studied—the evidence has been somewhat conflicting in this respect. However, this may be largely due to the methods of analysis employed; thus, throughout the day the overall amount of alkaloid or glycoside may not change to any extent but there may be an interconversion of the various alkaloids or glycosides present. Daily variations of the alkaloids of the poppy, hemlock, lupin, broom, the solanaceous plants and ergot have been reported, also with the steroidal alkaloids of 'industrial shoots' of *Solanum laciniatum*, the cardiac glycosides of *Digitalis purpurea* and *D. lanata*, the simple phenolic glycosides of *Salix* and the volatile oil content of *Pinus* and *Salvia*.

Generally speaking, leaves are collected as the flowers are beginning to open, flowers just before they are fully expanded, and underground organs as the aerial parts die down. Leaves, flowers and fruits should not be collected when covered with dew or rain. Any which are discoloured or attacked by insects or slugs should be rejected. Even with hand-picking, it is difficult, certainly expensive, to get leaves, flowers or fruits entirely free from other parts of the plant. In cases such as senna leaf and digitalis the official monographs allow a certain percentage of stalks to be present or a limited amount of 'foreign matter' (for definition, see *BP*/*EP* and Chapter 16). Similarly, with roots and rhizomes a certain amount of aerial stem is often collected and is permitted in the case of senega root. The harvesting of umbelliferous fruits resembles that of wheat. Reaping machines are used, and the plants, after drying in shocks, are threshed to separate the fruits. Special machines are used to harvest ergot and lavender flowers (illustrations will be found in earlier editions). Barks are usually collected after a period of damp weather, as they then separate most readily from the wood. For the collection of gums, gum resins, etc., dry weather is obviously indicated and care should be taken to exclude vegetable debris as far as possible.

Underground organs must be freed from soil. Shaking the drug before, during and after drying, or brushing it, may be sufficient to separate a sandy soil, but in the case of a clay or other heavy soil washing is necessary. For example, valerian collected from the wild is washed in the streams on the banks of which it usually grows. Before drying, any wormy or diseased rhizomes or roots should be rejected. Those of small size are often replanted. In certain cases the rootlets are cut off; rhubarb, ginger and marshmallow are usually peeled. All large organs, such as calumba root and inula rhizome, should be sliced to facilitate drying. Before gentian root is dried, it is

Table 11.1 Examples of the ontogenetic variation of some metabolites.

Example	Ontogenetic variation
Volatile oils	
Mentha piperita	Relatively high proportion of pulegone in young plants: replaced by menthone and menthol as leaves mature
M. spicata	Progression from predominance of carvone in young plants to dihydrocarvone in older ones
Cloves	Contain about 14–21% of oil; mother 'blown' cloves contain very little oil
Coriandum sativum	Marked changes in oil composition at the beginning of flowering and fruiting
Achillea millefolium	During flowering, monoterpenes (principally 1,8-cineole) predominate in oils from leaves and flowers. Oil obtained during the vegetative period contains principally sesquiterpenes (92%) with germacrane D the major component
Laurus nobilis	Highest yield: end of August (Portugal), July (China), Spring (Israel), coinciding with highest level of 1,8-cineole
Valeriana officinalis	Highest content in September (valerenic acid and derivatives, and the valepotriates reached maximum in February–March)
Cinnamomum camphora	Camphor accumulates in heartwood as tree ages; ready for collection at 40 years
Diterpenes	
Taxus baccata	Needles contain up to 0.1% 10-deacetylbaccatin which is replaced by large amounts of 2,4-dimethoxyphenol in winter
Cannabinoids	
Cannabis sativa	Young seedlings contain principally cannabichromene; Δ^9-tetrahydrocannabinol is major cannabinoid of adult plants
Cardioactive glycosides	
Digitalis purpurea	Glycoside content varies with age; purpurea-glycoside A is formed last but eventually reaches a constant maxiumum of 50% of the total glycoside
D. lanata	Although highest levels of total glycosides are observed in first-year leaves, those glycosides most important medicinally (e.g. lanatoside C) attain their highest levels in second-year plants
Cyanogenetic glycosides	
Linum usitatissimum seeds	Monoglucosides (linamarin and lotaustralin) and diglucosides (linustatin and neolinustatin) in developing embryos; diglucosides only accumulate in mature seeds
Steroidal sapogenins	
Agave sp.	Steroidal sapogenins isolated from young, mature, old and flowering plants had successively fewer hydroxyl groups
Yucca sp.	Similar to *Agave*
Dioscorea tokoro	Changes in sapogenin content in first season's growth
Alkaloids	
Papaver somniferum	Morphine content of capsule highest 2½–3 weeks after flowering; the secondary alkaloids (codeine, thebaine, narcotine and papaverine) reach their maximum somewhat earlier
Datura stramonium	The hyoscine/hyoscyamine ratio falls from about 80% in young seedlings to about 30% in mature fruiting plants
Duboisia myoporoides	The hyoscine/hyoscyamine ratio depends both on the developmental stage of the plant and on the position of the leaves on the stem
Ipomoea violacea seeds	Lysergic acid amide/chanoclavine ratio increases as the seed matures
Steroidal alkaloids	
Solanum dulcamara fruits	Solasodine content fluctuates during maturation of fruit; tomatidenol and soladulcidine eventually predominate
Citrus glycosides and limonoids	Limonin and naringin levels in grapefruit fall as fruit matures
Furanocoumarins	
Ammi visnaga	Unripe fruits richest in both khellin and visnagin
Tannins	
Liquidambar formosana	Seasonable variation of hydrolysable leaf tannis, most rapid changes in the Spring
Vanillin	
Vanilla planifolia	Highest rate of vanillin biosynthesis occurs 8 months after flower pollination

made into heaps and allowed to ferment. Seeds such as nux vomica and cocoa, which are extracted from mucilaginous fruits, are washed free from pulp before drying.

DRYING

If enzymic action is to be encouraged, slow drying at a moderate temperature is necessary. Examples of this will be found under 'Orris Rhizome', 'Vanilla Pods', 'Cocoa Seeds' and 'Gentian Root'. If enzymic action is not desired, drying should take place as soon as possible after collection. Drugs containing volatile oils are liable to lose their aroma if not dried or if the oil is not distilled from them immediately, and all moist drugs are liable to develop mould. For these reasons, drying apparatus and stills should be situated as near to the growing plants as possible. This has the further advantage that freightage is much reduced, as many fresh drugs contain a considerable amount (60–90%) of water.

The duration of the drying process varies from a few hours to many weeks, and in the case of open-air drying depends very largely on the weather. In suitable climates open-air drying is used for such drugs as clove, colocynth, cardamom and cinnamon. Even in warm and dry

climates arrangements have to be made for getting the drug under the cover of sheds or tarpaulins at night or during wet weather. For drying in sheds the drugs may be suspended in bundles from the roof, threaded on strings, as in the case of Chinese rhubarb, or, more commonly, placed on trays made of sacking or tinned wire-netting. Papers spread on a wooden framework are also used, particularly for fruits from which it is desired to collect the seeds.

Drying by artificial heat is more rapid than open-air drying and is often necessary in tropical countries (e.g. West Africa, where the humidity is very high, and Honduras for drying cardamom fruits). In Europe continuous belt driers are used for large crops such as digitalis. Alternatively heat may be applied by means of open fires (e.g. nutmegs), stoves or hot-water pipes. In all drying sheds there must be a space of at least 15 cm between superimposed trays, and air must circulate freely.

H. N. ElSohly *et al.* (*Planta Medica*, 1997, **63**, 83) have studied the effect of drying conditions on the taxane content of *Taxus* needles. When the length of drying extended up to 10 and 15 days as in a shadehouse or in the laboratory the recovery of taxanes was adversely affected. Drying in a tobacco barn, greenhouse or oven, and freeze-drying was generally satisfactory for taxol and cephalomannine recoveries but the recoveries for 10-deacetyltaxol and 10-deacetylbaccatin III were only 75–80% of those expected.

Rapid drying helps flowers and leaves to retain their colour and aromatic drugs their aroma, but the temperature used in each case must be governed by the constituents and the physical nature of the drug. As a general rule, leaves, herbs and flowers may be dried between 20 and 40°C, and barks and roots between 30 and 65°C. In the cases of colchicum corm and digitalis leaf it will be noted that the *BPC* and *BP* specify the temperatures at which drying is to be done. For rural tropical areas, solar dryers have some distinct advantages over conventional artificial heat dryers and have been introduced into some countries.

Exactly how far drying is to be carried is a matter for practical experience. If leaves and other delicate structures are overdried, they become very brittle and tend to break in transit. Drugs such as aloes and opium may require further drying after importation.

STORAGE

The large-scale storage of drugs is a considerable undertaking. Except in a few cases, such as cascara bark, long storage, although often unavoidable, is not to be recommended. Drugs such as Indian hemp and sarsaparilla deteriorate even when carefully stored. It has been reported that the content of taxol in *Taxus baccata* leaves and extracts stored at room temperature for one year decreased by 30–40% and 70–80% respectively; storage in a freezer and out of direct sunlight produced no adverse deterioration (B. Das *et al*., *Planta Medica*, 1998, **64**, 96).

Similarly the alkamides of the popular immunostimulant herb *Echinacea purpurea* decrease rapidly on storage; N. B. Perry *et al.* (*Planta Medica*, 2000, **66**, 54) have shown that although drying has little effect on the quantity of alkamides, storage for 64 weeks at 24° produces an 80% loss, and a significant loss even at −18°. Drugs stored in the usual containers—sacks, bales, wooden cases, cardboard boxes and paper bags—reabsorb about 10–12% or more of moisture. They are then termed 'air-dry'. Plastic sacks will effectively seal the contents. The permissible moisture contents of starch, acacia gum and others will be found in the *BP* and *European Pharmacopoeia*. The combined effects of moisture and temperature on humidity and the subsequent water-condensation when the temperature falls, must be considered in drug storage. Drugs such as digitalis and Indian hemp should never be allowed to become air-dry or they lose a considerable part of their activity. They may be kept in sealed containers with a dehydrating agent. For large quantities the bottom of a case may be filled with quicklime and separated from the drug by a perforated grid or sacking. If the lime becomes moist, it should be renewed. Volatile oils should be stored in sealed, well-filled containers in a cool, dark place. Similar remarks apply to fixed oils, particularly cod-liver oil. In the latter case the air in the containers is sometimes replaced by an inert gas. Air-dry drugs are always susceptible to the attack of insects and other pests, so they should be examined frequently during storage and any showing mould or worminess should be rejected.

In order to reduce undesirable microbial contamination and to prevent the development of other living organisms, some plant materials may require sterilization before storage.

EUROPEAN REGULATIONS

To ensure that satisfactory standards for the growing and primary processing of medicinal and aromatic (culinary) herbs are achieved throughout the European Union 'Guidelines for Good Agricultural Practice of Medicinal and Aromatic Plants' was issued as a final European version in August 1998. It covers seeds and propagation, cultivation, harvesting, primary processing, packaging, storage and transport, personnel and facilities, documentation, education and quality assurance. Details can be found in *ICMAP News*, No 6, April 1999 (ICMAP = International Council for Medicinal and Aromatic Plants). Legal requirements covering the manufacture of herbal medicines in Europe have now (2007) been implemented (see Chapter 16: Quality control).

12

Plant growth regulators

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The growth and development of plants is regulated by a number of chemical substances which together exert a complex interaction to meet the needs of the plant. Five groups of plant hormones are well established; they are the auxins, gibberellins, cytokinins, abscisic acid and its derivatives, and ethylene. These substances are of wide distribution and may, in fact, occur in all higher plants. They are specific in their action, are active in very low concentrations, and regulate cell enlargement, cell division, cell differentiation, organogenesis, senescence and dormancy. Their action is probably sequential. Other hormones concerned with flower formation and reproduction, but as yet uncharacterized, have also been envisaged. The essential role of these substances is illustrated by cell and tissue cultures; without the addition of suitable hormones no development or cell division occurs.

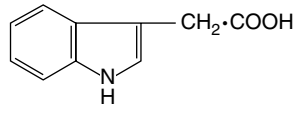
The effects of these very active substances on the production of secondary metabolites, particularly with a view to producing plants containing an enhanced proportion of active constituent, are of interest to pharmacognosists. In such studies the manner in which the results are recorded is all-important, particularly as the treatment may also influence the size of the test plant compared with the controls. For commercial purposes yield per hectare is an obvious criterion, whereas for biosynthetic studies yield per plant or per cent fresh weight may be of more significance. For final drug evaluation per cent dry weight is the most likely requirement.

In spite of the early enthusiasm for research on drug enhancement by the use of hormones applied to medicinal field crops, very little in the way of useful practical application emerged; the results were, however, of interest and selected examples of this older work continue to be retained in this chapter.

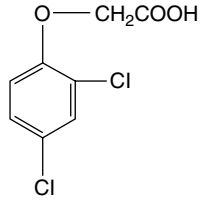
AUXINS

These growth-promoting substances were first studied in 1931 by Dutch workers who isolated two growth-regulating acids (auxin-a and auxin-b, obtained from human urine and cereal products, respectively). They subsequently noted that these had similar properties to indole-3-acetic acid (IAA), the compound now considered to be the major auxin of plants, and found particularly in actively growing tissues. Several similar acids, potential precursors of indoleacetic acid, have also been reported as natural products; they include indoleacetaldehyde, indoleacetonitrile and indolepyruvic acid. These compounds and IAA are all derived, in the plants, from tryptophan.

Typical effects of auxins are cell elongation giving an increase in stem length, inhibition of root growth, adventitious root production and fruit-setting in the absence of pollination. A number of widely used synthetic auxins include indole-3-butyric acid, naphthalene-1-acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D).



Indole-3-acetic acid



2,4-Dichlorophenoxyacetic acid

In the plant, oxidative degradation of IAA to give a number of products is controlled by IAA oxidase. Some substances such as the orthodiphenols (e.g. caffeic and chlorogenic acids and quercetin) inhibit the action of the enzyme and, hence, stimulate growth themselves. Conversely, monophenols such as *p*-coumaric acid promote the action of IAA oxidase and so inhibit growth. IAA may also be conjugated in the plant with aspartic acid, glutamic acid, glycine, sugars and cyclitols; such bound forms may represent a detoxication mechanism or are inactive storage forms of the hormone.

The main practical uses of auxins are: (1) in low concentrations to accelerate the rooting of woody and herbaceous cuttings; and (2) in higher concentrations to act as selective herbicides or weed-killers. Placed for 24 h in a 1:500 000 solution of NAA, cuttings will subsequently develop roots. This includes cuttings from trees such as holly, which were formerly very difficult to propagate in this way and had to be raised from seed or by grafting. Similarly, indole-3-butyric acid was successful with *Cinchona* cuttings, saving some 2 or 3 years compared with growth from seed. Similar results have been obtained with cuttings of *Carica, Coffea, Pinus* and other species. In biogenetic studies, use has been made of auxins to induce root formation on isolated leaves such as those of *Nicotiana* and *Datura* species. Auxins used in suitable concentration (usually stronger than when used for rooting cuttings) selectively destroy some species of plant and leave others more or less unaffected. They have, therefore, a very important role as selective weed-killers in horticulture and agriculture. Thus 2,4-D is particularly toxic to dicotyledonous plants while, in suitable concentration, having little effect on monocotyledons. It can, therefore, be used to destroy such dicotyledonous weeds as dandelion and plantain from grass lawns. (N.B. Certain carbamate and urea derivatives have an opposite effect and can be used to destroy grass without serious injury to dicotyledonous crops.)

There have been several reports on the effects of auxins on the formation of secondary metabolites on medicinal plants.

Seedlings and young plants of *Mentha piperita*, when treated with derivatives of NAA, gave in the mature plants an increased yield (30–50%) of oil which itself contained 4.5–9.0% more menthol than the controls. The study of the effects of auxins on alkaloid formation has concentrated principally on the tropane alkaloids of *Datura* species. Morphological changes in the plants were observed (2,4-D, for example, produced abnormal and bizarre forms of *D. stramonium*; an increase in trichome production, particularly in branched non-glandular forms; smooth fruits as distinct from those with spines; and a proliferation of vascular tissue). Generally, workers found no marked effect on alkaloid production or on the type of alkaloids produced, although a Russian paper records that with thornapple and scopolia tissue cultures a stimulating effect on alkaloid production was obtained with NAA and an inhibiting effect with 2,4-D; similar results were reported for *Rauwolfia serpentina* tissue cultures with these two hormones. An increased alkaloid production has been reported for submerged cultures of certain ergot strains when treated with various auxins (IAA; NAA; 2,4-D; indole propionic acid; indole butyric acid), whereas unpredictable irregular quantitative and qualitative effects on ergoline alkaloid production were observed with the same hormones in *Ipomoea, Rivea* and *Argyreia* (Convolvulaceae) suspension cultures. Experiments carried out in Hungary involving the injection of IAA into poppy capsules 1 and 2 days after flowering produced a relatively elongated capsule form and, in general, a reduced alkaloid content. In studies on anthraquinone production by cell suspension cultures of *Morinda citrifolia*, Zenk and co-workers have shown that cells grown in the presence of NAA have a substantial anthraquinone production but those with 2,4-D as sole auxin do not. IAA appears to have no beneficial effect on the production of sennosides in *Cassia angustifolia*.

GIBBERELLINS

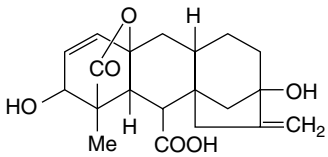
This group of plant growth regulators was discovered by Japanese workers in connection with the 'bakanae' (foolish seedlings) disease of rice. In this, the affected plants become excessively tall and are unable to support themselves; through a combination of the resulting weakness and parasite damage they eventually die. The causative organism of the disease is *Gibberella fugikuroi*, and in 1926 Kurosawa found that extracts of the fungus could initiate the disease symptoms when applied to healthy rice plants. Some 10 years later, Yabuta and Hayashi isolated a crystalline sample of the active material which they called 'gibberellin'. Preoccupation with the auxins by western plant physiologists, the existence of language barriers and the advent of World War II meant that a further 10 years elapsed before the significance of these findings was appreciated outside Japan. In the 1950s groups in Britain, the USA and Japan further investigated these compounds, which were shown to have amazing effects when applied to plants. It soon became apparent that a range of gibberellins was involved, and they are now distinguished as GA_1, GA_2, GA_3, etc. GA_3, commonly referred to as gibberellic acid and produced commercially by fungal cultivation, is probably the best-known of the series; its structure was finally determined in 1959. The first good indications that gibberellins actually existed in higher plants came with West and Phinney's observation in 1956 that the liquid endosperm of the wild cucumber (*Echinocytis macrocarpa*) was particularly rich in substances possessing gibberellin-like activity and Radley's report of a substance from pea-shoots behaving like gibberellic acid on paper chromatograms. Finally, in 1958 MacMillan and Suter isolated crystalline GA_1 from *Phaseolus multiflorus*. By 1980, 58 gibberellins were known of which about half were derived from the *Gibberella* fungus and half from higher plants. GA_{117} was characterized from fern gametophytes in 1998 (G. Wynne *et al*., *Phytochemistry*, 1998, **49**, 1837). Of these many GAs most are either dead-end metabolites or are intermediates in the formation of active compounds; only a limited number have hormonal activity *per se*. It is now considered possible that these substances are present in most, if not all, plants.

Gibberellins are synthesized in leaves and they accumulate in relatively large quantities in the immature seeds and fruits of some plants. The most dramatic effect of gibberellins can be seen by their application to short-node plants—for example, those plants producing rosettes of leaves (*Digitalis, Hyoscyamus*)—when bolting and flowering is induced; also, dwarf varieties of many plants, when treated with the hormone, grow to the same height as taller varieties. Other important actions of the gibberellins are the initiation of the synthesis of various hydrolytic and proteolytic enzymes upon which seed germination and seedling establishment depend.

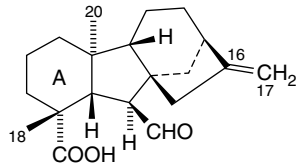
The growth effect of gibberellins arises by cell elongation in the subapical meristem region where young internodes are developing. The effects of gibberellins and auxins appear complementary, the full stimulation of elongation by either hormone necessitating an adequate presence of the other.

As with auxins, gibberellins appear also to occur in plants in deactivated forms; thus, β-D-glucopyranosyl esters of GA_1, GA_4, GA_8, GA_{37} and GA_{38} are known. As such they may serve a depot function. The glucosyl ester of GA_3 has been prepared in several laboratories.

The biogenetic pathways of the gibberellins appear to be similar in both higher plants and *Gibberella*. They arise at the C_{20} geranylgeranyl pyrophosphate level of the isoprenoid mevalonic acid pathway (q.v.) with cyclizations giving the C_{20} tetracyclic diterpernoid entkaurenoic acid, which by a multistep ring contraction furnishes the gibbane ring system as exemplified by the key intermediate GA_{12}-aldehyde. Several



Gibberellic acid (GA_3)



GA_{12} aldehyde.
Key diversification point for synthesis of GAs

pathways diverge from GA_{12}-aldehyde to give the known 90 or so gibberellins. All GAs have either the ent-gibberellane (C_{20} GAs) or the ent-20-norgibberellane (C_{19} GAs) (loss of C-20) carbon skeleton. Both types are modified by the position and number of OH groups, oxidation state of C-18 and C-20, lactone formation, presence and position of double bonds on ring A, epoxide formation, the presence of a carboxyl group and hydration of the C16–C17 double bond.

The gibberellins have been used to treat many plants which contain useful secondary metabolites. A summary of some of the findings, for different groups, is given below.

Volatile oils and terpenoids

Early work involving GA treatment of volatile oil-containing plants was concerned with resultant changes in morphological characters in genera such as *Citrus, Eucalyptus* and *Foeniculum*. GA spraying of the flowers of *Humulus lupulus* advanced the maturity of the hops by 10 days and gave a more evenly developed crop. Although the cones of treated flowers were more subject to wind burn than normal, their yield was increased by about 40%. The α-resin content of the hops, on which their commercial value depends, however, was 1.8% compared with 10.2% for the controls; the volatile oil composition also differed. Several studies have been made of the effects of GA treatment on *Mentha piperita*. The detailed results vary, but the general result is a lowering of the oil content (possibly by reduction of the number of glandular hairs) with little change in the oil composition. In contrast to the above, Kaul and Kapoor have reported favourably on the GA treatment of *Chenopodium ambrosioides* and *Anethum* spp. with respect to volatile oil content. The former afforded a 33% increase in volatile oil with no appreciable change in ascaridole content. With *Anethum graveolens* specific doses of GA increased the oil content by up to 50% and with *A. sowa* (Indian dill) by up to 30%. At the lower doses of GA treatment there was no significant change in the carvone content of the oil, but at higher concentrations there appeared to be a slight increase over the (then) official limit (53%, *BP* 1958). With *Foeniculum vulgare* and *Coriandrum sativum* Gjerstad found that foliar sprays of 100 parts/10^6 GA, applied bi-weekly, gave a difference in cauline length of 200–300% but no differences were detected in yield of fruits and quantity and quality of volatile oil.

Alkaloids

The seeds of the tropane alkaloid-producing species of *Atropa, Hyoscyamus* and *Datura* often exhibit protracted dormancy or erratic germination; GA treatment of the seeds can be used to assist in obtaining uniform germination and total emergence. Considerable work has been published on the effects of the hormone on the morphology and alkaloid content of treated plants. *Hyoscyamus niger* is a perennial and the hormone effects included stem elongation giving a two- to threefold increase in height; a spindly and vine-like growth; slightly chlorotic and narrow leaves; a more rapid onset of flowering; increases in the stem dry weights but decreases in the dry weights of leaves, tops and roots. The overall yield of alkaloids in the treated plants was reduced by about a half and the concentration of alkaloids in various morphological parts ranged from 43 to 84% of that of the controls, with the stems showing the greatest reduction. Subsequent experiments showed that with belladonna, increased alkaloid yields could be obtained by adjusting the dose of GA treatment to favour overall growth in the older plant.

Similar results have been reported with *Datura* spp., plants showing the predictable morphological effects and giving a reduced alkaloid yield. The effects were not transmitted to the second-generation plants. With *Nicotiana tabacum* and *Duboisia* hybrids, treatment again gave a generally reduced alkaloid content accompanied by characteristic morphological effects.

Other alkaloid-containing plants which have been subjected to GA treatment include *Catharanthus roseus* (generally a lowering of alkaloid content and some change in the relative proportion of vinblastine to other alkaloids); *Rauwolfia serpentina* (lowering of alkaloid concentration in the roots, the effect increasing with dose); and *Thea sinensis* (slight difference in caffeine content of leaves). It would appear, therefore, that with the alkaloid-containing plants so far tested, the substantial internodal growth produced by GA is offset by a lower overall accumulation of alkaloid.

Glycosides

In 1959 Sayed and Beal reported the effects of the daily GA treatment of first-year rosette *Digitalis purpurea* plants. Flowering occurred in the first year with treated plants and the leaves became longer and more linear, with an increase in dry weight. An increase in cardioactive glycosides was obtained but no increase in the digitoxose content was observed. Similar experiments by Burton and Sciuchetti in 1961 involving weekly treatments of *D. lanata* produced similar results, with 'bolting' after the twelfth week. The total glycoside per shoot of the treated plants was considerably increased in the first 8 weeks, and at harvest time showed about 30% increase with the lower dose of hormone (10 μg week^{-1}), and a 50% decrease with the higher (50 μg week^{-1}). The authors concluded that the effect of the treatment of glycoside production correlated more closely with the growth response than with the effect on carbohydrate formation. With both leaf and root cultures Lui and Staba (1981) found GA to have a positive effect on the production of digoxin.

Application of GA to *Cassia angustifolia* (Bhatia *et al*., *Planta Med*., 1978, **34**, 437) appeared to reduce the sennoside content of the leaves at all concentrations used, but slightly increased the dry weight of the shoot.

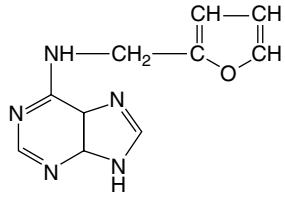
Fixed oils

Gjerstad obtained castor plants (*Ricinus communis*) five times the height of controls as a result of GA treatment. No significant differences were observed regarding the quantity and quality of the fixed oil of the seed.

CELL DIVISION HORMONES: CYTOKININS

Auxins and gibberellins are concerned largely with cell enlargement; and although they influence cell-multiplication processes, there are other substances which have a more specific effect on cell division (cytokinesis). The activity of the latter is not only confined to cell division in a tissue *per se*; they also regulate the pattern and frequency of organ production as well as position and shape. They have an inhibitory

effect on senescence. The presence of such compounds has been suspected for many years, the German botanist Haberlandt in 1913 having noted that phloem tissues contained water-soluble substances capable of promoting cell division in parenchymatous cells of wounded potato tubers. It was not until many years later (1954) that Miller, working at Wisconsin on tissue cultures, discovered that aged or autoclaved DNA from herring sperm stimulated cell division. This active degradation product was called kinetin and in 1955 was identified as 6-furfurylaminopurine (6-furfuryladenine). Kinetin itself has not been isolated from plants, but in 1964, after the indication of cytokinins in liquid endosperm of the coconut and in extracts of maize embryos at the milky stage, an active substance named zeatin was isolated from the latter source. Like most other cytokinins, it is a 6-substituted adenine derivative, 6-(4-hydroxy-3-methylbut-2-eny1)-aminopurine. It has since been shown to be associated in maize with zeatin riboside (1 β-D-ribofuranose) and with a phosphate ester of this compound. The hormone complex has been detected in the cambial region of various woody plants. Isopentenyladenine and dihydrozeatin are examples of cytokinin isolated from other sources; many more have been detected but not identified. The sidechain of cytokinins is of isoprenoid origin.



Kinetin

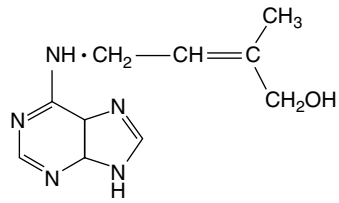
Cytokinins have been much employed in tissue culture work, in which they are used to promote the formation of adventitious buds and shoots from undifferentiated cells. In cell cultures, they have been shown to promote the biosynthesis of berberine (*Thalictrum minus*), condensed tannins (*Onobrychis viccifolia*) and rhodoxanthin (*Ricinus*).

A limited study only has been made of the effects of cytokinins on secondary metabolism in intact plants. Concerning plants producing tropane alkaloids, Ambrose and Sciuchetti have compared the action of kinetin and GA on *Datura meteloides*. Plants received weekly doses of 25 μg kinetin; in relation to the controls the following differences were noted:

Kinetin treatment	Gibberellic acid treatment
Shorter and bushier plants	Taller and spindly plants
Decreased growth	Increased growth
No change in alkaloid content in plant organs	Decreased alkaloid production
Delayed response	Rapid response

Luanratana and Griffin (*J. Nat. Prod.*, 1980, **43**, 546, 552; 1982, **45**, 270) observed the beneficial effects of a commercial seaweed extract containing cytokinin activity on *Duboisia* hybrids grown both hydroponically and in a commercial plantation. In the latter there was an 18% increase in leaf yield and a 16% increase in hyoscine content compared with the controls. A further pointer to the usefulness of the treatment was that in field plants it led to a delay in the usual seasonal fall in alkaloid content (February–April in Australia), thus permitting an economically useful, extended collection period. Shah *et al.* (1990) reported favourable increases in growth and alkaloid yield with *Hyoscyamus muticus* treated with kinetin (50 p.p.m.).

Verzár-Petri injected benzyladenine and kinetin separately into developing poppy capsules; the effects were similar to those observed with auxins. Leaves of the coffee plant after kinetin treatment developed a transient increase of up to 10% in their caffeine content. The effect was transitory and passed after 6–12 days. With *Cassia angustifolia* plants low concentrations of hormone were found to increase slightly the sennoside content and to favour an increase in the dry weight of shoots.

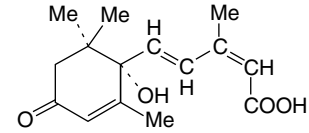


Zeatin

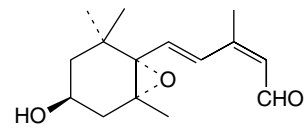
GROWTH INHIBITORS

Natural growth inhibitors are present in plants and affect bud opening, seed germination and development of dormancy. One such substance, abscisic acid [3-methyl-5-(1-hydroxy-4-oxo-2,6,6-trimethyl-2-cyclohexane-1-y1)-*cis*,*trans*-2,4-pentadienoic acid] was isolated and characterized in 1965; it has also been isolated from the fungus *Cenospora rosicola*.

The structural similarity of abscisic acid (ABA) to the carotenoids prompted research on the relationship of these two groups of compounds and it has now been demonstrated that some xanthophylls, particularly violaxanthin, produce a germination inhibitor on exposure to light. Evidence has now accumulated in support of an indirect 'apocarotenoid' pathway for ABA biosynthesis, the most likely precleavage precursors being 9′-*cis*-neoxanthin and 9-*cis*-violaxanthin which fracture across the 11,12 (11′,12′) double bond to produce xanthoxin which in plant tissues is readily transformed into ABA (see A. D. Parry and R. Horgan, *Phytochem.*, 1991, **30**, 815). However, it is also possible that ABA arises from farnesol at the C_{15} sesquiterpenoid level of the MVA pathway (q.v.). The theoretical postulation involves a number of steps in which, as with the apo-carotenoid pathway, *cis*-$\Delta^{2'}$-xanthoxin could also be involved. In accord with isoprenoid biosynthesis, mevalonic acid (MVA) has been demonstrated to be stereochemically incorporated into ABA in higher plants, and likewise labelled acetate in *Cenospora rosicola*. Other substances related to abscisic acid have



Abscisic acid (ABA)



cis-Δ^2-Xanthoxin

been isolated from plants and include vomifoliol (several sources), which lacks the 2,4-pentadiene side-chain; it has the same activity as abscisic acid in stomatal closure tests. Little or no work appears to have been reported on the effects of abscisic acid on the production of pharmacognostically interesting substances.

A number of synthetic growth inhibitors have been studied; the first to be described was maleic hydrazide in 1949. *N*-Dimethylaminosuccinamic acid can be considered as a hydrazine derivative and acts as a shoot-elongation inhibitor by suppressing the oxidation of tryptamine to IAA. Sciuchetti and colleagues showed that this compound sprayed on to *Datura stramonium* and *D. innoxia* plants reduced the eventual height of the plants and lowered, overall, their total alkaloid content; however, significant increases were noted in the concentrations of stem alkaloid (56% increase in the second week and 90% in the fourth week compared with the controls). The inhibitor, tributyl 2,4-dichlorobenzylphosphonium chloride (phosphon) produced similar results with *D. ferox*. Trigonelline, an alkaloid of fenugreek seeds, promotes cell arrest in G_2 (a specific period preceding mitotic division of the nucleus) in various legumes.

ETHYLENE

It has been known for many years that ethylene induces growth responses in plants, and in 1932 it was demonstrated that the ethylene evolved by stored apples inhibited the growth of potato shoots enclosed with them; it has a role in fruit ripening. Current thought maintains that this simple compound should be included among the natural plant hormones. Ethylene is synthesized in the plant from *S*-adenosylmethionine via the intermediate 1-aminocyclopropane-1-carboxylic acid (ACC). The gene for ACC synthase has been cloned from tomato squash (H. Klee and M. Estelle in *Annu. Rev. Plant Physiol.*, 1991, **42**, 529). One biochemical action of ethylene is the stimulation of the *de novo* synthesis and secretion of cell-wall dissolving enzymes such as cellulase during leaf abscission and fruit ripening. A compound that gives rise to a typical ethylene response in plants is (2-chloroethyl)phosphonic acid (ethephon) applied in aqueous solution in concentrations of the order of 100–5000 p.p.m. In the cell sap, at pH values above 4.0 it is broken down to ethylene and phosphate. It is marketed as Ethrel.

At low concentration ethylene has been shown to increase the sennoside concentration in *Cassia angustifolia*, and applied to tobacco leaves it stimulates production of the stress compounds phytuberin and phytuberol (these are compounds produced in response to tobacco mosaic virus); with *Digitalis lanata* tissue cultures, cardenolide accumulation is decreased. Ethephon is now increasingly used as standard practice for enhancing the flow of rubber latex. Sprayed on to the scraped bark (tapping groove) of the rubber tree it increases latex yields by from 36 to 130%.

Other growth regulators

In addition to the well-known plant growth substances discussed above, a very large number of other compounds have been isolated from natural sources which in some way influence plant growth. Some are widely distributed and others are of restricted occurrence. Generally they have a less specific action than the regulators already mentioned above. They have no common chemical structure and only a few recurrent functional groups (e.g. phenolic hydroxy groups and α-methylene-γ-butyrolactone moieties). This implies that these substances may be acting at many different sites along the growth regulatory process. Substances involved include aliphatic and aromatic carboxylic acids, phenolic and neutral compounds, salicylate, polyamines, *S*- and *N*-heterocyclic compounds, including alkaloids and terpenes. *Acorus calamus* produces a number of sesquiterpenes having the skeletal structures of cadinane, acorane and eudesmane which inhibit the germination of lettuce seeds (K. Nawamaki and M. Kuroyanagi, *Phytochemistry*, 1996, **43**, 1175). A new class of plant growth regulators known as brassinosteroids is found in the seeds, pollens, galls, leaves, flower-buds and shoots of a considerable range of plants. Some 40 of these compounds are known; they stimulate cell enlargement and cell division and influence gene expression and nucleic acid metabolism at the molecular level, see V. A. Khripach *et al*. (1999), *Brassinosteroids, a New Class of Plant Hormone*, San Diego: Academic Press.

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Plant cell and tissue culture; biochemical conversions; clonal propagation

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One of the rapidly expanding areas of pharmacognosy has involved the application of the artificial culture of plant cells, tissues and organs to the study of medicinal plants. Principal topics include the development of commercial production of expensive biomedicaments, the discovery of new metabolites, the selection of superior strains of medicinal plants, the elucidation of biosynthetic pathways of secondary metabolites with isolation of corresponding enzymes, and the improvement of medicinal plant species by genetic engineering.

INDUSTRIAL SIGNIFICANCE

A number of factors militate against dependence by the pharmaceutical industry on the use of botanical sources of drugs, and these have been, to some extent, responsible for the reluctance of industry to invest in the exploitation of the plant kingdom. These factors include the following.

1. *Availability of raw material*. Some plants, although highly desirable as sources of biochemicals, just cannot be produced in an economically sufficient quantity to satisfy demand. An example is *Strophanthus sarmentosus* seeds, which, early in the search for corticosteroid precursors (1950s), were known to contain a very suitable compound, sarmentogenin, conveniently substituted in the steroid C ring (see Chapter 23). But the plants, tropical lianes existing as different chemical races (Chapter 14), are not particularly abundant and are difficult to cultivate. More recent examples include the very limited supply of the Pacific yew (*Taxus brevifolia*) the principal source of taxol, a diterpene with considerable potential as a starting material for the semisynthesis of promising anticancer drugs and also *Coleus forskohlii*, an Indian species now listed as vulnerable to extinction in the wild as a result of indiscriminate collection for the isolation of forskolin, a diterpenoid used in the treatment of glaucoma and heart disease.
2. *Fluctuation of supplies and quality*. The production of crude drugs is subject to the vagaries of the climate, to crop disease, to varying methods of collection and drying which influence quality, and to the inherent variation of active constituents arising from plants of the same species having different genetic characteristics.
3. *Political considerations.* New compounds of promising medicinal value are increasingly reported in previously uninvestigated tropical or subtropical species situated in countries of uncertain political persuasion. Industry is not prepared to undertake the prolonged and expensive development of such material.
4. *Patent rights*. Generally, it is not possible to patent a naturally occurring plant metabolite as such, only a novel method for its extraction and isolation. Hence, there is little incentive for a pharmaceutical company to spend many years and vast sums of money on launching a new natural product over which it has no patent rights.

Following from the above, it is not surprising that industry worldwide takes a close interest in the commercial possibilities of cultivating particular species of plant cells, under conditions analogous to the production of antibiotics, that will yield biomedicinals. By this means, production could at all times be geared to demand, and a product of standard quality assured. Furthermore, a highly sophisticated and specific method of production can be patented.

Shikonin, a dye and antibacterial, is commercially produced by the cultivation of *Lithospermum* plant cells and the production of the ginsenosides and antitumour alkaloids of *Catharanthus roseus* are currently being developed; Japanese patents exist concerning the manufacture of many secondary metabolites including the purple pigment from *Melissa officinalis*, the production of catharanthine and ajmalicine

by cell cultures derived from *C. roseus* anthers, the manufacture of an analogue of taxol (q.v.) by callus cultures of *Taxus* spp., and tropane alkaloids from *Duboisia* tissue cultures. A vast amount of work has been reported during the last decade and the majority of common medicinal plants, and many less common ones, have been subjected to cell culture investigation. Nevertheless, in the majority of cases, yields of metabolites have been commercially disappointing. Staba, a pioneer in the investigation of medicinal plant cell culture, stated (*J. Nat. Prod*., 1985, **48**, 203) 'there are arguably as many gravestones as milestones along the way in developing plant tissue culture systems for the production of secondary metabolites'. Ultimately, of course, as Fowler pointed out over 25 years ago (*Chem. Ind*., 1981, 229), 'no matter how elegant the science, the fundamental criterion has to be price comparability coupled with profitability', a fact clearly obviated by subsequent events.

CULTIVATION OF PLANT CELLS

Although the feasibility of artificially cultivating plant cells had long been recognized, and White had propagated isolated tomato roots for periods of over 30 years, it was only some few decades ago that modern developments in the cultivation of cells of higher plants as a callus, or as a suspension liquid culture, really began. In this connection the publication of P. R. White's *Cultivation of Plant and Animal Cells* in 1954 and H. E. Street's developmental work at Leicester University deserve mention.

Cultures of single cells growing under controlled conditions in a liquid medium, or callus cultures consisting of undifferentiated masses of cells developing on a semi-solid medium, can be initiated from parenchymatous tissues of shoots, roots and other plant structures (see Fig. 13.1). The maintenance of such cultures depends on an adequate supply of nutrients, including growth factors, and a controlled sterile environment. The cells, although undifferentiated, contain all the genetic information present in the normal plant. By suitable manipulation of the hormone content of the medium, it is possible to initiate the development of roots, shoots, and complete plants from the callus cell culture and to encourage the division of cells in a suspension culture.

Several forms of suspension culture are commonly utilized, as follows.

1. *Batch suspension cultures*. In this technique the cells multiply in a liquid medium which is being continuously agitated to break up any cell aggregates. Except for the circulation of air, the system is 'closed' with respect to additions or subtractions from the culture. Typically, the original inoculation of cells into the medium is followed by a lag period and then, after increasing in mass, the cells undergo a period of exponential growth and division. Finally, a stationary growth phase is reached, at which point some component of the medium, essential for growth, has probably been exhausted. Growth will recommence when cells are transferred to a fresh medium or when more medium is added to the original culture.
2. *Semicontinuous cultures*. In this instance the system, an 'open' one, is designed for the periodic removal of culture and the addition of fresh medium, by which means the growth of the culture is continuously maintained.
3. *Continuous cultures*. Two forms of this 'open' system are the chemostat and turbostat systems, in which the volume of culture remains constant and fresh medium and culture are, respectively, continuously added and withdrawn. The essential feature of these two systems is that cell proliferation takes place under constant conditions. In the chemostat arrangement, a steady state is achieved by adding medium in which a single nutrient has been adjusted so as to be *growth-limiting*; this contrasts with the batch culture method, in which the transient conditions in which the cells find themselves lead to continuous changes in their growth rate and metabolism.

The problems associated with plant-cell culture are not completely identical with those encountered in the fermentation of microorganisms and fungi. With the former there is much slower cell proliferation and it takes about 3–6 weeks to progress from the shake-flask level (300 ml) to production capacity (20 000 litres). Also in plant cell cultures, large aggregates of cells may form, which then exist under different environmental conditions from the suspended cells. Dispersal of aggregates by the use of the usual fermenter paddles was originally considered too vigorous for fragile plant cells, resulting in their rupture. To overcome this, various designs of low-shear fermenters including the air-lift or drum types were designed. However, such refinements are not always necessary and the shikonin production mentioned above is, in fact, carried out in conventional stirred-tank vessels. As R. Verpoorte *et al*. have pointed out (*J. Nat. Prod*., 1993, **56**, 186), for the industrial application of plant cell cultures, the recognition that shear-tolerant plant cell cultures exist is important because the fermentation industry at present uses stirred tanks almost exclusively. Investment in new ingenious bioreactors, as reported for experimental cell cultures in recent years, would place a major constraint on the commercialization of plant cell biotechnology.

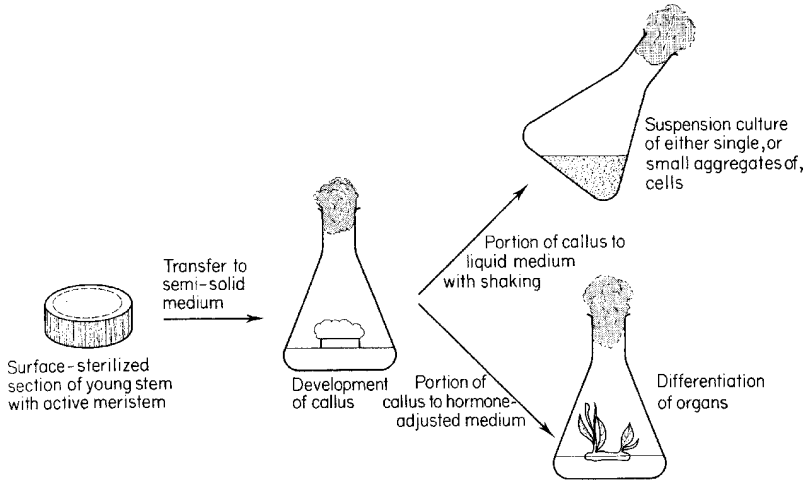


Fig. 13.1
Origin of plant cell cultures.

To maximize on the cell mass produced, the cell suspension culture eventually becomes very dense and this presents problems of even aeration.

Most pilot studies have utilized fermenters of 5–15 litres capacity and the reported scaling up to 20 000 and more recently 75 000 litres in West Germany and Japan presents chemical engineering problems of considerable complexity.

PRODUCTION OF SECONDARY METABOLITES

The genetic information required for the manufacture of secondary metabolites is also present in the undifferentiated cells of the species concerned, and when activated should lead to the production of these materials. Much interest has been aroused by this aspect of cell culture with the aim of growing particular plant cells on a commercial scale for the production of valuable metabolites.

A pioneer in the cell culture of medicinal plants was E. J. Staba of Minnesota University, and his group was the first to demonstrate that many medicinal plants did produce in cell culture their characteristic secondary metabolites, albeit often in low yield. Notable advances were made by Zenk and colleagues who in 1975 demonstrated a 10% (dry weight) production of anthraquinone derivatives in a *Morinda citrifolia* culture—at that date the highest yield of secondary metabolite achieved by cell culture. By 1991 (M. H. Zenk, *Phytochemistry*, 1991, **30**, 3861) almost 1000 species of callus were deposited in the collection at Braunschweig. Commercially orientated research has concentrated on those species that produce high-value speciality phytochemicals. Obvious examples are *Catharanthus roseus* (dimeric antitumour alkaloids), ginseng (ginsenosides) and *Taxus* species (taxol).

Apart from the general problem of low yield of product other factors which need to be addressed with cell cultures as a source of phytopharmaceuticals are: instability of cell lines, compartmentalization and isolation of the products, and the nature of the metabolites produced. Some points concerning these problems are given below.

Low production of desired metabolites

Knowledge of the enzymology of secondary metabolite formation, although rapidly expanding, is still incomplete. Secondary metabolic processes compete with primary metabolism for precursors and potential bottlenecks for the former may involve those enzymes linking the primary and secondary pathways, for example, tryptophan decarboxylase converting tryptophan to tryptamine in the formation of indole alkaloids and cyclase enzymes involved in the synthesis of cyclohexanoid monoterpenes from geranylpyrophosphate. With cell cultures, as distinct from whole plants, particular genes may be repressed and need to be activated by suitable elicitors, a technique which is currently an important area of research and is discussed below.

The **compositions of the media** in which culture cells are grown have been extensively investigated with a view to increasing both the biomass and secondary metabolites. Often, as reported with *Dioscorea deltoidea* for instance, rapidly dividing cells produce little or no metabolites of interest and a change from a growth medium (high biomass) to a production medium is required to effect the necessary biosynthesis. In this connection Zenk's 'alkaloid production medium' for ajmalicine in *C. roseus* may be noted, together with the effects of long-term starvation of phosphate on levels of purine nucleotides and related compounds (F. Shimano and H. Ashihara, *Phytochemistry*, 2006, **67**, 132).

Variations in the relative hormonal contents of the growth medium can also affect metabolism. It has been reported that reduced concentrations of 2,4-D increased alkaloid formation in *C. roseus* cultures and that abscisic acid and antigibberellin compounds have similar effects. With *Thalictrum minus*, ethylene has been shown to activate the production of berberine in cell cultures from the key intermediate (*S*)-reticuline and the ethylene-producing reagent 2-chloroethylphosphoric acid stimulates anthraquinone production in callus cultures of *Rheum palmatum*. Conversely, cardenolide accumulation in *Digitalis lanata* tissue cultures is decreased by ethylene. Cytokinins have been found to enhance secondary metabolite accumulation in a number of tissue culture studies—indole alkaloids (*C. roseus*), condensed tannin (*Onobrychis* sp.), coumarins (*Nicotiana* sp.), rhodozanthin (*Ricinus* sp.), berberine (*Thalictrum minus*). Rhodes *et al*. found a five-fold increase in alkaloid content of a culture of *Cinchona ledgeriana* occurs when cells are transferred from a 2,4-D, benzyladenine medium to one containing IAA and zeatin riboside. For information on plant hormones, see Chapter 12.

Although alkaloids from a wide range of medicinal plants have been produced satisfactorily by cell culture in the laboratory, a singular lack of success has been experienced in obtaining quinine and quinidine from *Cinchona* cultures and morphine and codeine from those of *Papaver somniferum* although, in both cases, other alkaloids are formed. To some extent the problems with the former are being overcome by the use of transformed roots (see below) but the growth rate is very slow. With morphine biosynthesis it appears that lack of developed laticiferous tissue in the unorganized cell culture may be responsible because cytodifferentiation leading to latificer-type cells leads to morphinan alkaloid production. One problem with *Catharanthus* cell cultures has been their inability to dimerize the requisite indole monomers to form the medicinally important anticancer alkaloids vinblastine and vincristine. In a similar way the accumulation of monoterpenes in cell cultures of some volatile oil-producing plants is severely limited, probably because of the absence of such storage structures as glands, ducts and trichomes. Thus, in *Rosa damascena* callus and suspension cultures, negligible amounts of monoterpenes are accumulated, although enzymes with high activity for the conversion of mevalonate and IPP into geraniol and nerol (see Chapter 18) are extractable from the apparently inactive callus. In this case, non-compartmentalization of the metabolites probably leads to their further metabolism. This is supported by the finding that, when added to cultures of *Lavandula angustifolia*, the monoterpenoid aldehydes geranial, neral and citronellal are reduced to their corresponding alcohols, geraniol, nerol and cirtronellol which, once formed, disappear from the cultures over about 15 h.

In studies on the phenolic antioxidant compounds produced by *in vitro* cultures of rosemary, A. Kuhlmann and C. Rohl (*Pharm. Biol.*, 2006, **44**, 401) find the content of carnosic acid, carnosol and rosmaric acid to be dependent on the differentiation grade of the cell culture type. Higher concentrations of rosmaric acid were measured in suspension cultures than in shoot and callus cultures, whereas the former on average produced three-fold less carnosic acid than the two latter cultures. Carnesol could not be detected in suspension cultures.

With *Ginkgo biloba* although a satisfactory biomass of undifferentiated cells could be produced on a manufacturing scale, the poor level of ginkgolides produced renders it of scant importance.

It has been observed that the **origin (stem, root, etc.) of the callus** can play an important part in determining the biochemistry of the subsequent culture.

Improved metabolite production may sometimes be achieved by the **addition of precursors** to the culture medium. Thus, addition of coniferin (a phenylpropane) to cell suspension cultures of *Podophyllum*

hexandrum improved podophyllotoxin production 12.8-fold and an increase in quinoline alkaloids was obtained with *Cinchona ledgeriana* cultures fed with L-tryptophan. With transformed root cultures of *Catharanthus roseus*, the addition of the precursor loganin to the culture medium has been shown to increase the production of both ajmalicine and serpentine at the early stationary phase of growth, although it produced no increases during the early and late exponential growth phases. Catharanthine production was unaffected but was increased, together with the other alkaloids, by multiple feedings of loganin (see E. N. Gaviraj and C. Veeresham, *Pharm. Biol.*, 2006, **44**, 371 and references cited therein).

Light intensity and selective wavelengths of light have been shown to have a stimulating effect on the production of some secondary metabolites in various tissue cultures. Thus, in one report (1990) blue light enhanced, whereas red light decreased, diosgenin production in *Dioscorea deltoidea* callus cultures. A recent example of the stimulant effect of UV-B radiation on secondary metabolism in callus cultures is the research of F. Antognoni *et al.* (*Fitoterapia*, 2007, **78**, 345) on *Passiflora quadrangularis*. Daily doses of UV-B radiation (12.6, 25.3, 37.9 KJ m^{-2}) produced increases in the flavonoid production of orientin, isoorientin, vitexin and isovitexin. Isoorientin accumulation in the callus after 7 days reached levels comparable to those found in the fresh leaves of greenhouse-raised plants. However, such beneficial treatments are difficult to accommodate with conventional stirred-tank fermentors.

The selection of **high-yielding cell lines** has been a major factor in countering low productivity. Such selection, perhaps involving a few plants from several thousand, has been greatly facilitated by the use of modern immunoassays (q.v.). In the case of *Catharanthus roseus* cultures, for example, recent research has concentrated on the production of the dimeric alkaloids vinblastine and vincristine (q.v.), the important anticancer drugs. The alkaloids are produced at the end of a complex biogenetic pathway in which the monomers are first produced. The latter, as corynanthe-, strychnos- and aspidosperma-type alkaloids can all be produced (0.1–1.5%) in culture using Zenk's alkaloid production medium. Different cell cultures derived from any one species of plant may vary enormously in their synthetic capacities, so that, in the above case, distinct high ajmalicine-producing and high serpentine-producing strains are possible.

Examples of other plants for which somaclonal variation has been exploited include *Nicotiana rustica* (nicotine) (of no commercial interest), *Coptis japonica* (berberine), *Anchusa officinalis* (rosmarinic acid), *Lithospermum erythrorhizon* (shikonin) and *Hyoscyamus muticus* (hyoscine). For *Thalictrum minus* (berberine) a strain giving a 350-fold increase in alkaloid production has been reported.

Instability of cell lines. It is well known that changes in the genetic characteristics of cells occur within a culture so that callus selected for specific biochemical properties may need reselection after a period of time. In a few cases, for example anthraquinone formation, selection may be achieved on a colour basis but, more usually, assays such as radio-immunoassay are necessary. Gross changes in chromosome number may occur in cultured cells; thus, Tabata and colleagues noted in 1974 that with a particular suspension culture of *Datura innoxia* cells there was a 32% level of diploid cells, with the remainder mostly at the tetraploid level, ranging in constitution from $4n-5$ to $4n+3$ (see Chapter 14 for extra chromosomal types). Another strain contained no diploid cells but cells with 46 or 44 chromosomes occurring in the proportions of 79% and 21%, respectively. Nevertheless, for alkaloid production, Kibler and Neumann in 1979 found that haploid ($1n$) and diploid ($2n$) cell suspension cultures of *D. innoxia* showed no difference in tropane alkaloid production (c.f. leaves of $1n$ and $2n$ plants; Table 14.1), but for protoplast-derived cell-culture clones of *Hyoscyamus muticus*, Oksman-Caldentey *et al*. have found that cultures from $1n$ plants are richer in hyoscine than those from $2n$ plants.

Isolation of product. For continuous cultivation and production of active metabolites it is preferable, for isolation purposes, that the metabolites be excreted into the medium rather than be retained within the cells. The biomass can then be separated from the nutrient liquid from which the active constituents are extracted. Two-phase culture systems have been described. With these an immiscible non-toxic liquid phase, e.g. a silicone product, is added to the fermentation tank to extract the metabolites and in this way the development of the culture is not disturbed. The removal of entrapped metabolites from immobilized cells (q.v.) without killing the cells is another innovation.

Nature of metabolites produced. Sometimes compounds not detected in the original plant appear in the cultures; thus, a new coumarin, rutacultin, has been isolated from suspension cell cultures of *Ruta graveolens*, two new chalcones have been characterized from static (callus) cultures of *Glycyrrhiza echinata*, sesquiterpene lactones from *Andrographis paniculata* cultures, new minor alkaloids and anthraquinones from *Cinchona ledgeriana* and *C. pubescens*, and tropane alkaloids, not previously obtained from the species, from belladonna root-cell suspension cultures. Recently the novel compound (2-glyceryl)-*O*-coniferaldehyde has been obtained from cultures of *Artemisia annua* and *Tanacetum parthenium* (L. K. Sy and G. D. Brown, *Phytochemistry*, 1999, **50**, 781) and the quinone-methide triterpenes, tingenone and 22-hydroxytingenone, from callus cultures of *Catha edulis* (E. Abdel Sattar *et al*., *Saudi Pharm. J*., 1998, **6**, 242). Other plants yield cultures which produce a different spectrum of secondary metabolites from those found in the intact plant. These aspects of cell culture, although generally unhelpful for the promotion of this technique for industrial purposes, have important implications for other areas of phytochemistry. However, in the case of cell cultures of *Papaver somniferum* and *P. bracteatum* which do not produce morphinan alkaloids, sanguinarine, a benzophenanthridine alkaloid of commercial importance, is obtained in yields sufficiently high to allow industrial exploitation.

INDUCED SECONDARY METABOLISM IN CELL CULTURES

Although the undifferentiated cells of a plant suspension culture are generally totipotent, i.e. they possess the complete genetic make-up of the whole plant, many genes, including those involved in secondary metabolism, are repressed with the consequence that the yields of desired compounds in such cultures are disappointingly low. However, it is becoming increasingly apparent that a large number of secondary metabolites belong to a class of substances termed phytoalexins. These are stress-related compounds produced in the normal plant as a result of damaging stimuli from physical, chemical or microbiological factors. When cell cultures are subjected to such elicitors, some genes are derepressed, resulting, among other things, in the formation of the secondary metabolites which are found in the entire plant. The technique is being increasingly employed in cell-culture studies and examples giving a range of both abiotic acid and biotic inducers are given in Table 13.1.

Table 13.1 Induced production of metabolites in cell cultures by various elicitors.

Elicitor	Plant-cell suspension culture	Effect
Arachidonic acid	*Taxus* spp.	Production of taxol
Chitosan	*Polygonum tinctorium*	Production of indirubin
Colchicine	*Valeriana wallichii*	Sixty-fold increase in valepotriates with six new compounds (not due to higher ploidy level)
Copper sulphate	*Lithospermum erythrorhizon*	Greatly increased shikonin production
	Various Solanaceae	Induced formation of sesquiterpene phytoalexins of lubimin type
Calcium	*Alkanna tinctora*	Stimulation of sanguinarine and chelerythrine biosynthesis
Acetylsalicylic acid	*Catharanthus roseus*	Increased production of tumour cell suspensions (505%), total phenolics (1587%), furanocoumarins (612%), anthocyanins (1476%)
Methyl jasmonate	*Sanguinaria canadensis*	Dihydrobenzophenanthridine oxidase activated in last step of sanguinarine biogenesis
	Cinchona robusta	Production of novel anthraquinones (robustaquinones) with a rare oxygenation pattern in ring A
	Nicotiana tabacum	Production of anatalline
Thiosemicarbazide	*Panax ginseng*	Promotes biosynthesis of saponins and inhibits phytosterol production
Sterilized fungal mycelia (*Pythium*, *Phytophthora*, *Verticillium*), etc. or extracts	*Pimpinella anisum*	Stimulation of coumarin synthesis
	Petroselinium crispum	
	Ammi majus	
	Catharanthus roseus	Production of catharanthine and other major indole alkaloids stimulated
	Cephalotaxus harringtonia	Dramatic increase in alkaloid content
	Cinchona ledgeriana	Increase in anthraquinone production
	Gossypium arboreum	One hundredfold increase in gossypol after 120 h incubation
Yeast, yeast extracts and carbohydrate preparations	*Eschscholtzia californica*	Large and rapid increase of benzophenanthridine alkaloid production
	Thalictrum rugosum	Up to fourfold enhancement of berberine
	Ruta graveolens	Increased production of acridone expoxides but not rutacridone
	Orthosiphon aristatus	Stimulation of rosmarinic acid production

BIOCHEMICAL CONVERSIONS BY PLANT CELL CULTURES

In much the same way that modification of a particular substrate can be effected by microbial fermentation so, too, can plant suspension cultures be employed for the same purpose.

Of possible commercial significance is the ability of some cell cultures of *Digitalis lanata* to effect glucosylations, hydroxylations, and acetylations. Reinhard and colleagues demonstrated that their cell culture, strain 291, cultivated in air-lift bioreactors, was particularly efficient in the conversion of β-methyldigitoxin into β-methyldigoxin (a 12β-hydroxylation). Commercial exploitation of this process would enable utilization of the large stocks of digitoxin which accumulate as a byproduct in the manufacture of digoxin from *D. lanata*. More recently, Stricker (*Planta Med.*, 1986, 418) reported on a highly efficient 12β-hydroxylation of digitoxin itself using *D. lanata* cell suspensions with a two-stage process involving first, the proliferation of cells in a growth medium and then a transfer to a suitable production medium. With cell cultures of both *D. lanata* and *Thevetia neriifolia* a number of new cardenolides have been biosynthesized from added precursors. Cell suspension cultures of *Strophanthus gratus* will effect various biochemical conversions of digitoxigenin, as will cultured ginseng cells.

Monoterpene bioconversions have been demonstrated with *Mentha* cell lines capable of transforming pulegone to isomethone, and (−)-menthone to (+)-neomenthol. Cultured cells of *Eucalyptus perriniana* have been shown to biotransform thymol, carvacrol and eugenol into glycosides (glucosides and gentiobiosides), which accumulated within the cells (K. Shimoda *et al.*, *Phytochemistry*, 2006, **67**, 2256).

The rue plant (*Ruta graveolens*) and its normal tissue cultures contain a number of constituents, including furanocoumarins derived from 7-hydroxycoumarin (Fig. 13.2, series A). In 1974, Steck and Constabel showed that two chemical mimics of the 7-hydroxycoumarin precursor, the 4-methyl and 8-methyl derivatives, when fed to the *Ruta* cell culture, gave rise to a number of the corresponding unnatural analogues (Fig. 13.2, series B and C).

Possibilities for the production of anticancer drugs are illustrated by the biotransformation of synthetic dibenzylbutanolides to lignans suitable for conversion to etopside (Chapter 27) by a semi-continuous process involving cultures of *Podophyllum peltatum* (J. P. Kutney

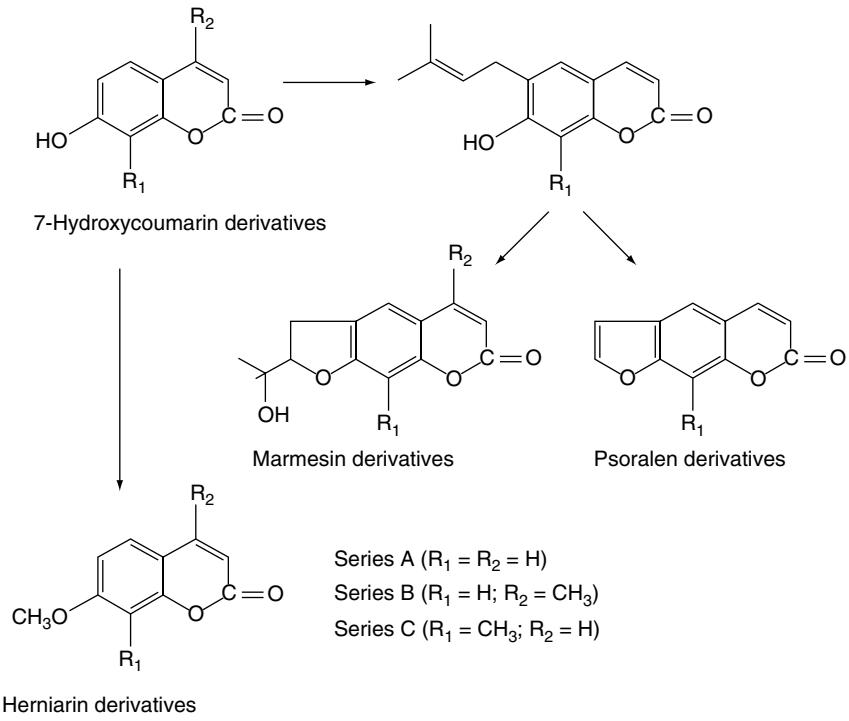


Fig. 13.2
Biochemical conversions involving *Ruta graveolens* cell cultures.

et al. Heterocycles, 1993, **36**, 13). Interesting and potentially useful hydroxylation and oxidation reactions have also been demonstrated for the biotransformation of podophyllum lignans in cell suspension cultures of *Forsythia intermedia* (A. J. Broomhead and P. M. Dewick, *Phytochemistry*, 1991, **30**, 1511).

The principal alkaloid produced by the cultivation of *Rauwolfia serpentina* cells is the glucoalkaloid raucaffricine. However, feeding with high levels of ajmaline leads to the production of a new group of alkaloids, the raumaclines.

Some biotransformations are stereo-specific and have potential for the isolation of optically active compounds from the racemate; thus, *Nicotiana tabacum* cell cultures can selectively hydrolyse the *R*-configurational forms of monoterpenes such as bornyl acetate and isobornyl acetate.

Many other biochemical transformations by cell cultures have been demonstrated, and include epoxidations, ester formation and saponification, glycosylation, hydroxylation, isomerization, methylation, and demethylation and oxidation.

For this technique to be commercially viable, the product must be sufficiently important, the substrate must be available in reliable amounts, and the reaction should not be one that is more easily performed by microorganisms or by chemical means.

IMMOBILIZED PLANT CELLS

Immobilized plant cells can be used in the same way as immobilized enzymes to effect chemically difficult reactions. Reinhard and coworkers have reported on the immobilization of *Digitalis lanata* cells by suspending them in a sodium alginate solution, precipitating the alginate plus entrapped cells with calcium chloride solution, pelleting, and allowing the product to harden. The granules catalysed the conversion of digitoxin to purpurea glycoside A (formula, see Table 23.7) and hydroxylated β-methyldigitoxin to give β-methyldigoxin. Although the hydroxylating activity of the entrapped cells was about half that of suspended cells, the pellets had the advantage that the biocatalyst was re-usable for periods extending to over 60 days. Other workers report the biotransformation of codeinone to codeine by immobilized cells of *Papaver somniferum* and the release of papaverine, codeine and morphine to the medium (3240–4050 mg l^{-1}); decarboxylation of L-tyrosine and L-DOPA has also been recorded. Japanese workers have studied seaweed immobilized cell cultures for the production of flavour constituents. Glyoxal cross-linked polyacrylamide-hydrazide has been used as the immobilizing agent for *Mentha* cells which effect the monoterpene reductions mentioned above and the technique has been further developed by arresting unwanted cell division of the entrapped cells by gamma irradiation. Other immobilization supports which have been used include polyurethane foam, acrylamide and xanthan-acrylamide.

A technique which has been found useful for the study of alkaloid formation in *Coffea arabica* is to use a membrane of polypropylene sheeting of specified pore size, porosity and thickness on which to immobilize the cells in a 3-mm thick layer; the nutrient medium circulates below the membrane. *Catharanthus roseus* cells have also been considerably studied and progress has been made in developing methods for the release of alkaloids sequestrated in the cell vacuoles without killing the culture.

It has been suggested that the immobilization of mycelia-forming microorganisms (e.g. *Claviceps paspali*) by alginic acid might be effective in the biotechnological production of complex metabolites such as the ergot alkaloids.

ORGAN CULTURE

In the same way that it is possible to culture undifferentiated plant cells, so aseptic suspension cultures of leaves and roots can be maintained. The organs can be obtained in the culture either by differenti-

ation from callus tissue cultures by suitable hormonal manipulation, or by the use of sterilized roots or growing points from whole plants or seedlings. Often, these cultured organs will synthesize secondary metabolites which may be either non-existent or in poor yield in the normal cell culture. Thus, cardenolide production in *Digitalis lanata* and *D. purpurea* cultures increases as tissue differentiation proceeds. Enhanced production of alkaloids occurs when roots develop from the callus cultures of the tropane alkaloid-producing Solanaceae. In contrast to the latter, in which leaf differentiation alone produces little alkaloid, leaf organ cultures of *Catharanthus roseus* and *Rauwolfia serpentina* synthesize a variety of alkaloids. Dimeric alkaloids have been detected in organ cultures of *C. roseus*, suggesting the possibility of an efficient production system for these valuable alkaloids. The dimers occurred only in those cultures which also contained vindoline and catharanthine. Whereas cell suspension cultures of *Papaver bracteatum* were found to synthesize orientalidine and sanguinarine, the root and shoot cultures produced thebaine. For ginsenoside production, ginseng root cultures have been grown in 20 000-litre bioreactors.

As with cell culture, elicitation can give increased yields of secondary metabolites. Thus both normal and hairy (q.v. below) root cultures of *Hyoscyamus muticus* when treated with jasmonic acid and its methyl ester at concentrations of 0.001 to 10 μM produced large increases in the levels of methyl putrescine and conjugated polyamines. However the increase of tropane alkaloid production was not remarkable (S.-Bionde *et al*., *Plant Cell Rep*., 2000, **19**, 691).

Transformed root culture—'hairy root' culture

Certain soil bacteria of the genus *Agrobacterium* cause a transformation of plant cells by introducing into their genome t-DNA from a bacterial plasmid. Such transformed roots, produced by inoculating the host plant, when grown in a hormone-free medium give rise to copious roots referred to as 'transformed roots' or 'hairy roots' (see Chapter 14). On removal of the *Agrobacterium* the roots continue to develop profusely and for some plants which normally produce secondary metabolites the hairy roots accumulate these metabolites in quantities comparable to those found in the normal intact plant.

Agrobacterium rhizogenes and *A. tumefaciens* are the bacterial species most commonly used to effect transformation.

Compared with the few examples given in the 13th edition of this book there is now a considerable bibliography on transformed root cultures of medicinal plants. Some examples are given in Table 13.2.

As with ordinary cell cultures and root cultures it is possible to utilize transformed roots to carry out biological conversions not normally associated with the intact plant. The rapid growth rate of hairy roots offers the possibility of rapid conversions. Ginseng hairy root cultures have been shown to convert digitoxigenin (the aglycone of a number of *Digitalis* cardiac glycosides, q.v.) into new compounds by esterification at C-3 with stearate, palmitate and myristate, and by the formation of gentiobiosides and sophorosides. Parr *et al*. (*Phytochemistry*, 1991, **30**, 2607) in studies on the biosynthesis of tropane alkaloids fed the *S*-analogue of tropinone (8-thiabicyclo[3.2.1] octan-3-one) to transformed root cultures of *Datura stramonium* and obtained the *S*-analogue of tropine, together with the 3-*O*-acetyl ester.

Cell cultures derived from *A. rhizogenes*-transformed *Papaver somniferum* tissue have been studied for alkaloid production (R. D. Williams and B. E. Ellis, *Phytochemistry*, 1993, **32**, 719). As with normal cultures, no morphinan alkaloids were produced but large amounts of sanguinarine were.

Table 13.2 Metabolites of some transformed (hairy) roots.

Alkaloid-containing plants	
Atropa belladonna	Increase in growth rate compared with untreated roots
Catharanthus roseus	Optimization of selected lines may give source of catharanthine and vindoline
Cinchona ledgeriana	Quinoline alkaloid production comparable with that of intact plants
Datura stramonium	Alkaloid content comparable with normal roots of intact plants; increased by use of various elicitors, e.g. methyl jasmonate
Hyoscyamus albus	New piperidone alkaloid obtained together with tropane alkaloids
H. muticus	Culture treated with chitosan accumulated hyoscyamine 2.5 to 3-fold compared with untreated hairy roots
Narcissus confusus	Improved production of galanthamine in shake cultures by use of methyl jasmonate as elicitor
Solanum laciniatum	Solasodine yield increased about four-fold
Flavonoids	
Glycyrrhiza glabra	Known and new flavonoids produced; also a new prenylated biaurone
Iridoids	
Valeriana officinalis	Yield of valepotriates four times that found in normal 9-month-old roots
Lignans	
Linum flavum	5-Methoxypodophyllotoxin production 2–5 times that of untransformed roots; 5–12 times higher than cell suspension culture; comparable with natural roots
Polyacetylenes	
Lobelia inflata	Polyacetylenes produced, some having a gentiobiose moiety. These compounds have anticancer activity
Quinones	
Sesamum indicum	Yield of antimicrobial naphthoquinone increased by more than 50 times that found in intact plant
Steroids	
Panax ginseng	Japanese patent for production of ginsenosides; diol and triol type ginsenosides produced. Production more effective than with ordinary root cultures
Trigonella foenum-graecum	By optimization of the cultural conditions, up to three times the production of diosgenin compared with non-elicited roots

CLONAL PROPAGATION

As noted earlier (Fig. 13.1), by adjustment of the plant growth regulators in the cell culture medium it is possible to promote differentiation of organs from callus tissues and to carry these forward to produce entire plants. As all cells of the callus are derived from a single meristem, all regenerated plants should be genetically identical. This fact has obvious commercial implications for the production, in a short period of time, of uniform crops derived from a small number of desirable plants (or even a single individual).

In 1980, Levy reported from Ecuador on the first large-scale commercial application of *in vitro* culture for the mass propagation of clones of pyrethrum plants. The scale of such projects is evident from the original objective, which was to produce, from 57 superior clones isolated over a 10-year research period up to 1976, 12 million plants per year to reach a plantation target of the number required for 1000 ha of land over 4 years. Further details of the process are given in the 15th edition of this book. Similar techniques are now practised for other crops of pharmaceutical interest, benefits being the ability to introduce selected high-yielding strains on a commercial scale in a short time, the creation of improved growth characteristics and a greater ease of plantation planning.

Clonal propagation is a potentially valuable method for producing high-yielding crops of species which tend to be variable when grown from seed. Fennel (*Foeniculum vulgare*), for instance, is genetically heterozygous and produces wide variations in oil yield and composition. In 1987, Miura *et al.* (*Planta Med*., 1987, **53**, 92) recorded a 2-year trial involving the production of uniform clonal plants derived from somatic embryoids of suitable parent plants. In the normal sexually propagated plants the anethol content of fruits varied from 0 to 12.9% (mean 2.82%), whereas in the clonal plants it showed a narrower distribution of 0.7–3.0% (mean 1.92%).

Genetic homogeneity

In practice, genetic changes can occur in cells during their artificial culture, and careful regulation of the medium is necessary. However, it has been shown, at least for *Datura innoxia*, that on differentiation it is the normal 2*n* plants, as distinct from the abnormal chromosomal types, that are favoured. Clonally propagated species for which the homogeneity of the secondary metabolites has been shown include *Aconitum carmichaelii*, *Angelica acutiloba*, *Bupleurum falcatum*, *Gentiana scabra*, *Rehmannia glutinosa* and *Stevia rehaubiana*.

Further reading

Verpoorte R, Heijden R van der, Schripsema J 1993 Plant cell biotechnology for the production of alkaloids: present status and prospects (*review with 130 refs*). Journal of Natural Products 56: 186

Zafar R, Aeri V, Datta A 1992 Application of plant tissue and cell culture for production of secondary metabolites (*review with 80 refs*). Fitoterapia 63: 33

14

Phytochemical variation within a species

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As was indicated in Chapter 3, the species represents the unit of plant classification; it may constitute a homogeneous taxon of plants with little variation from one specimen to another or it may include various varieties or races which each have some distinctive feature(s). Thus with *Datura stramonium* four varieties are described: var. *stramonium*—white flowers, thorny capsules; var. *inermis*—white flowers, bald capsules; var. *tatula*—lilac flowers, thorny capsules and var. *godronii*—lilac flowers, bald capsules. Often such varieties represent single gene mutations (in the above case two independent genes are involved) and are morphologically recognizable. In other instances the mutation gives rise to a variant having a different secondary metabolite profile—not necessarily discernible in the morphological form; these are termed chemical races or chemodemes. The mutation may involve the presence or absence of a single component or, if acting at an early stage of the biosynthetic route, may involve a whole series of compounds.

Knowledge of the existence of such chemical races is not new and A. Tschirch (1856–1939) in his *Handbuch der Pharmakognosie* deals with the various 'physiological forms' of *Vanilla planifolia*, *Cinchona* spp., fennel, *Piper nigrum* and the balsam-containing trees. Also, 60 years ago A. R. Penfold and co-workers were describing chemical races of various Australian oil-producing species of *Leptospermum*, *Boronia* and *Eucalyptus*.

In addition to the above which involve submicroscopic point mutations associated with an alteration in the DNA chromosomal material, there are other genetic variations which may affect chemical constituents of the species. These include (a), the existence of polyploids in which chromosome sets become doubled, tripled, quadrupled etc., (b), the addition of one or a few chromosomes above the normal complement (extrachromosomal types), (c), gross structural changes to a chromosome, (d), artificially produced transgenic plants.

Two consequences of the above are, on the positive side, it provides the possibility for the selection and establishment of superior strains with respect to the chemical constituents as described in this chapter. The negative aspect concerns the arrival on the drug market of material which is low in active constituents. This, together with other factors, necessitates the need for strict quality control (Chapter 16) which is being vigorously addressed by various legislative bodies (Chapter 2). Transgenic crops present their own problems.

CHEMICAL RACES, CHEMOTYPES, CHEMODEMES

The plant kingdom has been subjected to an extensive, but not exhaustive, chemical investigation. Thousands of samples have been screened for substances of medicinal value or for suitable precursors of therapeutically active compounds. Many other plants have been studied chemically from the viewpoints of manural treatments, plant resistance and biosynthesis of active constituents. From such observations has emerged evidence for the existence of 'chemical races', 'chemotypes' or 'chemodemes'. These are defined as chemically distinct populations within a species and have similar phenotypes but different genotypes and as such are identical in external appearance but differ in their chemical constituents.

Before the existence of a chemical race can be established, certain fundamental observations are necessary. A chemical analysis of a number of random samples of a particular species may show a variation between the samples but would be insufficient to demonstrate any genetical differences, since factors such as age, climate and soil can all exert profound effects on the result of the ultimate analysis. Samples of seed, or clones from different plants, must be raised together under uniform conditions,

and to exclude hybrids, which do not breed true, cultivation for a number of generations is desirable. It may then be possible to demonstrate that differences occur in either the nature or quantity of a particular constituent and that these differences are of a hereditary nature.

Such observations necessitate numerous assays and precise horticultural work, to which must be added the difficulties of dealing with plants which may take years to mature. Furthermore, many of the more important vegetable drugs cannot be successfully cultivated in temperate climates, so that it is not surprising that the number of medicinal plants fully investigated under ideal conditions is still limited. However, with the world-wide increase in genetic studies on medicinal plants, progress is being made towards the isolation of those enzymes associated with the existence of specific chemical races; recently, for example, the cloning of an enzyme involved in ginkolide biosynthesis (K. SangMin, *Phytochemistry*, 2006, **67**, 1435).

The clinical significance of chemical races is illustrated by Valerian; the plant normally contains both volatile oil and iridoid compounds, the latter with reported cytotoxic activity. As the sedative properties of the drug are ascribable to the valerenic acid and valerone constituents the cultivation of chemical races lacking the iridoids was introduced.

Fixed oils. Agriculturally, the cultivation of seed oil plants is second only in importance to that of cereals. Most of the fixed oil produced is used by the food industry but there are also important industrial and other, including pharmaceutical, uses. It is not surprising therefore that sustained breeding programmes for the improvement of yields and quality of oil have been in progress over many years. Normal rapeseed oil contains, as an acylglycerol, 20–40% of erucic acid, an acid having an extra long carbon chain (C_{22}) and one double bond. Its presence in quantity renders the oil unsuitable for edible purposes but varieties are now extensively grown which contain no erucic acid. The value of the crop has been further enhanced by coupling low erucic acid content with one giving low glucosinolates in the protein meal thus improving the animal feed properties. However, erucic acid is industrially important for the manufacture of lubricants, artificial fibres and plasticizers, so that varieties of rape developed for their high erucic acid content are also important agricultural crops.

The production of oil from sunflower seed has been improved by varieties that yield linoleic acid-enriched oil and which are more convenient for harvesting by having a large single flower head and no side-shoots. Groundnuts, the source of Arachis Oil *BP*, exist as various strains with different relative proportions of fatty acids.

Safflower constitutes an important oil-seed crop and its genetic variability has facilitated the breeding of varieties with widely differing oil constitutions. High oleic varieties are used for oil for human consumption and high linoleic varieties are important for oils used as industrial coatings and lubricants.

The above examples involve plants with a short life-span so that breeding by classical methods is a relatively rapid procedure. However, this is not so with plants such as the coconut palm, olive and cocoa so that in these cases modern techniques involving gene transfer would have an obvious advantage for the introduction of new or modified oil characteristics.

Cyanogenetic glycosides. A well-known chemical race in the cyanogenetic series is the almond. There are many varieties of *Prunus communis* showing different morphological forms, with and without amygdalin, but some varieties have similar characters and differ only in the presence or absence of the glycoside. Also in this group the clovers, especially *Trifolium repens*, have been extensively studied and *Linaria* has been shown by Dillemann to produce a chemical race by introgressive hybridization. *L. striata* contains cyanogenetic glycosides, and, crossed with the non-active *L. vulgaris*, gives rise to hybrids which, on repeated back-crossing with *L. vulgaris*, give some plants difficult to distinguish from *L. vulgaris* but which contain the cyanogenetic principles of *L. striata*.

Alkaloids. The *Duboisia* species form an important commercial source of the tropane alkaloids and have been extensively studied by Australian workers. With both *D. myoporoides* and *D. leichhardtii*, trees from natural stands in various locations were examined and their progeny were raised side by side in experimental plantations. The trees produce hyoscine, hyoscyamine, norhyoscyamine, tigloidine and valeroidine, and the proportion of any one alkaloid to total alkaloid may vary greatly. It was shown not only that seasonal and environmental factors are involved in this variation, but also that within a species there exists a wide range of alkaloid genotypes. Other varieties containing nicotine and nornicotine were also reported. Interspecific hybrids between the two species were studied and four hybrid clones were selected for possible exploitation as high alkaloid yielding strains. Thus, in this genus we have the possibility of two distinct types of chemical race—different alkaloid types within a species and different alkaloid types among hybrid phenotypes.

An example of the improvement of the morphine content of opium poppies by genealogical selection is furnished by the work of Lecat. The original seed gave capsules having an average morphine content of 0.385%. From this heterogeneous population were selected six individuals whose capsules analysed about 0.7% morphine. The seeds of these plants formed the heads of the lines cultivated in successive years, during which the best plants were collected and all those containing less than 0.7% morphine were rejected. The harvest of 1955 gave capsules with an average morphine content of 0.765%, thus doubling the original morphine content of the population. Such a method of breeding does not produce a race of plants surpassing individual morphine contents from the original heterogeneous population; it merely produces a homogeneous race of the alkaloid-rich plants.

Phillipson and colleagues reported at least three different chemical races of *Papaver fugax* and *P. armeniacum* in which either (1) 1-benzyltetrahydroquinoline, proaporphine, aporphine, (2) morphinane or (3) rhoeadine types are the major alkaloids; there are at least two different chemical strains of *P. tauricola* containing either the first or third types of the above. Three different isoquinoline alkaloid chemotypes of *Thalictrum minus* have been reported from Bulgaria. *Papaver bracteatum* is a species exhibiting races with respect to thebaine.

From *Claviceps purpurea* a number of races have been isolated containing different groups of ergot alkaloids and these have obvious implications for the commercial production of alkaloids.

One fodder crop in which the presence of alkaloids is undesirable is lupin seed. Ordinary wild forms are bitter and contain alkaloids of the lupinane series but over the years a number of sweet forms have been developed for commercial purposes in Europe. The strains depend for their low alkaloid content on the presence of a particular recessive gene. However, as a number of such genes exist, cross-fertilization between two different sweet strains will again give bitter progeny. To avoid this happening, considerable care is necessary in regions where different strains are grown side by side. Other plants for which there is evidence of alkaloid varieties include *Ephedra distachya* and the *Lycopodium* species.

Chemical races appear to be lacking in the pharmaceutically important indole alkaloid-containing genera *Strychnos*, *Rauwolfia* and *Catharanthus*.

Anthraquinones. The purgative anthraquinone drugs owe their activity to complex mixtures of the 1,8-dihydroxy derivatives of anthranols, their glycosides and free anthraquinones. The relative proportions of the constituents of the mixture, which greatly influence the pharmacological activity, depend not only on time of collection, age of plant, drying conditions and geographical source, but also on genetical

factors. In a programme involving *Rheum palmatum*, van Os produced races varying in their rhein/chrysophanol ratio and other hereditary strains for high- and low-yielding total anthraquinones. The analysis of individual *Cassia angustifolia* plants has indicated that selection of individuals for high sennoside B-yielding strains is a possibility.

Cardiac glycosides. With *Digitalis purpurea* the property of high glycoside content is hereditary. The proportion of glycosides derived from digitoxin and gitoxin is also very different in plants of different origin and remains so during subsequent cultivation under standardized conditions. The strains were distinguished chemically as digipurpurin, strospeside and digitoxin types. It now remains to prove that these characters are independent of the phenotype (i.e. that they are not inseparably associated with other characters of the parent plant). One race, 'Cambridge', which is relatively rich in digitoxin, is easy to distinguish; the other digitoxin race found in the Vosges differs little from the other selections. Variation in the proportion and quantity of glycosides in *D. lanata* has also been noted in mixed populations and, by the selfing of selected individuals, strains rich in a particular glycoside have been produced, the inherited character being strongly developed. Valuable physiological forms could thus be produced and Ligeti has recommended that these strains be designated by such names as '*D. lanata* Ehrh, chemo-varieties A and C', depending on the respective predominance of lanatosides A and C. It appears that with such inbred lines continuous selection is still required to prevent reversion to the normal character level of the species.

The great value of the radioimmunoassay (q.v.) for the rapid selection of high-yielding strains of *Digitalis lanata* has been demonstrated by Weiler and Westekemper. After two selection steps involving the analysis of over 10 000 individual plants, the average digoxin content of the plants could be raised two to threefold and several strains with average digoxin concentrations in the leaf of 0.6% were isolated. Individual plants were found with 0.9–1.0% digoxin content. As is usual with this type of selection, no plants better than the few best of the original selection were obtained.

Following intensive chemical investigation of the genus *Strophanthus*, Reichstein and his colleagues differentiated four chemical varieties of the polymorphous *S. sarmentosus* from different geographical sources. They are sarverogenin-, sarmentogenin- and sarmutogenin-producing types with glycosides of these, and a fourth form which has a low glycosidal content (Fig. 14.1). Although the locality of growth may produce quantitative differences in the constituents of the various races, the overall type is genetically controlled. Similar variation may exist among those plants that yield steroidal saponins, several thousand of which, from different localities have been screened for their sapogenin content.

Withanolides. The plant *Withania somnifera* (Solanaceae), in addition to producing alkaloids, contains steroidal lactones. Investigations over the years, carried out on various sources of plant material, and concerning the non-alkaloidal constituents, had given differing results, which were explained by the work of Abraham *et al*. (1968) on Israeli plants. Three chemotypes were discovered among 24 populations of *W. somnifera* collected in various parts of the country. Chemotype I contained predominantly withaferin A (0.2% of the dry weight), which is the principle responsible for the plant's bacteriostatic and antitumor properties. Chemotype II contains a compound of similar structure, and chemotype III a mixture of related compounds comprising a group

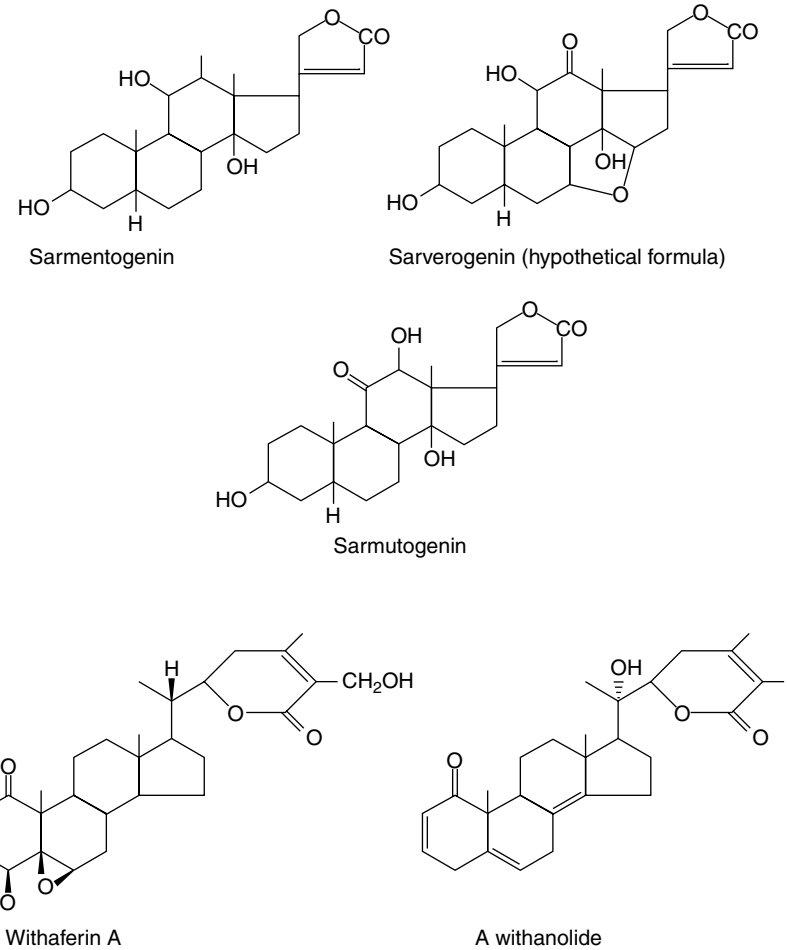
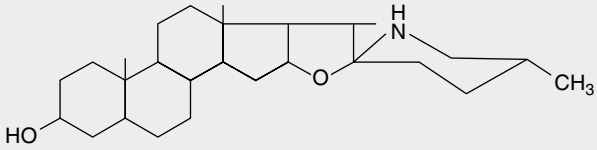
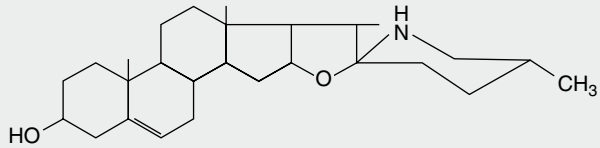
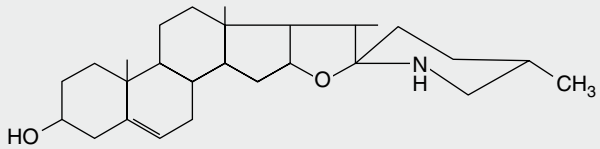


Fig. 14.1
Steroidal constituents of chemical races of *Strophanthus* and *Withania*.

Table 14.1 Chemical races of *Solanum dulcamara*.

Aglycone	Sugars	Glycoside
Soladulicidine (25D) 	Galatose (1 mol) Glucose (2 mol) Xylose (1 mol)	Soladulcidine-tetraoside
Solasodine (25D) 	Galactose (1 mol) Glucose (1 mol) Rhamnose (1 mol)	Solasonine
Δ5-Tomatidenol (25L) 	Galactose (1 mol) Glucose (1 mol) Rhamnose (1 mol)	α-Solamarine

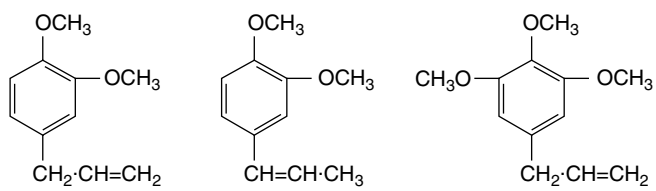
of steroidal lactones—the withanolides (Fig. 14.1). The only morphological difference observed between the chemotypes was a difference in flowering time (12 days early) for chemotype III. Since then, other chemotypes of *W. somnifera* have been reported from India and South Africa.

Steroidal alkaloids. *Solanum* spp. (Solanaceae) contain steroidal glycosidic alkaloids some of which have been investigated as potential intermediates in corticosteroid synthesis. In *S. dulcamara* (the woody nightshade) Sander has distinguished a west European tomatidenol group and an east European soladulcidine-solasodine group (Table 14.1). Although polyploid forms do occur in the genus, these chemical varieties all had $2n = 24$ chromosomes and were genetically stable. Subsequent work demonstrated that the different chemotypes can occur in the same locality. With the commercial species, about 3500 individual 6-month-old *Solanum laciniatum* and *S. aviculare* were analysed by radioimmunoassay (q.v.) and found to contain average leaf concentrations of 1.6–1.7% solasodine; from these a few individuals were selected for future breeding work.

Essential oils. The biochemical group of plants offering evidence of the largest number of chemical races is that containing volatile oils. Here, again, many of the differences within a species which have been reported may be due to factors other than genetic ones. Australia offers unique opportunities for the investigation of this problem as the flora is rich in oil-bearing plants. As an example, the common form of *Eucalyptus dives* contains piperitone as the chief constituent of the oil, but other races are known which produce principally phellandrene or cineole, while still others produce oils intermediate in composition.

There are three races of *Melaleuca bracteata* producing volatile oils containing chiefly methyl eugenol, methyl iso-eugenol and elemicin, respectively. They can be transformed one into the other by simple chemical steps, which suggests that one of the compounds (e.g. methyl eugenol) occurs in all the races. With the appropriate enzyme, methyl iso-eugenol could be formed by a simple double-bond shift and elemicin by the addition of a hydroxyl group and subsequent methylation. Because of this a one-gene-one-enzyme hypothesis suggests itself and it would be possible to test this by breeding experiments.

In the American turpentine industry, breeding investigations have shown that oleoresin yields in pines are inherited. Two chemotypes differing in their Δ^3-carene content of the oil have been recognized for *Pinus sylvestris*.



Methyl eugenol Methyl iso-eugenol Elemicin

Plants of the Labiatae have long been cultivated for their volatile oils and many varieties of a single species may exist; these are not, however, necessarily true chemical races because morphological differences may also be involved. Chemical races in the genera *Ocimum*, *Melissa*, *Micromeria* and *Thymus* have been studied. As one example, Rovesti's observations on the Ethiopian plant *Ocimum menthaefolium* are shown in Table 14.2. These forms occurred at different altitudes and exhibited a correlation between humidity of atmosphere and the constituents but cultivation of all four types at Asmara showed the chemical races to be stable and not transitory phenotypes varying with the environment. Similar wide variations of constituents of *Melissa officinalis* have been noted. Sixteen genotypes cultivated in the same field in former Yugoslavia for 2 years contained 0.046–0.246% oil. The constituents were the same for all oils but there was wide variation in relative amounts between the genotypes, namely citronellal 2.711–12.141%, linalool 1.501–6.380%, caryophyllene 1.210–19.073%, geranyl acetate + citronellol 9.710–26.913; (through *Chem. Abs.*, 1991, **114**, 58920). In Spain a 5-year selection and improvement programme for *M. officinalis* raised the essential oil content from 0.2–0.3% to more than 0.5% (T. Adzet *et al*., *Planta Med*., 1992, **58**, 558).

Chemotypes of *Acorus calamus*, sweet flag (Acoraceae), having differences in essential oil composition, have been DNA profiled.

Cinnamomum camphora (Lauraceae) exists as various chemical races which vary in their volatile oil composition, and a similar situation prevails for *C. zeylanicum* (*C. verum*). In the same family *Ocotea pretissa* gives oils of the sassafras type which may or may not contain camphor.

Chemical races appear to occur very widely in some Compositae. For *Tanacetum vulgare* (Tansy) ten different chemotypes have been reported for Finland with others for Piedmont (Italy) and Central Europe. Some principal components of these forms are thujone, isothujone, camphor, chrysanthenyl acetate and sabinene. *Achillea millefolium* (Yarrow) occurs as various chemical races and *Artemisia dracunculus* (Tarragon) has yielded, from plants originating mainly in France, an oil containing estragol, whereas from plants of Germany and Russia, sabinene, elemicin and *trans*-isoelemicin are the principal components. Wormwood *BP/EP* (*A. absinthium*) has a number of chemotypes with respect to its volatile oil content which may contain over 40% of any one of *p*-thujone, *trans*-sabinyl acetate *cis*-epoxyocimene or chrysanthenyl acetate. A clone of *Artemisia annua* giving a high yield of the important antimalarial artemisinin has been recorded (D. C. Jain *et al*., *Phytochemistry*, 1996, **43**, 1993).

Further examples of chemical races among volatile oil-containing drugs of the Labiatae can be found in Chapter 22.

Table 14.2 Oil composition of *Ocimum menthaefolium*.

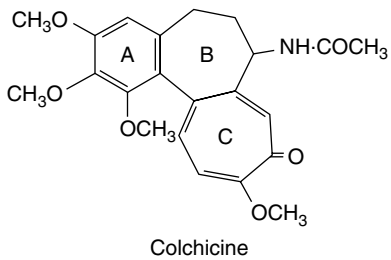
Race	Oil content	Composition of oil (%)
Var. *camphorata*	0.285	Camphor 23, cineole 13, pinene 10, estragol 40, sesquiterpene 10
Var. *estragolata*	0.248	Estragol 73, linalool 9, anethole 5, sesquiterpene 5, limonene 1.8
Var. *anisata*	0.236	Anethole 39, estragol 31, limonene 10, linalool 9, sesquiterpene 1.1
Var. *citrata*	0.212	Citral 56, estragol 20, terpene alcohol 10, terpene (as limonene) 9, sesquiterpene 5
Var. *intermedia* (between camphorata and estragolata)	0.267	Camphor 7, cineole 3, estragol 58

Miscellaneous. Other groups of active compounds which exist as chemical races are the phloroglucinol derivatives of *Dryopteris*, cannabinoids in cannabis, the bitter principles (e.g. amaragentin, sugars and volatile oil) of *Gentiana lutea* and the glycosides of *Salix*. The existence of two discrete chemotypes of *Equisetum arvense* with respect to flavonoid content is noted in the *British Herbal Compendium* Vol. 1. *Artemisia annua* plants raised in Holland from seeds obtained from a number of countries gave plants exhibiting distinct geographic chemotypes (T. E. Wallaart *et al.*, *Planta Medica*, 2000, **66**, 57).

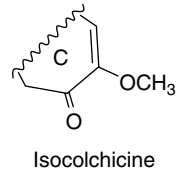
These examples serve to show that the occurrence of chemical races in plants, whether they be of natural origin or produced by plant breeding, can offer considerable scope for the improvement of the therapeutic value of the drug either by adjustment of the individual constituents or by increase in the overall yield.

CHANGES IN CHROMOSOME NUMBER

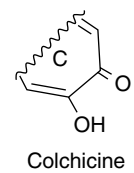
Polyploidy. In some organisms the chromosomes can be grouped, not in pairs but in threes, fours or higher numbers; these are polyploid individuals—triploid, tetraploid, octaploid, etc. Such polyploids can be derived by the multiplication of the chromosomes of a single species (*autoploids*) or as a result of the multiplication of the chromosomes following hybridization between two species (*alloploids*). The latter case furnishes a mechanism whereby a hybrid of two species, itself infertile, may give rise to a constant fertile type by polyploid formation. In the new polyploid form, pairing of the chromosomes at meiosis is possible which was probably not so in the original hybrid. Types such as this arise naturally: *Primula kewensis* formed as above from the infertile hybrid *P. verticillata* × *P. floribunda* was first recorded in 1912; since then many new species have been synthesized as well as some Linnean species (e.g. the production of the hemp nettle, *Galeopsis tetrahit* Linn. from *G. pubescens* × *G. speciosa*). Of pharmacognostical interest is the stabilization of the (fertile) F_1 hybrid of *Datura ferox* × *D. stramonium* by polyploid formation as indicated later in this chapter. In some species the somatic chromosome number



Colchicine



Isocolchicine



Colchicine

varies irregularly within wide limits (e.g. the blue grass *Poa pratensis* from 20 to over 100). Such plants, termed *aneuploids*, do not breed true and often exhibit apogametic (asexual) reproduction.

Among the natural polyploids of medicinal interest may be mentioned the mints and valerian; many of the former are alloploids. Valerian occurs across Europe in a variety of forms (2*n*, 4*n* and 8*n*); it is possible that the wide variability in pharmacological action of different samples of Valerianae Radix is associated with these different forms and a more rigid definition of the botanical source might be desirable. As indicated in Table 14.3, the oil compostion of *Acorus calamus*, the sweet flag, varies with the ploidy. For pharmaceutical purposes the 2*n* variety, containing no detectable toxic β-asarone is preferable.

Polyploidy can be artificially induced in many plants by suitable treatment with the alkaloid colchicine. The cytological effect of colchicine on dividing cells was reported by Dustin, Havas and Lits in 1937 and in the same year used practically by Blakeslee in his *Datura* studies. In the presence of colchicine, chromosomes in a cell undergoing mitosis will continue to divide without the formation of a mitotic spindle figure. Sister cells therefore are not formed, and in the growing root tips of onion (2*n* = 16), a 72-h treatment with colchicine solution has given rise to cells containing as many as 256 chromosomes. This 'C-mitotic' activity of colchicine may arise from its interaction with the disulphide bonds of the spindle protein and by inhibition of the conversion of globular proteins to fibrous proteins. On cessation of treatment, the spindle figure again forms in the normal way.

C-mitotic activity is greatly influenced by modifications of the colchicine molecule. Thus, colchicine is 100 times more active than its isomer isocolchicine and colchiceine is virtually inactive.

Modification of substituents in other rings may not have such a marked effect on activity—colcemid, which possesses a methylamino substituent in place of the acetylamido group of colchicine, is reported to have effects the same as colchicine but with toxicity to animal cells.

Plant materials can be treated with colchicine in a number of ways. Seeds are frequently soaked in an aqueous solution of colchicine (0.2–2.0% solution for 1–4 days) before planting, and seedlings can be inverted onto filter paper soaked in the solution so that the growing points are not damaged. Alternatively, the soil around the roots of young seedlings can be moistened with the alkaloid solution. Young buds and shoots can be treated by immersion, and lanolin pastes and agar gels are useful for general application to tissues.

Newly formed polyploids usually require a number of generations to stabilize themselves and treatments of the above type often fail to give a uniform plant regarding chromosome number; such mixochimeric conditions may involve different chromosome numbers in the three germ layers of the plant.

Typical effects of polyploidy compared with the diploid state are larger flowers, pollen grains and stomata. The influence of polyploidy on the constituents of a number of drug plants is indicated in Table 14.3; some figures quoted are taken from extensive studies and are given as an approximate indication of the differences obtained. As can be seen, the effects of polyploidy are not generally predictable and each species

Table 14.3 Influence of chromosome number on constituents of medicinal plants.

		Form		
Plant	Constituents	2n	4n	Others
Atropa belladonna	Total tropane alkaloids		Increase of about 68% over 2*n*	
Datura innoxia	Hyoscine, dry weight (%)	0.21		0.14, 0.11 (1*n*)
	Atropine, dry weight (%)	0.03		0.01, 0.01 (1*n*)
Datura stramonium	Total tropane alkaloids		Increases of about 60–150% over 2*n*	
Hyoscyamus niger	Total tropane alkaloids		Increase of 22.5% over 2*n*	Mainly 8n increase of about 34% over 2*n*
Cinchona succirubra	Quinine, dry weight (%)	0.53	1.12	0.27 (1*n*)
Opium poppy	Morphine yield per unit area			Increases of up to 100%: 3n plants especially high
Lobelia inflata	Alkaloid content: dry weight (%) per plant	0.25	0.32–0.46 52–152% that of 2*n*	
Acorus calamus	Volatile oil content (%)	2.1 (light oil, no detectable β-asarone)	6.8 (yellow-brown viscous oil. 2–8% β-asarone)	3.1 (3n) (yellow oil, 0.3% β-asarone)
Achillea millefolium complex	Azulene in volatile oil	Very variable	Most promising source	No azulene (8*n*)
Carum carvi	Volatile oil content (%)	6.0	10.0	
Mentha spicata	Volatile oil content (%)	0.48	0.05	
Fenugreek seeds	Diosgenin	0.68	0.60	
Digitalis purpurea	Total glycosides (%)		Lower or same as in 2*n*	
Digitalis lanata	Total glycosides (%)		Lower or same as in 2*n*. Relatively high content of lanatosides A and B	3.0 (3*n*)
Urginea indica	Proscillaridin A and scillaren (%)	0.004–0.26	0.02–0.45	0.04–0.07 (3*n*)
Capsicum sp.	Ascorbic acid (%)	0.04–0.09	0.04–0.15	
Cannabis sativa	Ratios of marihuana-like activity (toxicity to fish)	1.4	2.6	

must be examined individually. Care must be taken that the method used to express the results does not give a deceptive effect. Thus, with lobelia, tetraploid plants are smaller than diploid ones, so that, in spite of an increased percentage of alkaloid in 4*n* plants expressed on a dry weight basis, the total alkaloid content per plant may not exceed that of the 2*n* plants. A similar situation exists with tetraploid *Artemisia annua* with respect to artemisinin content (T. Wallaart *et al*., *Planta Medica*, 1999, **65**, 728). With tetraploid caraway plants, notwithstanding a 13% smaller crop of fruits from one plant, the total volatile oil content of this plant was increased by 100%; the 4*n* caraway was also found to be perennial (2*n* = biennial) and to possess an increased frost resistance.

Berkov reported (*Pharm. Biol.*, 2001, **39**, 329) that for autotetraploids of *Datura innoxia, D. stramonium* and *Hyoscyamus niger*, the 4*n* seeds contained, respectively, 1.8, 1.65 and 1.96 times the alkaloid content of the 2*n* seeds. Also (S. Berkov and S. Philipov, *Pharm. Biol.*, 2002, **40**, 617), for *D. stramonium* roots, concentrations of the principal alkaloids together with the 13 minor ones were higher in the 4*n*, compared with the 2*n*, roots.

In some species polyploidy does not affect the relative proportions of the individual constituents—for example, solanaceous herbs produce increased quantities of tropane alkaloids in the 4*n* state and reduced amounts as haploids but the proportion of hyoscine to hyoscyamine remains unaltered; the proportion of carvone in oil of caraway derived from 4*n* plants is also unchanged. However, 4*n Digitalis lanata* is reported to contain a relatively high proportion of lanatosides A and B compared with the 2*n* form. Haploid *D. lanata* plants raised from androgenic cell cultures are reportedly smaller than the 2*n* form, have some morphologically abnormal flowers and show very variable cardenolide contents (B. Diettrich *et al*., *Planta Medica*, 2000, **66**, 237). The sesquiterpene lactones of *Ambrosia dumosa*, family Compositae, exhibit marked differences between the diploid and polyploid forms.

V. Lebot and J. Levesque (*Phytochemistry*, 1996, **43**, 397) record that some 100 tons of kava root (*Piper methysticum*) (q. v.) are imported annually into Europe. The plants are all sterile decaploids (2*n* = 10*x* = 130) and are raised by smallholders throughout the Pacific Islands. Unfortunately the yields of kavalactones from different sources vary enormously and there is a real need for clonal selection for genetic improvement. The above authors have examined by HPLC the chemical composition of 121 cultivars originating from 51 Pacific Islands.

Extrachromosomal types. Sometimes plants occur with one or more chromosomes extra to the somatic number and these are known as extrachromosomal types. They were first noticed by Blakeslee's group in 1915, although their genetic constitution was not immediately apparent, when they sporadically appeared in pure line cultures of *Datura stramonium*. Such plants were later shown to possess 25 chromosomes in the somatic cell and with *Datura* (*n* = 12), twelve 2*n* + 1 types are possible, each one containing a different extra chromosome. The chromosomes were designated by numbering their halves (or ends), so that the largest chromosome is 1.2 and the smallest 23.24. All 12 types eventually appeared in Blakeslee's cultures and were originally named according to some obvious characteristic of the plant (e.g. Globe, Rolled, Ilex, etc.) although the end-numbering system can also be used to identify them; thus, Globe = 2*n* + 21.22. Other 2*n* + 1 types are also produced and are termed secondaries, tertiaries and compensating. Secondary types have the extra chromosome made up of two identical halves of a chromosome (e.g. 2*n* + 1.1) and in tertiary types it is composed of two halves of different chromosomes. Compensating types lack one of the normal chromosomes, which is compensated for by two others each carrying a different half of the missing one (e.g. 2*n* – 1.2 + 1.9 + 2.5). At meiosis 2*n* + 1 types produce a mixture of *n* and *n* + 1 gametes and so do not breed true; they proved particularly useful to geneticists for gene location.

Table 14.4 Alkaloids of some primary and secondary types of *Datura stramonium.**

Type		Alkaloid content, compared with controls, at vegetative state (calculated on a dry weight basis)
2*n* + 1.2	'Rolled'	136% increase
2*n* + 9.10	'Echinus'	143% increase
2*n* + 3.4	'Glossy'	155–227% increases
2*n* + 5.5	'Strawberry'	
2*n* + 11.11	'Wedge'	
2*n* + 17.17	'Dwarf'	
2*n* + 19.19	'Divergent'	
2*n* + 10.10	'Thistle'	35–40% decreases
2*n* + 13.14	'Microcarpic'	
2*n* + 21.22	'Globe'	

*From Mechler and Haun (*Planta Med.*, 1981, **42**, 102)

In 1963, Stary reported the analysis of the primary types Poinsettia (2*n* + 17.18) and Globe (2*n* + 21.22) and showed them to possess more hyoscine than hyoscyamine in the leaves of mature plants, whereas in diploid strains the reverse is true. Mechler and Haun have reported on the total alkaloid content of other 2*n* + 1 types, including some secondary types. Their abstracted results are given in Table 14.4.

ARTIFICIAL PRODUCTION OF MUTATIONS

The mutagenic properties of X-rays and radium emissions were exploited as early as 1921 by Blakeslee at the commencement of his classical studies on the genetics of the genus *Datura*. Since then, all types of ionizing radiation (α-particles, β-rays, γ-rays, thermal and fast neutrons) have been extensively studied in this respect and a number of new varieties of crop plants have been produced (barley, peas, soya beans, mustard and rape). In barley approximately one-fifth of all viable mutations produced by ionizing radiations are of the 'erectoides' (dense spike, stiff straw) type; in other crops increased yields, early maturity and mildew resistance have been achieved.

Subsequent to a small number of investigations on chemical mutagens dating from 1910, the avalanche of research on this subject started during and after World War II, following observations by Auerbach and Robson on the production of mutations in *Drosophila* (fruit fly) by mustard gas. Further stimulus was given to this work by the discovery that many chemical mutagens also possess carcinogenic or anticarcinogenic properties. These substances vary enormously in their molecular complexity and chemical properties, and it is only more recently that their mode of action has been elucidated.

Collectively, ionizing radiations and chemicals will produce a mutation spectrum which covers all of the groups listed earlier. The former, however, produce in the chromosomes aberrations of a more random nature than do chemicals, which often act principally at certain loci—particularly at those areas of the chromosome which stain differently at mitosis (heterochromatin). Also, the distribution of effects between nuclei is more random with X-rays than with chemicals.

Mutagenic agents act at various stages of nuclear organization. Thus, at that stage of the interphase (non-dividing) nucleus when DNA synthesis is taking place, aberrations involving chromatid exchanges and isochromatid breaks occur. These effects do not become immediately (0–8 h) manifest in the cell but appear as delayed effects 8–48 h after treatment. Ionizing radiations and most chemicals produce aberrations

of this type. Clearly, breaks which occur in the interphase nucleus chromosomes before DNA synthesis occurs (chromosomes unsplit) would be of the chromosome type and these are induced by X-ray treatment and by a few chemicals (e.g. ethoxycaffeine and streptonigrin). Other mutations may be induced during the DNA post-synthetic stage of the interphase nucleus and during mitosis itself—as in the production of polyploids by colchicine and in the inducement of binucleate or polynucleate conditions due to inhibition of cell plate formation by cyclic organic compounds (e.g. halogenated derivatives of benzene and toluene, hydrazinotropone compounds, aminopyrine). Most mutagens produce more than one type of fragmentation or exchange effect (e.g. ethoxycaffeine and streptonigrin, besides producing chromosome exchanges, also induce sub-chromatid exchanges). A few of the many known mutagens are given in Table 14.5.

Factors which may influence the effect of mutagenic treatment include oxygen tension within the tissues, temperature and pH. Chemical mutagens can be applied in a similar way to colchicine (q.v.). Seeds, whole plants, isolated organs, growing points, etc., are suitable for direct irradiation. In order to obtain single mutations in a plant, irradiation of pollen, which is subsequently used to fertilize a normal flower, is often advantageous. It is unlikely that a pollen grain will retain its viability if it undergoes more than one mutational change.

Among plants of medicinal interest, the production of polyploid forms has already been discussed. Blakeslee's radiation work on *Datura stramonium* resulted in the production of many single gene mutation types (e.g. Zigzag, Quercina, Bunchy, Equisetum—names derived from some characteristic aspect of the plant). These mutants are not isolated individuals but are produced regularly by radiation treatment. Some forms such as 'pale' (chlorophyll-deficient) are more frequent than others. In many cases Blakeslee was able to map the position of the genes responsible for these effects. Other mutants obtained in these studies were of the extra-chromosomal type (q.v.).

Several workers have studied, without a full genetic analysis, alkaloid production in various species of *Datura* raised from irradiated seeds; types have been produced which show differences in the relative proportions of the alkaloids synthesized but no new alkaloids have been detected by this treatment.

By the irradiation of poppy seeds with ^{60}Co a number of mutations have been produced, including ones producing plants with an increased morphine content; these increases were maintained in the X_2 generation with an average morphine content of 0.52% compared with 0.32% for the controls.

As mentioned earlier, races of sweet lupins (almost free of bitterness) can be obtained by selection. More recently, bitter lupin seeds of an X-ray-induced early-maturing mutant of *Lupinus digitatus* were treated with ethylmethanesulphonate solution, and, of the 440 progeny, 11 were mutants which could be classed as sweet. Four of these were of normal vigour and near-normal fertility.

Breeding experiments have been performed with irradiated *Mentha piperita* in the USA in an endeavour to produce a dominant mutation (bud sport) for *Verticillium* (wilt) resistance, a disease to which mints are particularly prone; a successful strain, *Todd's Mitcham Peppermint*, is now cultivated. A radiation-induced mutant of Scotch peppermint (*Mentha* × *gracilis*) has been shown to produce an oil typical of the ordinary peppermint (C-3 oxygenated monoterpenes) instead of the C-6 oxygenated monoterpenes characteristic of spearmint (see Fig. 22.4 for formulae). The results have given further insight into the biogenesis of these compounds (R. Croteau *et al.*, *Plant Physiol.*, 1991, **96**, 744).

In India mutant strains of *Capsicum annuum* with increased yields (20–60%) of capsaicin have been isolated from M_3 and M_4 generations originating from seed treated with sodium azide and ethylmethanesulphonate.

In the future, haploid plants will undoubtedly find increasing use for the study of induced mutations; in many cases whole plants can now be regenerated from haploid tissue cultures of pollen. Such material has the advantage that induced recessive mutations, which in the diploid organism require subsequent breeding experiments for their study, are immediately apparent in the phenotype.

HYBRIDIZATION

In plant breeding hybridization forms a possible means of combining in a single variety the desirable characters of two or more lines, varieties or species, and occasionally of producing new and

Table 14.5 Cytological action of some chemical mutagens.

Group	Examples	Cytological effect
DNA precursors and related compounds	Adenine	Inhibition of early stages of DNA synthesis, purine formation
	Deoxyadenosine	Inhibition of deoxyribonucleotide synthesis
	Ethoxycaffeine	Complex formation with inhibition of the DNA-polymerase reaction, degradation and denaturation of DNA
DNA base analogues	Thymidine analogue 5-bromodeoxyuridine	Production of abnormal DNA
Antibiotics	Streptomycin	As ethoxycaffeine
	Actinomycin	As ethoxycaffeine
Alkylating agents	Nitrogen mustard	Production of abnormal DNA
	Diepoxypropyl ether	
Nitroso compounds and chelating agents	Many compounds (e.g. cupferron, 8-hydroxy-quinoline)	Possibly combine with some trace heavy metals (Fe, Cu) which may be bound with the DNA molecule
Miscellaneous	Inorganic cyanides	Inhibition of cytochrome oxidase with resultant peroxide formation
	Maleic hydrazide	Reaction with sulphydryl groups
	Hydroxylamine	Combination with cytosine component of DNA

desirable characters not found in either parent. Hybridization, particularly between homozygous strains which have been inbred for a number of generations, introduces a degree of heterozygosity with resultant hybrid vigour (heterosis) often manifest in the dimensions and other characteristics of the plant. Several methods of breeding crops by the use of sexual hybridization are available and for these the reader is referred to standard works on the subject. Although this chapter is devoted principally to chemical variants of a particular species it is convenient to include here, in addition to intervarietal hybridization, interspecific hybridization in which hybrid vigour is also apparent.

The hybrid nature of a number of drugs (e.g. cinchona, q.v.) is well known. The commercial mints are hybrids and must therefore be propagated vegetatively, as the plants will not breed true and the progeny vary in their oil composition. Hegnauer considers that with the spearmint-type oil (high carvone content) genes of *Mentha longifolia* or of *M. rotundifolia* seem to be necessary. The cultivated peppermint (*M. piperita*) is probably a hybrid derived from *M. aquatica* and *M. spicata* and it is the former that contributes the menthofuran characteristics. Hybrids between various species of *Mentha* have been used to study the inheritance of a number of essential oil components, including menthol, carvone and pulegone.

Hybrids of species producing tropane alkaloids have received considerable study. In the genus *Datura* the effect of hybridization on chemical constituents is illustrated by the cross *D. ferox* × *D. stramonium*. The aerial organs of the latter normally contain hyoscyamine and hyoscine (2:1 ratio) at the flowering period; and those of the former, hyoscine with some meteloidine. The F_1 of the cross consists of plants larger than either of the parents, and containing hyoscine as the principal alkaloid with only small amounts of other bases. In the F_2, segregation occurs as regards both morphological characters and alkaloid constituents. With *D. leichhardtii* and *D. innoxia* the former plant produces hyoscyamine and hyoscine (2:1) and the latter species usually mainly hyoscine but sometimes, according to conditions of growth, appreciable quantities of hyoscyamine. In this instance the F_1 hybrid contains a hyoscyamine:hyoscine ratio intermediate between that of the two parents. Various hybrids of tree daturas have also given favourable alkaloid yields and have been subjected to field trials in Ecuador. These plants are self-sterile but can be propagated by cloning (Chapter 13).

In experiments performed in Australia on hybrids of *Duboisia leichhardtii* and *D. myoporoides* no uniform dominance of either hyoscine or hyoscamine in the F_1 was obtained, although the ratio generally favoured hyoscyamine even in geographical areas conducive to the production of hyoscine. Investigations of this type on *Duboisia* are complicated by the occurrence of chemical races and by the marked susceptibility of alkaloid content to environment. However, single desirable hybrids can be vegetatively propagated and have now become established as commercial crops (q.v.). Experiments in Japan on artificial crosses of *Duboisia* produced some F_1 hybrids which contained more than twice the amount of alkaloids (hyoscine and hyoscyamine) than that contained by the parents. Greatest increases were found when *D. myoporoides* was the female parent. Naturally occurring intergeneric hybrids involving *Duboisia* spp. and other Anthocercideae have been analysed (El-Imam *et al*., *Int. J. Pharmacog*., 1991, **29**, 263) and the alkaloid metabolism in callus and regenerating shoot cultures of the commercial hybrid *D. leichhardtii* × *D. myoporoides* studied (Gritsanapan and Griffin, *Phytochemistry*, 1992, **31**, 3069).

Nicotiana tabacum (Solanaceae), as now cultivated, must have been derived from at least two different parent species, and 'synthetic' tobaccos can be prepared by using suspected species as parents. Although it has not been possible to produce in this way species exactly comparable to *N. tabacum*, such synthetic plants are most useful for the study of alkaloid inheritance characteristics. This is important in the commercial production of tobacco, in which both the quantity and the nature of the alkaloid produced are important. Demethylation of nicotine may take place in the leaves of some species, and by hybrid studies this reaction has been shown to be due, in the groups of plants studied, to either one pair of dominant factors or two pairs of dominant and independent factors.

The inheritance of the opium alkaloids (morphine, codeine, thebaine, narcotine and papaverine) has been studied in the cross *Papaver somniferum* × *P. setigerum*. A heterotic increase in codeine and thebaine was found in different F_1 plants, and in the F_2 plants, with the exception of codeine, some increase in alkaloid content was noted. An absence of narcotine was generally dominant over its presence (Khanna *et al*., *Planta Med*., 1986, p. 157). A continuation of this work to the F_8 generation (Shukla *et al*., *Int. J. Pharmacognosy*, 1995, **33**, 228) resulted in a population that was completely diploid but which showed considerable diversity with regard to the opium contents of morphine, narcotine and papaverine. The pattern of alkaloids was closer to that of *P. somniferum* than to that of *P. setigerum* with morphine contents ranging from 8.0 to 30.0%. The authors envisaged that a suitable breeding programme could result in opium with a higher level of morphine than that normally encountered. F_1 hybrids of *P. bracteatum* and *P. orientale* contained a lower thebaine content and higher oripavine content than in either parent, a result which provided genetic evidence for the biosynthetic linkage between these alkaloids (Fig. 26.15).

In a continuation of the Israeli work on withanolides Eastwood *et al*. reported that a cross of a South African chemotype of *Withania somnifera* with the Israeli chemotype II produced three new withanolides not detected in either parent. The outstanding feature was the presence of a new oxidizing system in the hybrid which apparently oxidizes ring A to a diketone in the presence of either the saturated or the unsaturated lactone (Fig. 14.2). A further notable feature was the isolation of a compound having a saturated C2–C3 link, a feature which had only been noticed in a minor constituent of chemotype I. In *Datura*, too, hybrids may contain different withanolides from those of either parent and involve different oxidation states of ring C.

Solasodine occurs in *Solanum incanum* up to the extent of 1.8% whereas *S. melongena* contains only traces of steroidal alkaloids. Work in Jammu, India, has shown the F_1 hybrid of these species to be much more prolific in fruit-bearing than either of its parents, with a content of 0.5% solasodine. The diversity of glycoalkaloids found in *Solanum*

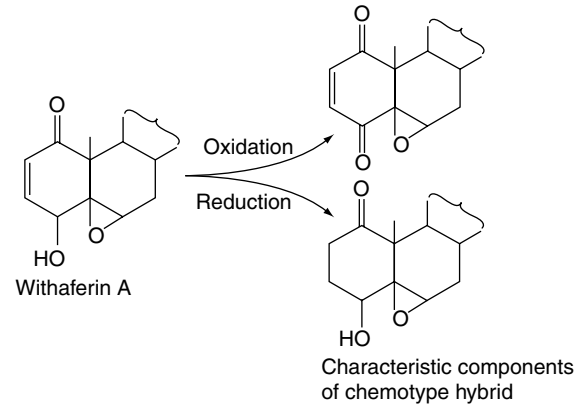


Fig. 14.2
Withanolide oxidations and reductions characteristic of a *Withania somnifera* chemotype hybrid (for complete structure of withaferin A see 'Withanolides').

Table 14.6 Lanatoside formation in *Digitalis* hybrids.

♀	Parents	♂	Glycosides of the F_1
D. purpurea	×	*D. lanata*	Lanatoside A (principal glycoside). Also lanatosides B and E. Small amount of glucogitaloxin. No lanatoside C or purpurea glycoside A
D. purpurea	×	*D. lutea*	Similar to the above
D. lutea	×	*D. lanata*	Same composition as the parent plants which resemble one another. Lanatoside A the principal glycoside but lanatoside C, which occurs in large amounts in *D. lanata*, could not be detected

species is, in part, the result of the independent inheritance of the aglycone and glycosidic moieties and explains the occurrence of a new alkaloid, sisuine, found in the hybrid of *S. acaule* and *S. ajanhuiri*. This group of hybrid clones is cultivated by the Aymara people of Western Bolivia.

In 1961, Calcandi and coworkers demonstrated (Table 14.6) that in the hybridization of *Digitalis* species, lanatoside formation is dominant to purpurea glycoside formation; in 1984 these observations were largely confirmed for some crosses and extended with more detailed analysis by Wichtl and Mangkudidjojo (*Pharm. Zeit*., 1984, **129**, 686). For the glycoside content of other *Digitalis* F_1 hybrids including those of *D. ferruginea* and *D. cariensis*, see Fingerhut *et al.*, *Planta Med.*, 1991, **57** (Suppl. No. 2), A70; A71.

Preliminary studies by Cornish *et al*. in 1983 indicated that foenugreek seed, a potential source of diosgenin (Chapter 23), is capable of genetic improvement regarding the monohydroxysapogenin yield by hybridization of various races of *Trigonella foenum-graecum*.

Pyrethrum hybrids have been used in Kenya for pyrethrin production (see Chapter 40); these hybrids are produced either by crossing two clones assumed to be self-sterile or by planting a number of desirable clones together and bulking the seed. The aim is to obtain progeny which, owing to hybrid vigour, will increase pyrethrin yield per acre. Unfortunately, it appears that increased production of pyrethrins is not always associated with hybrid vigour. Research at the Pyrethrum Marketing Board, Kenya, indicated that clonal selection, based on vigour and individual pyrethrin content of hybrids raised from seed, followed by vegetative propagation, would give better results.

TRANSGENIC MEDICINAL PLANTS

Until recently the transfer of genetic material from one plant to another was dependent on hybridization as has been discussed above and its application is limited to those relatively few species which are intercompatible and give fertile F_1 hybrids.

Developments in molecular biology and chemical genetics have provided new vistas for genetics and it is now possible to explain, in terms of the DNA molecule, chromosome duplication and the structure of the gene itself—two mysteries involving the nature of life which intrigued and baffled scientists for many years. From such fundamental studies the science of genetic engineering explosively emerged in 1973, when it became possible, by the formation of recombinant DNA, to transfer certain DNA sequences from the chromosomes of one organism to those of another. Since DNA assemblages represent the genes or hereditary characters of an organism, this led the way for the artificial transfer of a particular character from one organism to another.

Nature itself does effect genetic transfers between very dissimilar organisms as evidenced by the well-known crown gall and hairy root diseases of plants. Both are caused by bacterial infection of the plant and involve, respectively, the soil organisms *Agrobacterium tumefaciens* and *A. rhizogenes*. Crown gall disease is essentially a plant cancer and hairy root disease is also a proliferation manifestation in which there is an excessive development of the root system. A further characteristic associated with both diseases is the production, by the host, of a group of substances called opines, the actual compounds synthesized being specific for the particular parasite involved. Many opines are dicarboxylic acids, the majority being formed by condensation of an amino acid (lysine, arginine, ornithine, histidine) with either pyruvate or α-ketoglutarate; others involve simple sugars such as mannose and sucrose, see Fig. 14.3. (For a review on opines (129 refs) see Y. Dessaux *et al*., *Phytochemistry*, 1993, **34**, 31.) Another important feature concerning these diseases, first noted in the late 1960s, was that *Agrobacterium* strains would degrade only those opines for whose occurrence in the host they were responsible. Thus opines, of no value to the plant cell, constitute an exclusive food substrate for the particular bacterial strain. When, however, the bacterium is eliminated from the host plant (e.g. by treatment with an antibiotic) the characteristic morphological features and the production of opines continue to be maintained. This is because genetic material (T-DNA) of the bacterium carrying the genes which express the disease has been transported to, and integrated into, the plant DNA. These genes are carried on large plasmids which have been named Ti (Tumour-inducing) and Ri (Root-inducing) plasmids. Such transgenic plant material is of particular medicinal and phytochemical interest when grown as transformed cell cultures or hairy root cultures in studies involving the production of secondary metabolites and has already been discussed in some detail in Chapter 13.

A further development has been to utilise *Agrobacterium* in a vector system for transferring genes from one species of higher plant to another. Such transfers usually involve just a single enzyme system; multiple enzyme systems involved in secondary metabolism and originating from different chromosomes obviously present great problems. Of pharmacognostical significance is the work of Hashimoto's group at Kyoto University on *Atropa belladonna*: the conversion of

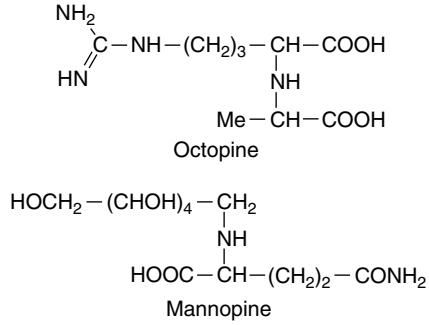


Fig. 14.3
Examples of opines.

hyoscyamine to hyoscine (Chapter 26) in the plant involves the enzyme hyoscyamine-6-hydroxylase and in the normal belladonna plant the gene is not strongly expressed and plants are hyoscyamine dominant. Conversely, although *Hyoscyamus niger* contains less alkaloid (% dry weight) than belladonna it contains a higher proportion of hyoscine. The hydroxylase gene of *H. niger* was placed under the control of the cauliflower mosaic virus 35S promoter and introduced to *A. belladonna* roots by a binary vector system using *Agrobacterium rhizogenes* (*Phytochemistry*, 1993, **32**, 713). The belladonna hairy roots produced showed increased amounts, and increased enzyme activities, of the hydroxylase and contained up to fivefold higher concentrations of hyoscine than the wild-type hairy roots. A second paper (*Proc. Natl. Acad. Sci. USA*, 1992, **89**, 11799) reported that whole plants regenerated from the transformed roots showed, in the first transformant and its selfed progeny, an alkaloid composition of the leaves and stems that was almost exclusively hyoscine. Such a plant, if developed commercially, would be a valuable temperate-region source of hyoscine.

In the same area K. Jouhikainen *et al*. (*Planta*, 1999, **208**, 545) have demonstrated enhancement of scopolamine production in *Hyoscyamus muticus* hairy root cultures by introducing the above 35S-*h6h* transgene that codes for the enzyme hyoscyamine-6β-hydroxylase (EC 1.14.11.11). *H. muticus* is a species producing up to 6% dryweight of tropane alkaloids, normally hyoscyamine; the introduction of the above transgene moved alkaloid production towards scopolamine (but not entirely) and indicated the potential for greater conversion by the use of more efficient promoter systems.

With belladonna, the first successful application of transferring an agronomically useful trait to a medicinal plant has been reported (K. Saito *et al*., *Plant Cell Rep*., 1992, **11**, 219). It involved the production of transgenic fertile *A. belladonna* plants integrated with a herbicide-resistant *bar* gene by means of *Agrobacterium*–Ri vector. The regenerated plants were produced via hairy roots and the *bar* trait was transferred to progeny, which showed resistance to bialaphos and phosphinothricin.

An example of the possible potential for increasing alkaloid yield in *Cinchona* is illustrated by the successful integration of two genes from *Catharanthus roseus* into the hairy roots of *Cinchona officinalis* (A.-Geerlings *et al*., *Plant Cell Reports*, 1999, **19**, 191). These two genes, responsible for the respective production of the enzymes tryptophan decarboxylase and strictosodine synthase, are crucial to the synthesis of both the cinchona and catharanthus alkaloids (Chapter 26). As a result of the transfer both the quinine and quinidine levels were raised above the normal for *C. officinalis* hairy roots; in this instance however, after one year, the roots had lost their capacity to produce alkaloids.

Transgenic plants of *Panax ginseng* which develop abundant roots have been produced and could therefore be of commercial significance (D.-C. Yang and Y.-E. Choi, *Plant Cell Reports*, 2000, **19**, 491).

Further reading

Lindsey K (ed) 2000 Transgenic plant research. Harwood Academic, Amsterdam

15

Deterioration of stored drugs

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The factors which must be considered in relation to drug deterioration are moisture content, temperature, light and the presence of oxygen; when these conditions are suitable, living organisms (bacteria, moulds, mites and insects) will rapidly multiply, using the drug as a source of nutrient. Drugs affected in this way are excluded by national pharmacopoeias.

PRIMARY FACTORS

As indicated in Chapter 11, air-dry drugs contain about 10–12% of moisture, and in some instances (e.g. digitalis) this may be sufficient to activate enzymes present in the leaves and bring about decomposition of the glycosides. Other drugs, such as powdered squill, which contain mucilage quickly absorb moisture and become a sticky mass. The containerized shipment of drugs which is now common practice can lead to spoilage due to excessive condensation of moisture on the inner metal walls. It is a particular problem with cargoes in transit from humid moist climates to temperate regions. An increase in temperature, in combination with moisture, may accelerate enzyme activity; a large temperature rise will obviously lead to a loss of volatile constituents (e.g. essential oils from dried plant material) and in the case of absorbent cotton-wool cause a reorientation of the small amount of fatty material present leading to non-absorbency or lower absorbency. Direct sunlight can cause decomposition of certain constituents (e.g. vitamins in cod-liver oil) as well as producing a bleaching of leaves and flowers. Oxygen assists in the resinification of volatile oils and in the rancidification of fixed oils.

MOULD AND BACTERIAL ATTACK

The moulds found in deteriorating drugs are usually the same as those associated with poorly stored food products. Species of *Rhizopus, Mucor, Penicillium* and *Eurotium* are common. Their presence is indicated by a mass of hyphae which bind the particles of drug and by a characteristic smell. Deterioration of drugs is only one aspect of the importance of moulds in pharmacognosy—see Chapter 30. Bacterial attack of crude drugs is less obvious unless chromogenic species are involved or effects produced such as dustiness in cotton-wool by attack on the fibres. Although not a cause of deterioration, certain pathogenic bacteria such as salmonellae and *Escherichia coli* are tested for in some crude drugs taken internally (digitalis, sterculia, tragacanth, gelatin). Also, as plant materials which have been dried under normal conditions contain viable bacteria and mould spores in variable amounts, the pharmacopoeias set limits for the total viable aerobic count per gram of drug (see Quality Control, Chapter 16).

COLEOPTERA OR BEETLES

Beetles are insects and constitute the largest order of the animal kingdom, comprising some 250000 species, of which about 600 have been found associated with stored food products or drugs. Not all of these utilize the stored product itself but may be found in the wood of packing-cases or living predaciously. Beetles have a body which is divided into head, thorax and abdomen. To the lower side of the thorax are attached three pairs of legs, while the upper surface usually bears two membranous hind-wings which are folded beneath horny elytra (forewings). They show complete metamorphosis of egg, larval, pupal and adult stages, and those which constitute pests in stored products cause damage both as adults and as larvae. Among the characters which distinguish the larvae of most species which attack foodstuffs and vegetable drugs are the well-developed biting mouth-parts and a head which is darker in colour than the rest of the body. Table 15.1 lists beetles commonly found in stored drugs; for illustrations see Fig. 15.1.

Table 15.1 Beetles commonly found in stored drugs.

	Description
Family Nitidulidae (sap-feeding beetles)	
Carpophilus spp., e.g. *C. hemipterus* (dried fruit beetles)	Obovate or oblong 2–4.5 mm long; 11-segmented antennae with a compact club. Elytra somewhat shortened exposing two–three apical abdominal segments. In this country about three species are found in granaries, food-stores and warehouses
Family Silvanidae	
Oryzaephilus mercator (merchant grain beetle)	Dark brown, narrow, distinctly flattened beetles, about 3 mm long. Clubbed antennae. Attack nuts and dried fruits
O. surinamensis (saw-toothed grain beetle)	
Family Curculionidae (weevils)	
Calandra granaria (granary weevil)	Dark brown to black insects, about 3–4 mm long. Hind-wings absent, characteristic snout and antennae. Bore into seeds and fruits and lay an egg in the cavity by means of the ovipositor. Larvae develop and pupate within the seeds
Calandra oryzae (rice weevil)	Similar, hindwings present. 2.3–4.5 mm long
Family Anobiidae ('furniture beetles')	
Stegobium paniceum (*Sitodrepa panicea)* *Anobium paniceum* (drugroom beetle)	Pale reddish-brown in colour, greyish hairs, 2–3 mm long. Antennae 11-segmented, with three terminal segments forming a loose club. Common in many stored vegetable drugs, formerly frequent in ships' biscuits
Anobium punctatum (common furniture beetle)	Similar to *Stegobium paniceum*, 3–5 mm long. Viewed laterally, the prothorax exhibits a distinct hump. Does not attack drugs but may occur in wood of packing-cases, floors, etc.
Lasioderma serricorne (tobacco, cigar or cigarette beetle)	Reddish colour, 2–2.5 mm long. Found in many stored products, including ginger and liquorice
Family Ptinidae ('spider beetles')	
Ptinus fur (white-marked spider beetle) *P. tectus* (Australian spider beetle) *P. hirtellus* (brown spider beetle) *Trigonogenius globulus* *Niptus hololeucus* (goldenspider beetle, cloth bug) *Gibbium psylloides*	All rather similar, somewhat resembling spiders, with long legs and antennae, stout bodies and hairy covering, 2–4 mm long. Some species (e.g. *Niptus hololeucus*) are densely covered with hairs; others (e.g. *Gibbium* spp.) are glabrous with a shining cuticle. Of wide occurrence in stored products—food, spices, cocoa, cereals, almonds, capsicum, ginger, nutmegs, etc.
Family Tenebrionidae	
Tribolium confusum (confused flour beetle) *T. castaneum* (rust red flour beetle)	Reddish-brown beetles, 2–4 mm long. Found in many foodstuffs including flour and nuts. Infested flour has lingering pungent odour. Reported as becoming more common in crude drugs e.g. rhubarb

LEPIDOPTERA

The Lepidoptera include the moths and butterflies, and although the number of species is large, only a relatively small number of moths cause injury to drugs. As in the case of the clothes moth, the damage is caused by the larva and not by the mature insect; but since moths are very mobile and lay eggs, infestation tends to spread rapidly. Moths reported in stored drugs are listed in Table 15.2.

Ephestia kuehniella, the Mediterranean flour moth; *E. cautella*, the fig moth; *E. elutella*, the cocoa moth, and *Plodia interpunctella*, the Indian meal moth, all belong to the same family, the Phycitidae. Various members of the family Tineidae, which cause damage to clothes, carpets, etc., are also found in drugs.

Ephestia kuehniella is about 25 mm long. It has dark brownish-grey scaly forewings and dirty-white hindwings. The larvae are whitish except for the brownish anterior and the dark hairs on each segment; when fully grown, they remain in the food material, where they overwinter and form pupae. In contrast, the grubs of *E. elutella* migrate away from the food and in so doing often leave the food containers completely 'webbed' with their silky threads; they enter cracks in walls, etc., where they spin cocoons and remain until they pupate in the spring to finally emerge as adults in May.

Table 15.2 Moths of stored drugs.

Moth	Products attacked
Ephestia kuehniella	Almond, capsicum, cocoa, cotton seed, ground-nut
E. cautella	Tonco bean, cocoa
E. elutella	Cocoa, tobacco, rose petals, pomegranate root bark
Plodia interpunctella	Cinnamon bark and yeast cake
Tinea pellionella	Aconite root, almonds, capsicums, mustard seed, ginger, linseed, orris, saffron and tobacco

ARACHNIDA

The arachnids or 'mites' differ from the true insects in that the mature forms have eight legs but possess no antennae. The members of the Tyroglyphidae (e.g. *Tyroglyphus dimidiatus*, the cheese-mite) are much smaller than the insects, and individuals can only be seen with a lens. If the suspected 'dust' is scraped into a pile, it will be seen to move and will gradually become flat if mites are present in considerable numbers.

Tyroglyphus farinae (*Acarus siro*) (Fig. 15.2A), the flour or meal mite, is 0.4–0.7 mm long. The body is oval in outline, with a truncated posterior, and is clearly divided into two regions—the anterior propodosoma and the posterior hysterosoma. It is common in cereal products, oil-seed cakes and many other commodities.

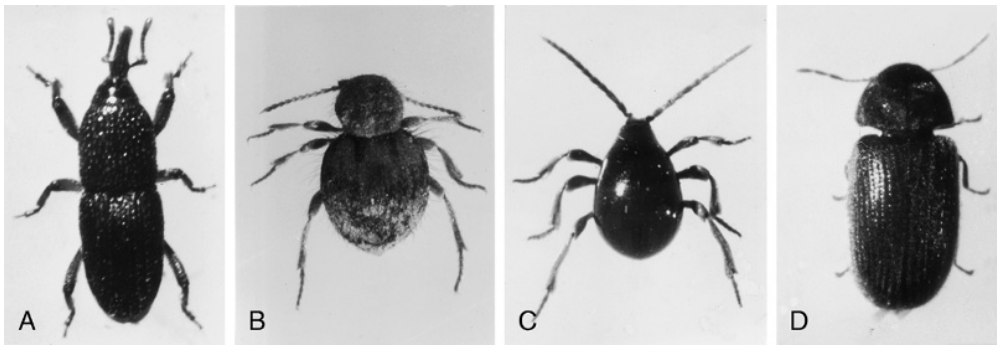


Fig. 15.1
Insect pests found in crude drugs. A, *Calandra granaria*; B, *Ptinus* spp; C, *Gibbium* spp.: (all × 10). D, *Stegobium paniceum* (× 12). (Photos: Dr M. E. Brown)

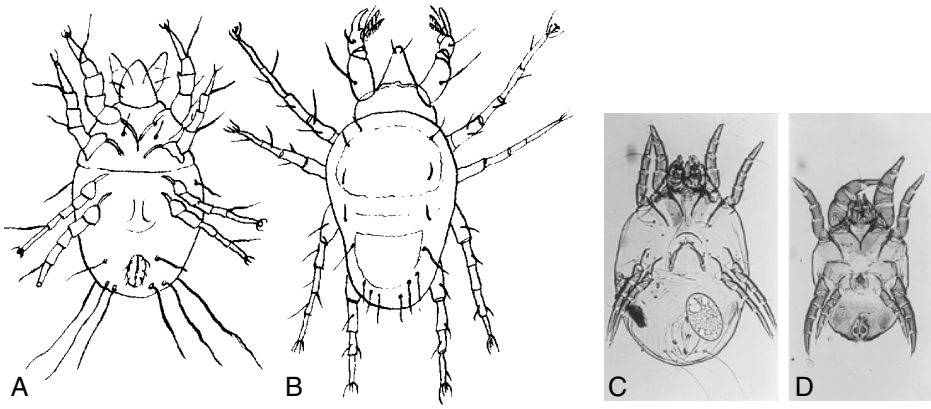


Fig. 15.2
Arachnids. A, *Tyroglyphus farinae*, female, ventral view, × 40. B, *Cheyletus eruditus*, female, dorsal view, × 40. C, *Dermatophagoides culinae*, female, ventral view. D, *D. culinae*, male, ventral view.

The Tyroglyphidae may themselves be attacked by other mites such as *Cheyletus eruditus* (Fig. 15.2B).

A number of different mites commonly attack cantharides, causing considerable damage. Ergot, quince and linseed are other drugs which seem very liable to attack.

Mites commonly exist in ordinary house dust as found under carpets, in mattresses, etc.; and these include *Dermatophagoides pteronyssinus, D. culinae, Glycyphagus domesticus* (the house mite) and *Tyroglyphus farinae* (the flour mite). It has been shown, comparatively recently, that these mites are the allergens responsible for house dust sensitivity, an allergy from which many people suffer. It is now possible to diagnose this condition by prick tests which utilize an extract prepared from *Dermatophagoides culinae*. This mite (Fig. 15.2C) does not show the marked division of the body into two parts exhibited by *Tyroglyphus* spp.

CONTROL OF INFESTATION

The detection, prevention and eradication of mite and insect infestation is an important hygienic and economic consideration for all who have occasion to store and use crude drugs. Effective preventive measures involve *good hygiene* in the warehouse (removal of spillages, old debris and packaging materials; elimination of sources of infections such as floor cracks and crevices), *effective stock control* (regular inspection, rotation of stock, early recognition of infestation), *optimum storage conditions* (maintenance of cool, dry environment) and *good packaging* (woven sacks and bags, multi-ply paper sacks stitched at the seams, paper, polythene film, flimsy cardboard are all penetrable by insects and mites)

If material becomes infested it may be wiser to sacrifice a small consignment rather than to risk contamination of other materials. After a contaminated drug has been removed, pallets, shelves, walls and floors should be thoroughly cleaned and sprayed with a contact insecticide such as chlorpyriphos-methyl or pirimiphos-methyl. Weekly air-spraying with pyrethrins or synthetic pyrethroids, and the use of slow-release dichlorvos strips, control air-borne insects. Various dust formulations may be used in cracks.

Fumigation is the only practical means of killing insects and mites in bulk consignments. Methyl bromide and ethylene oxide have been commonly used and the treatment needs to be applied under gas-proof sheets or in other suitable gas-tight enclosures or chambers. However in Europe the use of ethylene oxide has now been prohibited.

Owing to the possible hazards involved, the work should be performed by professional operators in compliance with the Health and Safety at Work Acts. Before drugs are subsequently used the fumigants must be completely removed as, if they are consumed, they may constitute a health hazard.

Low-temperature storage appears to offer great possibilities. It not only reduces insect attack, but also will, if a sufficiently low-temperature be employed, gradually destroy insects, larvae and eggs. The eggs of *Ephestia elutella* and *E. kuehniella* are rapidly destroyed at −15°C and more slowly at rather higher temperatures.

The eggs of the flour mite can withstand exposure to −10°C for up to 12 days or 0°C for several months; development is possible within the range 2.5–30°C, depending on humidity.

Studies on the effects of ionizing radiations (e.g. from a ^{60}Co source) on cereal pests such as *Tyroglyphus* mites and various beetles show that small doses inhibit reproductive ability and larger ones destroy both mites and their eggs.

The quantitative determination of insect infestation in powdered vegetable drugs is of some significance. Melville (*J. Pharm. Pharmacol*., 1949, **1**, 649; 1951, **3**, 926) developed a method based on the acetolysis of the weighed sample for the quantitative isolation of the insect fragments present. These fragments are suspended in a suitable medium with a weighed quantity of lycopodium spores. Using the fact that the number of strial punctures per elytron is characteristic for a particular beetle, one can, by counting the strial punctures evident on the fragments of elytra present under the microscope, arrive at a figure for the number of beetles present. The use of lycopodium spores (94 000 spores mg^{-1}) eliminates the necessity for counting all the strial punctures present in a particular volume (or weight) of the suspension; the use of lycopodium powder in this respect is described under 'Quantitative Microscopy' (Chapter 43).

SPOILAGE BY RODENTS

Rodent faeces usually contain the animal's hairs, so that drugs which on microscopical examination show the presence of these hairs should be rejected.

Further reading

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