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Quality control

The quality control of crude plant drugs is of paramount importance. In the past, the monographs of national pharmacopoeias adequately covered this aspect for drugs used in the allopathic system of medicine and the *British Herbal Pharmacopoeias* (1983, 1990, 1996) contained descriptions, tests and quantitative standards for those species commonly employed by medical herbalists. However, there was no control on the plant materials used in the many herbal products manufactured for general retail sale. Under current EU regulations, herbal products can only be manufactured under licence in conformity with the 'Rules and Guidance for Pharmaceutical Manufacturers and Distributors 2007', as set out by the Medicines and Healthcare products Regulatory Agency (MHRA) and published by the Pharmaceutical Press, London. Also, the *BP*/*EP* 2007 includes a monograph 'Herbal Drugs', which gives requirements relating to definition, production, identification, various tests, pesticide residues, heavy metal content, microbiological quality and, where necessary, limits for aflatoxins and radioactive contamination. Quality control personnel are required to have particular expertise in herbal medicinal products in relation to the above.

One possible problem in devising standards for crude drugs concerns the requirement for an assay of the active constituents when the latter may not have been precisely ascertained. Furthermore, one of the tenets of herbal medicine is that the maximum effectiveness of the drug derives from the whole drug or its crude extract rather than from isolated components. In cases where an assay is lacking it is therefore important that the crude drug is properly authenticated, its general quality verified and all formulations of it prepared in accordance with good manufacturing practice. Attention should also be paid to the shelf-life of the crude drug and its preparations.

Although official standards are necessary to control the quality of drugs their use does raise certain problems. Of necessity, to accommodate the considerable variation that occurs between different batches of a natural product it is necessary to set reasonable standards which allow the use of commercial material available in any season. This has resulted in a tendency for producers or manufacturers to reduce all of their material to the lowest requirement; for example, in a good year the majority of the alkaloid-rich leaves of belladonna herb may be removed and used for the economical manufacture of galenicals and the residue of the herb, containing much stem, used to give the powdered drug. Similarly, high-quality volatile oils may be mixed with lower grades and still remain within official limits.

STANDARDS APPLICABLE TO CRUDE DRUGS

There are a number of standards, numerical in nature, which can be applied to the evaluation of crude drugs either in the whole or the powdered condition.

Sampling

Before a consignment of a drug can be evaluated, a sample must be drawn for analysis; considerable care must be exercised to ensure that this sample is truly representative. With large quantities of bulky drugs a different method of sampling is required from that involving broken or powdered drugs. The *BP* gives no specific instructions for this but the methods of sampling used in the USA are fully described in older editions of the *USP*. EU guidelines specify that sampling should be carried out by those with particular expertise.

Preliminary examination

In the case of whole drugs the macroscopical and sensory characters are usually sufficient to enable the drug to be identified. The general

appearance of the sample will often indicate whether it is likely to comply with such standards as percentage of seed in colocynth, of ash in valerian or of matter insoluble in alcohol in asafoetida. However, drugs may comply with the descriptions given in the pharmacopoeias and yet be unsatisfactory, as it is often difficult specifically to describe deterioration of drugs owing to faulty harvesting, shipment or storage or deterioration due to age. In such cases the trained worker will be able to infer much of the history of the sample from its appearance. The following examples will serve to indicate the type of evidence to look for.

If leaves and similar structures are baled before being properly dried, much discoloured material may be found in the middle of the bale. Overdrying, on the other hand, makes leaves very brittle and causes them to break in transit. If starch-containing drugs break with a horny fracture, it may usually be inferred that the temperature of drying has been too high and that the starch has been gelatinized. A pale colour in the case of chamomiles indicates that the drug has been collected in dry weather and carefully dried, while the colour of the fractured surface of gentian is a good indication as to whether it has been correctly fermented. Some drugs are particularly liable to deterioration if, during shipment or storage, they become damp (e.g. cascara). Under moist condition moulds readily establish themselves on drugs having a high mucilage content (e.g. psyllium, linseed, squill and cydonia). Evidence of insect attack must also be looked for.

The price of certain drugs depends largely on such factors as size and colour, which are not necessarily related to therapeutic value. This applies to such important drugs as senna leaflets, senna pods, chamomile flowers, ginger, nutmegs and rhubarb.

Foreign matter

The difficulty of obtaining vegetable drugs in an entirely pure condition is fully recognized, and pharmacopoeias contain statements as to the percentage of other parts of the plant or of other organic matter which may be permitted. Table 16.1 gives examples of various official types of limit applicable to specific drugs. Drugs containing appreciable quantities of potent foreign matter, animal excreta, insects or mould should, however, be rejected even though the percentage of such substances be insufficient to cause the rejection of the drug on the percentage of foreign matter.

In the case of whole drugs a weighed quantity (100–500 g, according to the type of drug), of a carefully taken sample is spread in a thin layer on paper. It is examined at ×6 magnification and the foreign matter is picked out and weighed and the percentage recorded. Details will be found in the appropriate *BP* Appendix. For foreign organic matter in powdered drugs, see 'Quantitative Microscopy'.

Moisture content

Not only is the purchase of drugs (e.g. aloes, gelatin, gums) which contain excess water, uneconomical, but also in conjunction with a suitable temperature moisture will lead to the activation of enzymes and, given suitable conditions, to the proliferation of living organisms. As most vegetable drugs contain all the essential food requirements for moulds, insects and mites, deterioration can be very rapid once infestation has taken place.

A large number of methods are now available for moisture determination, many being employed in industries unrelated to pharmacy.

Loss on drying. This is employed in the *BP*/*EP* and *USP*. Although the loss in weight, in the samples so tested, principally is due to water, small amounts of other volatile materials will also contribute to the weight loss. For materials (digitalis, hamamelis leaf, yarrow, hawthorn berries, starch, aloes, fibres) which contain little volatile material, direct drying (100–105°C) to constant weight can be employed. The moisture balance combines both the drying process and weight recording; it is suitable where large numbers of samples are handled and where a continuous record of loss in weight with time is required. For materials such as balsams which contain a considerable proportion of volatile material, the drying may be accomplished by spreading thin layers of the weighed drug over glass plates and placing in a desiccator over phosphorus pentoxide. Vacuum drying over an absorbent may be utilized, possibly at a specified temperature.

Table 16.1 Examples of BP limits for foreign matter.

Drugs	Foreign matter limits
Leaves and herbs	
Bearberry leaf	⩾8% Foreign matter of which ⩾5% stems and ⩾3% other foreign matter. Leaves of different colour to official description ⩾10%
Birch leaf	⩾3% Fragments of female catkins, ⩾3% other foreign matter
Lemon balm	⩾10% Stems having a diameter 1 mm, ⩾2% other foreign matter
Wild thyme	⩾3% Foreign matter (involves recognition of *Thymus vulgaris* and *T. zygis*)
Wormwood	⩾5% Stems with diameter greater than 4 mm, ⩾2% other foreign matter
Fruits and seeds	
Hawthorn berries	⩾2% Foreign matter, ⩾5% deteriorated false fruits
Juniper berries	⩾5% Unripe or discoloured cone berries, ⩾2% other foreign matter
Psyllium seeds	⩾1% Foreign matter including greenish unripe fruits. No seeds of other *Plantago* spp.
Inflorescences	
Calendula flowers	⩾5% Bracts, ⩾2% other foreign matter
Elder flowers	⩾8% Fragments of coarse pedicels and other foreign matter, ⩾15% discoloured brown flowers
Lime flowers	⩾2% Foreign matter, absence of other *Tilia* spp.
Rhizomes and roots	
Couch grass rhizome	⩾15% Greyish-black pieces of rhizome in cut drug
Marshmallow root	⩾2% Brown deteriorated root, ⩾2% cork in peeled root
Valerian root	⩾5% Stem bases, ⩾2% other foreign matter
Barks	
Quillaia bark	⩾2% Foreign matter
Cascara bark	⩾1% Foreign matter

Separation and measurement of moisture. The 'loss on drying' methods can be made more specific for the determination of water by separating and evaluating the water obtained from a sample. This can be achieved by passing a dry inert gas through the heated sample and using an absorption train (specific for water) to collect the water carried forward; such methods can be extremely accurate, as shown in their use for the determination of hydrogen in organic compounds by traditional combustion analysis.

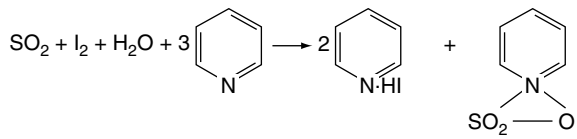
Methods based on distillation have been widely used for moisture determination. The sample to be analysed is placed in a flask together with a suitable water-saturated immiscible solvent (toluene, xylene, carbon tetrachloride) and pieces of porous pot and is distilled. The water in the sample has a considerable partial pressure and co-distils with the solvent, condensing in the distillate as an immiscible layer. A simple apparatus (Fig. 16.1A) originally devised by Dean and Stark permits the direct measurement of the water obtained and the less dense solvent (toluene, xylene) is continuously returned to the distillation flask. The method is employed in the *USP* and in the *BP/EP* for some volatile oil-containing drugs (Roman chamomile flowers, lovage root, eucalyptus, peppermint and sage leaves) and aniseed and star-anise fruits. To accommodate the loss of water due to solubility in the solvent the *BP* specifies a preliminary distillation of the solvent with added water (about 2 ml); the exact volume of water separating as a layer is read off and then the drug (sufficient to give a further 2–3 ml water) added to the flask and distillation resumed. Water separated from the drug is calculated from the combined final volume. Heavier-than-water solvents require the receiver shown in Fig. 16.1B. The method is readily applicable to crude drugs and food materials but has the disadvantage that relatively large quantities of the sample (5–10 g) may be required.

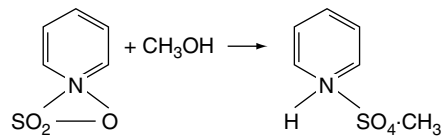
Gas-chromatographic methods have become increasingly important for moisture determination because of their specificity and efficiency. The water in the weighed, powdered sample can be extracted with dry methanol and an aliquot submitted to chromatography on a column on either 10% carbowax on Fluoropak 80 or Porapak, a commercial polymer suitable for gas–liquid chromatography (GLC). The water separated by this means is readily determined from the resulting chromatogram. Teflon-6 coated with 10% polyethylene glycol 1500, with *n*-propanol as an internal standard has also been employed for the determination of moisture in crude drugs.

Chemical methods. The most extensively employed chemical method for water determination is probably the Karl Fischer procedure, which finds use not only in the pharmaceutical, but also in the food, chemical and petrochemical industries. It is used in the *BP* and is particularly applicable for expensive drugs and chemicals containing small quantities of moisture [very small quantities of water (10 μg to 10 mg) are determined quantitatively by coulometric titration, see below]. Dry extracts of alkaloid-containing drugs, alginic acid, alginates and fixed oils (e.g. arachis, castor, olive and sesame oils for *BP* parenteral use) may usefully be evaluated. For crude drugs such as digitalis and ipecacuanha the powdered material can first be exhausted of water with a suitable anhydrous solvent (dioxan) and an aliquot taken for titration.

The Karl Fischer reagent consists of a solution of iodine, sulphur dioxide and pyridine in dry methanol. This is titrated against a sample containing water, which causes a loss of the dark brown colour. At the end-point when no water is available, the colour of the reagent persists. The basic reaction is a reduction of iodine by sulphur dioxide in the presence of water. The reaction goes to completion by the removal of sulphur trioxide as pyridine sulphur trioxide, which in turns reacts with the methanol to form the pyridine salt of methyl sulphate, see formulae below.

$$H_2O + I_2 + SO_2 \rightleftharpoons 2HI + SO_3$$





$$H_2O = I_2$$

In the absence of methanol, the pyridine sulphur trioxide reacts with another molecule of water. The reagent requires standardization immediately before use and this can be done by employment of a standard solution of water in methanol or by use of a hydrated salt—for example, sodium tartrate ($Na_2C_4H_4O_6 \cdot 2H_2O$). To eliminate interference from atmospheric moisture, the titration is carried out under an atmosphere

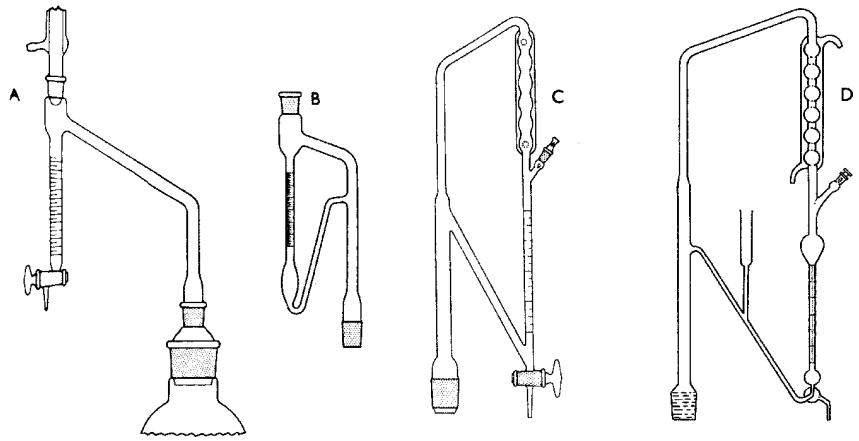


Fig. 16.1
A, Apparatus for the determination of moisture in crude drugs by distillation and for volatile oils heavier than water; B, receiver of apparatus for the determination of water in crude drugs (heavy entrainment) and for volatile oils in drugs; C, receiver for determination of volatile oil in drugs as used by the *BP* 1980; D, receiver for determination of volatile oil in drugs as used by both the *EP* and the *BP*.

of dry nitrogen, the end-point being recorded amperometrically. Equipment is now available for a completely automated determination, thus eliminating the manual aspects of sample handling and weighing, introduction to the Karl Fischer cell, titration and data completion. Although the *BP* Karl Fischer reagent contains pyridine, as above, the latter has been replaced by other bases (e.g. imidazoles) in some commercial reagents. Alternatives to methanol, such as diethylene glycol monoethyl ether, have been introduced to improve reagent stability.

The principal drawbacks of the Karl Fischer method are the instability of the reagent and the possibility of substances in the sample, other than water, which may react with the reagent.

The coulometric method for the quantitative determination of water relies on the same basic reactions as indicated above for the Karl Fischer procedure. However, the iodine is produced electrochemically at the anode by oxidation of iodide and reacts immediately with the sulphur dioxide and water from the sample. When all the water is used up, iodine is produced in excess and this is the electrochemical end-point. If necessary, moisture in a solid sample can be evaporated and passed into the reaction vessel in a stream of dry inert gas. The method is employed for the measurement of the very small amounts of water permissible in fixed oils used for the preparation of parenteral dosage forms; examples include a maximum of 0.1% for soya, olive and evening primrose oils.

Other chemical methods for water determination include treating the sample with various carbides, nitrides and hydrides and measuring the gas evolved; gas chromatography has been employed for the analysis of the liberated gas.

Spectroscopic methods. Water will absorb energy at various wavelengths throughout the electromagnetic spectrum and this fact can be made a basis for its quantitative determination (see later in this chapter for a general discussion on spectroscopy). Measurements can be made in both the infrared and ultraviolet regions; interfering substances must be absent. The method is particularly suitable for very small quantities of water (e.g. trace quantities in gases). Nuclear magnetic resonance (NMR) spectroscopy has been employed for the determination of moisture in starch, cotton and other plant products.

Electrometric methods. Conductivity and dielectric methods have both been utilized for moisture determination but have not, as yet, found extensive application to pharmaceutical products.

Extractive values

The determination of water-soluble or ethanol-soluble extractive is used as a means of evaluating drugs the constituents of which are not readily estimated by other means. But as suitable assays become available (e.g. with the anthraquinone-containing drugs), some of the previously used extractive tests are no longer required as pharmacopoeial standards. In certain cases extraction of the drug is by maceration, in others by a continuous extraction process. For the latter the Soxhlet extractor is particularly useful and has been in use for many years, not only for the determination of extractives (e.g. fixed oil in seeds) but also for small-scale isolations (Fig. 16.2). A development of the Soxhlet technique is also shown in Fig. 16.2; in this apparatus extraction is by boiling solvent followed by percolation; finally, evaporation yields the extract and the recovered solvent ready for the next sample. Some examples of the types of extractive used are given in Table 16.2.

Ash values

When vegetable drugs are incinerated, they leave an inorganic ash which in the case of many drugs (e.g. rhubarb) varies within fairly wide limits and is therefore of little value for purposes of evaluation. In other cases (e.g. peeled and unpeeled liquorice) the *total ash* figure is of importance and indicates to some extent the amount of care taken in the preparation of the drug. In the determination of total ash values the carbon must be removed at as low a temperature (450°C) as possible without producing flames. If carbon is still present after heating at a moderate temperature, the water-soluble ash may be separated and the residue again ignited as described in the *BP*, or the ash may be broken

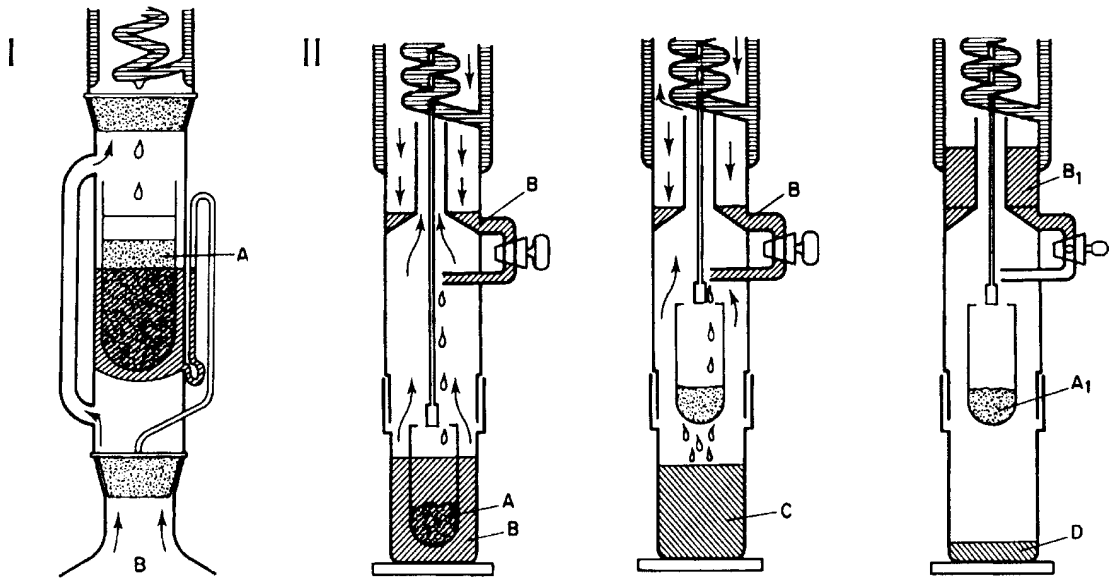


Fig. 16.2
I, Soxhlet continuous extraction apparatus. A, powdered drug for extraction in thimble and plugged with suitable fibre e.g. defatted tow or cotton wool; solvent refluxes into thimble and syphons into flask B, containing boiling solvent, when receiver is full. II, Three-stage continuous extraction and solvent recovery: left, extraction by boiling with solvent; centre, percolation stage; right, removal of solvent. A, sample for extraction; A_1, exhausted drug; B, solvent; B_1, recovered solvent; C, solvent containing soluble plant constituents; D, final extract. (Soxtec System, Tecator Ltd.)

Table 16.2 Extractives employed for drug evaluation.

Drug	Method of evaluation
Gentian, liquorice, many drugs of *BHP*	Percentage of water-soluble extractive
Quillaia	Percentage of ethanol (45%) extractive
Valerian, cocillana, asafoetida	Percentage of ethanol (60%) extractive
Ginseng, hop strobiole	Percentage of ethanol (70%) extractive
Ginger, ipomoea and jalap	Percentage of ethanol (90%) extractive
Benzoin, catechu and Tolu balsam, myrrh	Limits of ethanol-insoluble matter
Colocynth	Limit of light petroleum extractive
Crushed linseed	Percentage of ether-soluble extractive

up, with the addition of alcohol, and again ignited. The total ash usually consists mainly of carbonates, phosphates, silicates and silica.

To produce a more consistent ash, the *Pharmacopoeia* utilizes a *sulphated ash*, which involves treatment of the drug with sulphuric acid before ignition, whereby all oxides and carbonates are converted to sulphates. Two methods are given. The first, employed unless otherwise directed, involves moistening a weighed quantity of the drug with (concentrated) sulphuric acid followed by gentle ignition and then repeating the moistening of the charred drug with subsequent firing at 800°C. The ignition is repeated until a constant weight of ash is achieved. The second method utilizes sulphuric acid R for moistening the drug, followed by gentle heating until the drug is charred; after cooling, a second 1 ml of sulphuric acid R is added and the ignition is continued at 600°C ± 50°C until complete incineration is achieved. If the residue exceeds the prescribed limit the process is repeated until a constant weight (within 0.5 mg) is obtained or until the residue complies with the prescribed limit.

If the total ash be treated with dilute hydrochloric acid, the percentage of *acid-insoluble ash* may be determined. This usually consists mainly of silica, and a high acid-insoluble ash in drugs such as senna, cloves, liquorice, valerian and tragacanth indicates contamination with earthy material. Senna leaf, which may be used directly as the powdered drug, is required to have a low acid-insoluble ash (2.5%); hyoscyamus, however, which unavoidably attracts grit on to its sticky trichomes, is allowed a higher value (12%). Horsetail *BP*/*EP*, the dried sterile stems of *Equisetum arvense*, has a natural content of silica and the acid-insoluble ash value should lie within the limits 10–15%. In the case of ginger a minimum percentage of *water-soluble ash* is demanded, this being designed to detect the presence of exhausted ginger.

Crude fibre

The preparation of a crude fibre is a means of concentrating the more resistant cellular material of drugs for microscopical examination. It is particularly useful for rhizomes such as ginger which contain relatively large amounts of oleoresin and starch. The technique involves defatting the powder and boiling in turn with standard acid and alkali with suitable washing of the insoluble residue obtained at the different stages (see Chapter 43). The crude fibre so obtained can also be employed quantitatively to assay the fibre content of foods and animal feedstuffs and also to detect excess of certain materials in powdered drugs, e.g. clove stalk in clove. For further details see the 14th edition of this book.

Determination of volatile oil

Minimum standards for the percentage of volatile oil present in a number of drugs are prescribed by many pharmacopoeias. A distillation method is usually employed, and the apparatus first described by Meek and Salvin in 1937 is still widely used in laboratories; the receiver for this apparatus is very similar to that for water estimation by heavy entrainment (Fig. 16.1B). The weighed drug is placed in a distillation flask with water or a mixture of water and glycerin and connected to the receiver (cleaned with chromic acid), which is filled with water and connected to a condenser. On distillation, the oil and water condense and the volatile oil which collects in the graduated receiver as a layer on top of the water is measured. For oils with relative densities around or greater than 1.00, separation from the water is assisted by placing a known volume of xylene in the receiver and reading off the combined oil and xylene. Alternatively, for oils with relative densities greater than water (e.g. clove oil, 1.05), a receiver similar to the type shown in Fig. 16.1A can be used and no xylene is necessary. The *BP* 1980 employed the apparatus illustrated in Fig. 16.1C; it differs from the above in that the distillate passes through the condenser and so is cooler than with the reflux type. The *BP* (2007) employs an apparatus similar to that shown in Fig. 16.1D. The time taken to complete the distillation of the oil varies with the nature of the drug and its state of comminution but about 4 h is usually sufficient. Solution of the volatile oil in a fixed oil (e.g. in powdered fruits of the Umbelliferae) may retard distillation. Note that the pharmacopoeial standards for volatile oil contents of powdered drugs are lower than those for the corresponding whole drugs.

Tannin content

A number of drugs (Agrimony, Alchemilla, Hamamelis, Loosestrife, Oak bark, Rhatany, Tormentil) are assayed for their tannin contents (*BP* 2007). The method refers to those polyphenols adsorbed by hide powder and giving a colour reaction with sodium phosphomolybdotungstate reagent. See individual drugs.

Bitterness value

This standard is relevant to Bogbean leaf, Centaury, Gentian and Wormwood of the *BP*. These drugs are used for their bitter effect and specific directions for the determination of the standard are given under each monograph. The bitterness value is determined organoleptically by comparison with a quinine hydrochloride solution which acts as the standard.

Swelling index

This is defined in the *BP* as the volume in millilitres occupied by 1 g of a drug, including any adhering mucilage, after it has swollen in an aqueous liquid for 4 h. The drug is treated with 1.0 ml ethanol (96%) and 25 ml water in a graduated cylinder, shaken every 10 min for 1 h and allowed to stand as specified. In some instances, as with linseed and psyllium seed where the mucilage is in a layer near the surface of the drug, the standard can be determined on the whole drug; in other cases such as marshmallow root where the mucilage is distributed throughout the tissues, the powdered drug is used. Examples are: Agar ⩽10, Cetraria ⩽4.5, Fenugreek ⩽6, Fucus ⩽6.0, Ispaghula husk ⩽40 (determined on 0.1 g powder), Ispaghula seed ⩽9, Linseed ⩽4 (whole drug) and ⩽4.5 (powdered drug), Marshmallow root ⩽10, Psyllium ⩽10.0.

Some variations in the method of determination have given rise to other terminology. Thus Skyrme and Wallis in their original work on seeds of *Plantago* spp. in 1936 used the term *swelling factor* (24-h standing period) and the *BP* (1993) cites *swelling power* in respect of

Ispaghula husk (variation in shaking procedure and standing time). Yet again, for Iceland moss the *BP* 2007 cites *swelling value*, ⩽4.5.

R_F values

Pharmacopoeias are increasingly employing thin-layer chromatography as a means for assessing quality and purity. For a discussion of chromatographic analysis, see Chapter 17. It suffices to mention here that the R_F value (rate of flow, i.e., distance moved by solute divided by distance moved by solvent front) of a compound, determined under specific conditions, is characteristic and can be used as an aid to identity. R_F values vary from 0.0 to 1.00; the hR_F ($R_F \times 100$) values are in the range 0–100. Quantitative extracts of crude drugs are prepared and compared chromatographically with standard reference solutions of the known constituents. Intensities of the visualized chromatographic spots can be visually compared and the method can be used to eliminate inferior or adulterated drugs. In this way semiquantitative tests for the principles of drugs (peppermint, saffron, German chamomile, digitalis) not rapidly evaluated by other means have been developed.

In an analogous manner, gas chromatographic retention times and peak areas can be employed for the examination of volatile oils and other mixtures.

Microbial contamination

The *BP* requires a number of drugs (e.g. acacia, agar, pregelatinized starch, sterculia, tragacanth, powdered digitalis etc.) to be free of *Escherichia coli* in the quantity of material stated; others (e.g. alginic acid, cochineal, guar, tragacanth) are also tested for the absence of *Salmonella*. Upper limits for total viable aerobic count, commonly 10^3, 10^4 microorganisms g^{-1}, are being increasingly applied to crude drugs including the gums, Agar, Tragacanth, Acacia, Guar and Guar Galactomannan. Xanthan gum (q.v.) produced by fermentation has limits of 10^3 for bacteria and 10^2 for fungi, g^{-1}. Generally, manufacturers will ensure that, for crude drugs to be taken internally, the limits for bacterial and mould contamination as applied to foodstuffs are adhered to.

In an investigation by Lutomski and Kedzia (*Planta Med*., 1980, **40**, 212) of mould contamination of crude drugs, 246 samples were examined and 24% were contaminated at the level of 10 000 organisms g^{-1}. From 50 crude drugs, 75 *Aspergillus* and 28 *Penicillium* strains were isolated; other common genera were *Mucor*, *Rhizopus* and *Thamnidium*. There was no evidence to show that moulds were producing strongly toxic substances in the crude drug and herbal preparations, in contrast to the embryotoxic, teratogenic, mutagenic and carcinogenic substances produced by some species of the above in peanuts, corn, wheat and rice (see Chapter 39). However, in a series of papers Roy and colleagues (*Int. J. Crude Drug Res.*, 1990, **28**, 157; *Int. J. Pharmacognosy*, 1991, **29**, 197 and references cited therein) have shown that roots (e.g. *Acorus calamus*, *Picrorrhiza kurroa*) and seeds (Neem and *Datura*) stored under traditional storage conditions in India develop unacceptable levels of mycotoxins, principally aflatoxin B_1; the effects of temperature, relative humidity and light on the elaboration of aflatoxin B_1 have also been studied. By the use of mass spectrometry, mycotoxins in food products such as cereals, oil seeds and milk are regularly determined at levels of one part per billion and below.

For a review on the microbial contamination of medicinal plants, see W. Kneifel *et al.*, *Planta Medica*, 2002, **68**, 5.

Toxic residues

These may arise in crude drugs as a result of pesticide application during cultivation of the drug and at a later stage from fumigation of the stored product. The problems and the nature of the toxic residues are essentially those encountered in the food industry and in a number of countries regulations exist to cover limits of these residues in foods, cosmetics, drugs and spices. Appendix XI L of the *BP* 2007 gives the requirements relating to pesticide residues for herbal drugs; specific directions for the sampling of bulk materials are given and the various insecticides and their assays listed. In certain instances it may be necessary to test for aflatoxins and radioactive impurities. It has been reported that many spices, chamomile and valerian obtained commercially contain pesticide residues, but mainly within acceptable levels.

Thin-layer chromatography (TLC) and gas chromatographic methods are available for the determination of organochlorine and urea derivatives, enzymatic methods for organophosphorus compounds, colorimetric methods for urea derivatives, and spectroscopic techniques for paraquat, triazines and heavy metals.

Toxic residues may be substantially reduced or eliminated by the use of infusions of the dried plant material and by the extraction of the useful plant constituents. Storage at 30 ºC has been shown to reduce rapidly the ethylene oxide residues in senna pods to tolerable levels. Much research has been devoted to the harmful effects of ethylene oxide which is a very effective insecticide and acaricide; for an in-depth report see Golberg (1986), *Hazard Assessment of Ethylene Oxide*, Boca Raton, FL., USA: CRC Press.

Heavy metal accumulation

Herbal drugs, like foods, should comply with the WHO guidelines, and the Pharmacopoeial monograph 'Herbal Drugs', with respect to heavy metal content. Small quantities of trace elements are invariably present in plant materials and, indeed, some, such as zinc, copper and molybdenum, appear to be necessary microcomponents of a normal diet. However, under certain circumstances the levels of some metals, particularly those of lead, cadmium, copper and mercury, can increase to unacceptable concentrations. This may arise either by the deliberate inclusion of, for example, mercury compounds in a particular herbal formulation or by the natural accumulation of heavy metals in herbs growing under particular environmental conditions. Mercury was once accepted as a common ingredient of Western medicines (see the *BPC* of 1949) and, more recently, has appeared in Asian medicines exported world-wide to immigrant communities. In the second instance, increased levels of heavy metals can arise from the nature of the soil and via atmospheric pollution. It is of interest to note that one method of prospecting for metals has involved the analysis of the above-ground flora of the area involved; in this respect, some plants are more prone to metal accumulation than others.

The published work on this aspect of herbal drugs is somewhat limited. For recent studies readers are referred to research by V. Rai *et al.* (*Pharm. Biol.*, 2001, **39**, 384) on the accumulation of lead, cadmium, copper and zinc in nine important drugs of Indian medicine. Samples were obtained from various localities in India and consisted of both authenticated material as well as market samples. In most cases, the concentrations of lead and cadmium exceeded the permissible WHO limits; concentrations varied in the same plant obtained from different localities and the authors attributed this to the industrial activity of the region and possible vehicular pollution.

Limitations for particular metals are placed on some products that have been chemically manipulated, for example, nickel in hydrogenated soya and arachis oils. There are pharmacopoeial limits for iron, chromium and zinc in gelatin, cadmium in linseed oil and iron in pilocarpine salts. Determination is by atomic adsorption spectroscopy after acid digestion of the sample with concentrated nitric, hydrochloric and sulphuric acids. Adsorption is measured at the following wavelengths: Cd 228.8 nm, Cu 324.8 nm, Fe 248.3 nm, Ni 232 nm, Pb 283.5 nm and Zn 213.9 nm; separate techniques are given for As 193.7 nm and Hg 253.7 nm.

Test for aristolochic acids

This test is included in the *Pharmacopoeia* primarily to detect the presence of these acids in unlicensed herbal medicines. They are present in various species of *Aristolochia* and *Asarum*, which may be used either as substitutes or adulterants in certain traditional Chinese medicines. Because of the nephrotoxic and carcinogenic properties of aristolochic acid, the use of *Aristolochia* has been prohibited in the UK since 1991, see 'Serpentary'; with a further statutory prohibitive order in 2001. Other regulatory orders are in force world-wide. In spite of this, such products periodically appear on the market, a recent example being the availability of Xie Gan Wan pills in South Wales (see MHRA warning, *Pharm. J*., 2007, **279**, 224).

For the pharmacopoeial test, the sample is shaken with 0.1 M sodium hydroxide for at least 2 hours and the filtered solution purified using a solid-phase extraction column. It is then subjected to liquid chromatography and the eluate monitored at 225 nm. No peaks due to aristolochic acid I and arictolochic acid II should appear, as evidenced by comparison with the chromatogram of a reference solution of these two acids.

STANDARDS APPLICABLE TO VOLATILE AND FIXED OILS

Certain standards are particularly appropriate to volatile oils and fixed oils.

Refractive index

The refractive index of a substance is the ratio between the velocity of light in air and the velocity in the substance under test. For light of a given wavelength (it is usual to employ the D line of sodium, which has a doublet of lines at 589.0 nm and 589.6 nm), the refractive index of a material is given by the sine of the angle of incidence divided by the sine of the angle of refraction. The refractive index varies with the temperature, and pharmacopoeial determinations are made at 20°C.

A convenient instrument is the Abbé refractometer, in which the angle measured is the 'critical angle' for total reflection between glass of high refractive index and the substance to be examined. By this means, and by selecting a particular wavelength of light at which to make the measurements, it is possible to calibrate the instrument directly in terms of refractive index. It is the emergent beam that is viewed in the instrument, and the critical angle is indicated by the edge of the dark part of the field of view. In this instrument the need for a monochromatic light source is eliminated by the inclusion of a dispersion 'compensator' placed at the base of the telescope tube of the refractometer. This consists of two direct-vision prisms made accurately direct for the D sodium line; the prisms can be made to rotate in opposite directions. The system of variable dispersion which these prisms form can be made to counterbalance the resultant dispersion of the refractometer prism and the material being examined. The temperature of the sample is adjusted by a water jacket.

Automatic refractometers such as the Leica Auto Abbé refractometer are now available. Advantages over the traditional 'visual' transmitted light instruments are: (1) they measure refractive index with precision to the fifth decimal place, compared with four decimal places for the visual refractometer (however, current pharmacopoeias require only three decimal places in standards for volatile oils); (2) a reflected light principle is employed meaning that light is not transmitted through the sample and hence problems with dark or coloured samples are avoided; (3) the shadow-line location is determined by the instrument software, eliminating variations in readings caused by individual subjective interpretations of the placement of the shadowline border on a crosswire; (4) no mechanical components are involved, thus reducing wear with time. However, if the increased sensitivity of such instruments is to be fully utilized then additional care and consideration must be given to the measurement of temperature and to the correction for effect of temperature on refractive index.

Measurements of refractive index are particularly valuable for purity assessments of volatile and fixed oils, and many values can be found in the *EP*, *BP*, *BPC* and other pharmacopoeias. Oils of cassia, cinnamon and cinnamon leaf have refractive indices of about 1.61, 1.573–1.600 and about 1.53, respectively, making possible the differentiation of the oils. The refractive index of lemon oil is 1.474–1.476 and that for terpeneless lemon oil 1.475–1.485.

Optical rotation

The optical rotation of a liquid is the angle through which the plane of polarization of light is rotated when the polarized light is passed through a sample of the liquid; this rotation may be either clockwise or anticlockwise. Along with the fundamental effects of the molecules of liquid under investigation, the observed rotation is dependent on the thickness of the layer examined, its temperature and the nature of the light employed. The *BP* uses the D-line of sodium (λ = 589.3 nm), a layer 1 dm thick and a temperature of 20°C. With 'half-shadow' or 'triple-shadow' polarimeters in which the two or three fields are viewed simultaneously and matched to the same intensity, rotations can be measured with an accuracy of at least ± 0.01 degree.

Most volatile oils contain optically active components and the direction of the rotation, and its magnitude, is a useful criterion of purity. Examples to illustrate the range of values found are caraway oil, +74° to +80°; lemon oil, +57° to +70°; terpeneless lemon oil, −5° to +2°; cinnamon oil, 0° to −2°; citronella oil, Java, −5° to +2°; citronella oil, Ceylon, −9° to −18°; nutmeg oil, +8° to +18°; peppermint oil, −10° to −30°; spearmint oil, −45° to −60°.

Many solid materials of natural origin are optically active and the rotation of their solutions (water, ethanol and chloroform are common solvents) can be measured in a similar way to the above. The specific rotation of the solid is given by

$$\frac{\text{Angular rotation per dm of solution}}{\text{Grams of optically active substance per ml of solution}} = \frac{100\alpha}{lc} = \frac{100\alpha}{ldp}$$

where α is the observed rotation in degrees, l is the length of the observed layer in dm, c is the number of grams of substance contained in 100 ml solution, d is the density and p is the number of grams of substance contained in 100 g of solution. The record of the specific rotation of a compound should include the solvent used and the concentration, in addition to the type of light employed (sodium D line of 589.3 nm or mercury green line of 546.1 nm)—for example, $[\alpha]_{\mathrm{D}}^{20°}$ (2.0% in ethanol) = −15°.

For examples of the use of specific rotation as a physical constant, students can consult the *BP* or other pharmacopoeial monographs on alkaloidal salts.

The visual polarimeter requires the use of solutions which are not highly coloured or to any extent opaque, and sometimes, with plant extracts, such solutions are difficult to obtain. Because angular rotation falls off linearly, whereas absorption does so exponentially with decrease in path length, the use of short sample tubes has an obvious advantage if it is possible to measure accurately the correspondingly small rotations. Automatic polarimeters employ a sample tube about 1/10th the length of that required by a visual polarimeter. Measurement

of small rotations is made possible by utilization of the Faraday electro-optic effect; this involves the rotation of the plane of a polarized beam of light in a magnetic field, the degree of rotation being proportional to the field strength. The instrument is zeroed by means of two solenoids through which passes the polarized beam. The insertion of an optically active solution between the solenoids affords a recordable signal, either (−) or (+), which is generated by a photomultiplier at the end of the light path.

Chiral purity

Optical rotation as described above arises within molecules having at least one asymmetric carbon atom. Such molecules have two possible configurations (enantiomers), which are non-superimposable mirror images of one another and exhibit opposite light-rotational properties [(−) and (+)]. Plants synthesize just one enantiomer, which, under certain conditions, may partially change to the opposite isomer. Equal quantities of both are known as racemic mixtures and have zero rotation; thus, for the alkaloid hyoscyamine, $[\alpha]_D$ in 50% ethanol = −22° and its racemate atropine is optically inactive. Synthetically produced compounds are normally racemates. The relatively recent introduction of chiral chromatography has provided a method for the quantitative separation of enantiomers and has found use as a standard for volatile oils, which are susceptible to adulteration with the synthetic product. The *Pharmacopoeia* uses chiral gas chromatography employing fused silica columns 30 m in length with a stationary phase of modified β-cyclodextrine. Chiral purity tests are specified for the volatile oils of caraway, neroli and lavender, and also for carvone.

Quantitative chemical tests

A number of quantitative chemical tests—acid value, iodine value, saponification value, ester value, unsaponifiable matter, peroxide value, anisidine value, acetyl value, volatile acidity—are mainly applicable to fixed oils and are mentioned in Chapter 19. Some of these tests are also useful in the evaluation of resins (acid value, sulphated ash), balsams (acid value, ester value, saponification value), volatile oils (acid value, acetyl value, peroxide value, ester value) and gums (methoxyl determination, volatile acidity).

ASSAYS

A crude drug may be assayed for a particular group of constituents—for example, the total alkaloids in belladonna or the total glycosides of digitalis. Alternatively, it may be necessary to evaluate specific components—for example, the reserpine content, as distinct from the total alkaloid content, of *Rauwolfia* spp. Biological assays, which can be time-consuming, were at one time employed for the assay of those potent drugs (e.g. digitalis) for which no other satisfactory assay was available. In pharmacopoeias these have now been largely replaced by chemical and physical assays for routine standardization. However, the biological assay remains important for screening plant materials and their fractionated extracts in the search for new drugs. In this respect there is a role for simple biological assays (e.g. brine shrimp toxicity) which can be carried out by the phytochemist without the specialist procedures used by pharmacologists. Some types of assay commonly employed are given in Table 16.3. Often a preliminary purification or fractionation of the active constituents of the drug is required and for this chromatography is finding increasing use. Examples of chromatographic systems employed are listed in Table 16.4 and are more fully explained in Chapter 17. Spectrometric methods, particularly in conjunction with chromatography, are finding increasing use and are dealt with more fully below.

Spectroscopic analysis

The electromagnetic vibrations utilized in spectroscopic analysis can be roughly divided, according to wavelength, into the ultraviolet (100–400 nm), the visible (400–800 nm), the near-infrared (800–3000 nm) and the infrared (3–40 μm) regions. The ultraviolet region can be subdivided into three further categories—UVC (100–290 nm), UVB (290–320 nm) and UVA (320–400 nm). These are often quoted in connection with sunlight—all UVC is absorbed by the ozone layer of the atmosphere, UVB is present in small amount but is primarily responsible for major skin damage and UVA, although the major component, is far less damaging. In spectroscopic analysis we are concerned with the capacity of certain molecules to absorb vibrations

Table 16.3 Types of assay employed for crude drugs.

Types of assay	Examples
Separation and weighing of active constituents	Colchicine in colchicum corm and seed. Resins of podophyllum and of the Convolvulaceae. Crude filicin in male fern. Total balsamic esters of Peru balsam
Chemical	'Total alkaloids' of many drugs (e.g. acid–base titrations). Non-aqueous titrations of alkaloid salts. Strychnine in nux vomica. Morphine in opium. Cinnamic aldehyde in oil of cinnamon. Free alcohols in peppermint oil. Carvone in oil of caraway. Assay of fumitory
Physical	Cineole in eucalyptus oil (f.p. of *o*-cresol complex)
Spectrometric, including colorimetric and fluorescence	Most groups of active constituents
Biological	Cardioactive drugs, antibiotics, vitamins, taenicides, anthraquinone derivatives, mydriatic drugs, saponins, antitumour drugs, antiamoebic drugs, ginkgolides (anti-PAF activity)
Radioimmunoassay (RIA)	Hesperidin, limonin and naringin (*Citrus*), cardenolides (*Digitalis lanata*), loganin (plant cell cultures), sennosides (*Cassia angustifolia*), tropane alkaloids (medicinal Solanaceae), morphine and related alkaloids (poppy capsules), lysergic acid derivatives (ergot), quinine (cultured plant tissues), ajmaline (*Rauwolfia* spp.), vincristine and related alkaloids (*Catharanthus roseus*), solasodine (*Solanum* spp.)
Enzyme-immunoassay (ELISA)	Quassin, neoquassin, 18-hydroxyquassin (*Quassia* and *Picrasma* spp.), podophyllotoxin, tropane alkaloids (Solanaceae), artemisinin in *Artemisia annua*, pyrrolizidine alkaloids, ergot alkaloids, galanthamine in *Leucojum aestivum*, saikosaponina in *Bupleurum*, ginsenosides

Table 16.4 Chromatographic systems employed in the analysis of drugs.

Type	Employment
Liquid chromatography; *BP* uses stainless steel columns of varying size; typical packing is chromatographic octadecylsilyl silica gel	Arnica flower, Cola, Devil's claw, Garlic, Goldenseal root, Opium, Papaveretum, triglycerides of fixed oils (e.g. Refined sesame oil)
Gas chromatography	Volatile oils; *BP* gives chromatographic profiles for some drugs e.g. to aid distinction between Aniseed oil from star-anise and that from *Pimpinella anisum*. Separation of fatty acids derived from fixed oils. Fatty acid content of fruits, e.g. Saw Palmetto Fruit
Thin-layer chromatography	Extensively used in *BP* and *BHP* as an identification test and test for purity. Separated constituents can be removed from chromatogram and determined spectrometrically

at specific wavelengths. Thus, the butenolide side-chain of cardiac glycosides is responsible for a strong absorption at 215–220 nm, the conjugated double bonds of lycopene (a pigment of tomatoes and other fruits) give rise to the absorption of light at a wavelength of 470 nm, thus giving a red colour, and the carbonyl group of ketones, carboxylic acids and esters is responsible for a strong absorption in the infrared at about 5.7–6.1 μm. In the ultraviolet and visible regions the characteristic absorption spectrum of a molecule is produced by changes in the electronic energy levels associated with various chromophoric groups within the molecule. These changes involve the absorption of relatively high amounts of energy (in precise quanta), and they are also accompanied by changes in vibrational and rotational energy changes within the molecule. The result is a banded absorption spectrum showing no sharply defined peaks. By comparison, the absorption spectrum of a molecule in the infrared region is much more complex, because here the energies involved are too small to bring about electronic transitions but large enough to produce numerous vibrational and associated rotational energy changes. Each of these changes is associated with a characteristic wavelength and the spectrum shows a much finer structure than in the visible or ultraviolet regions. The infrared spectrum of a molecule can be divided into the 'fingerprint' region (7–11 μm), which is characteristic of the molecule under examination but in which it is difficult to assign peaks to specific vibrations, and the remainder of the spectrum, in which many functional groups can be recognized. The *BP* employs the comparison of infrared spectra of phytochemicals (pilocarpine, physostigmine, etc.) with *European Pharmacopoeia Chemical Reference Substances* (EPCRS) as a test of identity.

The *BP* uses ultraviolet absorption characteristics as standards for benzylpenicillin, lanatoside C and a number of alkaloids—for example, morphine, reserpine, cocaine, colchicine and tubocuraine chloride.

If light of a particular wavelength is passed through a solution of a substance, the transmission $T = I/I_0$, where I_0 is a measure of the light reaching the detector (a photoelectric cell) when solvent alone is used in the light-path and I is the light reaching the detector when a solution of the substance under investigation is examined. T is measured in experiments but the most useful value is $\log_{10}(I_0/I)$, the decimal optical density or simply the optical density (E). The optical density, but not the transmission, is proportional to the number of absorbing units in the light-path. For solutions this is Beer's law. The absorption spectrum of a pure substance under defined conditions of solvent and temperature is a set of values of E observed at different wavelengths in a solution of unit concentration (1 mol l^{-1}) when the thickness of the layer traversed by the light is 1 cm. Alternatively, any other solution of known strength may be used. For example, for a 1% w/v solution with a layer thickness of 1 cm the optical density is indicated by $E_{1cm}^{1\%}$. Such absorption spectra are valuable for the identification, determination of the structure and purity and analysis of compounds. Some substances will absorb ultraviolet light of a certain wavelength and during the period of excitation re-emit light of a longer wavelength and often in the visible region. This is fluorescence, and the fluorescence spectrum is characteristic for those substances which exhibit the phenomenon. The applications of fluorescence analysis are discussed below.

For the *quantitative evaluation* of a substance, a standard curve is first prepared by measuring the optical densities of a series of standard solutions of the pure compound by use of light of a suitable wavelength, usually that at which the compound gives an absorption maximum. The solutions must be sufficiently dilute to obey Beer's law. The optical density of the solution to be evaluated is then determined and its composition ascertained from the standard curve. Individual components of a mixture can be determined by ultraviolet absorption, provided that the different compounds exhibit different absorption maxima. Thus, for strychnine and brucine the reported $E_{1cm}^{1\%}$ values at the wavelengths (λ) indicated are:

	$E_{1cm}^{1\%}$	
λ	Strychnine	Brucine
262 nm	322	312
300 nm	5.16	216

By measurement of the extinctions of the mixed alkaloid solution at the above wavelengths, a two-point spectrophotometric assay is available for the determination of both alkaloids. This method, official in the *BP* (1980) for the assay of nux vomica seeds and preparations, replaced the older, chemical method. A similar type of assay is employed by the *EP* for quinine-type alkaloids and cinchonine-type alkaloids in cinchona bark; measurements are made at 316 and 348 nm. Occasionally it is useful to examine the ultraviolet spectrum of a more complex mixture; thus, the *USP* includes an ultraviolet absorbance test for the absence of foreign oils in oils of lemon and orange and the *BP* an extinction limit test between 268 and 270 nm for castor oil.

In most cases it is essential that no interfering substances are present during the measurements; these can be particularly troublesome in the ultraviolet region, particularly with materials extracted from thin-layer and paper chromatograms. For this reason, if pure solutions are not available for analysis, some form of colorimetric analysis is often preferable, particularly if the reaction used to produce the colour is highly specific for the compound under consideration.

Colorimetric analyses can be carried out with a suitable spectrophotometer—most instruments which operate in the ultraviolet range also have facilities for work in the visible region—but much simpler colorimeters in which suitable filters are used to select the correct wavelengths of light required are quite satisfactory for most purposes.

In these instruments a simple light-source is used and, between the lamp and the solution to be analysed, a filter is placed which transmits that range of wavelengths absorbed by the compound under test (i.e. a colour complementary to that of the solution under test). The transmitted light is recorded by a photoelectric cell and the composition of the solution determined by reference to a standard curve. The *BP*/*EP* tests the colouring power of Roselle (*Hibiscus sabdariffa*) by measurement of the absorbance of a water-soluble extract at 520 nm; similarly for an acid extract of Red Poppy Petals at 525 nm.

Characteristic absorption maxima from the more complex infrared spectra can also be utilized in quantitative analysis in the same way as ultraviolet and visible absorptions. Mixtures of substances can also be evaluated; thus, by measurements at 9.80, 9.15 and 9.00 μm it is possible to evaluate separately the 25β- and 25α-epimeric steroidal sapogenins present in plant extracts. A few of the many examples of the application of spectrometric analysis to constituents of drugs are given in Table 16.5.

Table 16.5 Some examples of the application of spectrometric analysis to the constituents of drugs.

Region of spectrum	Constituents	Wavelength for measurement of optical density
Ultraviolet	Alkaloids:	
	Lobeline	249 nm
	Reserpine	268 nm
	Vinblastine	267 nm
	Vincristine	297 nm
	Tubocurarine chloride	280 nm
	Morphine	286 nm
	Colchicine	350 nm
	Cardioactive glycosides with butenolide side-chain	217 nm
	Saponins—glycyrrhizinic acid	250 nm
	Iridoids—harpagoside in Devil's claw	278 nm
	Quassinoids	254 nm
	Cassia oil—aldehyde content	286 nm
	Bergamot oil—bergapten content	313 nm
	Flavaspidic acid from male fern	290 nm
	Capsaicin	248 and 296 nm
	Vanillin	301 nm
	Allicin—garlic	254 nm
	Vitamin A (cod-liver oil)	328 nm
Visible	Alkaloids:	
	Ergot (total alkaloids)	550 nm by the use of *p*-dimethylaminobenzaldehyde reagent; 532 nm by the reaction with vanillin in concentrated hydrochloric acid
	Morphine	442 nm by the nitroso reaction
	Reserpine	390 nm by the treatment of alkaloid with sodium nitrite in dilute acid
	Tropic acid esters of hydroxytropanes	555 nm by treatment of alkaloid with fuming nitric acid followed by evaporation to dryness and addition of methanolic potassium hydroxide solution to an acetone solution of the nitrated residue (Vitali–Morin reaction)
	Anthraquinones	500 nm after treatment with alkali (see 'Senna leaf *BP*' for the determination of sennoside; also aloes (512 nm), cascara and rhubarb (515 nm)); 530 nm for the cochineal colour value
	Capsaicin in capsicum	730 nm after reaction with phosphomolybdic acid and sodium hydroxide solution; 505 nm after treatment with diazobenzene-sulphonic acid in 10% sodium carbonate solution
	Cardioactive glycosides:	
	based on digitoxose-moiety	590 nm by Keller–Kiliani reaction
	based on lactone ring	620 nm by reaction with *m*-dinitrobenzene
	Ouabain	495 nm by reaction with alkaline sodium picrate
	Cyanogenetic glycosides:	
	(cyanide determination)	630 nm by the pyridine-parazolone colour reaction
	Tannins:	
	Rhatany, hamamelis leaf	715 nm using phosphotungstic acid and sodium carbonate solution (see *BP*)
	Procyanidins (as cyanidin chloride) in hawthorn berries	545 nm
	Volatile oils:	
	Menthol from peppermint oil	500–579 nm (green filter) by use of *p*-dimethylaminobenzaldehyde reagent
	Proazulenes (as chamazulene) in yarrow	608 nm measured on the blue oil obtained by distillation

(Continued)

Table 16.5 Some examples of the application of spectrometric analysis to the constituents of drugs. (Cont'd)

Region of spectrum	Constituents	Wavelength for measurement of optical density
	Flavonoids:	
	Birch leaf, calendula flowers (hyperoside), elder flowers (isoquercitrin)	425 nm with aluminium chloride and glacial acetic acid
Infrared	Alkaloids:	
	Quinine and strychnine mixtures	6.2 and 6.06 μm
	Steroidal sapogenins	11.11 μm and 10.85 μm; see text
	Volatile oils:	
	o-Methoxycinnamaldehyde in cassia oil	Measurements at 7.18 μm and 7.62 μm to distinguish bark oil from leaf and twig oils
	Water	1.9 μm; fairly specific for water without interference from other –OH groups

Fluorescence analysis

Many substances—for example, quinine in solution in dilute sulphuric acid—when suitably illuminated, emit light of a different wavelength or colour from that which falls on them. This emitted light (fluorescence) ceases when the exciting light is removed.

Analytical tests based on fluorescence *in daylight* are not much used, as they are usually unreliable, owing to the weakness of the fluorescent effect. An exception to this is the well-known umbelliferone test, which can be applied to ammoniacum, galbanum and asafoetida. A strongly fluorescent solution of umbelliferone can be prepared by boiling galbanum with acid and filtering into an excess of alcoholic ammonia. Other fluorescent solutions are those of quinine (in dilute acid), aesculin (by infusing horse chestnut bark), chlorophyll (from nettle or parsley leaves), β-naphthol (dissolved in alkali) and aqueous solutions of the dyes eosin and fluorescein.

A very important generalization made by Stokes in 1852 stated that 'in fluorescence the fluorescent light is always of greater wavelength than the exciting light'. Light rich in short wavelengths is very active in producing fluorescence and for this reason strong ultraviolet light (such as can be obtained from a tungsten arc or mercury vapour lamp) produces fluorescence in many substances which do not visibly fluoresce in daylight. Fluorescence lamps are usually fitted with a suitable filter which eliminates visible radiation from the lamp and transmits ultraviolet radiation of the desired wavelength. Convenient long- and short-wave ultraviolet hand lamps are available for chromatographic observations; **it is most important that the eyes are properly protected in the presence of ultraviolet radiation**.

For examination, *solids* may be placed directly under the lamp, whereas *liquids* may be examined in non-fluorescent dishes or test-tubes or after spotting on to filter paper. Many alkaloids in the solid state show distinct colours—for example, aconitine (light blue), berberine (yellow) and emetine (orange). Pieces of cinchona bark when placed under the lamp show a number of luminous yellow patches and a few light blue ones. If the inner surface of the bark is touched with dilute sulphuric acid the spot immediately turns blue. Ipecacuanha root has a brightly luminous appearance wherever the wood is exposed, while the wood of hydrastics rhizome shines golden yellow. Areca nuts when cut show a light blue endosperm. Slices of calumba appear intensely yellow, with the cambium and phloem distinguished by their dark-green colour. Precipitated and prepared chalks may readily be distinguished from one another.

Most *oils, fats* and *waxes* show some fluorescence when examined in filtered ultraviolet light. In general, fixed oils and fats fluoresce least, waxes more strongly, and mineral oils (paraffins) most of all.

Powders may be examined macroscopically as above or microscopically by means of a fluorescence microscope. In connection with powdered drugs may be mentioned the detection of ergot in flour, of cocoa shells in powdered cocoa, and of rumex in powdered gentian. Different varieties of rhubarb may be distinguished from one another. The *BP* 1973 included a fluorescence test on the entire or powdered drug for the detection of rhapontic rhubarb but this is now replaced by a TLC test.

The location of separated compounds on paper and thin-layer chromatograms by the use of ultraviolet light has been extensively employed. With plant extracts it is often worthwhile to examine the chromatogram in ultraviolet light even if the constituents that one is investigating are not themselves fluorescent. In this way the presence of fluorescent impurities may be revealed which, if otherwise undetected, could interfere with a subsequent absorption analysis. Sometimes fluorescence-quenching can be employed to locate non-fluorescent substances on thin-layer chromatograms. For this, an ultraviolet fluorescent background is produced by the incorporation of a small amount of inorganic fluorescent material into the thin layer. The separated substances cause a local quenching of the background fluorescence and they therefore appear as dark spots on a coloured background.

Quantitative fluorescence analysis. This technique utilizes the fluorescence produced by a compound in ultraviolet light for quantitative evaluation. The instrument employed is a fluorimeter and consists of a suitable ultraviolet source and a photoelectric cell to measure the intensity of the emitted fluorescent light. Within certain limits of concentration the intensity of the fluorescence for a given material is related to its concentration. It is usual to select a narrow range of wavelengths for measurement by inserting a filter between the fluorescing solution and the photoelectric cell. The concentration of a substance in solution is obtained by reference to a standard curve prepared by subjecting standard solutions to the fluorimetric procedure. With plant extracts it is important to ascertain that (1) the substance being determined is the only one in the solution producing a fluorescence at the measured wavelength and (2) there are no substances in the solution which absorb light at the wavelength of the fluorescence. Refined instruments are now available in which the fluorescence spectrum is automatically analysed and in which the wavelength of the incident radiation can also be varied.

Quinine can be conveniently assayed by the measurement of the blue fluorescence (366 nm) produced by irradiation of the alkaloid in a dilute sulphuric acid solution at about 450 nm. The method can be used for the assay of quinine in the presence of other alkaloids (e.g. strychnine). Alexandrian senna has been assayed by measurement of the fluorescence produced in the Bornträger reaction under specified conditions. The hydrastine content of hydrastis root may be determined by oxidizing an extract of the drug with nitric acid and measuring the fluorescence of the hydrastinine produced; by this method berberine and canadine, other alkaloids of hydrastis, are excluded from the assay. Emetine and papaverine may be determined fluorimetrically after oxidation with acid permanganate and noscapine after oxidation

with persulphate. Fluorimetric methods have also been published for the estimation of the ergot and rauwolfia alkaloids, umbelliferone, aflatoxin and a number of drugs in body fluids.

NMR spectroscopy

Although this technique is usually associated with structure-determinations of organic compounds the use of ^{1}H-NMR spectroscopy has been described for the assay of atropine and hyoscine in extracts of belladonna, hyoscyamus and stramonium. It has also been used for the quantitative determination of strychnine and brucine in *Strychnos nux-vomica*, affording a number of advantages over other methods (M. Frédérich *et al.*, *Planta medica*, 2003, **69**, 1169). Another application has been the classification and correlation of extracts of St John's wort, involving multivariate data analysis and pharmacological activity (G. Ross *et al.*, *Planta medica*, 2004, **70**, 771).

^{13}C-NMR spectroscopy has been used to distinguish the exudates of various resin-producing families and, together with ^{1}H-nmr spectroscopy, to characterize those of *Pinus* (Pinaceae) and of other Coniferae families (Cupressaceae, Araucariaceae and Podocarpaceae) (J. B. Lambert *et al.*, *J. Nat. Prod.*, 2005, **68**, 625; 2007, **70**, 1283).

Immunoassays

Such assays are highly sensitive and usually very specific and have been developed as a powerful analytical tool for the quantitative determination of many compounds in biological fluids.

Radioimmunoassays (RIA). The assay depends on the highly specific reaction of antibodies to certain antigens. There are various modifications of the technique and it is the saturation method that has been developed for phytoanalysis. Usually the relatively small molecules (below MW 1000) constituting the secondary plant metabolites are not involved in such immunoresponses, but when bound covalently to protein carriers, as haptens, they do become immunogenic. (Haptens are molecules which combine with antibodies but do not stimulate their production unless linked to a carrier molecule.) If such a hapten is prepared in the labelled condition (e.g. ^{3}H- or ^{125}I-labelled) with a known specific activity, mixed with an unknown amount of unlabelled hapten and added to a limited amount of antibody in the form of a serum, then there will be competition between the labelled and unlabelled antigen for the restricted number of binding sites available. This results in some bound and some unbound hapten; these can be separated and a determination of the radioactivity in either fraction, with reference to a standard curve, enables the amount of unlabelled antigen to be calculated. The antiserum is raised in suitable animals (e.g. rabbits).

Following the rapid development of RIA procedures in clinical analyses, and largely owing to the work of Weiler, Zenk and colleagues in Germany since 1976, the method has been satisfactorily applied to a range of plant medicinals as illustrated in Table 16.3.

RIA has the advantage that only small amounts of plant material are required; it is usually specific for a single, or small range of metabolites; relatively crude, unprocessed plant extracts can usually be used; and the process can be mechanized. Thus, it is an efficient tool for the screening of large numbers of plants, some 200–800 specimens being assayed in 1 day. For the application of the method to the selection of high-yielding strains of *Digitalis* and *Solanum*, see Chapter 14. With herbarium material, assays can be performed on quantities of sample ranging from 0.5 mg to a few milligrams and in the examination of individual plants, structures as small as anther filaments (e.g. in digitalis) can be accommodated.

Possible disadvantages of the method are the considerable specialized expertise required to set up the assays and the possibility of cross-reactions with components of the plant extract other than those under investigation. Problems arising from the latter need to be ascertained before the assay. The RIA for hyoscine, for example, is highly specific but norhyoscine will react even more strongly; the cross-reaction with 6-hydroxyhyoscyamine is considerably less, and with hyoscyamine, very much less. Similarly, in the assay for solasodine, tomatidine, if present, will cross-react.

Enzyme-linked immunosorbent assays (ELISA). In this method, competition for an immobilized antibody takes place with a modified form of the compound under analysis that has an enzyme bound to it. Release of the compound–enzyme complex from the binding site and determination of the enzyme activity enables the original solution to be quantified.

Examples of applications to medicinal plants are given in Table 16.3. As with RIAs, the method is very sensitive; thus, for the pyrrolizidine alkaloid retronecine it can be measured in the parts per billion range and one sclerotium of ergot is detectable in 20 kg of wheat.

Tandem mass spectroscopy (MS–MS)

In phytochemistry to date, mass spectroscopy is usually associated with the structure elucidation of compounds rather than with their assay. However, by the simultaneous use of two mass spectrometers in series it is possible to determine quantitatively the amount of a particular targeted compound in complex mixtures, plant extracts or even in dried plant material. Plattner and Powell in their report on maytansinoid identification (*J. Nat. Prod.*, 1986, **49**, 475) refer to it as an important analytical tool for 'needle-in-a-haystack' analytical problems. Sensitivity to picograms of targeted compounds can be achieved with high specificity and nearly instantaneous response; for sensitivity it compares with RIA but is much more rapidly performed. The method has been used for the analysis of cocaine in plant materials, pyrrolizidine in *Senecio* and other genera, taxanes from single needles of *Taxus cuspidata*, aflatoxin B_1 in peanut butter, xanthones, steroids and antibiotics. Hoke *et al*. (*J. Nat. Prod.*, 1994, **57**, 277) consider it the best overall method for the determination of taxol, cephalomannine and baccatin in *T. brevifolia* bark and needle extracts. The chemotaxonomy of the Cactaceae has been investigated by this method.

Quantitative microscopy

Powdered drugs or adulterants which contain a constant number, area or length of characteristic particles/mg (e.g. starch grains, epidermis, trichome ribs respectively) can be determined quantitatively by microscopy using lycopodium spores as an indicator diluent. The method, formerly official in the *BP*, is described in Chapter 43.

PART 4

Phytochemistry

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17

General methods associated with the phytochemical investigation of herbal products

Before about 1800 only slow progress was made in phytochemistry. A few compounds such as cane-sugar, starch, camphor and benzoic acid had long been known, as their preparation was extremely simple; also complex mixtures such as fats, fixed oils, volatile oils, tars and resins had been prepared and used, although virtually nothing was known of their composition. The early scientific workers in the phytochemical field failed to appreciate the extreme complexity of the materials they were trying to investigate and almost entirely lacked the techniques necessary for real progress. Many hundreds of plants were burnt to yield ashes and these early investigators were disappointed to find no significant differences between the ashes of poisonous and those of non-poisonous plants. Expression, aqueous extraction and evaporation had long been used for the preparation of sugar from sugar-cane and the French apothecary Nicholas Leméry (1645–1715) extended the use of extraction processes and made use of alcohol as a solvent. Robert Boyle (1627–91) disposed of the ancient theory of Aristotle that matter was composed of four elements, and although he never isolated an alkaloid, he was obviously moving in the right direction when he treated opium with potassium carbonate and alcohol. In 1747 sucrose was isolated from many plants, including sugarbeet, by the German apothecary A. S. Marggraf (1709–80). K. W. Scheele (1742–86) was highly successful in the phytochemical field and isolated citric, gallic, malic, oxalic, tartaric and prussic acids.

In the nineteenth century progress became more rapid. In 1803 narcotine, the first alkaloid, was isolated; morphine, strychnine, emetine and many others followed rapidly. Between 1813 and 1823 Chevreul elucidated the chemical nature of fats and fixed oils. Until well into the middle of the twentieth century the main emphasis in natural-product chemistry remained the isolation and structure determination of a wide variety of compounds. At this point it became apparent that the principal structural types commonly found in plants had been largely elucidated. Indeed, by this time the attention of natural-product chemists was turning to the elucidation of the actual biosynthetic pathways found in the plant. Such studies were made possible by the introduction of new techniques of separation and analysis. This emphasis has continued until today, when most of the major pathways, including stereochemical aspects, have been studied in some depth. Interest has now moved on to plant biochemistry involving enzymatic and DNA studies related to the biosynthesis of natural products. There has also developed a renewed interest in the patterns of occurrence of compounds in plants (comparative phytochemistry).

Not all the chemical compounds elaborated by plants are of equal interest to the pharmacognosist. Until relatively recently the so-called 'active' principles were frequently alkaloids or specific glycosides usually with pronounced pharmacological properties; these therefore received special attention, and in large measure constituted the principal plant drugs of the allopathic system of medicine. It is now realized that many other constituents of plants, particularly those associated with herbal medicine, have medicinal properties which manifest themselves in more subtle and less dramatic ways than the obviously poisonous plants. This has considerably widened the scope of plant metabolites considered worthy of more detailed investigation. Other groups such as carbohydrates, fats and proteins are of dietetic importance, and many such as starches and gums are used in pharmacy but lack any marked pharmacological action. Substances, such as calcium oxalate, silica, lignin and colouring matters, may be of assistance in the identification of drugs and the detection of adulteration.

As a result of the recent interest in the plant kingdom as a potential source of new drugs, strategies for the fractionation of plant extracts based on biological activity rather than on a particular class of compound, have been developed. The chemical examination follows after the isolation of the active fraction.

The phytochemical investigation of a plant may thus involve the following: authentication and extraction of the plant material; separation and isolation of the constituents of interest; characterization of the isolated compounds; investigation of the biosynthetic pathways to particular compounds; and quantitative evaluations. Parallel to this may be the pharmacological assessment of the separated components, which may, in some investigations, precede the characterization.

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EXTRACTION OF PLANT MATERIAL

All plant material used should be properly authenticated, as much time and money can be wasted on the examination of material of doubtful origin. The choice of extraction procedure depends on the nature of the plant material and the components to be isolated. Dried materials are usually powdered before extraction, whereas fresh plants (leaves, etc.) can be homogenized or macerated with a solvent such as alcohol. The latter is also particularly useful for stabilizing fresh leaves by dropping them into the boiling solvent. Alcohol is a general solvent for many plant constituents (most fixed oils excepted) and as such may give problems in the subsequent elimination of pigments, resins, etc. Water-immiscible solvents are widely used—light petroleum (essential and fixed oils, steroids), ether and chloroform (alkaloids, quinones). The extraction of organic bases (e.g. alkaloids) usually necessitates basification of the plant material if a water-immiscible solvent is to be used; for aromatic acids and phenols acidification may be required. Extraction itself may be performed by repeated maceration with agitation, percolation or by continuous extraction (e.g. in a Soxhlet extractor, Fig. 16.2). Special methods for volatile oils, such as the *enfleurage* process, are considered in Chapter 22. Ultrasound may enhance the extraction process for some plant materials and the *BP* uses this in the preparation of a 50% ethanolic solution of opium for the assay of alkaloids and in the assay procedure of Agnus Castus. Its use has been studied for the extraction of atropine from *Hyoscyamus muticus* using various solvent systems (A. Djilana and B. Legseir *Fitoterapia*, 2005, **76**, 148).

Spouted bed extraction

In certain instances, as in the production of annatto powder from the seeds of *Bixa orellana*, the physical removal of the pigment layer of the seed-coat can yield a less impaired product than that produced by solvent extraction. Such methods can involve the use of a ball mill or a spouted bed unit. A development of the latter, the conical spouted bed extractor, has been investigated for annatto production. Basically it consists of a cylinder tapered at both ends and containing the seeds at the lower end through which a jet of hot air is forced. Seeds and pigment-loaded fine particles are propelled into the space above from whence the seeds fall back to be recirculated and the annatto powder moves to a cyclone from which it is collected. For full details see M. L. Passos *et al*., *Drying Technology*, 1998, **16**, 1855.

Supercritical fluid extraction

The use of supercritical fluids for the extraction of a range of materials including plant products of medicinal, flavouring and cosmetic interest has, during the last decade, become of increasing economic and research interest.

In 1822, Cagniard de la Tour reported that above a certain temperature, and pressure, single substances do not condense or evaporate but exist as a fluid. Under these conditions the gas and liquid phases both possess the same density and no division exists between the two phases. This is the critical state. For water, the critical conditions for temperature (t_c) and pressure (p_c) are 374°C and 220 atmospheres respectively and for carbon dioxide $t_c = 31$°C and $p_c = 74$ atm. In practice conditions somewhat above the critical temperature and pressure for a particular substance are usually used and these *supercritical fluids* exhibit properties intermediate between those of the liquid and gaseous phases. In phytochemistry these properties can be exploited to maximize the extraction of plant constituents. For industrial purposes supercritical fluid carbon dioxide has an environmental advantage over many common organic solvents and leaves no solvent residues in the product. It also allows a low temperature process and has proved of value for the extraction of labile expensive fragrances and medicinal phytochemicals. To render it more polar a small amount of modifier, e.g. methanol, may be added to the carbon dioxide. The high pressures, and for some substances the high temperatures, involved in supercritical fluid extraction are the principal disadvantages of the technique.

Pioneer work on medicinal plants was carried out by Stahl and coworkers (*Planta Med*., 1980, **40**, 12, and references cited therein). They studied the use of liquefied and supercritical carbon dioxide and liquefied nitrous oxide for the extraction of various plant constituents, including various types of alkaloids, the pyrethrins and the components of chamomile. With pyrethrum flower extract, the content of pyrethrins is substantially higher (up to 50%) than in commercially available petroleum ether extracts. By a two-step precipitation the active ingredients can be raised to up to 60% without decomposition of the thermolabile pyrethrins.

Further examples involving the extraction of phytochemicals with supercritical carbon dioxide follow:

1.	Acylphloroglucinols:	Oxygenated hyperforin derivatives of *Hypericum*
2.	Alkaloids:	Decaffeination of green coffee (industrial application) Isolation of vindoline from *Catharanthus roseus*
3.	Diterpene:	Extraction of taxol from *Taxus brevifolia* (extraction more selective than conventional ethanol extraction), also from *T. cuspidata*
4.	Fixed oils:	Extraction of oil from evening primrose (subtle shift in triglyceride composition; oxidation of γ-linolenic acid during extraction reduced)
5.	Pigments:	Extraction of annatto seeds
6.	Sapogenins:	*Smilax china* (increased yield of diosgenin)
7.	Sesquiterpene lactones:	In conjunction with gas chromatography for the isolation of parthenolide from feverfew. Addition of methanol or methyl cyanide as CO_2 modifiers gave higher yields but produced co-extractives The use of 10% methanol in CO_2 for the extraction of trilactones from ginkgo could be of commercial significance
8.	Volatile oils and resins:	Hops (commercial application); frankincense and myrrh (efficient extraction); juniper berries (significant difference in composition compared with distilled oil, the latter being significantly more enriched with monoterpenoid hydrocarbons) *Piper nigrum muntok* (superior aroma of oil; yield 2.8 per cent volatile oil compared with 0.6 per cent by steam

distillation); rose petals (product richer in relevant fragrance compounds compared with steam distillation); rosemary (aroma more closely resembles plant fragrance than distilled oil); also studied: angelica root, celery, coriander, *Illicium verum*, *Maytenus illicifolia*, pimento

For a review covering the extraction of flavour and fragrance compounds, see M. Gotto *et al.*, *Aroma research* 2007, **8**, 110

For additional information on the method, consult the 'Further reading'.

Solid phase microextraction

The method is suitable for some volatile oil-containing drugs. T. J. Betts (*Planta Medica*, 2000, **66**, 193), using methyl polysiloxane solid phase microextraction fibres, has extracted the volatile oil from the headspace above fresh cut eucalyptus leaves (37° for 10 min.). The fibres were then desorbed at 200° for capillary gas chromatography of the oil. Not surprisingly, the oil composition differs from that of steam-distilled oils. The method, coupled with gas chromatography and mass spectrometry, has been recently employed for the analysis of the flowers and essential oils from *Lavandula angustifolia* cultivated in N.E. Italy (C. Da Porto and D. Decorti, *Planta Medica*, 2008, **74**, 182).

SEPARATION AND ISOLATION OF CONSTITUENTS

As the instrumentation for the structure elucidation of organic compounds becomes ever more effective, and allows the use of increasingly small amounts of material, the most difficult operation in phytochemical research becomes that of the isolation and purification of plant constituents. Although the chemical properties of functional groups and moieties contained in compounds such as acids, aldehydes, phenols and alkaloids can be exploited for their separation from other materials, such methods might not fractionate components of the same class; it is in this latter area that new techniques are constantly being developed.

Sublimation

Sublimation may sometimes be possible on the whole drug, as in the isolation of caffeine from tea or for the purification of materials present in a crude extract. Modern equipment employs low pressures with a strict control of temperature.

Distillation

Fractional distillation has been traditionally used for the separation of the components of volatile mixtures; in phytochemistry it has been widely used for the isolation of the components of volatile oils. On a laboratory scale it is not easy by this method to separate minor components of a mixture in a pure state and gas chromatography is now routinely used (q.v.).

Steam distillation is much used to isolate volatile oils and hydrocyanic acid from plant material. The TAS oven (see 'Thin-layer chromatography') involves steam distillation on a semi-micro scale for the direct transfer of volatile materials from a powdered drug to a thin-layer plate.

Fractional liberation

Some groups of compounds lend themselves to fractional liberation from a mixture. As an example, a mixture of alkaloid salts in aqueous solution, when treated with aliquots of alkali, will give first the weakest base in the free state followed by base liberation in ascending order of basicity. If the mixture is shaken with an organic solvent after each addition, then a fractionated series of bases will be obtained. A similar scheme can be used for organic acids soluble in water-immiscible solvents; in this case, starting with a mixture of the acid salts, it is possible to fractionally liberate the acids by addition of mineral acids.

Fractional crystallization

A method much used in traditional isolations and still valuable for the resolution of often otherwise intractable mixtures. The method exploits the differences in solubility of the components of a mixture in a particular solvent. Frequently, derivatives of the particular components are employed (picrates of alkaloids, osazones of sugars).

Adsorption chromatography

Of the various methods of separating and isolating plant constituents, the 'chromatographic procedure' originated by Tswett is one of the most useful techniques of general application. The use of charcoal for the decolorization and clarification of solutions is well known; coloured impurities are *adsorbed* by the charcoal and a colourless solution results on filtration. All finely divided solids have the power to adsorb other substances on their surfaces to a greater or lesser extent; similarly, all substances are capable of being adsorbed, some much more readily than others. This phenomenon of selective adsorption is the fundamental principle of adsorption chromatography, the general process of which may be described with reference to one of Tswett's original experiments.

A light petroleum extract of green leaves is allowed to percolate slowly through a column of powdered calcium carbonate contained in a vertical glass tube. The pigmented contents of the solution are adsorbed on the substance of the column and undergo separation as percolation proceeds. The more strongly adsorbed pigments, xanthophyll and the chlorophylls, accumulate in distinct, characteristically coloured bands near the top of the column, while the less strongly adsorbed pigments, the carotenes, accumulate lower down.

Frequently, complete separation of all the constituents into distinct bands does not result during the first 'adsorption stage', but the bands remain crowded together near the top of the column. Such a column may be *developed* by allowing more of the pure solvent to percolate through the column when the adsorbed materials slowly pass downwards and the separate bands become wider apart. In many cases the process may be rendered more efficient by the use of a different solvent, one from which the substances are less strongly adsorbed. If, for example, light petroleum containing a little alcohol is percolated through the chromatogram obtained in the experiment described above, the bands become wider apart and pass down the column more rapidly than when pure light petroleum is used. As percolation continues, the lower bands reach the bottom of the column and disappear; the pigment is then obtained in the solution leaving the bottom of the column. This process of desorption is termed *elution* and the solution obtained is the *eluate*.

It was from such classic experiments of Tswett on the separation of coloured compounds that the term 'chromatography' arose and it has remained to describe this method of fractionation although its application to colourless substances is now universal.

Substances are more readily adsorbed from non-polar solvents such as light petroleum and benzene, while polar solvents—alcohol, water and pyridine, for example—are useful eluting media; many substances are adsorbed at one pH and eluted at another.

Various substances may be used as adsorbing materials; alumina is the most common and other materials include kaolin, magnesium oxide, calcium carbonate, charcoal and sugars.

When colourless substances are chromatographed, the zones of adsorbed material are not visible to the eye, although they may, in some cases, be rendered apparent as fluorescent zones when the column is examined under ultraviolet light. Failing this, it becomes necessary to divide the chromatogram into discrete portions and elute or extract each portion separately. Sometimes it is more convenient to collect the eluate from the whole column in fractions for individual examination.

The apparatus required is simple and consists essentially of a vertical glass tube into which the adsorbent has been packed; a small plug of glass wool or a sintered glass disc, at the base of the tube, supports the column. With volatile developing solvents it is usually preferable to use a positive pressure at the head of the column. Numerous modifications of the apparatus are used for large-scale operations, for use with heated solvents and for chromatography in the absence of air or oxygen.

Adsorption chromatography has proved particularly valuable in the isolation and purification of vitamins, hormones, many alkaloids, cardiac glycosides, anthraquinones, etc. It is commonly employed as a 'clean-up' technique for the removal of unwanted materials from plant extracts prior to assay.

Thin-layer chromatography with adsorbents such as alumina is an adaptation of the method and is discussed separately in this chapter.

Partition chromatography

Partition chromatography was introduced by Martin and Synge in 1941 for the separation of acetylated amino acids and was first applied to the separation of alkaloids by Evans and Partridge in 1948. The method has now been largely superseded by the more sophisticated HPLC (see below) but it retains the advantage of being inexpensive to set up and operate. The separation of the components of a mixture is, as in counter-current extraction, dependent on differences in the partition coefficients of the components between an aqueous and an immiscible organic liquid.

The aqueous phase is usually the stationary phase and is intimately mixed with a suitable 'carrier' such as silica gel, purified kieselguhr or powdered glass and packed in a column as in adsorption chromatography. The mixture to be fractionated is introduced on the column, in a small volume of organic solvent, and the chromatogram is developed with more solvent or successively with different solvents of increasing eluting power. When water is the stationary phase, the solutes undergoing separation travel down the column at different speeds depending on their partition coefficient between the two liquid phases; the use of a buffer solution as aqueous phase widens the scope of the technique, as ionization constants and partition coefficients are exploited in effecting separation.

The separated zones may be located by methods similar to those employed in adsorption chromatography. With water as the aqueous phase, the positions of separated zones of acids or alkalis may be shown by employing a suitable indicator dissolved in the water. This method is clearly not applicable to buffer-, acid- or alkali-loaded columns, and in these cases complete elution (elution development) of the separated zone is often necessary. The eluate is collected in aliquot portions and estimated chemically or physically for dissolved solute. A graph of the analytical figure (titration, optical rotation, optical density, refractive index, etc.) for each fraction of eluate may then be plotted to show the degree of separation of the solutes.

The fractionations obtained in partition chromatography are influenced to a considerable degree by the displacement effect of one solute on another and advantage is taken of this in displacement development, in which the chromatogram is developed with a solution of an acid or a base that is stronger than any in the mixture to be separated. The effect is for the stronger acids or bases to displace the weaker ones, resulting in a rapid clear-cut separation of the constituents. For the elution development of these separated zones it is essential that there is no distortion of the zones, since the front of one band follows immediately on the tail of the preceding less acidic or less basic component.

There have been several theoretical treatments of partition chromatography, all involving certain approximations, since a theory taking into account all known variables would be extremely complicated. For general purposes, one of the most satisfactory treatments of columns loaded with water is that of Martin and Synge, in which the theoretical plate concept of fractional distillation is applied to partition chromatography. In this theory, diffusion from one plate to another is taken as negligible and the partition of solute between two phases is independent of concentration and the presence of other solutes.

Partition chromatography on paper

In 1944 Consden, Gordon and Martin introduced a method of partition chromatography using strips of filter paper as 'carriers' for the analysis of amino acid mixtures. The technique was extended to all classes of natural products, and although to a large measure replaced by thin-layer chromatography (TLC), it remains the method of choice for the fractionation of some groups of substances.

The solution of components to be separated is applied as a spot near one end of a prepared filter-paper strip. The paper is then supported in an airtight chamber which has an atmosphere saturated with solvent and water, and a supply of the water-saturated solvent. The most satisfactory solvents are those which are partially miscible with water, such as phenol, *n*-butanol and amyl alcohol. Either the paper may be dipped in the solvent mixture so that the solvent front travels up the paper (ascending technique) or the trough of solvent may be supported at the top of the chamber, in which case the solvent travels down the paper (descending technique). The *BP* 2007 gives details of both methods. As the solvent moves, the components also move along the paper at varying rates, depending mainly on the differences in their partition coefficients between the aqueous (hydration shell of cellulose fibres) and organic phases. After the filter-paper strips have been dried, the positions of the separated components can be revealed by the use of suitable developing agents: ninhydrin solution for amino acids; iodine solution (or vapour) or a modified Dragendorff's reagent for alkaloids; ferric chloride solution for phenols; alkali for anthraquinone derivatives; antimony trichloride in chloroform for steroids and some components of volatile oils; aniline hydrogen phthalate reagent for sugars. The relative positions of the components and the size of the spots depend upon the solvent, and this should be selected to give good separation of the components with well-defined, compact spots. Improved separation of mixtures can often be obtained by adjusting the acidity of the solvent with ammonia, acetic acid or hydrochloric acid or by impregnating the paper with a buffer solution or formamide solution.

For the separation of some substances it is necessary to use a two-dimensional chromatogram: first one solvent is run in one direction, then, after drying of the paper, a second solvent is run in a direction at right angles to the first—this is particularly applicable to mixtures of amino acids.

The ratio between the distance travelled on the paper by a component of the test solution and the distance travelled by the solvent is termed the R_F value and, under standard conditions, this is a constant for the particular compound. However, in practice, variations of R_F often occur and it is desirable to run reference compounds alongside unknown mixtures.

The quantity of substance present determines the size of the spot with any one solvent and can be made the basis of quantitative evaluation. Also, the separated components of the original mixture can be separately eluted from the chromatogram, by treating the cut-out spots with a suitable solvent, and then determined quantitatively by some suitable method—for example, fluorescence analysis, colorimetry or ultraviolet adsorption. Drugs so evaluated include aloes, digitalis, ergot, hemlock, lobelia, nux vomica, opium, rauwolfia, rhubarb, broom, solanaceous herbs and volatile oils.

High-performance liquid chromatography (HPLC)/ high-speed LC

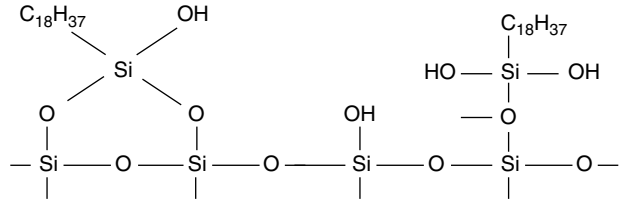
HPLC is a liquid column chromatography system which employs relatively narrow columns (about 5 mm diameter for analytical work) operating at ambient temperature or up to about 200°C at pressures up to 200 atm (20 000 kPa).

The columns are costly and it is usual to employ a small precolumn containing a cartridge of packing material to remove adventitious materials which might otherwise damage the main column. Normal flow rates of eluate are 2–5 ml min^{-1} but can be up to 10 ml min^{-1}, depending on the diameter of the column and the applied pressure. The apparatus is suitable for all types of liquid chromatography columns (adsorption, partition by the use of bonded liquid phases, reversed phase, gel filtration, ion exchange and affinity). The arrangement of such an apparatus, suitable for use with two solvents and giving graded elution, is illustrated in Fig. 17.1. Detection of the often very small quantities of solute in the eluate is possible by continuous monitoring of ultraviolet absorption, mass spectrum, refractive index, fluorescence and electrical conductance; nuclear magnetic resonance can now be added to this list. To improve detection, solutes may be either derivatized before chromatography (this technique can also be used to improve separations) or treated with reagents after separation (post-column derivatization). A transport system for monitoring is commercially available; in this a moving wire passes through the flowing eluate (coating block) and the dissolved solute, deposited on the wire, is pyrolysed and its quantity automatically recorded. It will be noted that, for any particular fractionation, some detector systems would be selective for certain groups of compounds and others would be universal.

HPLC can give much improved and more rapid separations than can be obtained with the older liquid chromatography methods and it is therefore finding increasing use in numerous areas. As with GLC apparatus, it is available from many manufacturers and can be completely automated.

Many stationary phases are available, the most widely used being silica based. In these, which consist of porous particles 5–10 μm in diameter, the silanol groups (Si-OH) afford a polar surface which can be exploited in separations using an organic mobile phase as in ordinary adsorption chromatography.

Reversed-phase packing material (Spherisorb ODS) is produced by the bonding of octadecylsilyl groups ($C_{18}H_{37}Si$–) to silica gel. In the commercial material there appears to be a considerable proportion of residual silanol–OH groups, so that adsorption and partition effects may operate during separation. The hydrocarbon chains probably allow non-polar interaction to take place. The structure of the packing material might be represented as:



To reduce tailing effects which might be caused by the remaining free hydroxyl groups the latter can be masked by treatment with a short-chain silane, usually trimethylchlorosilane. Silica-based columns are restricted to use in the range pH 3–8 and to overcome this polymer phases operating at pH 1–13 are available; these, however, have the disadvantage of exhibiting a high column back-pressure.

Of relatively recent introduction are chiral stationary phases which are utilized to separate the enantiomers of racemic mixtures. These have great potential for the study of natural products, many of which are optically active, and for isolating the pharmacologically active enantiomer from the racemic mixture of a synthetic drug. Pharmacognostical examples include, among many others, the assay of partially racemized hyoscine extracted from plants, the separation of aromatics and the resolution of mixtures of (+)- and (−)-epicatechin and other proanthocyanidin enantiomers of *Cassia fistula* and *C. javanica*. The mode of action of chiral stationary phases is not fully understood; typical materials are cyclodextrins, cellulose- and amino acid-derivatives suitably bonded. Two products are sorbents made of spherical silica gel particles to which β-cyclodextrin (ChiraDex®) or γ-cyclodextrin (Chira-Dex® GAMMA) are covalently bonded via a carbamate bond and long spacer.

For a review on the rapid detection and subsequent isolation of bioactive constituents of crude plant extracts with schemes for LC/UV, LC/MS, LC/MS/MS and LC/NMR see K. Hostettman *et al*., *Planta Medica*, 1997, **63**, 2.

Supercritical fluid chromatography

This technique, developed over recent years for both the qualitative and quantitative analysis of medicinal products, utilizes supercritical

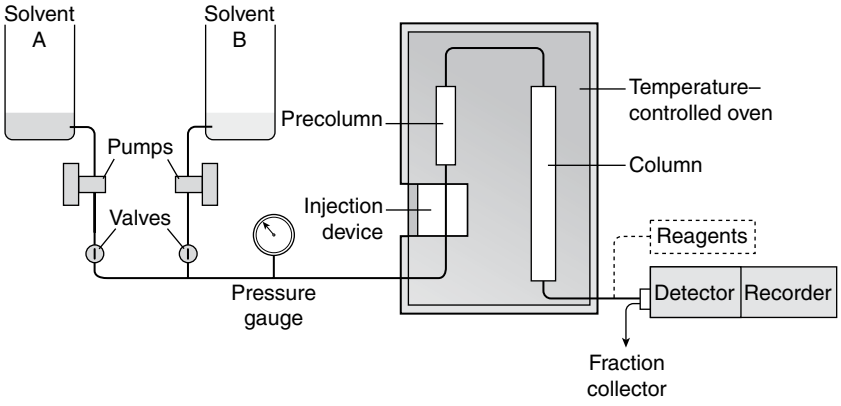


Fig. 17.1
Schematic representation of apparatus for high-performanance liquid chromatography utilizing two solvents.

fluids, particularly carbon dioxide, as the mobile phase in liquid chromatography. The low viscosity of the supercritical liquid provides a faster flow rate than standard HPLC and higher diffusivity of the materials to be separated. The plate height in the column, the length of the column and the time required for a particular separation are all reduced compared with the established technique. Sharper elution peaks give increased separation efficiency. Waste-disposal of used solvents is eliminated. Changes to the instrumental set-up have been devised to accommodate the properties of a supercritical liquid.

Counter-current extraction

This is a liquid–liquid extraction process and the principles involved are similar to those of partition chromatography. Developed by Craig in 1944, the extraction machine used (for that time) small amounts of extract and overcame the tedious multiple extraction processes then employed. Now, however, for research purposes, only the smallest of samples for fractionation and analysis are required, making the use of the cumbersome counter-current apparatus largely unnecessary.

Briefly, a lower, stationary phase is contained in a series of tubes and an upper, moving immiscible liquid is transferred from tube to tube along the series, the immiscible liquids being shaken and allowed to separate between each transference. The mixture to be fractionated is placed in the first tube containing the immiscible liquids and the apparatus is agitated and the layers are allowed to separate. The components of the mixture will be distributed between the two layers according to their partition coefficients. The upper phase is moved along to the second tube containing lower phase and more moving phase is brought into contact with the lower phase of tube 1. Shaking and transference again takes place and continues along a sufficient number of tubes to give a fractionation of the mixture.

The applications of counter-current extraction covered many fields of plant chemistry, including alkaloids, amino acids, antibiotics, antitumour compounds, phenols including anthraquinone derivatives, cardiac glycosides, essential oils, fatty acids, plant auxins, prostaglandins, steroids and vitamins.

Other more recent developments involving the counter-current principle are high-speed counter-current chromatography (planet coil centrifugal CCC), droplet counter-current chromatography (DCCC) and centrifugal droplet CCC. Details of these can be found in the 15th edition of this book. For a review (245 refs) giving the background and up-to-date methodology employed in the counter-current separation of natural products see G. F. Pauli *et al*., *J. Nat. Prod*., 2008, **71**, 1489–1508.

Thin-layer chromatography

In 1958 Stahl demonstrated the wide applicability of TLC, a technique which had been known in principle for many years but was never developed. It has now achieved remarkable success in the separation of mixtures of all classes of natural products and is established as an analytical tool in modern pharmacopoeias.

In outline the method consists of preparing, on a suitable glass plate, a thin layer of material, the sorbent, which may be either an adsorbent as used in column adsorption chromatography or an inert support which holds an aqueous phase as in column partition chromatography. The mixtures to be resolved are dissolved in a suitable solvent and placed as a series of spots on the film towards one end of the plate; this end is then dipped in a suitable solvent mixture and the whole enclosed in an airtight container. The solvent front travels up the film and after a suitable time the plate is removed, the solvent front is marked, the solvent is allowed to evaporate and the positions of the separated compounds are determined by suitable means.

TLC has certain advantages over paper chromatography. Fractionations can be effected more rapidly with smaller quantities of the mixture; the separated spots are usually more compact and more clearly demarcated from one another; and the nature of the film is often such that drastic reagents, such as concentrated sulphuric acid, which would destroy a paper chromatogram, can be used for the location of separated substances.

With adsorption TLC various substances exhibit different adsorptive capacities and any one material may vary in its activity according to the pretreatment. The adsorbent must be chosen in relation to the properties of the solvent and the mixture to be fractionated. In general, for a given substance, if a highly active adsorbent is used, then a solvent with a correspondingly high power of elution for this substance is required. Alumina (acid, basic and neutral) of different activity grades is very commonly employed. In order to produce a film with reasonable handling properties, the adsorbent may be mixed with about 12% of its weight of calcium sulphate ($CaSO_4.1/2H_2O$) to act as a binder. Ready-mixed powders are obtainable commercially; they require mixing with a given quantity of water and the slurry needs to be spread by a mechanical device or with a glass rod on to glass plates. The film sets within a few minutes and is then activated by heating at a suitable temperature (105°C for 30 min is common). Commercial ready-spread plates are available. The thickness of the film is characteristically of the order of 250 μm, but for preparative work layers of up to several millimetres thickness are employed. The thick films must be carefully dried to avoid cracks.

Solutions of substances to be examined are applied to the film with the aid of capillary tubes or, for quantitative work, with microsyringes and micrometer pipettes which permit volumes to be read off to ±0.05 μl. A useful innovation for applying steam-volatile components of a powdered drug directly to a thin-layer plate is the Stahl TAS oven. The drug sample is placed in a cartridge together with a suitable propellant (e.g. hydrated silica gel) and inserted in the TAS oven maintained at a predetermined temperature. The tapered exit of the cartridge is situated a short distance from the base-line of a TLC plate and 'steam-distilled' components from the drug are deposited ready for immediate development (for illustration see 12th edition). Stahl (*Planta Med*., 1976, **29**, 1) has described a development of this apparatus whereby 18 samples can be simultaneously deposited on a plate under uniform conditions. A more recent application has involved the study of tannin-containing drugs.

The solvents used for running the chromatogram must be pure, and common ones are methanol, ethanol and other alcohols, chloroform, ether, ethyl acetate, *n*-hexane, cyclohexane, petroleum spirit and mixtures of these. For routine assays automatic multiple development with polarity graduation of the developing solvent can be used. It must be remembered that chloroform ordinarily contains up to 1% of ethanol, which gives it quite different elution properties from those of pure chloroform. Benzene, formerly frequently used as a component of the mobile phase, has now for health reasons been routinely replaced with other non-polar solvents. Similarly, inhalation of chloroform-containing mixtures should be avoided.

TLC, which involves the partition of a substance between two immiscible phases, is again analogous to the column procedure and to paper chromatography. In the latter, the hydration shell of the cellulose fibres forms the stationary phase and thin-layer chromatograms utilizing powdered cellulose give comparable results. Kieselguhr and silica gel are also commonly employed, and their properties as thin layers can be modified by the inclusion of acids, bases and buffer solutions. The thin layers may also include a substance fluorescent in ultraviolet light and this facilitates the detection of solutes which cause a quenching of the background fluorescence. Much used is Kieselgel GF_{254} 'Merck' which gives a background green fluorescence when irradiated with ultraviolet light of wavelength 254 nm. Small quantities of ammonia solution, diethylamine, acetic acid, dimethylformamide and pyridine are often present as constituents of the developing solvents for silica plates. Other layers used include polyamide, which is particularly suitable for phenolic compounds, 'Sephadex' (Pharmacia,

Uppsala), a cross-linked dextran used as a molecular sieve (gel filtration), and ion exchangers. 'Reversed-phase' plates are now available commercially and are stated to be useful in that they will indicate the type of separation which a mixture would undergo by reversed-phase HPLC (q.v.).

As with paper chromatography, the method can be extended to two-dimensional chromatography, to electrophoretic separations, to quantitative evaluations and to work involving radioactive substances.

Compounds resolved on the TLC plate are visualized using either general or specific methods; thus, ultraviolet light will indicate fluorescent compounds (these should be examined in both long- (*c*. 365 nm) and short- (*c*. 263 nm) wavelength ultraviolet light. Fluorescence-quenching compounds (a very large number) are detected by the use of impregnated sorbents (see above). Iodine and Dragendorff's reagents are used in the form of sprays for the general detection of alkaloids although they (iodine in particular) are not absolutely specific for alkaloids. For indole alkaloids, the reagent *p*-dimethylaminobenzaldehyde is useful for ergot and a phosphomolybdic acid reagent for others. Antimony trichloride in chloroform is used as a spray reagent for steroidal compounds and other terpenoids, similarly anisaldehyde in sulphuric acid; both these require the sprayed chromatograms to be heated at 100°C for varying times (5–20 min) in order to develop the colours. Ammonia vapour can be used for free anthraquinone compounds and Fast Blue Salt B 'Merck' for cannabinoids and phloroglucides. Sugars separated by TLC using phosphate buffered amino layers (e.g. precoated plates of silica gel–Merck NH_2) can be located by *in-situ* thermal reaction (150°C for 3–4 min.) and fluorescence monitoring.

The *European* and *British Pharmacopoeias* employ thin-layer chromatographic tests for most vegetable drugs; illustrations of the chromatograms of the fatty acids derived from fifteen different fixed oils are given.

Preparative TLC. As mentioned above, thicker layers of sorbent are employed for preparative work and the separated bands of compounds are scraped from the plate and subjected to solvent extraction. With modern spectrometric methods for structure-determination available, this technique generates quantities of material sufficient for a complete analysis.

To speed up separations and to make them on-line for continuous recording, various modifications of preparative TLC have been developed. These include *centrifugally accelerated layer chromatography* and *overpressure layer chromatography*. The latter involves the complete covering of the sorbent layer with an elastic membrane under external pressure thus eliminating the vapour phase from the chromatographic plate. The mobile phase is forced up the sorbent layer through a special inlet. This method, introduced by Tyihák *et al*. in 1979–80, was subsequently adapted to *on-line overpressure layer chromatography* for the preparative separation of a number of natural products (the isolation of frangula-emodin; noscapine and papaverine fractionation; furocoumarin isomers of *Heracleum sphondylium* (Umbelliferae); the preparation of the secoiridoid glycosides of *Gentiana purpurea*). See J. Pothier *et al*. (*Fitoterapia*, 1997, **68**, 42) for the semi-preparative isolation of the alkaloids of *Strychnos nux-vomica*, opium, *Datura stramonium* and *Lupinus*.

Gas–liquid chromatography

The use of a liquid stationary phase and a mobile gaseous phase in chromatography was first suggested by Martin and Synge in 1941 and developed by James and Martin in 1952 for the separation of the lower fatty acids. Gas–liquid chromatography is now extensively used in all branches of analytical chemistry. Many commercial instruments are available, the more sophisticated being completely automated. For a schematic diagram see Fig. 17.2.

The *empty columns* are made of glass or metal and are either straight, often up to about 1.3 m in length, or coiled and up to 16 m in length. James and Martin used a tube of 4 mm internal diameter. The liquid *stationary phase* is held on an *inert material*, commonly partially fused diatomite. A uniform particle size, with a minimum of dust, is essential, for the inert support and the particles should be as small as possible to give a large surface area but sufficiently large to allow even packing of the column. The choice of stationary phase is governed by the temperature at which the column is to operate and the nature of the material to be fractionated; it should be non-volatile at the operating temperature and should not react with either the stationary and mobile phases or the solutes. Some materials commonly used, with their recommended temperature of operation, include: (1) non-polar compounds—silicone oils 200–250°C, apiezon oils and greases 275–300°C, silicone gum rubber 400°C, high-boiling-point paraffins such as mineral oil 100°C, squalene 75°C; (2) moderately polar compounds—high boiling point alcohols and their esters 100–225°C; (3) strongly polar compounds—polypropylene glycols and their esters 225°C. Up to 25% by weight of stationary phase is commonly employed on columns; one method of dispersing the stationary phase over the inert support is to dissolve it in a low boiling point solvent such as ether, mix thoroughly with the support and spread out the powder to allow the solvent to evaporate. The powder is then packed into the empty column a little at a time and as evenly as possible and then enclosed in a uniformly heated oven.

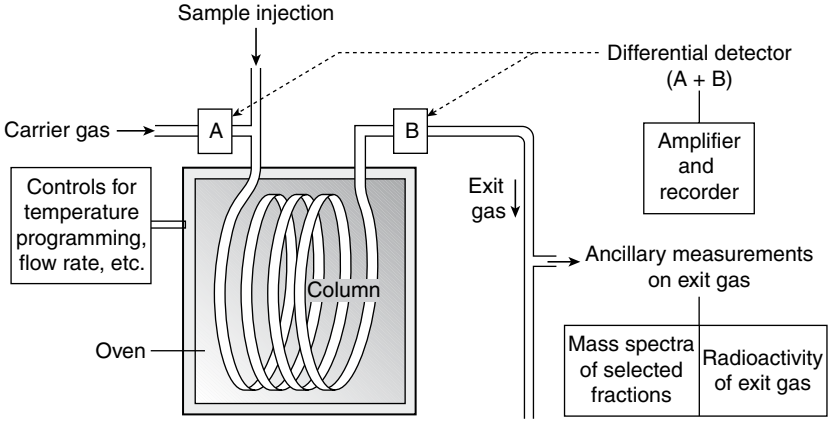


Fig. 17.2
Schematic diagram of gas–liquid chromatograph with ancillary attachments.

A combined support and stationary phase which can replace the above is furnished by cross-linked polymers of specified pore size. These are produced as beads from styrene-like compounds and are marketed under the trade name 'Porapak'. Advantages of such columns are that they remove undesirable adsorption sites present in diatomite, bleeding of the column (gradual leakage of stationary phase from the column) is reduced, and the rapid elution of water and other highly polar molecules is achieved with little or no tailing.

The *operating temperature* of the column is critical. Mixtures of low-boiling-point substances can be fractionated at low temperatures; some ethers, for example, can be dealt with at room temperature. Other materials require much higher temperatures—volatile oils 150–300°C, steroids 250°C and pesticides 400°C. Modern instruments can be temperature-programmed so that the column temperature increases as chromatography proceeds. This has the advantage that good separations of mixtures containing compounds with widely different properties can be obtained in one operation and long waits for the emergence of the more strongly retained fractions, with correspondingly less resolution, are lessened.

The *mobile phase* is a gas which is inert in so far as the other components of the chromatogram are concerned. The choice of gas is dependent on the detector system (see below), and gases commonly used are hydrogen, nitrogen, helium and argon. The *flow rate* of the gas is important; too high a flow rate will give incomplete separations and too slow a rate will give high retention times and diffuse peaks. Typical flow rates for short columns are 10–50 ml min^{-1}.

By means of a suitable injection device, the sample to be analysed is introduced on to the top of the column; 1.0–5.0 μl is a typical volume but with some detectors it can be considerably less. The measurement of such small volumes is difficult, especially if quantitative results are required, and often the sample is dissolved in a low-boiling-point solvent such as ether; the ether passes rapidly through the column and emerges with the gas front. The mixture to be analysed should volatilize immediately it comes into contact with the stationary phase. Some compounds, not themselves volatile, may be converted into volatile derivatives before chromatography. Thus, sugars, flavonoids including anthocyanins, morphine, codeine and the cardioactive glycosides and aglycones can be chromatographed as their trimethylsiloxy derivatives, which are formed as below:

$$2ROH + (CH_3)_3SiNHSi(CH_3)_3 \rightarrow 2ROSi(CH_3)_3 + NH_3$$

Non-volatile plant acids can first be converted to their methyl esters by treatment with diazomethane.

Along with the analytical columns of the above type, larger preparative columns can be used for the isolation of the separated components in quantities sufficiently large for subsequent examination. Sample sizes of 0.1–20 ml and column lengths of up to 60 m and internal diameters of 1–2 cm illustrate the dimensions involved.

The *detector system* analyses the effluent gas from the column. It may be of the integral type, in which some property—for example, titration value—of the eluate is recorded or it may be of the differential type, in which some property of the effluent gas is compared with that of a reference gas, often the mobile phase. The latter type is the most commonly used and examples are the katharometer, gas density balance, flame ionization, β-argon ray and electron-capture detectors. For details of these detectors the student is referred to one of the several standard books on gas chromatography. All these differential detectors give an electrical signal which is recorded graphically by a suitable recorder. Because not all detectors give the same relative response to given compounds under the same conditions, some columns are fitted with a double detector system.

The volume of gas that emerges from the column before the arrival of the gas front into which the sample was introduced at the head of the column is termed the 'hold-up' volume and it is dependent on the capacity of the column. It is obtained by multiplying the time which the gas front takes to pass through the column by the flow rate; the arrival of the front is often indicated on the recorder by a negative peak caused by the small amount of air injected with the sample. The observed retention volume $V_{R,obs.}$ of a component is calculated from its retention time, and the $V_{R,obs.}$ less the 'hold-up' volume is the adjusted retention volume ($V_{R,ad.}$). By taking into account the pressure drop along the length of the column the net retention volume ($V_{R,net}$) is obtained. This volume is characteristic for a given compound under the defined conditions of the amount of stationary phase and temperature. Of more universal value is the specific retention volume ($V_{R,sp}$), which is the $V_{R,net}$ reduced to 0°C g^{-1} of stationary phase:

$$V_{R,sp} = \frac{V_{R,\,net} \times 273}{\text{weight of stationary phase on column} \times \text{temperature (K) of column}}$$

Reference compounds are used to aid the identification of components of a mixture. For quantitative work with differential detectors, the areas enclosed by the peaks are proportional to the quantities of compounds which they represent. To obtain the percentage composition of components within a mixture, without the necessity of placing known amounts of sample on the column, internal standards can be used. These are pure substances, mixed in known proportion with a sample of material to be analysed, which give sharp peaks on the chromatogram not overlapping those of the mixture. Before use, internal standards must be calibrated for detector response against individual components of the mixture.

Sophisticated attachments are available for some equipment. It is thus possible to record the radioactivities and mass spectra of the separated components of a mixture as they emerge from the column. Such instruments have immense potential in biological research. Data storage and retrieval units are now available as standard accessories.

Some pharmacognostical examples of the applications of gas chromatography include the examination of many volatile oils (see, for example the *BP* assay of Clove Oil), camphor, plant acids, some alkaloids (opium, tobacco and *Conium* and tropane derivatives), the resins of the Convolvulaceae and of *Cannabis*, and steroidal compounds such as the sapogenins and cardioactive glycosides and aglycones. The *BP* test for foreign oils in fixed oils involves the gas-chromatographic separation of the methyl esters of the fatty acids produced by hydrolysis of the sample. The detection and estimation of cocaine and its metabolites in the body is an important forensic application. The estimation of pesticide residues on crops is of utmost importance, and here the sensitivity of detector systems, such as the electron capture detector, has made possible the determination of the chlorinated pesticides down to the parts-per-billion range.

Capillary-column gas chromatography

As the name implies, capillary bore columns are used rather than the standard columns described above. They afford marked improvements in resolving power and in speed of analysis.

The internal diameters of the columns range from about 0.15 mm to about 0.53 mm and the columns can be 1 to 60 m in length. They were originally made of stainless steel and then glass, but fused-silica columns are now considered the obvious choice as they are strong, easy to use, highly inert and give excellent performance. They can be conveniently used in the coiled condition, held, for example, in a 150 mm diameter cage. Such columns hold the stationary phase in a number of ways.

(1), Wall-coated open tubular (WCOT) columns have the inner wall of the tube coated with stationary phase up to about 1 μm in thickness. Greater thickness leads to column bleeding in which the stationary phase moves down the column and eventually leaks into the detector. Thicker layers, and hence increased sample capacity, can be achieved with silica columns having specially bonded phases. WCOT columns have the highest efficiency but a low sample capacity. (2), Support-coated open tubular (SCOT) columns have the inner wall lined with a thin layer of support material coated with immobile phase. This has the effect of increasing the available area of immobile phase, affording the column a greater load capacity. The efficiency, while lower than that of the WCOT columns is much higher than that for packed columns. (3) Micropacked columns involve a coated support packed into narrow-bore columns. In all ways they represent a compromise, being more efficient than the normal packed columns but having the same problem in that column length is restricted by the high back-pressure.

Another difference between capillary column chromatography and standard gas chromatography concerns the method of introducing the sample to the column. The volume of sample dissolved in solvent for analysis by the capillary method is too small for a microsyringe and so special injection heads are necessary which either split the sample (e.g. 25:1 with the smaller portion passing to the column) or are of the so-called splitless-injector type which are able to accommodate the relatively large volume of solvent and deliver the dissolved solute to the column.

As with other chromatographic techniques (see HPLC) the introduction of chiral stationary phases has given an added dimension to gas chromatography. Examples include the separation of the two enantiomers of linalool enabling the detection of reconstituted bergamot oil in the genuine oil (A. Cotroneo *et al.*, *Flavour Frag. J*., 1992, 7, 15) and the detection of added reconstituted lemon oil in the genuine cold-pressed essential oil (G. Dugo *et al*., *J. Essent. Oil Res*., 1993, 5, 21). Some volatile oils of the *Pharmacopoeia* are tested for chiral purity (q.v).

Gel filtration chromatography, gel permeation chromatography (molecular sieves)

These techniques are used for the separation of substances in solution according to their molecular size. The former refers to the use of aqueous mobile phases and the latter to organic mobile phases.

Hydrophilic gels such as those prepared from starch, agar, agarose (a component of agar), polyacrylamide, polyvinylcarbitol and cross-linked dextrans have been used for the fractionation of proteins, peptides, amino acids and polysaccharides.

The particles of these gels possess pores formed by the molecular structure of the gel, and when packed into a column and percolated with a solution, they permit large molecules of solute, which do not enter the pores, to pass rapidly down the column with the solvent via the intergranular interstices. Conversely, small molecules which are able to enter the gel pores become evenly distributed (on equilibrium) across the column and pass more slowly down its length. Thin layers of the sorbent can be used as in TLC.

The dextran gels (Sephadex) are formed by cross-linking dextrans (polymers of glucose in which linkages are almost entirely of the 1,6-α type) with α-epichlorohydrin (Fig. 17.3).

Individual pore sizes are determined by the distance apart of the cross-links, and gels covering a molecular weight range of up to about 200 000 are produced. Each gel type possesses a range of pore sizes, so that below the size limit of complete exclusion of the large molecules, different-sized solute molecules will enter the gel to a greater or lesser extent and so will vary in their elution rates. Similar principles are involved in the use of controlled-pore glass as sorbent; this gives a rigid column with continuous uniform pores. Obviously, the method is most applicable to mixtures containing large molecules of various sizes and to the separation of large molecules from small ones (as in desalting operations on partially hydrolysed proteins). The technique is important in DNA analysis for the separation of those fragments that result from the treatment of DNA with specific restriction enzymes.

Under the heading 'Size-exclusion chromatography', the *Pharmacopoeia* also uses rigid supports as packing material for columns; these may consist of glass, silica or a solvent-compatible cross-linked organic polymer.

Size-exclusion chromatography is used for the determination of those smaller fatty acids (oligomers) that need to be limited in fish oils, such as Fish Oil, Rich in Omega-3-Acids *BP*/*EP*, (oligomers, maximum 1.5%).

Electrochromatography

For the electrophoretic separation of mixtures, a filter-paper strip is impregnated with a solution of an electrolyte (usually a buffer solution) and supported in the centre; its two ends are dipped into solutions in which electrodes are immersed. A spot of the material to be fractionated is placed on the paper, the whole apparatus sealed and a potential difference of about 2–10 volts per centimetre applied along the paper. Some separations are carried out at much higher voltages than the above. According to the nature of the charge on the ions of the solute mixture, the solutes will move towards either the anode or the cathode. Thus, the amino acids can be separated either into groups (acid group, neutral group, basic group) or into individual amino acids. The migration velocity for a given substance depends on the magnitude of the ionic charge and the size and shape of the particular molecule. If preferable, paper can be replaced by thin layers of the gels described under 'Gel filtration', above. Many alkaloidal mixtures have been separated by this method and also plant acids, the component sugars of cardiac glycosides and anthraquinone derivatives.

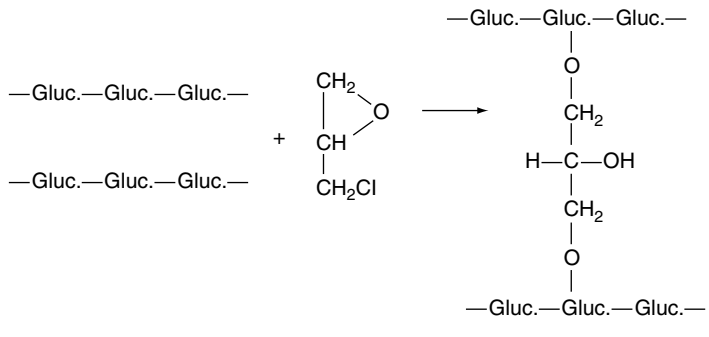


Fig. 17.3
Formation of dextran gels.

A development which combines the advantages of both gel filtration and electrophoresis is that of polyacrylamide gradient gel electrophoresis. It is a two-dimensional electrophoresis system which separates according to mobility of solutes in one direction and according to size in the other.

Capillary electrophoresis is a technique of relatively recent introduction and can give separation efficiencies of the order of 4×10^5 theoretical plates. It provides a more rapid analysis than gel electrophoresis and with detector systems such as the laser-induced fluorescence detector combines a high resolution with a 500-fold increase in sensitivity over UV detection. The method has been used for the analysis of flavonoids.

Affinity chromatography

This method has been developed largely for the resolution of protein mixtures, and it depends on the specific, reversible binding of individual proteins with a particular ligand such as an enzyme substrate or inhibitor. The ligands are coupled with a suitable carrier (cellulose, beaded agarose, controlled-pore glass, polyacrylamide or cross-linked dextrans), possibly with the introduction of a spacer—a suitable chemical moiety such as a hydrocarbon chain—between the ligand and matrix. Excess ligand is removed by washing and the material is packed in a column. A protein mixture in a suitable buffer solution is passed down the column and a protein with sufficient affinity for the bound ligand is retarded and may later be eluted in a purified state by a change in ionic strength or pH of the column buffer. The method has the advantage of preparing in one step a particular component in a high state of purity.

Affinity chromatography has been applied to the purification of enzymes for potential clinical application, for the isolation of certain antibodies and for the specific fractionation of different types of cells (e.g. erythrocytes and lymphocytes).

CHARACTERIZATION OF ISOLATED COMPOUNDS

It is outside the scope of this book to consider in any detail the structure elucidation of natural products. It is sufficient to state that although still utilizing classical chemical methods of degradation, chemists are coming to rely more and more on the use of physical techniques to establish structures of new compounds and to identify known compounds in plant sources. Ultraviolet, infrared, mass and nuclear magnetic resonance spectroscopy together with X-ray crystallographic and optical rotatory dispersion methods have all played a significant role in these developments. Various modifications of mass spectrometry (MS) have become of increasing importance for the structural characterization and determination of the active constituents of plants; these include electron ionization MS, chemical ionization MS, field desorption MS, fast atom bombardment MS and electrospray ionization MS. For an example of the application of electrospray MS combined with sequential tandem mass spectrometry to the investigation of the steroidal saponin mixture of the Chinese and Indian drug *Tribulus terrestris*, see S. Fang *et al*., *Planta Medica*, 1999, **65**, 68. Many problems of structure elucidation which 40 years ago were incapable of investigation, either through paucity of material or through lack of suitable chemical methods, can now readily be solved in a standard research laboratory. J. Schmidt *et al*. (*Phytochemistry*, 2007, **68**, 189), using liquid chromatography and similar combined MS techniques to the above, have demonstrated the importance of such methods for evaluating biosynthetic pathways and for studying the fate of distant natural product precursors in specific plants. They fed [ring-^{13}C-6] tyramine to *Papaver somniferum* seedlings and elucidated the structures of some twenty alkaloids into which the tyramine was incorporated; the alkaloids included those of the morphinan, benzoisoquinoline, protoberberine, benzo[*c*]phenanthridine, phthalide, isoquinoline and protopine classes. The routine analytical application of some of these techniques to plant drug analysis has been considered in Chapter 16.

BIOGENETIC INVESTIGATIONS

The living material used in biochemical research is extremely varied. Some work is possible which utilizes the whole organism with a minimum of disturbance—for example, bacteria, yeasts and moulds can be cultivated and investigated biochemically, and with animals, test substances can be added to the food and the blood and excreta analysed. With intact higher plants, however, the ultimate destruction of the plant for analysis is usually necessary. Minces, breis and homogenates are examples of preparations in which the tissues and the cell wall structures have been destroyed but in which the intracellular particles are still intact. The components of such a mixture can be isolated by centrifugation and the biological activity of each fraction can be tested. The penultimate stage in a biogenetic study is the isolation of the enzymes involved in the pathways under consideration and the *in vitro* demonstration of their properties. Finally, it is becoming increasingly possible to locate and clone the gene responsible for the synthesis of a particular enzyme. Now that the principal overall pathways associated with secondary metabolism have been largely established, it is the enzymic studies that currently receive considerable attention.

The techniques discussed below have been used for the study of secondary metabolism and their application dates from the middle of the last century; they relate principally to the search for the intermediates involved in particular pathways rather than to reaction mechanistics. It must be remembered however that many of the primary metabolic pathways, e.g. the Krebs (TCA) cycle, were established using classical biochemical methods.

TRACER TECHNIQUES

Tracer technology, now widely employed in all branches of science, had its origin in the early part of the last century, when it was realized that elements existed with identical chemical properties but with different atomic weights. Such isotopes may be stable (^{2}H, ^{13}C, ^{15}N, ^{18}O), or the nucleus may be unstable (^{1}H, ^{14}C) and decay with the emission of radiation. If it is possible to detect these isotopes by suitable means, then they can be incorporated into presumed precursors of plant constituents and used as markers in biogenetic experiments.

Radioactive tracers. In biological investigations the use of radioactive carbon and hydrogen, and to a lesser extent and for more specific purposes sulphur, phosphorus and the alkali and alkaline-earth metals, enables the metabolism of compounds to be followed in the living organism. For studies on proteins, alkaloids and amino acids a labelled nitrogen atom may give more specific information than a labelled carbon, but the two available isotopes of nitrogen are both stable, necessitating the use of a mass spectrometer for their use as tracers.

Natural carbon possesses two stable isotopes with mass numbers 12 and 13, the latter having an abundance of 1.10 atoms per cent. Radioactive isotopes of carbon have mass numbers of 10, 11 and 14. ^{10}C has a half-life of 8.8 s and ^{11}C a half-life of 20 min., which limits their usefulness in biological research. However, ^{14}C has an estimated half-life of over 5000 years and in the atomic pile it may be produced by the bombardment of ^{14}N with slow neutrons, the target material usually being aluminium or beryllium nitride.

The immense possibilities in biological research for the use of organic compounds with specific carbon atoms labelled led to the synthesis of many compounds from the inorganic carbon compounds produced in the pile by routes not before commercially utilized. In these syntheses the purity of the product is of great importance, since a small proportion of a strongly radioactive impurity might seriously jeopardize the results of any subsequent experiments.

Many compounds which are most conveniently prepared from natural sources (e.g. certain amino acids by the hydrolysis of proteins) are produced by growing *Chlorella* in an atmosphere containing $^{14}CO_2$. All the carbon compounds of the organism thus become labelled, each compound possessing a uniform labelling of its carbon atoms.

Many tritium (^3H)-labelled compounds are commercially available. Tritium labelling is effected by catalytic exchange (platinum catalyst) in aqueous media, by irradiation of organic compounds with tritium gas and by hydrogenation of unsaturated compounds with tritium gas. Tritium is a pure β-emitter of low toxicity, half-life 12.43 years, with a radiation energy lower than that of ^{14}C.

Detection and assay of radioactively labelled compounds. When radioactive tracers are used in biogenetic studies, adequate methods for the detection and estimation of the label are essential. For the soft and easily absorbed radiation from ^3H-and ^{14}C-labelled compounds the instrument of choice is the liquid scintillation counter. It depends on the conversion of the kinetic energy of a particle into a fleeting pulse of light as the result of its penetrating a suitable luminescent substance. Rutherford successfully used this method in his early studies on radioactivity and he counted the flashes of light produced by bombardment with α-particles on a fluorescent screen prepared from zinc sulphide. The usefulness of the detector was tremendously heightened by the development of the photomultiplier tube, which replaced the human eye in recording the scintillation. Liquid scintillation media, consisting of a solvent in which the excitation occurs and a fluorescent solute which emits the light to actuate the photomultiplier, have also been devised for the purpose of enabling the sample to be incorporated in the same solute, and, hence, attain optimum geometry between sample and scintillator.

Modern instruments are fully automatic (e.g. for 100 samples at a time) and will also measure mixed radiations such as ^3H and ^{14}C (this is possible because, although both are β-emitters, they have different radiation energies. With all counters, the instrument is connected to a suitable ratemeter which records the counts over a given time. With ^{14}C, because of its long half-life, no decay corrections are necessary for normal biogenetic experiments. However the half-life is important in carbon dating of old materials. With ^3H-labelled material some correction for decay may be necessary if samples are stored for any length of time.

The traditional unit of radioactivity has been the *curie*, defined as that quantity of any radioactive nuclide in which the number of disintegrations per second is 3.7×10^{10}. Subunits are the millicurie (3.7×10^7 disintegrations per second) and the microcurie (3.7×10^4 d.p.s.). The SI unit now used for radioactive disintegration rate is the *becquerel* (Bq), which has a disintegration rate of 1 s^{-1}, and its multiples include the gigabecquerel (GBq), at 10^9 s^{-1} (27.027 millicuries), and the megabecquerel (MBq), at 10^6 s^{-1} (27.027 microcuries).

For information on the theoretical basis of radioactive isotope utilization and the regulations governing the use of radioactive substances in universities and research establishments the reader is referred to the standard works and official publications on these subjects.

Autoradiography. A technique used for the location of radioactive isotopes in biological and other material is autoradiography. In this the specimen is placed in contact with a suitable emulsion (e.g. X-ray sensitive film) and after exposure the latter is developed in the usual manner. The resulting autoradiograph gives the distribution pattern of the radioactive substances in the specimen. The method can be applied to whole morphological parts (e.g. leaves) or to histological sections, for which the resulting negative is viewed under a microscope. In a similar manner, radioactive compounds on paper and thin-layer chromatograms can also be detected and the relative amounts of radioactivity in different spots determined by density measurements or by the use of calibrated films.

Precursor–product sequence. For the elucidation of biosynthetic pathways in plants by means of labelled compounds, the precursor–product sequence is commonly invoked. In this a presumed precursor of the constituent under investigation, in a labelled form, is fed to the plant and after a suitable time the constituent is isolated and purified and its radioactivity is determined. If specific atoms of the precursor are labelled, it may be possible to degrade the isolated metabolite and ascertain whether the distribution of radioactivity within the molecule is in accordance with the hypothesis under test. Radioactivity of the isolated compound alone is not usually sufficient evidence that the particular compound fed is a direct precursor, because substances may enter the general metabolic pathways of the plant and from there become randomly distributed through a whole range of products. If this happens, degradation of the isolated constitutent and the determination of the activity of the fragments would probably show that the labelling was random throughout the molecule and not indicative of a specific incorporation of the precursor. Further evidence for the nature of the biochemical incorporation of precursors arises from double- and triple-labelling experiments; either different isotopes, or specific labelling by one isotope at two or more positions in the molecules, are employed. The method has been applied extensively to the biogenesis of many plant secondary metabolites. Leete, in his classical experiment, used two doubly labelled lysines to determine which hydrogen of the lysine molecule was involved in the formation of the piperidine ring of anabasine in *Nicotiana glauca*. His feeding experiments gave the incorporations shown in Fig. 17.4, indicating the terminal *N* to be involved.

It will be appreciated that with the above lysine precursors it is not necessary to have *individual* molecules labelled with both ^{14}C and

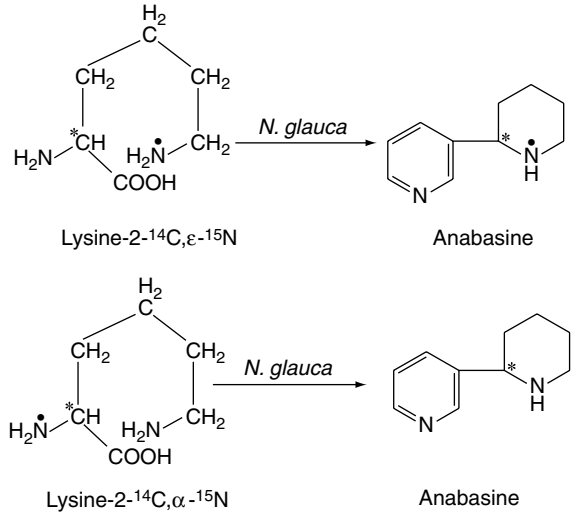
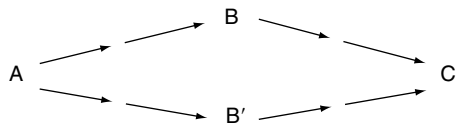


Fig. 17.4
Incorporation of doubly-labelled lysines into anabasine.

^{15}N but that the same result is obtained by using standard mixtures of specifically labelled [^{14}C]lysine and [^{15}N]lysine. (Note: ^{15}N is a stable isomer.) Extensive use has been made of ^{3}H/^{14}C ratios in the study of stereospecific hydrogen elimination reactions.

Competitive feeding. If incorporation is obtained, it is still necessary to consider whether this is in fact the normal route of synthesis in the plant and not a subsidiary pathway, invoked as a result of the atypical availability to the plant of the administered compound. Competitive feeding experiments can be of value in determining which of two possible intermediates is normally used by the plant. In its simplest form, without taking into account a number of other factors, competitive feeding could distinguish whether B or B′ was the normal intermediate in the formation of C from A as below:



Inactive B and B′ are fed with labelled A to separate groups of plants and a control is performed by feeding labelled A only to another group. If the incorporation of activity into C is inhibited in the plants receiving B, but is unaffected in the group receiving B′, then we may conclude that the pathway from A to C probably proceeds via B. In such experiments involving intact plants, the biological variation between normal plants is often so great that it is difficult to perform a controlled investigation. In comparative studies on the rates of demethylation of codeine and unnatural codeine derivatives in *Papaver orientale*, Kirkby and colleagues in 1972 overcame this problem by using the same plant simultaneously as the control and as the test plant. They did this by administering to the plant a mixture of ^{3}H-labelled codeine and ^{14}C-labelled unnatural codeine derivative. The products of the conversion of both could be independently followed by their characteristic radiations, even when the metabolites produced were chemically identical. (For the use of competitive feeding in the elucidation of the biogenesis of tropane alkaloids, see Beresford and Woolley, *Phytochemistry*, 1975, **14**, 2209.)

Administration of precursors. Negative results arising from feeding experiments must also be interpreted with caution; thus, the administered precursor may never have reached the necessary site of synthesis in the plant, or the plant may not, at the time of the experiments, have been synthesizing the constituent under investigation. Two examples of the latter situation which, until discovered, were the cause of misleading results in alkaloid studies were the cessation of hordenine production in barley seedlings 15–20 days after germination and the restricted synthesis of hyoscine, as distinct from hyoscyamine, in old plants of *Datura stramonium*.

Often, the actual weight of labelled material fed to the plant is extremely small and contamination of the solution by microorganisms, during infiltration, can lead to a loss, or even the complete disappearance, of the original compound. This situation is very likely to arise with infiltrations into non-sterile roots—it can, in some instances, be controlled by the use of broad-spectrum antibacterial agents.

A number of methods can be employed to introduce labelled substances into plants. Root feeding is particularly suited to plants which can be grown in hydroponic culture solution and which synthesize the compounds under investigation in the roots. Direct injection of precursor solutions is sometimes possible; this is particularly applicable to plants with hollow stems (Umbelliferae) and to capsules (opium poppy). For the introduction of [1-^{13}C]glucose solution into chamomile flowers K.-P. Adam and J. Zapp (*Phytochemistry*, 1998, **48**, 953) used a microsyringe, injected into the hollow receptacle of the inflorescence. With rigid tissues it is difficult to avoid a loss of solution from the site of injection by this method. Infiltrations can be made into rigid stems by using a wick consisting of a thread drawn through the stem and dipping into the labelled solution; alternatively, a flap can be cut in the stem and this dipped into the solution to be inflitrated.

Sequential analysis. A second method of investigation with ^{14}C is to grow plants in an atmosphere of $^{14}CO_2$ and, by analysis of the plants at given time intervals, to obtain the sequence in which various related compounds become labelled. From the results obtained, certain biosynthetic routes may become apparent and others rejected. Here, again, degradation of the isolated radioactive compounds is important, because some units of the molecule may become labelled more rapidly than others. This method has been very successfully used in the elucidation of the path of carbon in photosynthesis and also for determining the sequential formation of the opium, hemlock and tobacco alkaloids. Exposure periods to $^{14}CO_2$ as short as 5 min. have been used to obtain evidence of the biosynthetic sequence piperitone → (−)-menthone → (−)-menthol in *Mentha piperita*. In a number of instances the pathways suggested by these experiments have been at variance with those obtained by feeding labelled intermediates; it would seem that the latter are therefore examples of non-obligatory intermediates.

Use of stable isotopes. The stable isotopes ^{2}H, ^{13}C, ^{15}N, and ^{18}O, which have a low natural occurrence, can be used in the same way as radioactive elements for labelling compounds to be used as possible intermediates in biosynthetic pathways. The usual methods of detection are mass spectroscopy (^{15}N and ^{18}O) and nuclear magnetic resonance (NMR) spectroscopy (^{1}H and ^{13}C). It is the latter which is becoming of increasing significance for biosynthetic studies (the use of mass spectroscopy and NMR spectroscopy in biogenetic studies should not be confused with their extensive use in structural analysis of organic compounds).

ISOLATED ORGANS, TISSUES AND CELLS

The cultivation of isolated organs and tissues of plants eliminates interference from other parts of the plant which may produce secondary changes in the metabolites. It can be used for feeding experiments in conjunction with labelled compounds and is also useful for the determination of the site of synthesis of particular compounds.

Isolated shoots of plants, when placed in a suitable solution or in water, will usually remain turgid for some days and during this time presumably have a normal metabolism; soon, however, a pathological metabolism commences. The technique can be refined by aseptically connecting the cut end of the shoot to a reservoir of suitable sterile nutrient, when the shoots will remain normal for much longer periods. Such shoots often develop roots at the cut ends—a factor which could invalidate the results of an experiment. The technique has been recently (2007) used for the study of the biogenesis of hyperforin and adhyperforin in *Hypericum performatum* (q.v.). Isolated leaves can be similarly maintained. *Rooted leaves* have been used in studies on *Nicotiana* and *Datura*. By this method a large quantity of root is obtained with a relatively small amount of aerial parts. It has the advantage that the nutrient solution requires no sugar, as sufficient starch is synthesized in the leaf and consequently bacterial and fungal growth in the nutrient solution is minimized. *Petal discs* have been used in the investigation of the biosynthesis of oil of rose. *Isolated roots* have been extensively used. Surface-sterilized seeds

are germinated under aseptic conditions, and the root is severed and transferred to a sterile nutrient solution. Under suitable conditions a rapid growth is obtained and subcultures may be produced as necessary. In this way strains of tomato roots have been maintained for many years. For biogenetic and growth studies, selected compounds can be added to the culture as necessary. It has been demonstrated by this method that tropane alkaloids are formed in the roots of a number of Solanaceae. Also, the incorporation of a number of precursors into the alkaloids has been followed.

The technique can be extended to the cultivation of *isolated tissues and cells* (see Chapter 13), the use of which affords considerable potential in the investigation of biogenetic pathways. They offer the prospect of absolutely uniform plant material, obtainable at all times and manageable under regulated and reproducible conditions—factors rarely possible in work with entire living plants. Furthermore, potential precursors of the metabolite under investigation can easily be added to the system and samples can be taken repeatedly for analysis. The aseptic nature of the culture means that bacterial and fungal modifications of the precursor are eliminated. The method has recently been used to study the bifurcation of the taxoid biosynthetic pathway in *Taxus caspidata* by the administration of early precursors to the cell culture (R. E. B. Ketchum *et al*., *Phytochemistry*, 2007, **68**, 335).

GRAFTS

Grafting techniques have considerable use in biosynthetic studies, particularly for the determination of the sites of primary and secondary metabolism of some secondary plant products.

Alkaloid formation by grafted plants has been extensively studied in *Nicotiana* and the tropane alkaloid-producing Solanaceae. Thus, tomato scions grafted on to *Datura* stocks accumulate tropane alkaloids, whereas *Datura* scions on tomato stocks contain only a small amount of tropane alkaloids. This suggests the main site of alkaloid synthesis to be the *Datura* roots. However, interspecific grafts involving *D. ferox* and *D. stramonium* demonstrate that secondary modifications of alkaloids do occur in the aerial parts of these plants. This has been demonstrated conclusively by feeding alkaloid-free scions of *D. ferox* on *Cyphomandra betacea* stocks with hyoscyamine; on subsequent analysis, hyoscine was isolated from the leaves. More details of this conversion are given in the appropriate section.

In other genera it has been shown by grafts involving tomato that the alkaloid anabasine is produced in the leaves of *Nicotiana glauca*, nicotine in the roots of *N. tabacum* and the pungent principle of capsicum, capsaicin, in the developing fruits. Reciprocal grafts of high and low resin-yielding strains of *Cannabis* have shown this biochemical character to be determined by the aerial parts.

MUTANT STRAINS

Large numbers of mutant strains of microorganisms have been produced which lack a particular enzyme, which results in their metabolism being blocked at a particular stage. Such an organism may accumulate the intermediate compound immediately before the block and for its survival may require an artificial supply of another intermediate which arises after the block. Such organisms are obviously useful materials in biosynthetic studies and have proved of major importance in some investigations.

A mutant of *Lactobacillus acidophilus*, by its ability to utilize a constituent of 'brewer's solubles' but not acetate, led to the isolation of mevalonic acid, an important intermediate of the isoprenoid compound pathway.

Ultraviolet-induced mutants of ergot auxotrophic with respect to a number of amino acids have been produced; cultures of these have been used to inoculate rye and the resulting alkaloid contents of the sclerotia have been investigated. *Gibberella* mutants have been used to obtain novel isoprenoid compounds. With higher plants, in spite of repeated attempts with chemicals and irradiation, less success has been obtained in producing mutants useful for the study of biogenesis of therapeutically active compounds; the production of mature haploid plants from cell cultures of pollen (see Chapter 14) may offer a more satisfactory starting material for future work.

Further reading

Harborne JB 1998 Phytochemical methods—a guide to modern techniques. Chapman and Hall (now under Kluwer Academic Publishers imprint), Dordrecht, Nl

Houghton PJ, Raman A 1998 Laboratory handbook for the fractionation of natural products. Chapman and Hall (now under Kluwer Academic Publishers imprint), Dordrecht, Nl

Khaledi MG 1998 High performance capillary electrophoresis. J. Wiley, New York, NY

Meyer V 1999 Practical high-performance liquid chromatography, 3rd edn. J. Wiley, Chichester, UK

Mukhopadhyay M 2000 Natural extracts using supercritical carbon dioxide. CRC Press, Boca Raton, FL

Phillipson JD 2007 Phytochemistry and pharmacognosy. Phytochemistry 68: 2960–2972. *Review with 63 references*

Reich E, Schibli A 2007 High-performance TLC for the analysis of medicinal plants. Thieme, Stuttgart

Taylor LT 1996 Supercritical fluid extraction. J. Wiley and Sons, New York, NY

Wagner H, Bladt S, Rickl V 2003 Plant drug analysis. A thin-layer chromatography atlas. Springer, Berlin

18

Basic metabolic pathways and the origin of secondary metabolites

The biosynthesis of both primary and secondary metabolites is dependent on the highly organized structure of the plant and animal cell. Unlike animal cells, those of plants possess a rigid cell wall and are separated one from another by an intercellular structure, the middle lamella. Direct connection between adjacent cells is maintained by primary pit fields through which pass the plasmodesmata. Within the cell wall is the protoplast consisting of cytoplasm, nucleus and various organelles.

The light microscope shows the nucleus to contain various inclusions such as nucleoli, chromosomes (stainable during cell division) and the nuclear sap. The nucleus appears to be suspended within the cell by the cytoplasm, in which there may be large vacuoles with their own characteristic contents (crystals, aleurone grains, etc.). Other cytoplasmic inclusions are mitochondria, Golgi bodies, lysosomes and plastids (chloroplasts, chromoplasts, leucoplasts), but their structure is not resolvable, because of their small size. Electron microscopy shows a number of the subcellular organelles to have a highly organized fine structure suited to the many and varied biochemical processes which they perform.

Although, because of varied form and function, it is not possible to illustrate a 'typical' plant cell Fig. 18.1A shows diagrammatically the structures that might be expected in an unspecialized young root cell. Such a cell possesses a rigid wall, which immediately distinguishes it from an animal cell, but no chloroplasts and only small vacuoles are present. In a green plant cell (Fig. 18.1B) the same components are present but the large vacuole has oppressed the nucleus and cytoplasm towards the wall; green plastids often with starch granules are common.

The organelles allow the creation of different chemical environments within one cell, and furthermore, by their structure they increase the area available for surface reactions which are all-important in biological systems. A description of the various organelles is given in the 14th edition of this book and Fig. 18.1C illustrates some aspects of their interdependence in the normal functioning of the cell. The molecular structures of these bodies have been extensively studied and details will be found in standard botanical texts.

Some basic metabolic pathways appear to be similar in both plants and animals, whereas others are more restricted in their occurrence. It is to the secondary plant products (i.e. those not necessarily involved in the essential metabolism of the cell) that the majority of vegetable drugs owe their therapeutic activity and so it is in these that pharmacognosists are particularly interested. However, as illustrated in Fig. 18.2, the production of these secondary metabolites is dependent on the fundamental metabolic cycles of the living tissue and so a brief indication of the latter will also be given; for fuller accounts of these, the student should consult a standard work on plant biochemistry.

ENZYMES

Many reactions occurring in the cell are enzyme-dependent, and before anything was known of the chemical nature of these substances it was recognized that they were organic catalysts produced by animal and vegetable cells. Their wide distribution and the delicacy of their operation has long been appreciated; they engineer reactions at normal temperatures and at pH values around neutral in a manner not possible in the laboratory.

An enzyme usually acts on one substance or class of substances, since it is specific for a particular atomic group or linkage. Specificity, however, varies; lipases are, in general, not highly specific, whereas fumarase acts only upon L-malate and fumarate, while D-malate is a competitive inhibitor of fumarase. Enzymes are also stereo- and regiospecific in their actions and, as it becomes possible to prepare more rare examples, organic chemists are becoming increasingly aware of enzyme potential for carrying out single-step transformations with complete

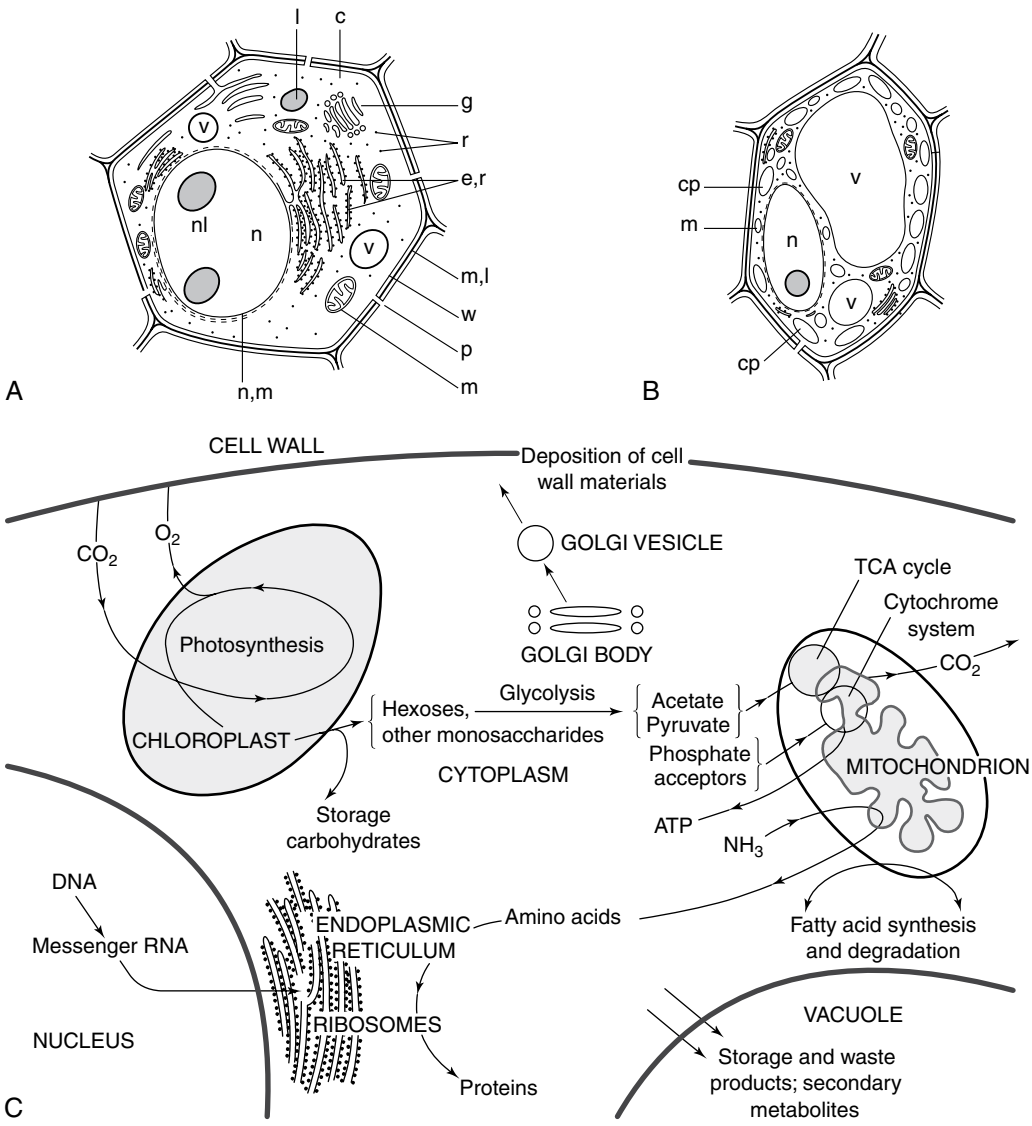


Fig. 18.1
The plant cell. A, diagrammatic representation of an undifferentiated cell: c, cytoplasm; e.r, endoplasmic reticulum; g, Golgi apparatus; l, lysosome; m, mitochondrion; m.l, middle lamella; n, nucleus; nl, nucleolus; n.m, nuclear membrane; p, pit in wall; r, ribosomes, w, primary cell wall; v, vacuole. B, green plant cell: cp, chloroplast; m, mitochondrion, n, nucleus; v, vacuole. C, Flow chart of some cell metabolites.

stereochemical exactitude, an aspect important in the synthesis of many drugs. An enzyme will convert many thousand times its own weight, and the gradual diminution in activity which takes place is probably due to secondary reactions which bring about destruction of the enzyme.

The enzymology of the secondary metabolic pathways in plants has still been little investigated but progress is being made and here again cell cultures have proved useful in that they are often a better source for the isolation of enzymes than is the differentiated plant. In some cases, e.g. cell cultures of volatile oil-containing plants, little or no oil accumulates in the culture owing to the absence of storage receptacles but the relevant enzymes for terpenoid synthesis are still manufactured and preparations of them can be made.

New horizons for the study of the enzymology of secondary metabolism have now opened up as a result of advances in gene technology. In suitable instances, by cloning, the cDNA responsible for an enzyme's synthesis can be expressed in another organism such as a bacterium and large amounts of enzyme prepared. By conventional methods only very small amounts of purified enzyme could be obtained from the original plant material. Of particular interest has been the isolation, characterization and cloning of the enzyme strictosidine synthase. This governs the key reaction for the commencement of the biosynthesis of the very many monoterpenoid indole alkaloids, namely the condensation of tryptamine and secologanin to give 3α(S)-strictosidine (see Chapter 26); for a review (126 references) on this enzyme see T. M. Kutchan, *Phytochemistry*, 1993, **32**, 493.

By means of relatively new technology, enzymes can be immobilized on a suitable carrier either in whole plant cells or as the isolated enzyme. In this way these biocatalysts can be repeatedly used in analytical and clinical chemistry, or to effect specific chemical transformations.

Like other catalysts, enzymes influence the rate of a reaction without changing the point of equilibrium. For example, lipase catalyses either the synthesis of glycerides from glycerol and fatty acids or the hydrolysis of glycerides, the final point of equilibrium being the same in either case. Similarly, β-glucosidase (prunase) has been used for both the synthesis and the hydrolysis of β-glucosides. In plants such reversible reactions may proceed in one direction or the other under different conditions, often resulting in daily and seasonal variations in the accumulation of metabolites.

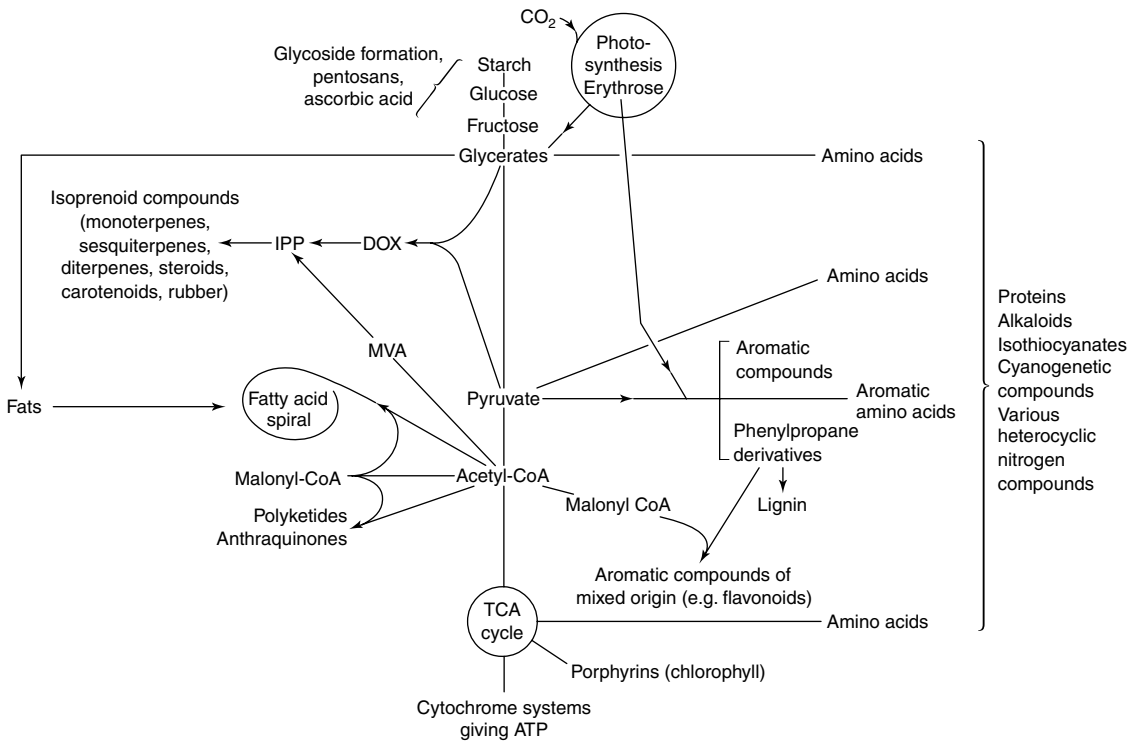
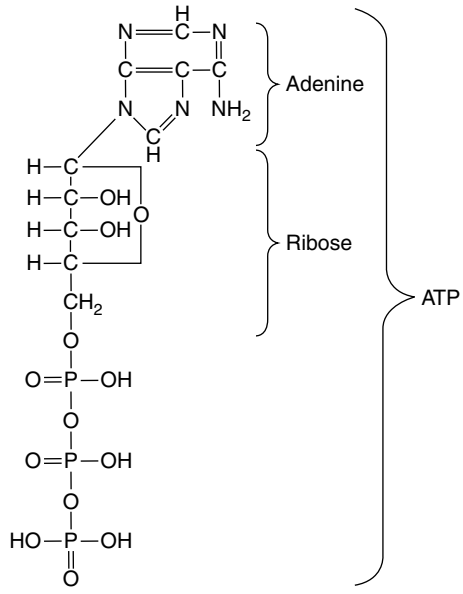


Fig. 18.2
Origins of some secondary metabolites in relation to the basic metabolic pathways of plants. DOX=deoxyxylulose; IPP=isopentenyl diphosphate; MVA=mevalonic acid.

Enzymes are colloidal in nature and consist of protein or contain protein as an essential part. They may be partly purified, and in some cases isolated, by the methods of protein chemistry (i.e. by fractional precipitation, dialysis and, more recently, gel and affinity chromatography, see Chapter 17). Most enzymes are soluble either in water or in dilute salt solutions and are precipitated by alcohol or acetone (acetone powders) and by high concentrations of salts. They are inactivated by heat, ultraviolet light and X-rays or by any treatment which brings about denaturation of proteins.

The activity of enzymes is markedly affected by the reaction of the medium and the presence of substances such as salts. It is well known, for example, that pepsin works only in an acid medium and trypsin in an alkaline one. In general, carbohydrases have pH optima of 3.8–7.5 and lipases optima of pH 5–8, while enzymes which act on bases all have optima more alkaline than pH 7.

The effect of heat on enzymes is of considerable importance in the drying of drugs. At low temperatures enzymic changes are not usually marked, although the proteolytic or protein-splitting enzymes in cod livers do bring about some hydrolysis at temperatures approaching zero. The optimum working temperatures of different enzymes vary, but they usually lie between 35 and 50°C. At temperatures of about 60°C destruction of the enzymes is usually fairly rapid, although considerable loss may take place below this temperature. When dry, enzymes show increased resistance to heat; thus, zymase, which in the presence of moisture is rapidly inactivated at 50°C, will, when dry, resist a temperature of 85°C.



Chemical nature

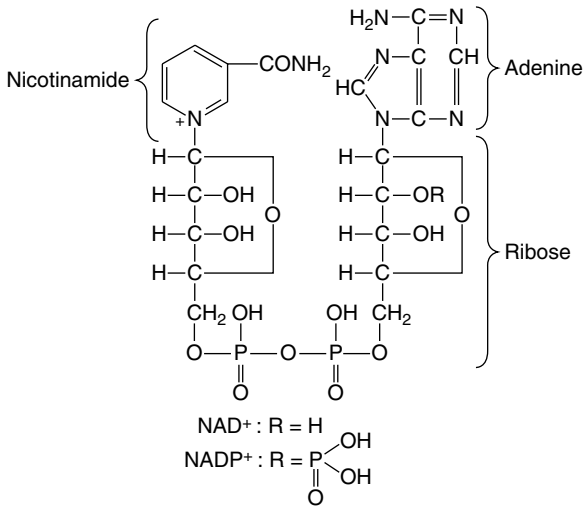
Following the isolation of urease in crystalline form by Sumner in 1926, many other enzymes have been prepared in a crystalline form and their protein nature has been established. Their molecular weights are high, but vary from about 9000 (hydrogenase) to about 1 000 000. In many cases the component amino acids are known and in some cases (e.g. ribonuclease) the amino acid sequence within the molecule has been determined. During the isolation of an enzyme, the purified protein (apoenzyme) may be inactive but regains its activity in the presence of an essential coenzyme or activator, which may be organic or inorganic.

Coenzymes

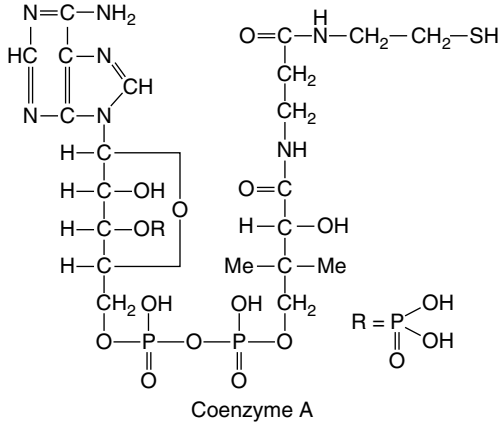
Some enzymes which require the presence of smaller organic molecules, called coenzymes, before they can function are of very common occurrence and participate in a large number of important biochemical reactions. One group of coenzymes consists of esters of phosphoric acid and various nucleosides. The adenosine and uridine phosphates contain one basic unit each (mononucleotides); they serve to transport energy in the form of high-energy phosphate bonds and this energy is made available for biochemical reactions in the presence of the appropriate enzyme by hydrolysis of the bond. Thus, the terminal phosphate bond of the adenosine triphosphate (ATP) on hydrolysis to adenosine diphosphate (ADP) affords 50 000 J mol^{-1}.

Uridine triphosphate (UTP) is involved in the synthesis of sucrose via diphosphate glucose which is also associated with the formation of uronic acids and cellulose.

Nicotinamide-adenine dinucleotide (NAD) and nicotinamide-adenine dinucleotide phosphate (NADP) contain two basic units each and are termed dinucleotides. They function in oxidation–reduction systems with appropriate enzymes; the oxidized forms are written NAD^+ and $NADP^+$ and the reduced forms NADH and NADPH respectively.



Another important coenzyme is coenzyme A (CoA), which contains the units adenosine-3,5-diphosphate, pantothenic acid-4-phosphate and thioethanolamine. It participates in the transfer of acetyl and acyl groups, acetyl-CoA (active acetate) having a central role in plant and animal metabolism.



Riboflavine (Fig. 31.2) is a component of the two coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). They participate in the biological oxidation–reduction system, and FAD facilitates the transfer of H^+ ions from NADH to the oxidized cytochrome system.

Other coenzymes are the decarboxylation coenzymes thiamine, biotin and pyridoxine (Fig. 31.2). Folic acid (Fig. 31.2) derivatives participate in enzymatic reactions which involve one-carbon fragment transfers.

A series of quinones e.g. plastoquinone and ubiquinone are widely distributed in plants, animals and microorganisms and function in biological electron transfer processes.

Classification of enzymes

As more enzymes are isolated, it becomes important to have a precise scheme of classification and nomenclature. Many long-established names such as pepsin, prunase, diastase and names for enzyme mixtures, such as emulsin and zymase, continue in use. A step towards uniform nomenclature was made when they were denoted by the name of the substrate and the termination '-ase'. Classes of enzymes were named similarly. Thus, the general term 'esterase' includes lipases, which hydrolyse fats; chlorophyllase hydrolyses chlorophyll; etc. The 1961 *Report of the Commission on Enzymes** made the recommendation that the chemical reactions catalysed be generally adopted for classification and nomenclature. This, of course, presupposes that the exact chemical reaction is known; see also the 1984 International Union of Biochemistry publication (London, UK: Academic Press) *Enzyme Nomenclature* and any later reports. Enzymes are classified into six main groups (Table 18.1).

Oxidoreductases. As any oxidation implies a simultaneous reduction, the names 'oxidase' and 'reductase' may be applied to a single type of enzyme. Eleven different groups of oxidoreductases are recognized. Many oxidases (that is, enzymes that utilize molecular oxygen as acceptor) convert phenolic substances to quinones. They act on guaiacum resin to produce a blue colour which is used as a test for their detection. Oxidases include laccase (1.10.3.2), present in lac, and ascorbate oxidase (1.10.3.3), which is widely distributed in plants. Oxalate oxidase (1.2.3.4), a flavoprotein present in mosses and the leaves of higher plants, oxidizes oxalic acid into carbon dioxide and hydrogen peroxide. Peroxidases (1.11) are distinguished by the fact that they use hydrogen peroxide and not oxygen as the hydrogen acceptor. Catalase (1.11) must be regarded as an exception, since it catalyses the decomposition of hydrogen peroxide. An oxidoreductase of the morphine biosynthetic pathway (Chapter 26) has recently been purified and characterized; it catalyses the stereoselective reduction of salutaridine to 7(*S*)-salutaridinol using NADPH as cosubstrate.

Hydrolases. These include many different types, of which the following are some of pharmaceutical importance.

1. Hydrolysing esters. These include lipases (3.1), which may be of vegetable or animal origin and which hydrolyse glycerides.

* This report has a numbered classification for each enzyme. For example, the enzyme present in garlic, alliine-lyase, is numbered 4.4.1.4. The first number denotes the fourth of the six main groups already mentioned and the other numbers further subdivisions. Thus:

4 = lyases
4.4 = carbon-sulphur lyases
4.4.1 = In this example a further subdivision is not required.
4.4.1.4 = the fourth enzyme listed in the group, namely alliine-lyase (formerly known as alliinase) or allyl sulphinate-lyase.

Table 18.1 Classification of some enzymes.

Group	Trivial name	Systematic name
1. Oxidoreductases	Glucose dehydrogenase	β-D-Glucose: NAD(P)-oxidoreductase
	p-Diphenyl oxidase (lactase)	*p*-Diphenol: O_2 oxidoreductase
	Peroxidase	Donor: H_2O_2 oxidoreductase
	Catalase	H_2O_2: H_2O_2 orthoreductase
2. Transferase	α-Glucan phosphorylase	α-1,4-Glucan: orthophosphate glucosyl-transferase
3. Hydrolases	Lipase	Glycerol ester hydrolase
	Chlorophyllase	Chlorophyll chlorophyllidohydrolyase
	Tannase	Tannin acyl-hydrolase
	α-Amylase	α-1,4-Glucan 4-glucano-hydrolyase
	Inulase	Inulin 1-fructanohydrolase
4. Lyases	Aldolyase	Ketose-l-phosphate aldehydelyase
	Decarboxylase	L-Tryptophan-decarboxylase
5. Isomerases	Maleate isomerase	Maleate *cis-trans*-isomerase
6. Ligases (Synthetases)	Asparagine synthetase	L-Aspartate: ammonia ligase (ADP)

Mammalian lipases also hydrolyse other esters, such as phenyl salicylate, acetylcholine and atropine. Other esterases are chlorophyllase (3.1.1.14), which hydrolyses chlorophyll, and tannase (3.1.1.20), which hydrolyses ester links in tannins. Other esterases probably occur in many drugs which contain esters in their volatile oils (e.g. valerian).

2. Hydrolysing sugars and glycosides. To the important group of glycoside hydrolases (3.2.1) belong all those enzymes that hydrolyse sugars, carbohydrates and glycosides. Those hydrolysing sugars include β-fructofuranosidase (sucrase or invertase), lactase, maltase, gentiobiase and trehalase. Polysaccharide enzymes are represented by α-amylase (3.2.1.1), β-amylase, cellulase, lichenase, inulase. Among the glycoside-hydrolysing enzymes are β-glucosidase or β-D-glucoside glucohydralase (3.2.1.20), which has a wide specificity for β-D-glucopyranosides. More specific glucoside-hydrolysing enzymes are those acting on salicin, amygdalin, sinigrin and cardiac glycosides.
3. Hydrolysing the C–N linkage. Many enzymes act on peptide bonds. To this group belong the well-known animal enzymes pepsin, rennin, trypsin, thrombin and plasmin, and the vegetable enzymes papain (from *Carica papaya*) and ficin (from species of fig). All the above belong to the group 3.4.4. Other enzymes acting on linear amides (3.5.1) are asparaginase (present in liquorice and many other plants) and urease. Cyclic amides are acted on by penicillinase (3.5.2.6).

Lyases. Two important decarboxylases in the biosynthesis of monoterpenoid indole alkaloids and benzylisoquinoline alkaloids are L-tryptophan-decarboxylase (EC 4.1.1.27) and L-tyrosine/L-dopa-decarboxylase (EC 4.1.1.25), respectively.

Isomerases. Two enzymes important in pathways leading to medicinally important metabolites are isopentenyl diphosphate (IPP) isomerase (EC 5.3.3.2) amd chalcone isomerase (EC 5.5.1.6). The former is involved in the isomerization of IPP and dimethylallyl diphosphate (Fig. 18.18), an essential step in the formation of terpenoids, and the latter is a key enzyme catalysing the isomerization of chalcones to their corresponding flavanones (Fig. 18.11).

PHOTOSYNTHESIS

Photosynthesis, by which the carbon dioxide of the atmosphere is converted into sugars by the green plant, is one of the fundamental cycles on which life on Earth, as we know it, depends. Until 1940, when investigations involving isotopes were undertaken, the detailed mechanism of this 'carbon reduction' was unknown, although the basic overall reaction,

$$CO_2 + H_2O \xrightarrow{\text{light}} (CH_2O) + O_2$$

had been accepted for many years.

Photosynthesis occurs in the chloroplasts: green, disc-shaped organelles of the cytoplasm which are bounded by a definite membrane and which are autoreproductive. Separated from the rest of the cell, the chloroplasts can carry out the complete process of photosynthesis. Bodies with similar properties are found in cells of the red algae but these contain, in addition to chlorophyll as the principal pigment, other tetrapyrrole derivatives—the phycobilins.

The light microscope reveals no definite internal structure of the chloroplasts, but electron microscopy shows these bodies to have a highly organized structure in which the chlorophyll molecules are arranged within orderly structures (grana), each granum being connected with others by a network of fibres or membranes. According to one theory, the flat chlorophyll molecules themselves are orientated between layers of protein and lipid molecules so that the whole chloroplast can be looked upon as a battery containing several cells (the grana), each cell possessing layers of plates (the chlorophyll molecules).

Two fundamental processes which take place in photosynthesis, both of which require light, are the production of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and phosphate and the light-energized decomposition of water (the Hill reaction; named after the English biochemist Robert Hill, 1899–1991):

$$H_2O \xrightarrow{h\nu} 2[H] + \tfrac{1}{2}O_2$$

ATP is a coenzyme and the high energy of the terminal phosphate bond is available to the organism for the supply of the energy necessary for endergonic reactions. The Hill reaction produces free oxygen

and hydrogen ions which bring about the conversion of the electron carrier, NADP, to its reduced form NADPH (see 'Coenzymes').

In this complicated process, two systems, Photosystem I and Photosystem II (also known as pigment systems I and II) are commonly referred to; they involve two chlorophyll complexes which absorb light at different wavelengths (above and below $\lambda = 685$ nm). Photosystem II produces ATP and Photosystem I supplies all the reduced NADP and some ATP. In these light reactions the chlorophyll molecule captures solar energy and electrons become excited and move to higher energy levels; on returning to the normal low-energy state, the electrons give up their excess energy, which is passed through a series of carriers (including in the case of Photosystem II plastoquinone and several cytochromes) to generate ATP. Photosystem I involves an electron acceptor and the subsequent reduction of ferredoxin in the production of NADPH. Reference to the current literature indicates that the nature and organization of the photosystems remains a very active research area. Students will have observed that an alcoholic solution of chlorophyll possesses, in sunlight, a red fluorescence—no carriers are available to utilize the captured energy and it is re-emitted as light. Hill first demonstrated in 1937 that isolated chloroplasts, when exposed to light, were capable of producing oxygen, provided that a suitable hydrogen acceptor was present. Work with isotopes has since proved that the oxygen liberated during photosynthesis is derived from water and not from carbon dioxide.

Following the light reactions, a series of dark reactions then utilize NADPH in the reduction of carbon dioxide to carbohydrate.

Current research suggests that terrestrial plants can be classified as C_3, C_4, intermediate C_3–C_4 and CAM plants in relation to photosynthesis.

C_3 plants

The elucidation of the carbon reduction cycle (Fig. 18.3), largely by Calvin and his colleagues, was in large measure determined by methods dependent on exposing living plants (*Chlorella*) to ^{14}C-labelled carbon dioxide for precise periods of time, some very short and amounting to a fraction of a second. The radioactive compounds produced were then isolated and identified. In this way a sequence for the formation of compounds was obtained. 3-Phosphoglyceric acid, a C_3 compound, was the compound first formed in a labelled condition but it was only later in the investigation, after a number of 4-, 5-, 6- and 7-carbon systems had been isolated, that ribulose-1,5-diphosphate was shown to be the molecule with which carbon dioxide first reacts to give two molecules of phosphoglyceric acid. An unstable intermediate in this reaction is 2-carboxy-3-ketopentinol.

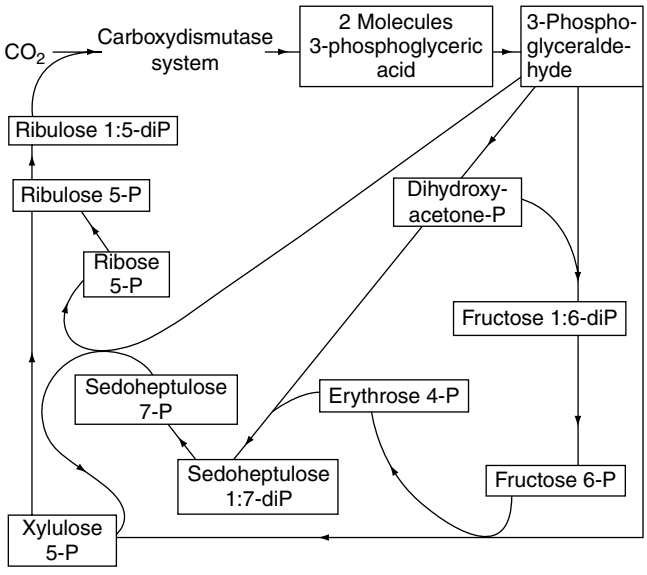


Fig. 18.3
The path of carbon in photosynthesis (after Calvin); the formulae of the sugars involved are given in Chapter 20.

C_4 plants

Some plants which grow in semi-arid regions in a high light intensity possess an additional carbon-fixation system which, although less efficient in terms of energy utilization, is more effective in its use of carbon dioxide, so cutting down on photorespiration and loss of water. Such plants are known as C_4 plants, because they synthesize, in the presence of light, oxaloacetic and other C_4 acids. Carbon assimilation is based on a modified leaf anatomy and biochemistry. Its presence in plants appears haphazard and has been linked with the so-called Kranz syndrome of associated anatomical features. In the mesophyll cells pyruvate is converted via oxaloacetate to malate, utilizing carbon dioxide, and the malate, or in some cases aspartate, is transported to the vascular bundle cells, where it is oxidatively decarboxylated to pyruvate again, carbon dioxide and NADPH, which are used in the Calvin cycle. Pyruvate presumably returns to the mesophyll cells. Plants possessing this facility exhibit two types of photosynthetic cells which differ in their chloroplast type. Intermediate C_3–C_4 pathways are also known.

CAM plants

This term stands for 'crassulacean acid metabolism', so called because it was in the Crassulaceae family that the distinctive character of a build-up of malic acid during hours of darkness was first observed. Other large families, including the Liliaceae, Cactaceae and Euphorbiaceae, possess members exhibiting a similar biochemistry. As with C_4 plants, this is an adaptation of the photosynthetic cycle of plants which can exist under drought conditions. When water is not available, respiratory carbon dioxide is recycled, under conditions of darkness, with the formation of malic acid as an intermediate. Carbon dioxide and water loss to the atmosphere are eliminated, a condition which would be fatal for normal C_3 plants.

From fructose, produced in the Calvin cycle, other important constituents, such as glucose, sucrose and starch, are derived: erythrose is a precursor in the synthesis of some aromatic compounds. Glucose-6-phosphate is among the early products formed by photosynthesis and it is an important intermediate in the oxidative pentose phosphate cycle and for conversion to glucose-1-phosphate in polysaccharide synthesis.

CARBOHYDRATE UTILIZATION

Storage carbohydrate such as the starch of plants or the glycogen of animals is made available for energy production by a process which involves conversion to pyruvate and then acetate, actually acetyl-coenzyme A, the latter then passing into the tricarboxylic acid cycle (Fig. 18.4). As a result of this, the energy-rich carbohydrate is eventually oxidized to carbon dioxide and water. During the process, the hydrogen atoms liberated are carried by coenzymes into the cytochrome system, in which energy is released in stages, with the possible formation of ATP from ADP and inorganic phosphate. Eventually the hydrogen combines with oxygen to form water.

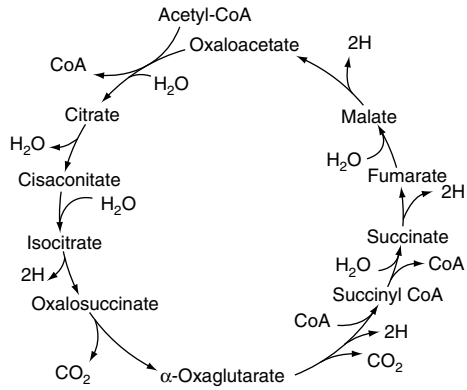


Fig. 18.4
The tricarboxylic acid cycle (TCA) or Krebs cycle.

A number of pathways for the initial metabolism of glucose are known for various living tissues. One involves compounds which are found in the photosynthetic cycle, and it appears as a reversal of this cycle but the mechanism is apparently quite different. Another pathway is the Embden–Meyerhoff scheme of glycolysis (Fig. 18.5).

The kinetics and enzyme systems involved in the above pathways have been studied extensively. It will be noted that one molecule of glucose can give rise to two molecules of pyruvate, each of which is converted to acetate, and one molecule of carbon dioxide. One turn of the TCA cycle represents the oxidation of one acetate to two molecules of carbon dioxide, giving rise to twelve molecules of ATP. The overall reaction for the metabolism of one molecule of glucose in terms of ADP and ATP is

$$C_6H_{12}O_6 + 6CO_2 + 38ADP + 38P\ (inorganic) \longrightarrow 6H_2O + 6CO_2 + 38ATP$$

The above schemes, given in barest outline, are fundamental not only for the building up and breaking down of reserve foodstuffs, but also in that the intermediates are available for the biosynthesis of all other groups of compounds found in plants. The levels at which some of the important groups arise are indicated in Fig. 18.2.

GLYCOSIDES

Glycosides are formed in nature by the interaction of the nucleotide glycosides—for example, uridine diphosphate glucose (UDP-glucose)—with the alcoholic or phenolic group of a second compound. Such glycosides, sometimes called *O*-glycosides, are the most numerous ones found in nature. Other glycosides do, however, occur in which the linkage is through sulphur (*S*-glycosides), nitrogen (*N*-glycosides) or carbon (*C*-glycosides).

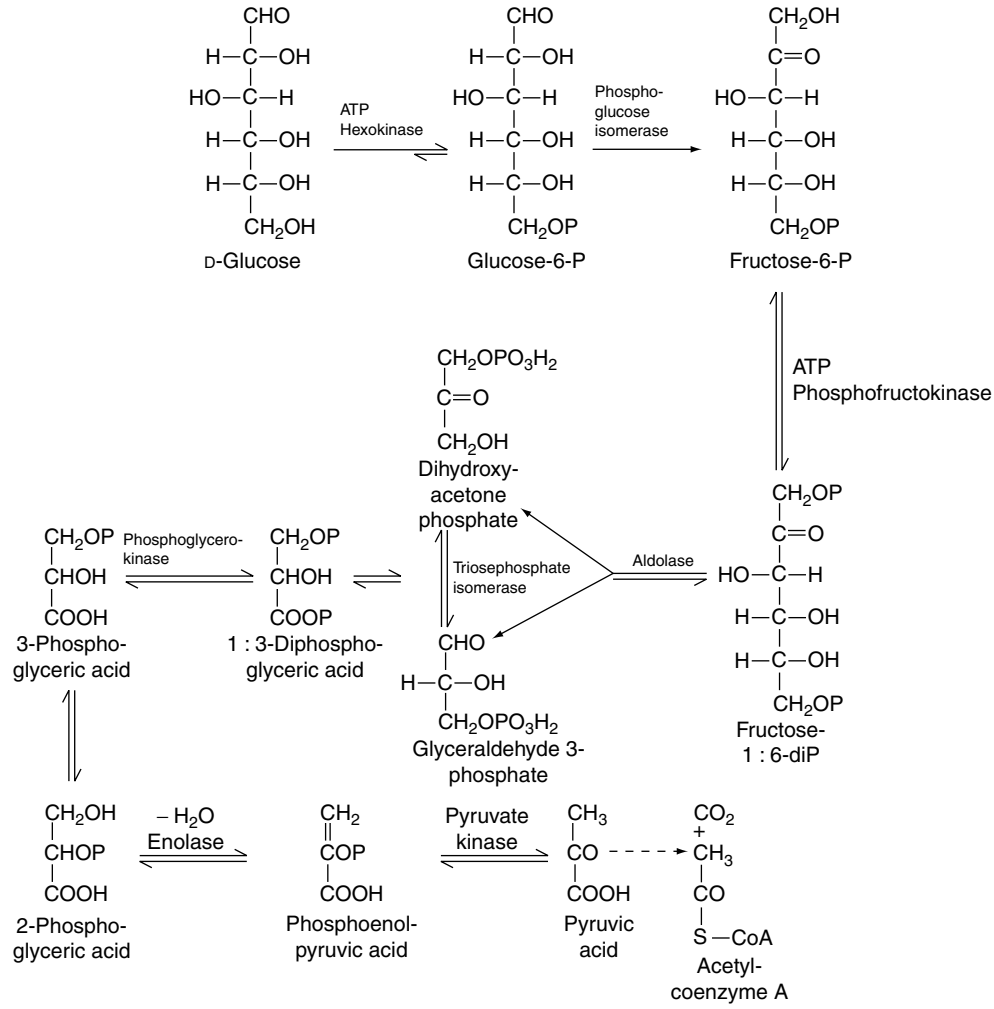
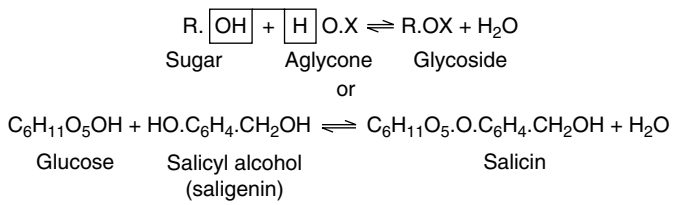


Fig. 18.5
Outline of the Embden–Meyerhoff scheme of glycolysis.

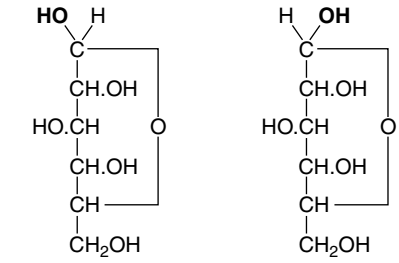
The formation and hydrolysis of an *O*-glycoside such as salicin may be represented:



It will be noted that such reactions are reversible and in plants glycosides are both synthesized and hydrolysed under the influence of more or less specific enzymes. While glycosides do not themselves reduce Fehling's solution, the simple sugars which they produce on hydrolysis will do so with precipitation of red cuprous oxide.

The sugars found in glycosides may be monosaccharides such as glucose, rhamnose and fucose or, more rarely, deoxysugars such as the cymarose found in cardiac glycosides. More than one molecule of such sugars may be attached to the aglycone either by separate linkages, which is rare, or, more commonly, as a di-, tri- or tetrasaccharide. Such complex glycosides are formed by the stepwise addition of sugars to the aglycone molecule.

Since sugars exist in isomeric α- and β-forms, both types are theoretically possible. Practically all natural glycosides, however, are of the β-type, although the α-linkage is found in nature in some carbohydrates such as sucrose, glycogen and starch. In *k*-strophanthoside, a glycoside formed from the aglycone strophanthidin and strophanthotriose (cymarose + glucose + glucose) the outer glucose molecule has the α-linkage and the inner glucose the β-linkage. Isomeric glycosides may be prepared synthetically; for example, from glucose and methyl alcohol one obtains both the α- and β-methyl glucosides by introducing methyl groups into the OH groups printed in heavy type in the formulae of glucose below.



β-Glycoside forming structure

α-Glycoside forming structure

The term 'glycoside' is a very general one which embraces all the many and varied combinations of sugars and aglycones. More precise terms are available to describe particular classes. Some of these terms refer to the sugar part of the molecule, others to the aglycone, while others indicate some well-defined physical or pharmacological property. Thus, a *glucoside* is a glycoside having glucose as its sole sugar component; a *pentoside* yields a sugar such as arabinose; *rhamnosides* yield the methyl-pentose rhamnose; and *rhamnoglucosides* yield both rhamnose and glucose. Terms used for aglycones are generally self-explanatory (e.g. phenol, anthraquinone and sterol glycosides). The names 'saponin' (soap-like), 'cyanogenetic' (producing hydrocyanic acid) and 'cardiac' (having an action on the heart), although applied to these substances when little was known about them, are useful terms which do in fact bring together glycosides of similar chemical structure.

The older system of naming glycosides using the termination '-in' (e.g. senegin, salicin, aloin, strophanthin) is too well established to be easily changed. It is not ideal, however, since many non-glycosidal substances (e.g. inulin and pectin) have the same termination. Modern workers more frequently use the termination '-oside' (e.g. sennoside) and attempt to drop the older forms by writing sinigroside for sinigrin, salicoside for salicin and so on.

Although glycosides form a natural group in that they all contain a sugar unit, the aglycones are of such varied nature and complexity that glycosides vary very much in their physical and chemical properties and in their pharmacological action. In Part 5 they are classified according to that aglycone fragment with which they often occur in the plant.

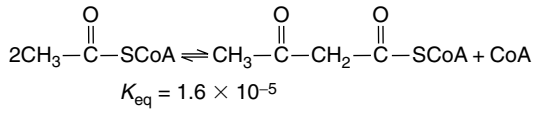
FATS AND FATTY ACIDS

Fats, considered in more detail in Chapter 19, are triglycerides involving long-chain saturated or unsaturated acids and, as such, constitute an important food reserve for animals and plants (particularly in seeds). Related to the simple fats are the complex lipids, most of which are diesters of orthophosphoric acid; they have the status of fundamental cellular constituents.

The fatty acids, on liberation from the fat, are available for the production of acetyl-CoA by the removal of C_2 units. The elucidation of this spiral (Fig. 18.6) owes much to the work of Lynen and it was primarily investigated with animal tissues. In this β-oxidation sequence one turn of the spiral involves four reactions—two dehydrogenations, one hydration and a thiolysis liberating a two-carbon unit of acetyl-CoA. All these reactions can be shown by isolated enzyme studies to be reversible, and until about 1953 it was generally assumed that the biosynthetic route of fatty acids operated in the reverse direction, starting from acetyl-CoA. This, however, is not precisely so, as indicated below.

Biosynthesis of saturated fatty acids

From the above it was generally assumed that the biosynthetic route for fatty acids operated in the reverse direction to the β-oxidative sequence for the degradation of these acids and started with acetyl-CoA. However, the unfavourable equilibrium of the initial thiolase reaction in such a pathway:



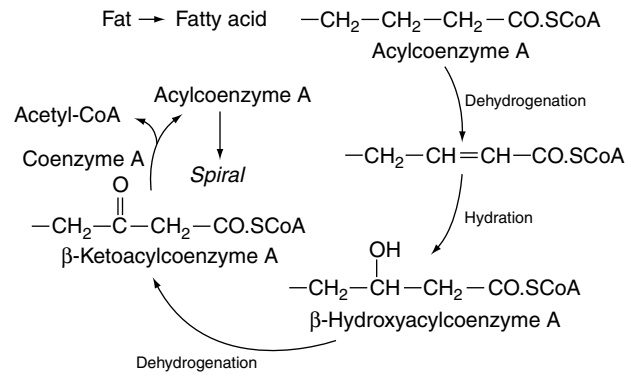


Fig. 18.6
Degradation of fatty acids. Reactions involved in one turn of the spiral.

and the poor yield of long-chain fatty acids obtained with purified enzymes of the β-oxidation pathway together with other observations suggested the possibility that this was not necessarily the principal biosynthetic pathway. It was then shown, with animal tissues, that malonyl coenzyme A was necessary for the initial condensation with acetate. In fact, in palmitic acid (C_{16},) only one of the eight C_2 units (i.e. the C15 + C16 carbons) is derived directly from acetyl-CoA; the other seven C_2 units are attached by malonyl-CoA, which is formed by carboxylation of acetyl-CoA.

One feature of this anabolic pathway is the involvement of the acyl carrier protein (ACP) to produce fatty acyl thioesters of ACP. This conversion serves (1) to activate the relatively unreactive fatty acid and (2) to provide a carrier for the fatty acid acyl group. Thus, these ACP thioesters appear to be obligatory intermediates in fatty acid synthesis, whereas it is the fatty acid thioesters of coenzyme A which operate in the catabolic oxidation pathway. The synthesis is explained by the reactions shown in Fig. 18.7. Reactions 4–7 are then repeated, lengthening the fatty acid chain by two carbons (derived from malonyl-*S*-ACP) at a time. Eight enzymes have been identified as being involved in the synthesis of palmitoyl-ACP and stearoyl-ACP from acetyl-CoA.

In purified enzyme systems, when propionyl-CoA replaced acetyl-CoA, the product was a C_{17} acid and, similarly, butyryl-CoA gave primarily a C_{18} (stearic) acid. Thus, the formation of these C_{16}, C_{17} and C_{18} acids may well depend on the availability of acetyl-, propionyl- and butyryl-CoA.

The system of biosynthesis of fatty acids in plants appears to operate in the same way as in animal tissues, but whereas in the latter the enzymes are located in the cytoplasm, in plants they are found in the mitochondria and chloroplasts. The mitochondria derived from the mesocarp of the avocado fruit have been used to demonstrate the incorporation of [^{14}C]acetate into esterified long-chain fatty acids.

Biosynthesis of unsaturated fatty acids

As a number of unsaturated fatty acids have a particular significance in pharmaceutical products, a treatment of their formation is deferred until Chapter 19.

AROMATIC BIOSYNTHESIS

The shikimic acid pathway

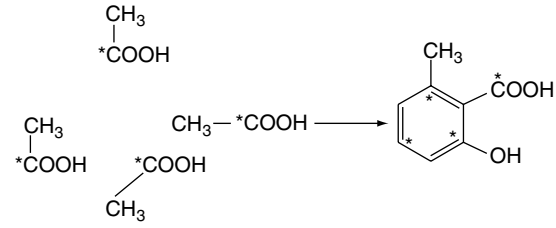
This appears to be an important route from carbohydrate for the biosynthesis of the C_6–C_3 units (phenylpropane derivatives), of which phenylalanine and tyrosine are both examples. A scheme of biogenesis for these aromatic amino acids, as elucidated in various organisms, is given in Fig. 18.8; for higher plants, the presence of the enzyme system responsible for the synthesis of shikimic acid has been confirmed. An important branching point arises at chorismic acid; anthranilate synthase uses chorismic acid as a substrate to give anthranilic acid which is a precursor of tryptophan. The synthesis is controlled by the latter acting as a feedback inhibitor; chorismate mutase converts chorismic acid to prephenate, the precursor of phenylalanine and tyrosine, and a variety of control mechanisms appear to operate at the branching point. The opium alkaloids are synthesized via this pathway (Chapter 26) and two isoforms of chorismate mutase have been isolated and characterized from poppy seedlings (M. Benesova and R. Bode, *Phytochemistry*, 1992, **31**, 2983).

Although there are only small differences in the sequence of reactions for the shikimate pathway in bacteria, fungi and plants there are considerable differences in the molecular organization of the pathway. (For a review (123 refs), see J. Schmid and N. Amrhein, *Phytochemistry*, 1995, **39**, 737.)

The shikimic acid pathway is also important in the genesis of the aromatic building blocks of lignin and in the formation of some tannins, vanillin and phenylpropane units of the flavones and coumarins (see Chapter 21). For a review see 'Further reading'.

The acetate hypothesis

The central position of acetate in relation to the general metabolism of plants has already been indicated and it is possible to devise many possible routes by which acetate condensation could occur to give a variety of aromatic compounds. The general validity of the mechanism has been established by the use of labelled compounds but the detailed steps for many compounds remain to be established. Thus, the incorporation of [1-^{14}C]acetate into 6-methylsalicylic acid by *Penicillium griseofulvum* takes place as below:



and the production of the mould anthraquinone metabolite endocrocin, from eight C_2 units, is represented in Fig. 18.9. Decarboxylation of endocrocin affords emodin. The original chain lengthening, which can be represented as a condensation of acetate units, is the same as in fatty acid production (q.v. above) but does not require the reduction of =CO to =CH_2. Thus, malonic acid plays a similar role in this aromatic synthesis to that in fatty acid formation and the chain is built up from the combination of malonyl units with a terminal acetyl (the starter) unit. Sometimes the starter unit is not acetate, as indicated with the

1. $CH_3CO\text{-}S\text{-}CoA + CO_2 + ATP \xrightarrow[\text{Biotin enzyme}]{Mn^{2+}} HOOCCH_2CO\text{-}S\text{-}CoA + ADP + Pi$
2. acetyl-*S*-CoA + ACP-SH ⟶ acetyl-*S*-ACP + CoASH
3. malonyl-*S*-CoA + ACP-SH ⟶ malonyl-*S*-ACP + CoASH
4. malonyl-*S*-ACP + acetyl-*S*-ACP ⟶ acetoacetyl-*S*-ACP + CO_2 + ACP-SH
5. acetoacetyl-*S*-ACP + NADPH + H^+ ⟶ D(–)-3-hydroxybutyryl-*S*-ACP + $NADP^+$
6. D(–)-3-hydroxybutyryl-*S*-ACP ⟶ crotonyl-*S*-ACP + HOH
7. crotonyl-*S*-ACP + NADPH + H^+ ⟶ butyryl-*S*-ACP + $NADP^+$

Fig. 18.7
Initial stages in the biosynthesis of fatty acids.

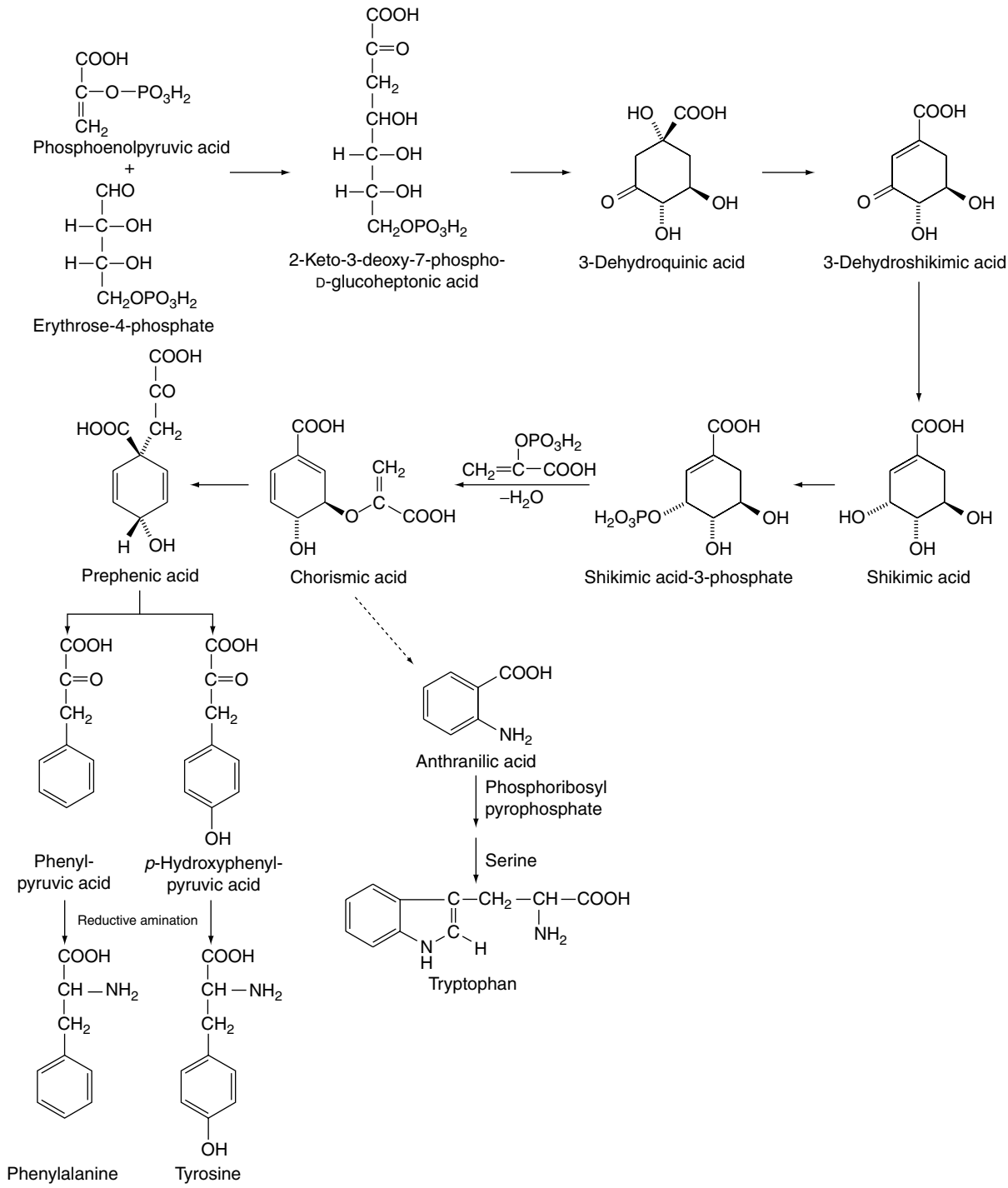


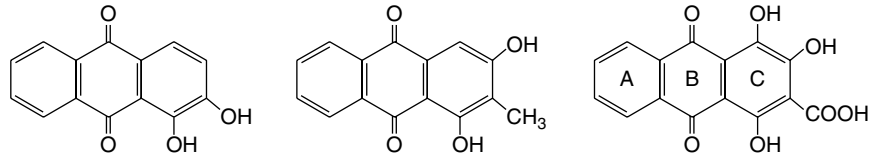
Fig. 18.8
Biosynthesis of aromatic compounds via the shikimic acid pathway.

formation of the tetracycline antibiotics from nine units; here malonamide-SCoA is the starter unit and by invoking standard biochemical reactions tetracycline is formed (Fig. 18.10). Higher plants also utilize the polyacetate–malonate pathway for the biosynthesis of emodin-type anthraquinones, such compounds all having substituents on both outer rings.

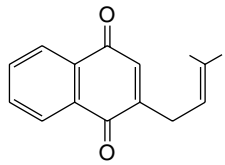
From the anthraquinones listed in Chapter 21, it will be seen that the structures of some anthraquinones are not fully explained by the acetate–malonate pathway and these are discussed below. However, there appear to be many exceptions to the rules in applying the general pathways to specific anthraquinones.

Compounds containing aromatic rings of different origin

The structures of anthraquinones such as alizarin, rubiadin, pseudopurpurin and morindadiol, pigments of the Rubiaceae and other families, cannot be readily explained on the acetate hypothesis.



Circumstantial evidence that these compounds might be formed from naphthoquinones with the participation of mevalonic acid (a key precursor in the formation of isopentenyl units, q.v.) was provided by their co-occurrence in teak and other plants with naphthoquinones having an isopentenyl residue (e.g. formula below).



That mevalonate (itself derived from acetate) is involved in this formation has been shown by tracer experiments with *Rubia tinctorum*; ring C and carbon side-chain of pseudopurpurin and rubiadin are derived from mevalonate, and in the same plant shikimic acid has been shown to be incorporated into ring A of alizarin.

The aromatic rings of some compounds can be derived from both the shikimic acid and the acetic acid pathways. Thus, a phenylpropane formed by the former route may undergo chain lengthening by the addition of acetate units (via malonyl; see 'Fatty Acids', above) to give a polyketide and then, by ring closure, give a flavonoid derivative (Fig. 18.11).

Isoflavones are formed in the same manner, with a rearrangement of the aryl-B ring in relation to the three carbons (Fig. 18.11). The flavonoids, in addition to their importance in plants as pigments, have interesting medicinal properties and are discussed in more detail in Chapter 21.

Rotenoids (insecticides of *Derris* and *Lonchocarpus* spp.) show a structural relationship to the isoflavones; see Chapter 40.

Stilbenes (q.v.) are also compounds containing aromatic units of different biogenetic origin.

AMINO ACIDS

Amino acids occur in plants both in the free state and as the basic units of proteins and other metabolites. They are compounds containing one or more amino groups and one or more carboxylic acid groups.

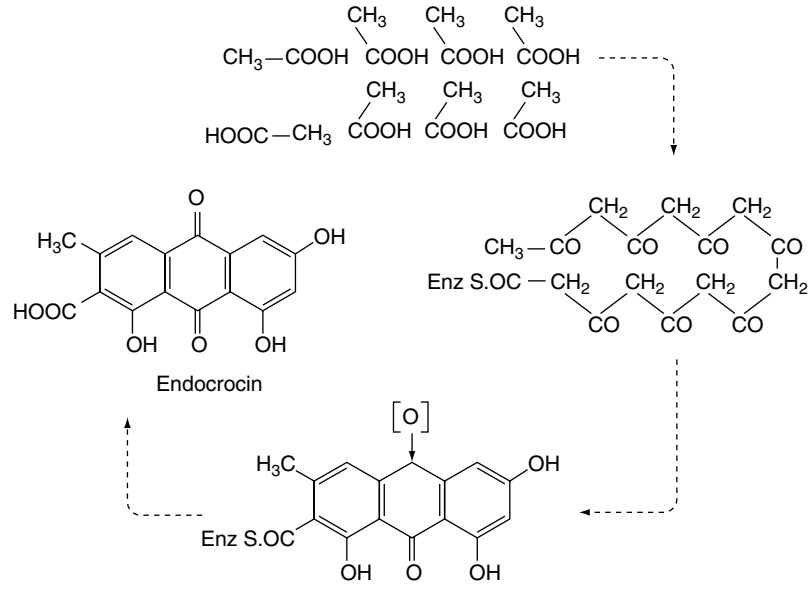


Fig. 18.9
Biogenesis of endocrocin.

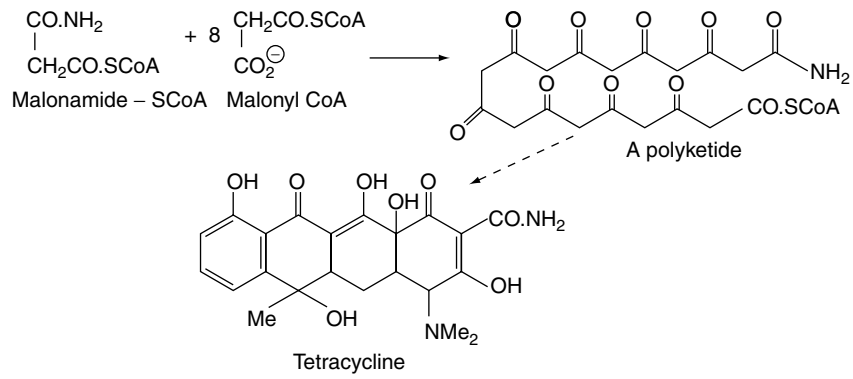


Fig. 18.10
Formation of tetracycline.

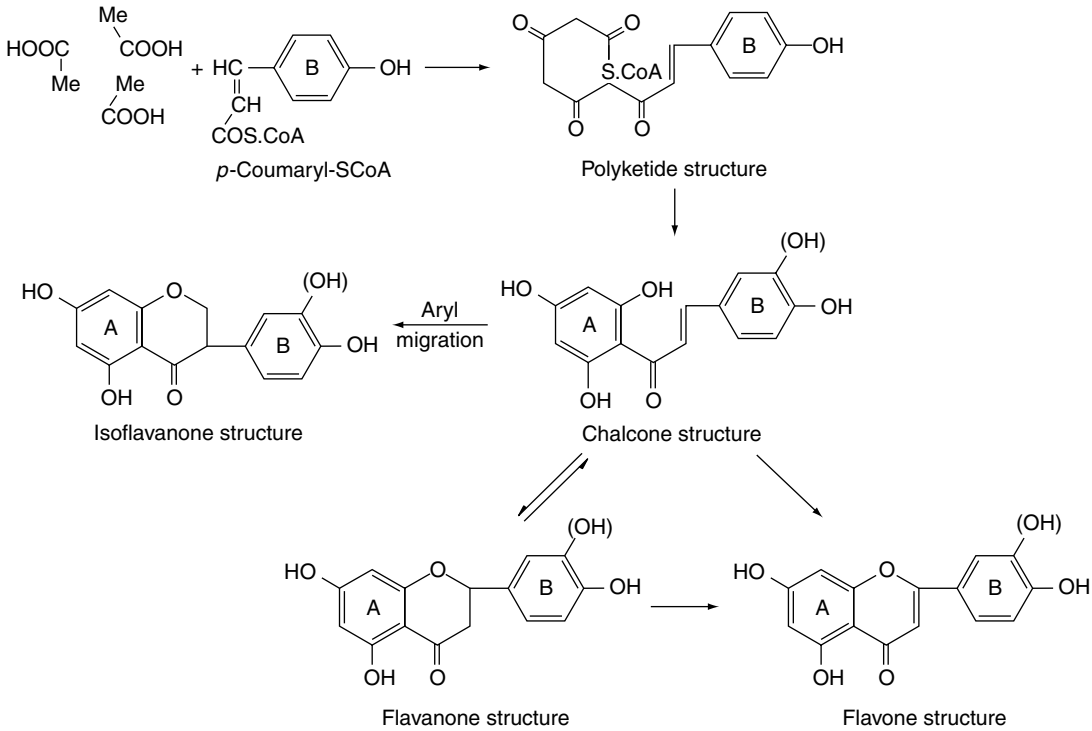
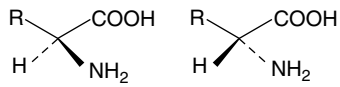


Fig. 18.11
Origin of flavanones, isoflavanones and flavones.

Most of those found in nature are α-amino acids with an asymmetric carbon atom and the general formula R–CH(NH_2)COOH. Some 20 different ones have been isolated from proteins, all having an L-configuration. Other amino acids occur in the free state and some having the D-configuration have been isolated from plants and microorganisms, where they may form antibiotic polypeptides.



Many amino acids contain only carbon, hydrogen, oxygen and nitrogen, but other atoms may be present (e.g. sulphur in cystine, and iodine in thyroxin). As already mentioned, more than one amino group may be present (e.g. lysine, diaminocaproic acid) and more than one carboxylic acid group (e.g. aspartic or aminosuccinic acid). Some amino acids are aromatic such as phenylalanine, or heterocyclic such as proline (pyrrolidine nucleus), tryptophan (indole nucleus) and histidine (imidazole nucleus).

Amino acids are generally soluble in water but only slightly soluble in alcohol. A general test is to warm with ninhydrin, when, with the exception of proline, which gives a yellow, they give a pink, blue or violet colour. Amino acids do not respond to the biuret test (compare polypeptides and proteins). Certain amino acids are detected by more specific tests (e.g. histidine gives colour reactions with diazonium salts).

Amino acids found in proteins

These include α-alanine; arginine; asparagine (amide of aspartic acid), abundant in many plants, particularly etiolated seedlings; aspartic acid; aminosuccinic acid, involved in the biosynthesis of purines; cysteine, which contains sulphur; cystine or dicysteine (in hair and insulin); 3,5-di-iodotyrosine (in thyroid); glutamic acid (a component of the folic acid vitamins); glutamine (free in animals and plants, e.g. sugar-beet); glycine (aminoacetic acid); histidine; δ-hydroxylysine (in gelatin); hydroxyproline (in gelatin); leucine (α-aminocaproic acid); isoleucine; lysine; methionine (contains a sulphur atom); 3-monoiodotyrosine (in thyroid); phenylalanine; proline; serine (in phosphoproteins such as casein); threonine (in casein); thyroxin (the iodine-containing thyroid-hormone); 3,5,3′-triiodothyronine (in thyroid); tryptophan; tyrosine and valine.

Amino acids found free and not occurring in proteins

Following the pioneer research of Fowden, several hundred of these amino acids have been characterized and only a few (e.g. γ-aminobutyric acid, α-aminoadipic acid, pipecolic acid and δ-acetylornithine) are of wide occurrence. Seeds or fleshy organs of plants are the principal sites for the accumulation of these compounds where they may serve as a nitrogen reserve. Other examples in this class are: β-alanine (β-aminopropionic acid); citrulline (an intermediate in the cycle of urea synthesis); creatine; ergothioneine (a sulphur-containing constituent of some animal tissues and of ergot); and taurine (a component of bile acids). A number have teratogenic properties (see Chapter 39). 3-*N*-Oxalyl-L-2, 3-diaminopropanoic acid is a neurotoxin present in the seeds of *Lathyrus sativus*, the grass pea; it is the causal agent of human neurolathyrism, an irreversible paralysis of the lower limbs, which occurs in some drought prone areas of Ethiopia, India and Bangladesh (see Yu-Haey Kuo *et al.*, *Phytochemistry*, 1994, **35**, 911 for references and biosynthetic studies). Other non-protein amino acids provide plant protection from insects.

Non-protein amino acids, considered oddities of plant biosynthesis 40 years ago, now constitute a group receiving increasing attention because of their possible physiological and ecological significance.

Biosynthesis of amino acids

As amino acids are also the precursors of some secondary metabolites, their biosyntheses will be considered below. They arise at various levels of the glycolytic and TCA systems.

Nitrogen appears to enter the metabolism of the organism by reductive amination of α-keto acids; pyruvic, oxalacetic and α-ketoglutaric acids give alanine, aspartic acid and glutamic acid, respectively (Fig. 18.12).

By transamination reactions with other appropriate acids, alanine, aspartic acid and glutamic acid serve as α-amino donors in the formation of other amino acids. The general transamination reaction may be written:

$$R—CH(NH_2)—COOH + R'—CO—COOH \rightleftharpoons R—CO—COOH + R'—CH(NH_2)—COOH$$

Glutamic acid, in particular, appears to be a central product in amino-acid metabolism and glutamic acid dehydrogenase has been reported in a number of plant tissues. The enzyme functions in conjunction with NAD:

$$\alpha\text{-ketoglutaric acid} + NH_3 + NADPH \rightleftharpoons \text{glutamic acid} + NAD$$

That the nitrogen of ammonia first appears in the dicarboxylic amino acids and is later transferred to other nitrogen compounds was demonstrated in some of the earliest plant biochemistry experiments (around 1940), which utilized ^{15}N.

Proline, hydroxyproline, ornithine and arginine

These amino acids are of importance in the secondary metabolism of some plants in that they are precursors of a number of alkaloids. They are metabolically connected to glutamic acid (Fig. 18.13) and their formation in plant cells is complex in that the reactions are strictly compartmentalized. The enzymes involved have been characterized, and for the formation of ornithine it is the *N*-acetyl derivatives which are involved. Arginine appears to be synthesized from ornithine in all organisms via the reactions of the urea cycle. (For a review giving the role of the mitochondria, cytoplasm and plastids in these reactions, see P. D. Shargool *et al*., *Phytochemistry*, 1988, **27**, 1571.)

Serine and glycine

Together with cysteine and cystine, these amino acids arise at the triose level of metabolism. Preparations from rat liver use the pathway indicated in Fig. 18.14 for the formation of serine.

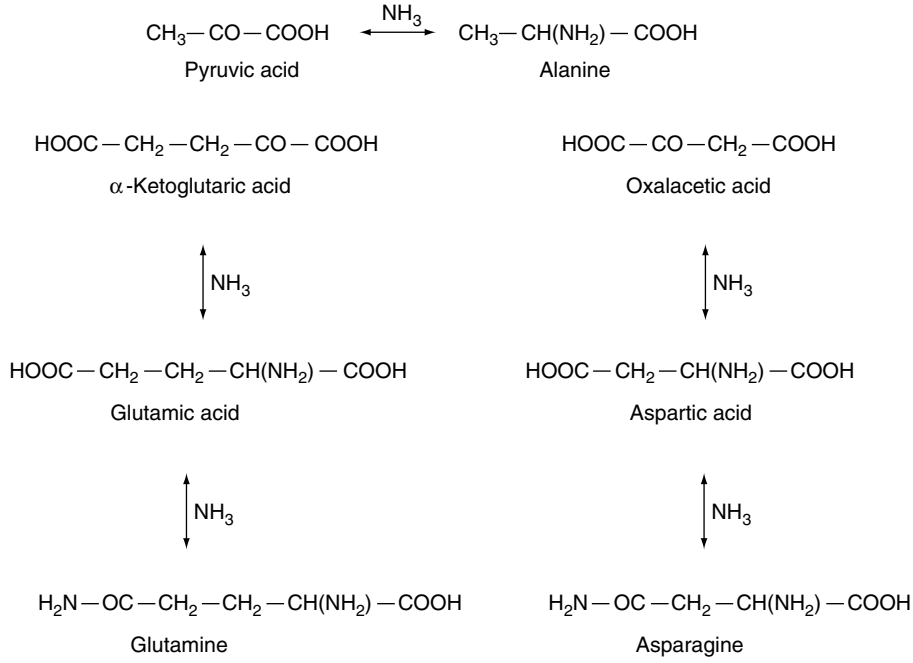


Fig. 18.12
Reductive- and trans-amination in the formation of amino acids.

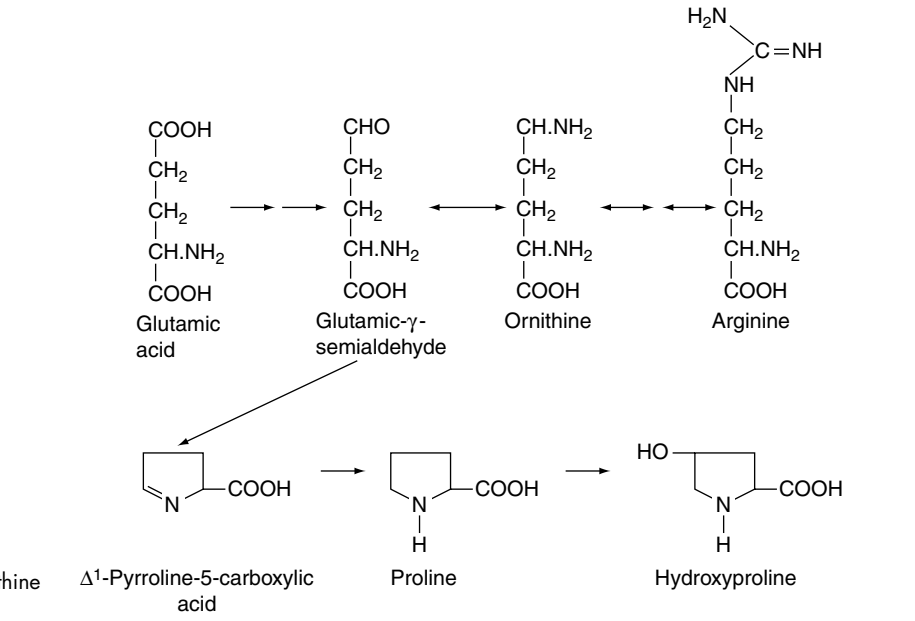


Fig. 18.13
Origin of proline, hydroxyproline, ornithine and arginine.

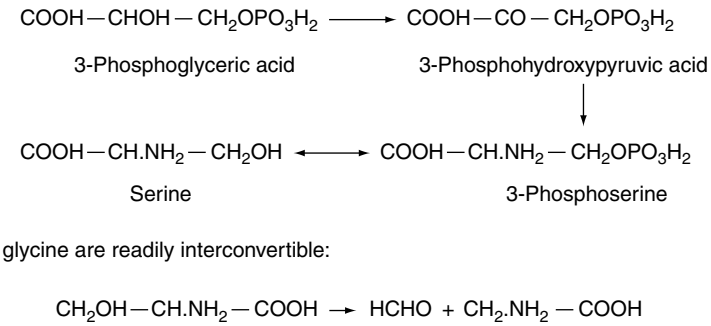


Fig. 18.14
Formation of serine and glycine.

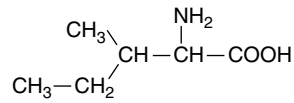
In animal tissues it has been shown that tetrahydrofolic acid (THFA) is responsible for the removal of the hydroxymethyl group of serine to form hydroxymethyltetrafolic acid. As this compound serves as a source of formate and methyl groups in many reactions, the β-carbon of serine may be their original source; this applies to the formation of methionine (Fig. 18.15), itself an important methyl donor in plant biochemistry.

Alanine, valine and leucine

Studies with microorganisms and yeasts have shown these amino acids to be derived from pyruvate. There is evidence that α-ketoisovaleric acid is aminated to form valine and that it can also condense with acetate to form an intermediate which on decarboxylation and amination affords leucine (Fig. 18.16).

Isoleucine

This amino acid



is formed by a similar series of reactions to valine but commencing with α-aceto-α-hydroxypropionic acid instead of α-acetolactic acid.

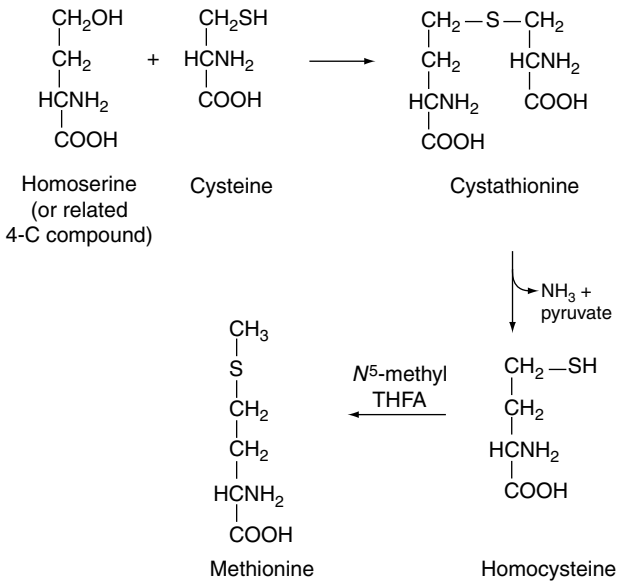


Fig. 18.15
Origin of methionine.

Lysine

Lysine, $H_2N–(CH_2)_4–CH(NH_2)–COOH$, is derived, in plants, from aspartate involving a pathway utilizing 2,3-dihydropicolinic acid and diaminopimelic acid. It is the precursor of some alkaloids of *Nicotiana*, *Lupinus* and *Punica*.

For a review of the biosynthesis and metabolism of aspartate-derived amino acids (lysine, threonine, methionine, *S*-adenosyl methionine) see R. A. Azevedo *et al*., *Phytochemistry*, 1997, **46**, 395.

Aromatic amino acids

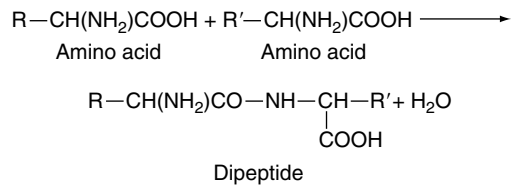
These have already been mentioned in the discussion of the biosynthesis of aromatic compounds.

PEPTIDES AND PROTEINS

The term 'peptide' includes a wide range of compounds varying from low to very high molecular weights and showing marked differences in physical, chemical and pharmacological properties. The lowest members are derived from only two molecules of amino acid, but higher members have many amino-acid units and form either peptides, simple proteins (albumins, globulins, prolamines, glutalins, etc.) or more complex proteins, conjugated proteins, in which other groupings form part of the molecule—for example, carbohydrate in mucoproteins, the very complex chlorophyll molecule in the protein of chloroplasts, phosphorus-containing proteins such as casein, nucleoproteins, in which proteins are combined with nucleic acid, and the lipoproteins of the cytoplasm, in which protein is combined with lipids. Among such substances with relatively low molecular weight are some antibiotics which have a cyclic polypeptide structure (e.g. gramicidin, bacitracin and polymyxin); peptide hormones such as oxytocin and vasopressin from the posterior pituitary gland; and glutathione, which is found in nearly all living cells.

All these more or less complex compounds have two or more molecules of amino acid united by a peptide linkage which results from the elimination of water, an OH coming from one amino acid and an H from the other.

Thus, a dipeptide is formed:



A dipeptide of the Sapindaceous plant *Blighia sapida* has hypoglycaemic properties and although more complex, penicillin also has a

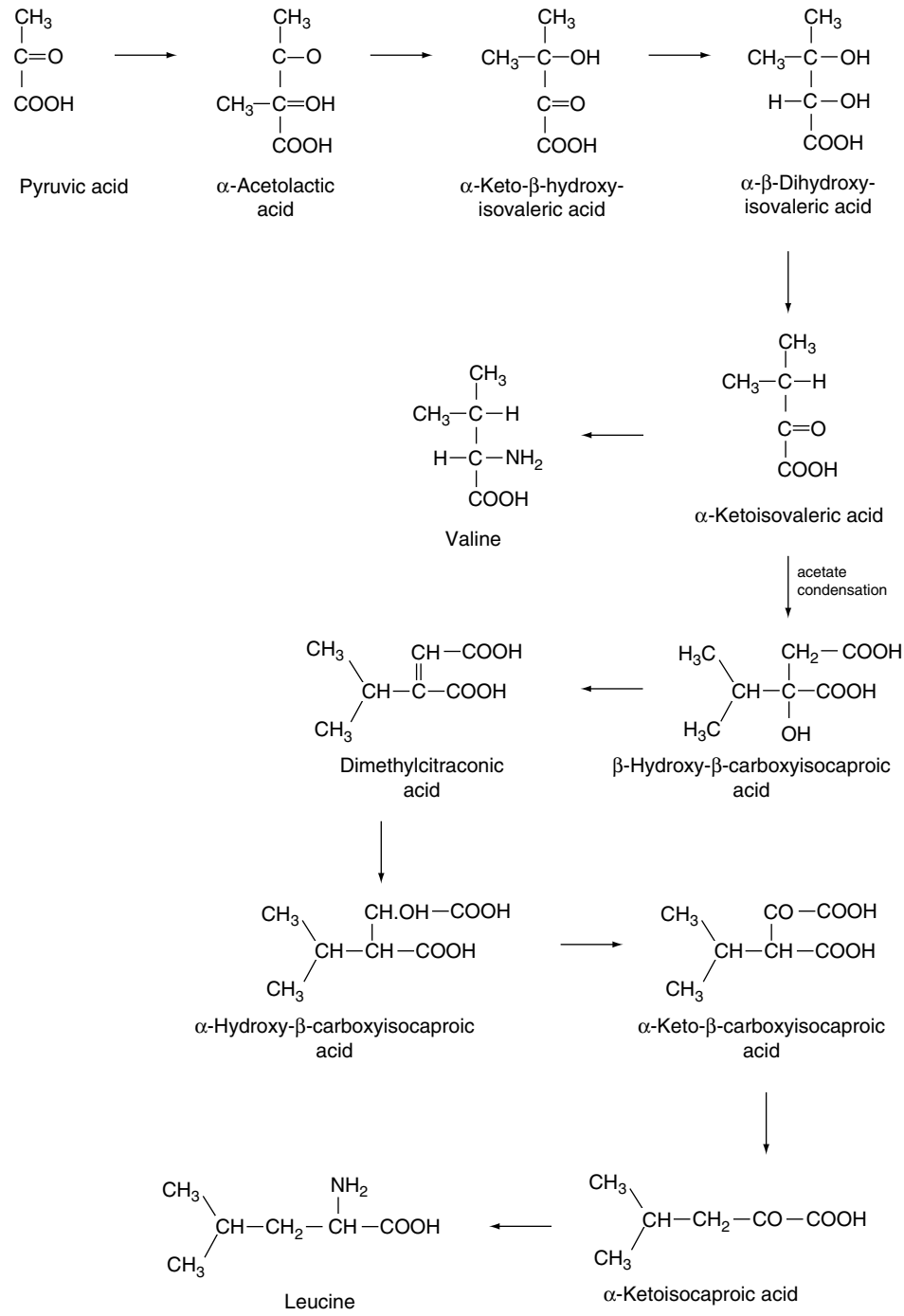


Fig. 18.16
Formation of valine and leucine.

dipeptide structure. Tripeptides have three amino-acid components and polypeptides from ten upwards. Peptides are usually defined as protein-like substances having molecular weights below 10 000. In typical proteins the molecular weight is higher, ranging from about 30 000 to 50 000 in the relatively simple prolamines and glutelins and reaching very high values, sometimes several million, in the complex proteins such as those in sheep's wool.

Protein synthesis takes place in association with the ribosomes, which are small bodies found in the cytoplasm and particularly in the endoplasmic reticulum area (see Fig. 18.1). The amino acids are brought to the ribosomes associated with a transfer-RNA molecule and by the action of the ribosomes, using a sequence dictated by a particular messenger-RNA molecule, are linked to form the peptide chains of the particular protein. Although not directly relevant to most pharmacognostical studies, the story of the nucleic acids and their vital role in the control of cell metabolism is a fascinating one which it is suggested students study from a standard work on biochemistry.

ISOPRENOID COMPOUNDS

Studies on the pyrogenic decomposition of rubber led workers in the latter half of the nineteenth century to believe that isoprene could be regarded as a fundamental building block for this material. As a result

of the extensive pioneering investigations into plant terpene structures, Ruzicka published in 1953 his 'biogenetic isoprene rule', which indicated that the apposition of isoprenoid units could be used to explain not only the formation of rubber and the monoterpenes, but also many other natural products, including some, such as sterols and triterpenes, with complex constitutions. The value of the rule lay in its broad unifying concept, which allowed the postulation of a rational sequence of events which might occur in the biogenesis of these otherwise unrelated compounds. Examples of various structures to which the rule can be applied are indicated in Fig. 18.17.

The task set biochemists was to investigate the validity of the rule, and the work on this subject constitutes a brilliant example of modern biochemistry; however, as will be seen below, this chapter of research is still unfinished. By 1951 it had been established that acetic acid was intimately involved in the synthesis of cholesterol, squalene, yeast sterols and rubber. The use of methyl- and carboxyl-labelled acetic acid with animal tissues indicated that the methyl and carboxyl carbons alternated in the skeleton of cholesterol or squalene and that the lateral carbon atoms all arose from the methyl group of acetic acid. The discovery, in 1950, of acetyl-coenzyme A, the so-called 'active acetate', gave further support to the role of acetate in biosynthetic processes.

The mevalonic acid pathway. The next major advance in the elucidation of the isoprenoid biosynthetic route was the discovery in 1956 of mevalonic acid and the demonstration of its incorporation, by living tissues, into those compounds to which the isoprene rule applied. Mevalonic acid (3,5-dihydroxy-3-methylvaleric acid) is a C_6 acid and, as such, is not the 'active isoprene' unit which forms the basic building block of the isoprenoid compounds. During the next four years, by research involving the use of tracer techniques, inhibitor studies, cell-free extracts, partition chromatography and ionophoresis as well as synthetic organic chemistry, it was established that the C_5 compound for which biochemists had been seeking so long was isopentenyl pyrophosphate; it is derived from mevalonic acid pyrophosphate by decarboxylation and dehydration. Isoprenoid synthesis then proceeds by the condensation of isopentenyl pyrophosphate with the isomeric dimethylallyl pyrophosphate to yield geranyl pyrophosphate. Further C_5 units are added by the

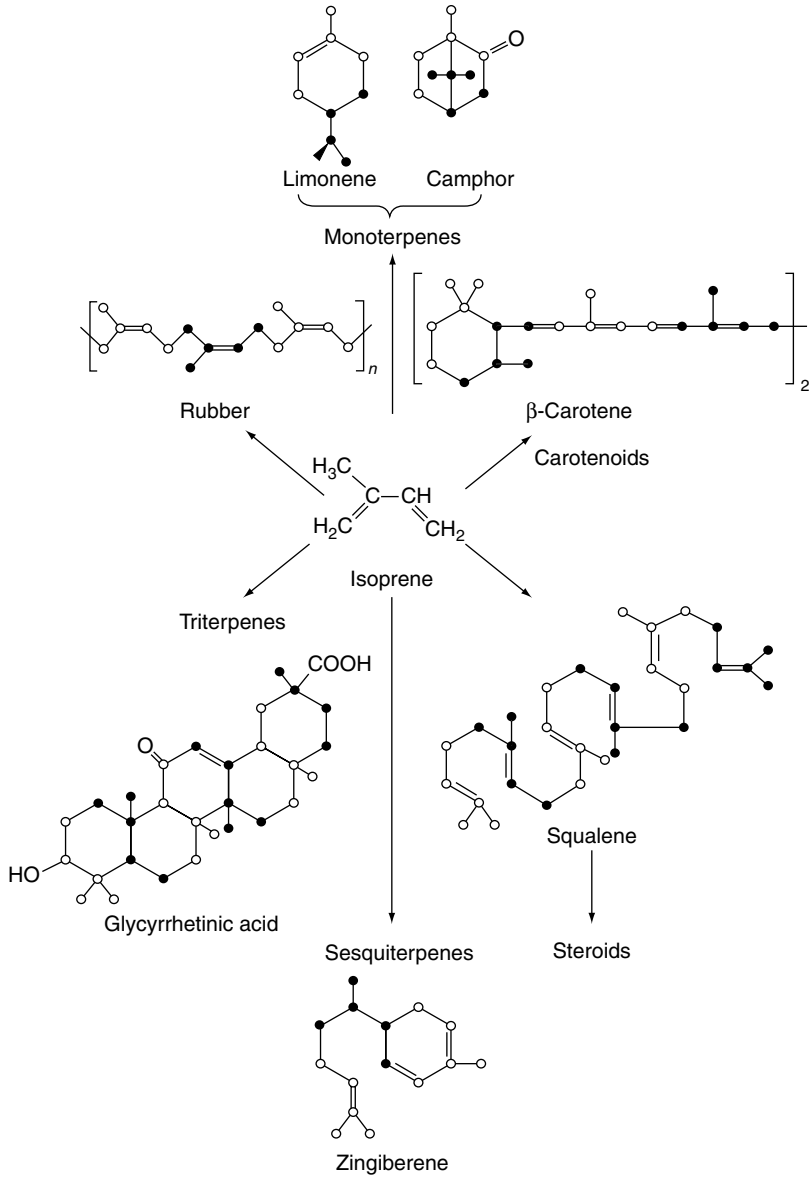


Fig. 18.17
Application of the isoprene rule illustrating incorporation of C_5 units.

addition of more isopentenyl pyrophosphate. These preliminary stages in the biosynthesis of isoprenoid compounds are shown in Fig. 18.18.

From geranyl and farnesyl pyrophosphates various structures can be built up (see Fig. 18.19).

Studies, particularly by Cornforth and Popják, involving the use of stereospecifically ^{3}H- and ^{14}C-labelled mevalonic acid, have demonstrated the stereochemical mechanism of the initial stages of isoprenoid formation. Only the (*R*)-form of mevalonic acid gives rise to the terpenoids, the (*S*)-form appearing to be metabolically inactive. In the formation of isopentenyl pyrophosphate, the elimination is *trans* and the elimination of the proton in the isomerization to the dimethylallyl pyrophosphate is also stereospecific (Fig. 18.20A).

In the subsequent additions of the C_5 isopentenyl pyrophosphate units to form the terpenoids the elimination of hydrogen is *trans*. Figure 18.20B shows the stereochemistry of the addition of one isopentenyl pyrophosphate unit. In the biogenesis of rubber, however, the hydrogen elimination produces a *cis* double bond (Fig. 18.20C).

It is considered that a simple change in orientation of the isopentenyl pyrophosphate on the enzyme surface could produce this change without altering the reaction mechanism. In neither rubber nor gutta are hybrid molecules containing both types of bond detectable. The first direct evidence for the presence of isopentenyl diphosphate isomerase in rubber latex was reported in 1996 (T. Koyama *et al*., *Phytochemistry*, 1996, **43**, 769).

In recent years the enzymology of the isoprenoid pathway has been extensively studied and for details the reader is referred to a standard text on plant biochemistry. One key regulatory enzyme is hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34, mevalonate kinase); it has been extensively studied in animals and more recently in plants. As with many enzymes the situation is complicated by the existence of more than one species of enzyme and a plant may possess multiple forms each having a separate subcellular location associated with the biosynthesis of different classes of terpenoids. For a review of the functions and properties of the important isomerase enzyme isopentenyl diphosphate isomerase see 'Further reading'.

It should be noted that some metabolites of mixed biogenetic origin involve the mevalonic acid pathway; prenylation for example is common, with C_5, C_{10} and C_{15} units associated with flavonoids, coumarins, benzoquinones, cannabinoids, alkaloids, etc.

The validity of the mevalonate pathway in the formation of all the major groups noted in Fig. 18.19 has been shown, and until recently no other biosynthetic route to isoprenoids had been discovered.

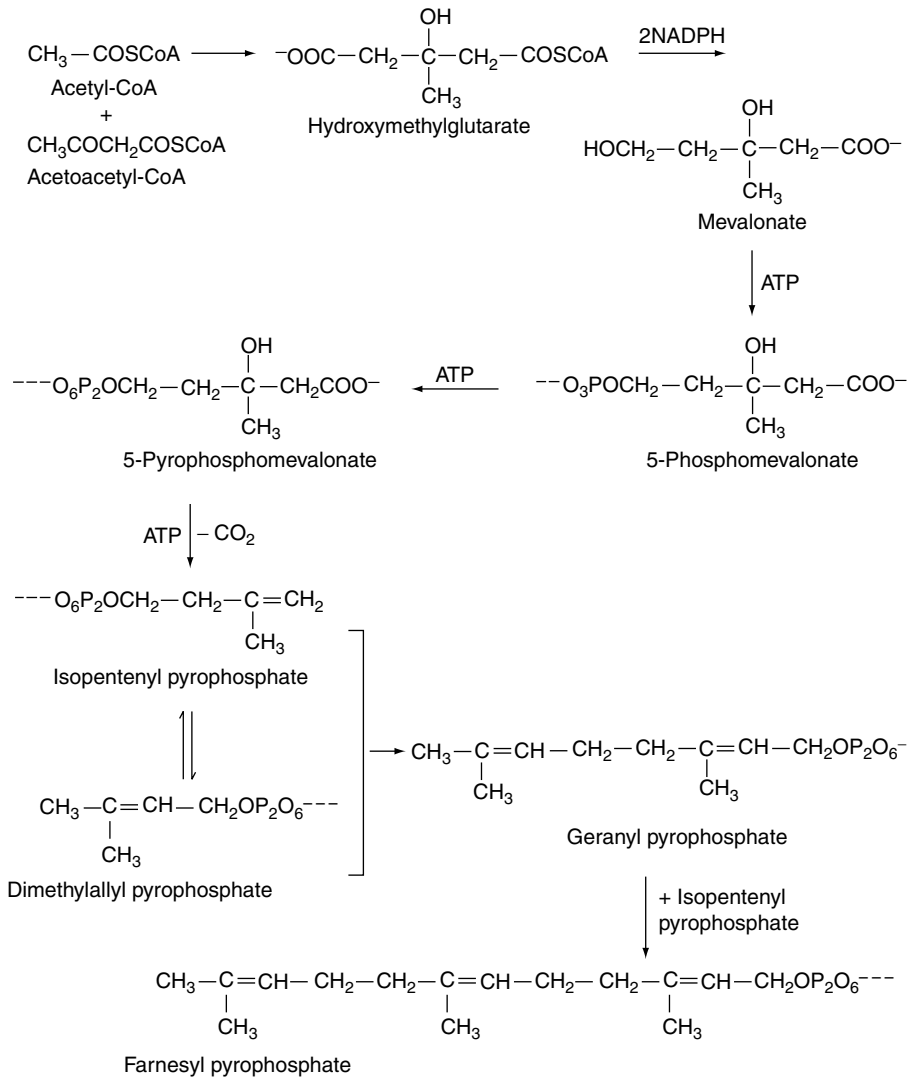


Fig. 18.18
Preliminary stages in the biosynthesis of isoprenoid compounds.

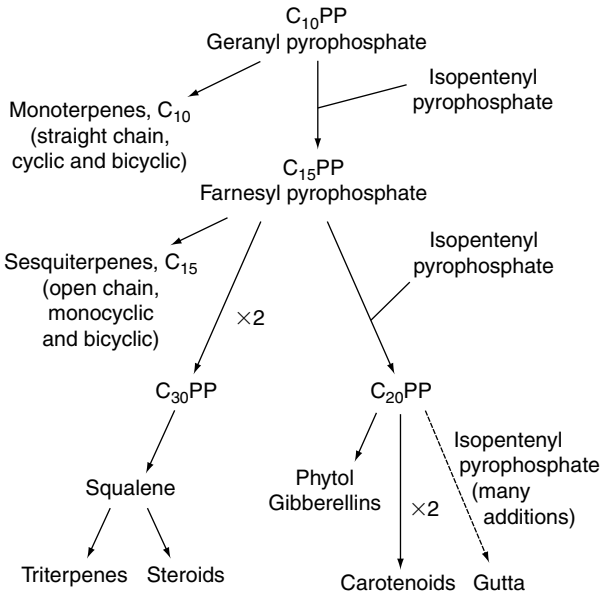


Fig. 18.19
Biosynthesis of isoprenoid compounds.

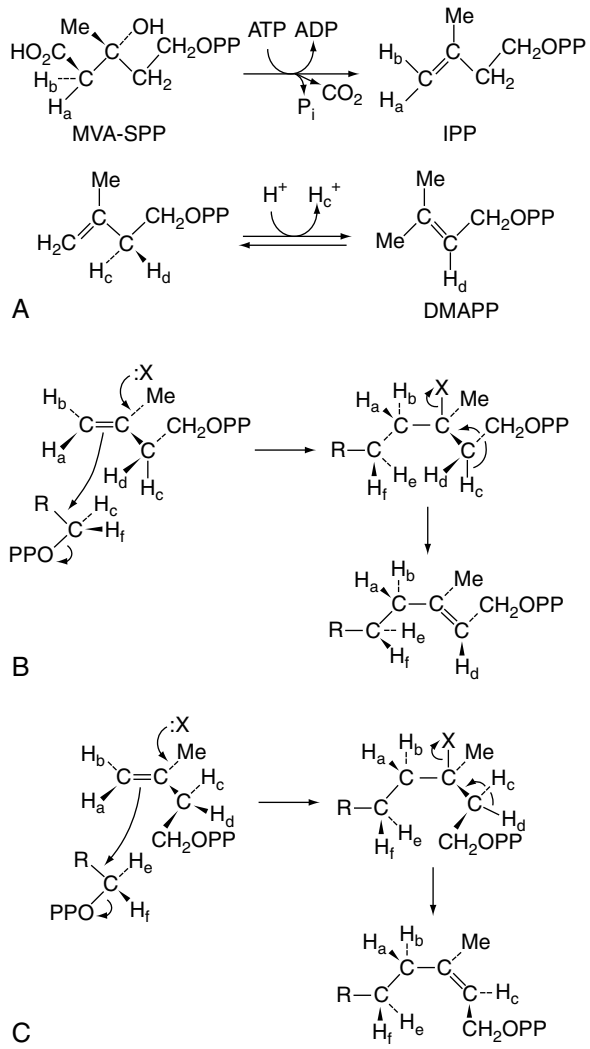


Fig. 18.20
Stereospecific reactions in terpenoid biogenesis. A, Formation from mevalonic acid (MVA) of isopentenyl pyrophosphate (IPP) by *trans* elimination; isomerization to dimethylallyl pyrophosphate (DMAPP). B, Association of the two 5-C units with *trans* elimination of hydrogen. C, As B but involving formation of *cis* double bonds as found in rubber.

The 1-deoxy-D-xylulose (triose/pyruvate) pathway. Following its discovery in 1956 mevalonic acid came to be considered the essential precursor for all isoprenoid syntheses. However, in 1993 M. Rohmer *et al*., (*Biochem. J*., 1993, **295**, 517) showed that a non-mevalonate pathway existed for the formation of hopane-type triterpenoids in bacteria. The novel putative precursor was identified as 1-deoxy-D-xylulose-5-phosphate, formed from glucose via condensation of pyruvate and glyceraldehyde-3-phosphate. Subsequent steps including a skeletal rearrangement afford isopentenyl pyrophosphate—the same methyl-branched isoprenoid building block as formed by the MVA route.

It was soon demonstrated that this novel route to IPP was also operative in the formation of monoterpenes (*Mentha piperita*, *Thymus vulgaris*), diterpenes (*Ginkgo biloba*, *Taxus chinensis*) and carotenoids (*Daucus carota*). This raised the question of to what extent the two pathways co-existed in the plant and it was hypothesized that the classical acetate/mevalonate pathway was a feature of cytoplasmic reactions whereas the triose/pyruvate sequence was a characteristic of the plastids. This did not exclude either the movement of plastid-synthesized IPP and DMAPP from the organelle to the cytoplasm or the translocation of a suitable C_5-acceptor to the plastid. Evidence accumulating indicates a cooperative involvement of both pathways. Indeed recent work on the biosynthesis of the isoprene units of chamomile sesquiterpenes (K.-P. Adam and J. Zapp, *Phytochemistry*, 1998, **48**, 953) has shown that for the three C_5 units of both bisaboloxide A and chamazulene, two were mainly formed by the non-mevalonate pathway and the third was of mixed origin.

The deoxyxylulose (DOX) pathway has helped explain the previously reported rather poor incorporations of MVA into certain isoprenoids. Thus V. Stanjek *et al*. (*Phytochemistry*, 1999, **50**, 1141) have obtained a good incorporation of labelled deoxy-D-xylulose into the prenylated segment of furanocoumarins of *Apium graveolens* leaves, suggesting this to be the preferred intermediate.

Fig. 18.21 illustrates how [1-^{13}C]-glucose, when fed to plants, can be used to differentiate IPP and subsequent metabolites, formed either by the MVA pathway or the DOX route.

SECONDARY METABOLITES

As indicated earlier in Fig. 18.2, the basic metabolic pathways constitute the origins of secondary plant metabolism and give rise to a vast array of compounds; some of these are responsible for the characteristic odours, pungencies and colours of plants, others give a particular plant its culinary, medicinal or poisonous virtues and by far the greatest number are, on current knowledge, of obscure value to the plant (and to the human race). However, a number of modern authors suggest that secondary metabolites, rather than constituting waste products of metabolism, are biosynthesized to aid the plant's survival. Notwithstanding, it may be that in some instances the purpose for which these compounds were produced no longer exists but the biosynthetic pathway has survived. For an interesting discussion and references, see E. J. Buenz and S. J. Schepple, *J. Ethnopharmacol*, 2007, **114**, 279. The possible functions in the plant of one large group of secondary metabolites, alkaloids, are discussed in Chapter 26.

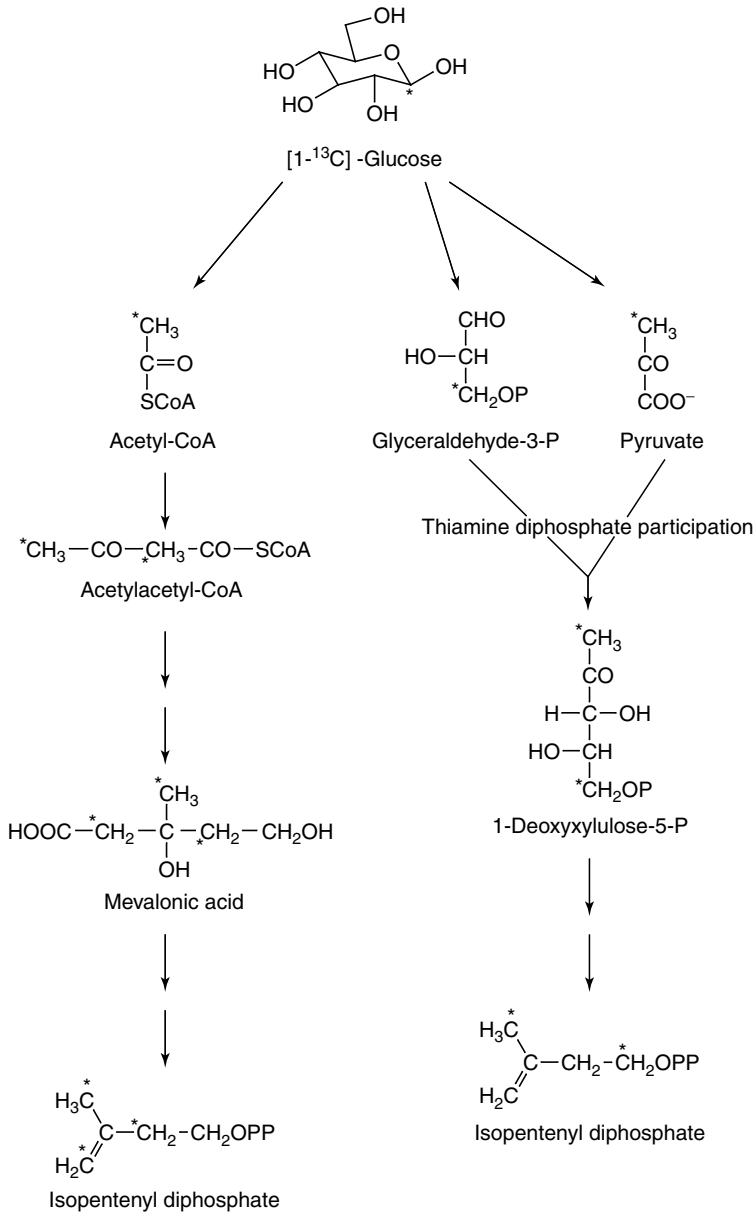


Fig. 18.21
The incorporation of [1-^{13}C]-glucose into isopentenyl diphosphate: left, via the mevalonic acid pathway; right, via the deoxyxylose pathway.

Recently, considerable attention has been directed to the possible ecological implications of secondary metabolites not only in relation to plant–plant interaction but also concerning the interrelationship of plants and animals. Various insects sequester specific alkaloids, iridoids, lactones and flavonoids which serve as defensive agents or are converted to male pheromones. The literature concerning chemical ecology is regularly reviewed and the volatile isoprenoids that control insect behaviour and development have been reported on (J. A. Pickett, *Nat. Prod. Rep*., 1999, **16**, 39). The enzymology associated with secondary metabolism is now receiving considerable attention and, with respect to alkaloid formation, a number of enzymes associated with the biosynthesis of the tropane, isoquinoline and indole groups have been prepared. The biosynthetic origins of those metabolites of medicinal interest are considered in more detail in Part 5, which is arranged principally according to biogenetic groups.

Although a number of the biogenetic groups are characterized by particular skeletal structures, the actual chemical properties of particular compounds are determined by the acquisition of functional groups. Thus, terpenes may occur as alcohols (menthol), ethers (cineole), ketones (carvone), etc., and as such have similar chemical properties to non-terpenoid compounds possessing the same group; aldehydes, as an example of a functional group, may be of aliphatic origin (citronellal), aromatic (cinnamic aldehyde), steroidal (some cardioactive glycosides); and resulting from the introduction of a heterocyclic system one biogenetic group may possess some of the chemical properties of another (e.g. steroidal alkaloids).

A particular group of compounds may also involve different biogenetic entities; thus, the complex indole alkaloids contain moieties derived from both the shikimate and isoprenoid pathways. In contrast, the same structure, as it occurs in different compounds, may arise from different pathways, as has been previously indicated with the formation of aromatic systems.

Stress compounds

These are compounds which accumulate in the plant to a higher than normal level as a result of some form of injury, or disturbance to

the metabolism; they may be products of either primary or secondary metabolism. Common reactions involved in their formation are the polymerization, oxidation or hydrolysis of naturally occurring substances; many however, are entirely secondary in their formation. A number of environmental and biological factors promote the synthesis of stress compounds and these include mechanical wounding of the plant, exposure to frost, ultraviolet irradiation, dehydration, treatment with chemicals, and microbial infection (see phytoalexins below). The production of such compounds has also been observed in cell cultures subjected to antibiotic treatment and in cells immobilized or brought into contact with calcium alginate. Examples of the latter include the formation of acridone alkaloid epoxides by *Ruta graveolens*, and the increased production of echinatin and the novel formation of a prenylated compound by *Glycyrrhiza echinata* cultures.

Stress compounds are of pharmaceutical interest in that they may be involved in various crude drugs formed pathologically (e.g. some gums and oleoresins) and potential drugs (gossypol); they are implicated in the toxicity of some diseased foodstuffs and they play a role in the defensive mechanism of the plant. In the latter area, the phytoalexins have received considerable attention in recent years and can be regarded as antifungal compounds synthesized by a plant in greatly increased amounts after infection. The antifungal isoflavonoid pterocarpans produced by many species of the Leguminosae are well known, see 'Spiny restharrow'. Other phytoalexins produced in the same family are hydroxyflavanones, stilbenoids, benzofurans, chromones and furanoacetylenes. Sesquiterpene phytoalexins have been isolated from infected *Ulmus glabra* and *Gossypium*. In the vine (*Vitis vinifera*) the fungus *Botrytis cinerea* acts as an elicitor for the production of the stilbenes resveratrol (q.v.) and pterostilbene.

Chemically, stress compounds are, in general, of extreme variability and include phenols, resins, carbohydrates, hydroxycinnamic acid derivatives, coumarins, bicyclic sesquiterpenes, triterpenes and steroidal compounds. For the promotion of stress compounds in cell cultures see Chapter 13 and Table 13.1.

Further reading

Facchini PJ, Huner-Allnach KL, Tari LW 2000 Plant aromatic L-amino acid decarboxylases: evolution, biochemistry, regulation and metabolic engineering applications. Phytochemistry 54(2): 121–138

Grayson DH 2000 Monoterpenoids (*a review covering mid-1997 to mid-1999*). Natural Product Reports 17(4): 385

Knaggs AR 1999, 2000 The biosynthesis of shikimate metabolites. Natural Product Reports 16(4): 525–560; 17(3): 269–292

Kruger NT, Hill SA, Ratcliffe RG (eds) 1999 Regulation of primary metabolic pathways in plants. Kluwer, Dordrecht, Netherlands

Ramos-Valdivia AC, Van der Heijden R, Verpoorte R 1997 Isopentenyl diphosphate isomerase: a core enzyme in isoprenoid biosynthesis. Natural Product Reports 14(6): 591–604

Rohmer M 1999 The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Natural Product Reports 16(5): 565–574

Seigler DS 1998 Plant secondary metabolism. Kluwer, Dordrecht, Netherlands

Singh BK 1999 (ed) Plant amino acids—biochemistry and biotechnology. Marcel Dekker Inc, New York

Wink M (ed) 1999 Biochemistry of plant secondary metabolism. Sheffield Academic Press

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PART 5

Pharmacopoeial and related drugs of biological origin

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Introduction

Drugs of the European and British Pharmacopoeias together with some related plant materials are considered in some detail in Chapters 19–26. They are arranged according to the biosynthetic origins of their principal constituents. An indication of how these drugs can also be grouped on a pharmacological/clinical usage basis is given in Chapter 6.

A large number of plant species, particularly those used in various traditional medicines, have been, and still are being, screened for specific pharmacological activities. Overviews involving some areas of this research, with its notable successes, are given in Chapters 26–29 and illustrate in conjuction with Chapters 8 and 9 the current approach to the search for 'new' drugs of potential value to the allopathic system of medicine.

Some products such as nutraceuticals, vitamins, antibiotics, hormones and flavourings are conveniently treated as heterogeneous biochemical groups (Chapters 31–33).

In Part 5, to help distinguish the monographs on drugs of the *BP* 2007 and *EP* from those of non-official drugs, the usual conventions relating to hierarchical headings have not been strictly observed; headings for the former are printed in bold capitals whereas the latter are in smaller typesize.

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19

Hydrocarbons and derivatives

Hydrocarbons contain carbon and hydrogen only and, from these, by the addition of functional groups and by interaction, all other natural compounds can theoretically be derived. In a particular class of compounds such as volatile oils, the components of any one member may be biosynthetically related (e.g. menthol and menthone in oil of peppermint) although because of their different functional groups they may undergo different sets of chemical reactions and possess different pharmacological properties. Among the most common functional groups are carboxylic acids, alcohols, ketones, aldehydes and phenols; biochemical interactions produce esters, lactones etc.

In this book most examples of medicinal plants containing the above are considered under their respective biogenetic groupings and in this chapter the detailed description of drugs is restricted to those examples in which simple acids, alcohols and esters comprise the principal medicinal components.

HYDROCARBONS

Although not featuring strongly in the pharmaceutical armamentarium, hydrocarbons are important in nature as components of cuticular waxes. The majority of these are odd-numbered long-chain alkanes within the range $C_{25–35}$ formed by decarboxylation of the next higher, even-numbered, free fatty acid. In recent years the long-chain polyenes of the Compositae have been systematically investigated in relation to their chemotaxonomic importance. Isoprene (C_5H_8), the unsaturated hydrocarbon moiety from which the terpenoids (isoprenoids) can be constructed (Fig. 18.17), has not to date been found free in nature. A number of cyclic terpenoid hydrocarbons including limonene, pinene, phellandrene and cadinene are components of essential oils. Rubber, gutta and the carotenes are polyunsaturated terpenoids.

MONOBASIC ACIDS

Organic acids possess one or more carboxyl groups and a monobasic acid may be represented as RCOOH. The very high frequency of the biochemical occurrence of the carboxyl group means that acids are found in all living organisms and as derivatives of all the major metabolic groups. They participate in essential metabolism and in this capacity range from the simple acids of the respiratory sequence to the complex deoxyribonucleic acids associated with the storage and transmission of hereditary characters. In the metabolic cycles they frequently function in association with coenzymes, and may accumulate as simple salts, esters and amides, or less frequently in the free state. Amino acids are discussed in Chapter 18.

C_1–C_6 Monocarboxylic acids

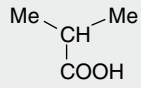
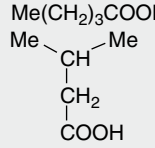
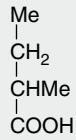
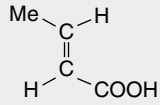
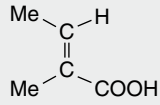
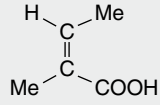
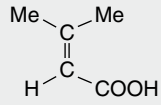
A number of these acids together with hydroxy- and keto-derivatives are intermediates in the early stages of the biosynthesis of fats, isoprenoid compounds and various amino acids (q.v.). In the free state they are not found abundantly in nature but occur scattered throughout the plant kingdom in the esterified form as a feature of some volatile oils, resins, fats, coumarin derivatives and alkaloids.

Some common acids are illustrated in Table 19.1.

Fatty acids

These acids are important as components of plant oils (acyl lipids) in which they occur as esters with the trihydric alcohol glycerol. They are also components of the resins of the Convolvulaceae and of waxes in which they are esterified with long-chain alcohols. Most are C_{10} to C_{20}

Table 19.1 Examples of C_1–C_6 monocarboxylic acids.

Name	Structure	Comments
Formic acid	HCOOH	Name derives from its first isolation from the ant, *Formica rufa*. A decomposition product of many vegetable materials. Occurs free in the hairs of the stinging nettle; combined in the gitaloxigenin series of cardioactive glycosides. *N*-formyl-L-methionine is involved in the initiation of protein synthesis on ribosomes
Acetic acid	MeCOOH	An essential primary metabolite, particularly as acetyl-CoA. Common in the esterified form
Propionic acid	$MeCH_2COOH$ $Me(CH_2)_2COOH$	Produced in the fatty acid oxidative cycle when an acyl-CoA with an odd number of carbon atoms is involved. Esterified as a tropane alkaloid
n-Butyric acid		Occurs in traces in many fats
iso-Butyric acid		Occurs free in carob beans (*Ceratonia siliqua*) and as its ethyl ester in croton oil. Component of resins of the Convolvulaceae and minor tropane alkaloids. Intermediate in the metabolism of valine
n-Valeric acid	$Me(CH_2)_3COOH$	Not common; component of Convolvulaceous resins
iso-Valeric acid		Free and esterified in *Valeriana* spp. Combined in some tropane alkaloids (e.g. valeroidine) and in the pyranocoumarin, dihydrosamidin. Intermediate in the metabolism of leucine
2-Methylbutyric acid		Component of some tropane and *Veratrum* alkaloids, Convolvulaceous glycosides and the pyranocoumarin visnadin
Caproic acid	$Me(CH_2)_4COOH$	Occurs in traces in many fats
Crotonic acid (*trans*-butenoic acid)		Constituent of croton oil
Tiglic acid		Occurs in croton oil (glycoside) from *Croton tiglium*. The acid of many minor tropane alkaloids, e.g. tigloidine. Component of Convolvulaceous resins and *Symphytum* alkaloids. Biosynthetically derived from isoleucine
Angelic acid		Occurs in the rhizome of *Angelica*. Esterifying acid of the *Schizanthus* alkaloid schizanthine X and of some volatile oils, e.g. chamomile oils. Component of the Cevadilla seed alkaloid cevadine and *Symphytum* alkaloids
Senecioic acid		First isolated from a species of *Senecio* (Compositae). Occurs as the esterifying acid of some alkaloids of *Dioscorea* and *Schizanthus*. Component of the pyranocoumarin samidin

straight-chain monocarboxylic acids with an even number of carbon atoms. Over 200 have been isolated from natural sources but relatively few are ubiquitous in their occurrence. They may be saturated (e.g. palmitic and stearic acids) or unsaturated (e.g. oleic acid). The double bonds, with a few minor exceptions such as the seed oil of pomegranate, are *cis*.

Less commonly they are cyclic compounds such as hydnocarpic acid and the prostaglandins. The latter are a group of physiologically active essential fatty acids found in most body tissues and are involved in the platelet-aggregation and inflammatory processes. They promote smooth muscle contraction making them of clinical use as effective abortifacients and for inducing labour. All the active natural prostaglandins are derivatives of prostanoic acid (see Table 19.4). A rich source of prostaglandin A_2 (PGA_2) is the soft coral *Plexaura homomalla*. Although recognized in the 1930s, and their structures determined in 1962, it was not until 1988 that prostaglandins were unequivocally established as components of some higher plants (cambial zones and buds of *Larix* and *Populus* spp.)

The characteristic acid of castor oil, ricinoleic acid (hydroxyoleic acid) has both a hydroxyl group and an unsaturated double bond. A range of acetylenic fatty acids occurs throughout the plant kingdom and some of them possess antifungal and antibacterial properties. The biogenetic relationship between these, the olefinic fatty acids and the saturated fatty acids is outlined later in this chapter.

Examples of fatty acids are listed in Tables 19.2–19.4. It will be noted that some have more than one unsaturated bond, the bonds

being interspersed by methylene groups. These polyunsaturated acids have received much attention in recent years both regarding their role in dietary fats and as medicinals. All the common acids have trivial names but in order to indicate more precisely their structures without recourse to the full systematic chemical name each can be represented by a symbol. Thus α-linolenic, systematic name all-*cis*-$\Delta^{9,12,15}$-octadecatrienoic acid, has 18 carbons and three double bonds which can be represented by 18:3. The position of the double bonds is then indicated by the *n-x* convention where *n* = number of carbon atoms in the molecule and *x* is the number of inclusive carbon atoms from the methyl (ω) end to the first carbon of the first double bond, in this case 3, so that the symbol for α-linolenic acid is 18:3(*n*-3). The positions of the two remaining double bonds are deduced by the fact that they will follow on from each other being separated only by one methylene (-CH_2-) group. In this area students may find the literature situation somewhat confusing because in some texts the acids may be symbolized on the basis of conventional chemical systematic numbering—for fatty acids the carboxyl carbon being C1. For α-linolenic acid this is represented as 18:3(*9c.12c.15c*), *c* indicating a *cis*-bond. The advantage of the first system is that it indicates any bioequivalence of the double bonds in acids of different chain-length, bearing in mind that chain elongation *in vivo* proceeds at the carboxyl end of the molecule by the addition of 2C units. Thus it can be seen from Table 19.3 that γ-linolenic acid and arachidonic acid both fall into the biochemical ω-6 family of unsaturated fatty acids and their respective symbols 18:3(*n*-6) and 20:4(*n*-6) reflect this whereas symbols based on chemical nomenclature for these acids *viz* 18:3(*6c,9c,12c*) and 20:4(*5c,8c,11c,14c*) do not. A comparison of symbols for some common unsaturated acids is shown in Table 19.5.

Under certain conditions, which are specified in pharmacopoeias, iodine or its equivalent is taken up at these double bonds and the so-called *iodine value* is thus a measure of unsaturatedness. The iodine value is the number of parts of iodine absorbed by 100 parts by weight of the substance. Near-infrared spectroscopy can also be used to determine this value as it is directly related to the HC=CH stretch bands at 2130 nm in the spectrum. Iodine values are useful constants for acids, fixed oils, fats and waxes, and help to indicate the composition of complex mixtures as well as pure substances.

Table 19.4 Cyclic unsaturated acids.

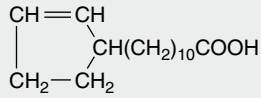
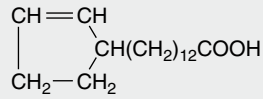
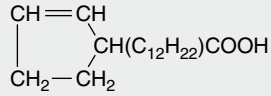
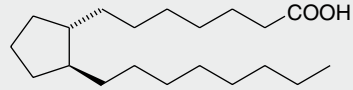
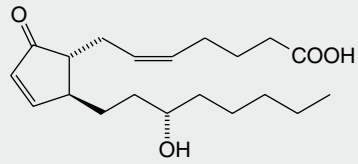
Common name	Structural formula
Hydnocarpic	
Chaulmoogric	
Gorlic	
Prostanoic	
PGA2	

Table 19.2 Straight-chain saturated acids.

Common name	Systematic name	Structural formula
Caprylic	*n*-Octanoic	$CH_3(CH_2)_6COOH$
Capric	*n*-Decanoic	$CH_3(CH_2)_8COOH$
Lauric	*n*-Dodecanoic	$CH_3(CH_2)_{10}COOH$
Myristic	*n*-Tetradecanoic	$CH_3(CH_2)_{12}COOH$
Palmitic	*n*-Hexadecanoic	$CH_3(CH_2)_{14}COOH$
Stearic	*n*-Octadecanoic	$CH_3(CH_2)_{16}COOH$
Arachidic	*n*-Eicosanoic	$CH_3(CH_2)_{18}COOH$

Table 19.5 Comparison of symbols ascribed to unsaturated fatty acids.

Common name of acid	Symbol employing biochemical equivalence of double bonds	Symbol based on chemical nomenclature
Palmitoleic	16:1 (*n*-7)	16:1 (9*c*)
Oleic	18:1 (*n*-9)	18:1 (9*c*)
Petroselinic	18:1 (*n*-12)	18:1(6*c*)
Ricinoleic	18:1 (*n*-9) (hydroxy at *n*-7)	D(+)-12h-18:1(9*c*) (*h* = hydroxy)
Erucic	22:1 (*n*-9)	22:1 (13*c*)
Linoleic	18:2 (*n*-6)	18:2 (9*c*,12*c*)
Eicosadienoic	20:2 (*n*-6)	20:2 (11*c*,14*c*)

Table 19.3 Straight-chain unsaturated acids.

Common name	Number of unsaturated bonds	Structural formula
Palmitoleic	1	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$
Oleic	1	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$
Petroselinic	1	$CH_3(CH_2)_{10}CH{=}CH(CH_2)_4COOH$
Ricinoleic	1	$CH_3(CH_2)_5CH(OH)CH_2CH{=}CH{-}(CH_2)_7COOH$
Erucic	1	$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}COOH$
Linolenic	2	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH{-}(CH_2)_7COOH$
α-Linoleic	3	$CH_3CH_2CH{=}CHCH_2CH{=}CHCH_2{-}CH{=}CH(CH_2)_7COOH$
γ-Linolenic	3	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2CH{=}CH(CH_2)_4COOH$
Arachidonic	4	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2{-}CH{=}CHCH_2CH{=}CH(CH_2)_3COOH$

Biosynthesis of saturated falty acids. See Chapter 18.

Biosynthesis of unsaturated fatty acids. Before the elucidation of the overall chemistry of formation of polyunsaturated fatty acids such as linoleic in the early 1960s by Bloch, knowledge concerning the biosynthesis of these compounds lagged behind that of the saturated acids. Recent progress has been much more rapid and, in general, it now appears that in aerobic organisms, monoenoic acids with the double bond in the 9,10-position arise by direct dehydrogenation of saturated acids. In higher plants, for this reaction, coenzyme A may be replaced by the acyl carrier protein (ACP), and Bloch has demonstrated that stearoyl-*S*-ACP is an effective enzyme substrate of the desaturase system of isolated plant leaf chloroplasts. The reduced forms of nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen are cofactors.

$$CH_3(CH_2)_{16}CO{-}S{-}ACP + O_2 + NADPH \longrightarrow$$
(stearoyl-S-ACP)

$$CH_3(CH_2)_7CH{=}CH(CH_2)_7{-}CO{-}S{-}ACP + H_2O + NADP$$
(oleoyl-S-ACP)

The position of the introduced double bond in respect to the carboxyl group is governed by the enzyme; hence, chain length of the substrate acid is most important. The hydrogen elimination is specifically *cis* but a few unusual fatty acids such as that in the seed oil of *Punica granatum* with the structure 18:3 (9*c*,11*t*,13*c*) have *trans* bonds. As illustrated in Fig. 19.1, further double bonds may be similarly introduced to give linoleic and linolenic acid.

Unsaturated fatty acids can also be formed in plants by elongation of a medium-chain-length unsaturated acid. This appears to occur by the formation of an intermediate, β,γ-unsaturated acid rather than the α,β-unsaturated acid normally produced in saturated fatty acid biosynthesis; the β,γ-bond is not reduced and more C_2 units are added in the usual way (Fig. 19.2).

Sterculic acid, a component of seed oils of the Malvaceae and Sterculiaceae, is a cyclopropene and is also derived from oleic acid, with methionine supplying the extra carbon atom to give, first, the cyclopropane. Ricinoleic acid is a hydroxy fatty acid found in castor oil seeds and is again biosynthesized from oleic acid (Fig. 19.3).

Some of the natural acetylenes and acetylenic fatty acids have obvious structural similarities to the more common fatty acids. The hypothesis that triple bonds are formed from double bonds by a mechanism analogous to that for the formation of double bonds and involving structurally and stereochemically specific enzymes has now received experimental support. By this means (Fig. 19.4) the range of acetylenes found in Basidiomycetes and in the Compositae, Araliaceae and Umbelliferae can be derived from linoleic acid via its acetylenic 12,13-dehydroderivative, crepenynic acid, an acid first isolated from seeds oils of *Crepis* spp.

Aromatic acids

Two common aromatic acids are benzoic acid and cinnamic acid (unsaturated side-chain), which are widely distributed in nature and

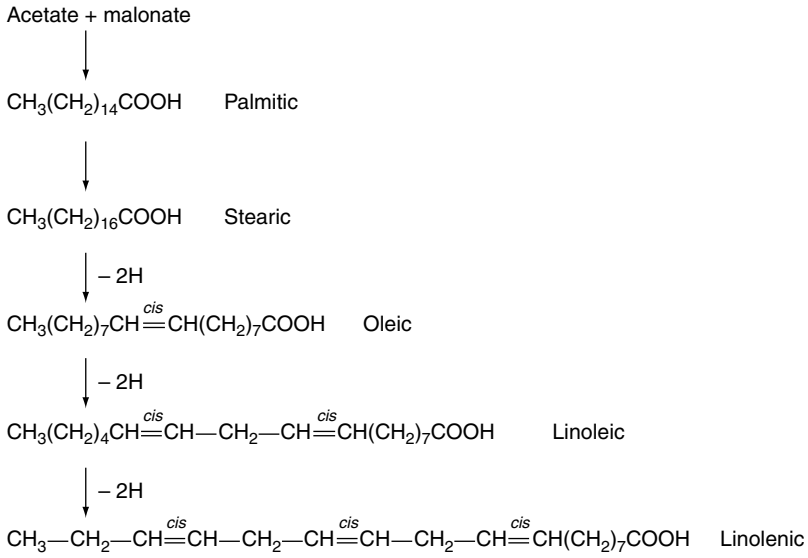


Fig. 19.1
Sequence of formation of olefinic fatty acids in plants.

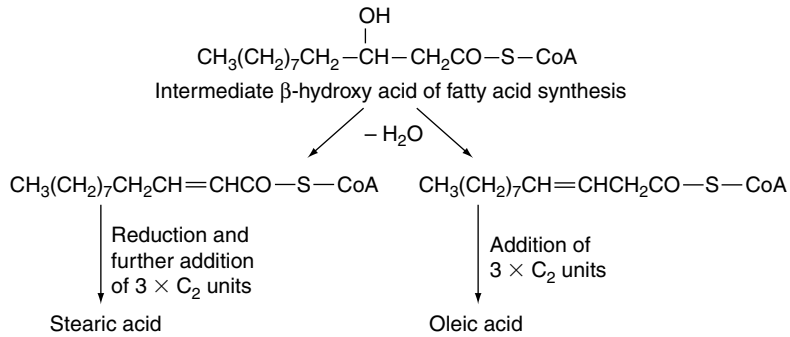


Fig. 19.2
Alternative pathways for synthesis of unsaturated fatty acids.

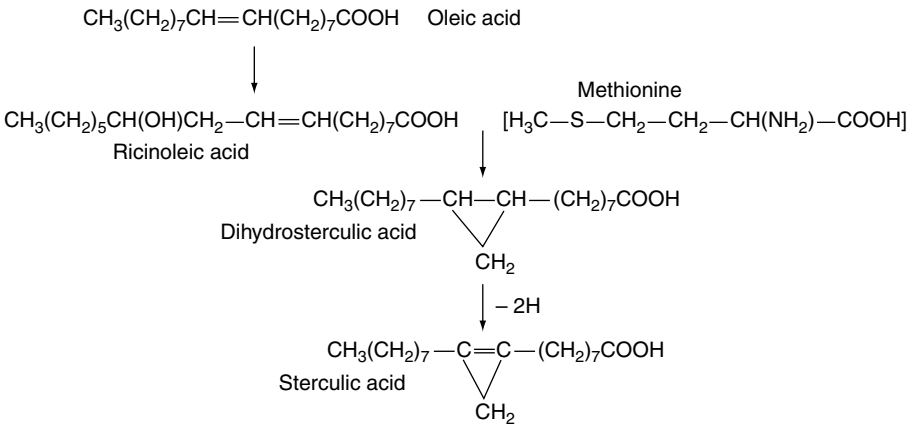
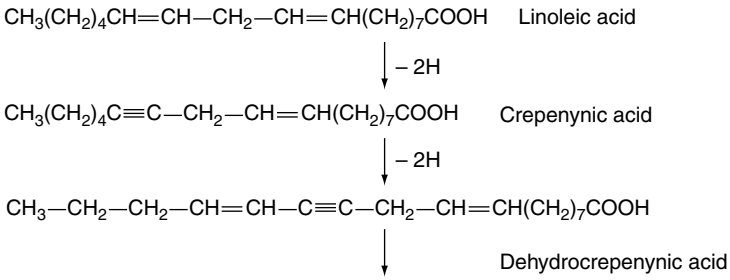


Fig. 19.3
Oleic acid as the precursor of ricinoleic and sterculic acids.



Range of acetylenes formed by further introduction of acetylenic bonds at the 'distal' part (furthermost from carboxyl group) of molecules and by chain shortening in 'proximal' part of molecule.

Fig. 19.4
Formation of acetylenic fatty acids.

often occur free and combined in considerable amounts in drugs such as balsams. Truxillic acid, a polymer of cinnamic acid, occurs in coca leaves. Other related acids of fairly common occurrence are those having phenolic or other groupings in addition to a carboxyl group; such are: salicyclic acid (*o*-hydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), veratric acid (3,4-dimethoxybenzoic acid), gallic acid (3,4,5-trihydroxybenzoic acid) and 3,4,5-trimethoxybenzoic acid. Similarly, derived from cinnamic acid, one finds *p*-coumaric acid (*p*-hydroxycinnamic acid), ferulic acid (hydroxymethoxycinnamic acid), caffeic acid (hydroxycinnamic acid) and 3,4,5-trimethoxycinnamic acid. Unbelliferone, which occurs in combination in asafoetida, is the lactone of dihydroxycinnamic acid.

Acids having an alcohol group are quinic acid (tetrahydroxyhexahydrobenzoic acid), which occurs in cinchona bark and in some gymnosperms; mandelic acid, $C_6H_5CHOHCOOH$, which occurs in combination in cyanogenetic glycosides such as those of bitter almonds and other species of *Prunus*; and shikimic acid, an important intermediate metabolite (see Fig. 18.8). Shikimic acid has itself acquired recent pharmaceutical importance as the starting material for the semi-synthesis of the antiviral drug oseltamivir (Tamiflu®) for use against bird flu infections in humans. Its principal source has been star-anise fruits (q.v.), leading to a supply shortage of the plant material. Other natural sources rich in this acid and of potential future use are needles of the Pinaceae (S. Marshall, *Pharm. J.*, 2007, **279**, 719) and the fruits (gumballs) of the American sweet gum tree *Liquidamber styraciflua* (q.v.). The acid is also produced commercially by the fermentation of genetically modified *Escherichia coli*. Tropic acid and phenyllactic acid are two aromatic hydroxy acids that occur as esters in tropane alkaloids (q.v.). For examples of the above see Fig. 19.5.

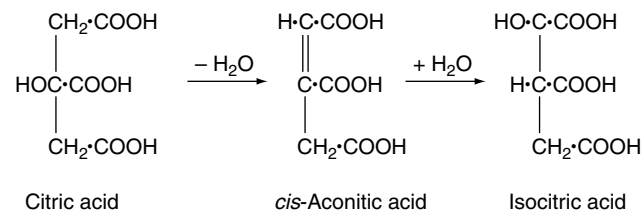
Chlorogenic or caffeotannic acid is a condensation product of caffeic acid and quinic acid. It occurs in maté, coffee, elder flowers, lime flowers, hops and nux vomica and is converted into a green compound, which serves for its detection, when an aqueous extract is treated with ammonia and exposed to air. See also 'Pseudotannins' (Chapter 21).

The biogenesis of the aromatic ring has been discussed in Chapter 18.

DIBASIC AND TRIBASIC ACIDS

Oxalic acid, $(COOH)_2$, forms the first of a series of dicarboxylic acids which includes malonic acid, $CH_2(COOH)_2$, and succinic acid, $(CH_2)_2(COOH)_2$. Closely related to malonic acid is the unsaturated acid fumaric acid, COOH–CH=CH–COOH. Malic acid contains an alcohol group and has the formula COOH–CH_2–CHOH–COOH. It is found in fruits such as apples and tamarinds. A high percentage of tartaric acid, COOH–$(CHOH)_2$–COOH, and its potassium salt occurs in tamarinds and other fruits.

The tribasic acids, citric, isocitric and aconitic are closely related to one another. The Krebs' citric acid cycle, which is discussed in Chapter 18, is very important. Citric acid is abundant in fruit juices, and aconitic acid, which occurs in *Aconitum* spp., is anhydrocitric acid. It forms part of the Krebs' cycle and the glyoxalate cycle in microorganisms.



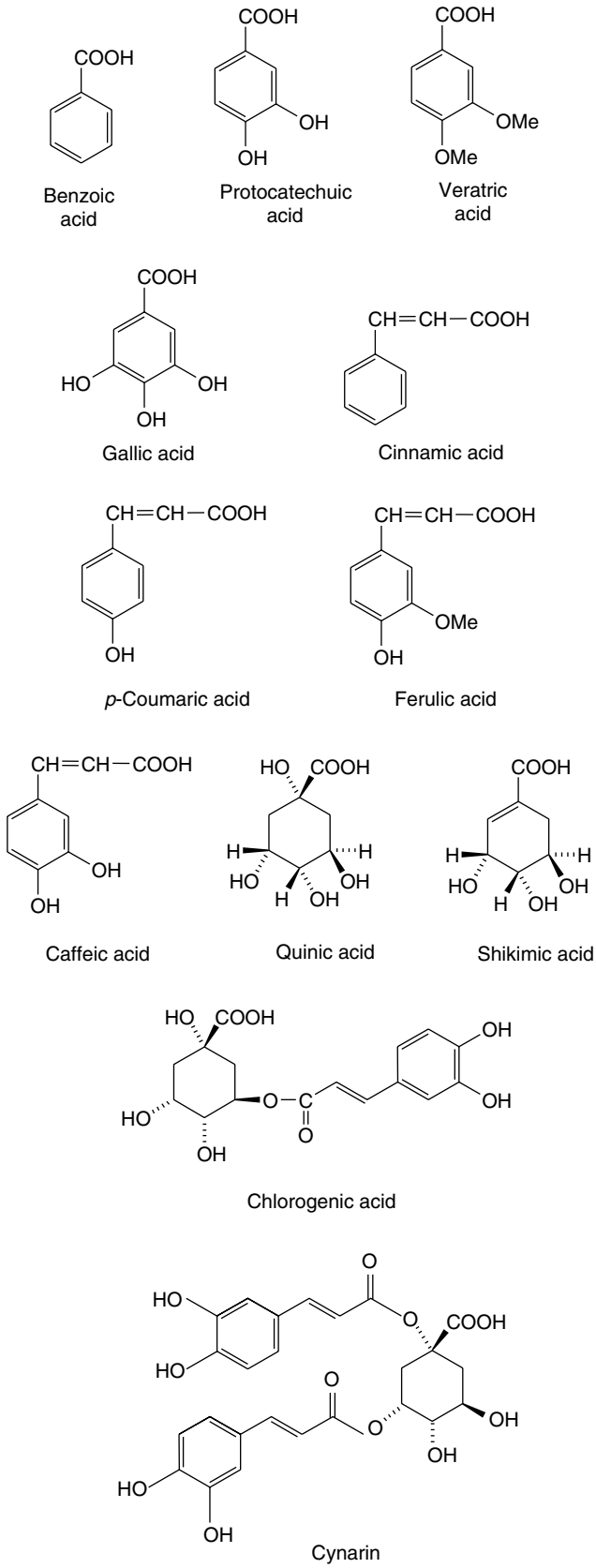


Fig. 19.5
Aromatic and other cyclic acids.

Of interest are opines, a group of substances formed by a host plant after infection with *Agrobacterium* spp.; a number of these compounds are di- and tri-carboxylic acids. For further details see Chapters 13 and 14.

ALCOHOLS

Alcohols possess one or more hydroxyl groups and exist naturally in either the free state or combined as esters. Like phenols they generally have common names ending in 'ol' (e.g. ethanol, glycerol and mannitol). They can be classed according to the number of hydroxyl groups present: monohydric alcohols-one hydroxyl, dihydric-two, trihydric-three and polyhydric-four or more. Furthermore each hydroxyl group may be classed as *primary*: $-CH_2OH$ (e.g. ethanol), *secondary*: –CHOH– (e.g. isopropanol) or *tertiary*: ≡COH (e.g. *t*-butanol). The remainder of the molecule may be saturated or unsaturated, aliphatic or aromatic. Numerous examples will be encountered throughout the text.

Monohydric aliphatic alcohols

Lower members of the series are found principally combined as esters e.g. methyl salicylate in oil of wintergreen and methyl and ethyl esters responsible for some fruit aromas. Esterified long-chain alcohols are constituents of some pharmaceutically important animal waxes and include cetyl alcohol ($C_{16}H_{33}OH$), ceryl alcohol ($C_{26}H_{53}OH$) and myricyl alcohol ($C_{30}H_{61}OH$). Such alcohols also participate in the formation of esters which are constituents of leaf cuticular waxes; an example is Carnauba Wax *BP* which contains myricyl cerotate.

Monohydric terpene alcohols

These are alcohols associated with that large group of compounds which arise from mevalonic acid and have isoprene as a fundamental structural unit. Pharmacognostically they are particularly evident as constituents of volatile oils namely: (1) non-cyclic terpene alcohols occur in many volatile oils—for example, geraniol in otto of rose, its isomer nerol in oils of orange and bergamot and linalol both free and combined as linalyl acetate in oils of lavender and rosemary; (2) monocyclic terpene alcohols are represented by terpineol and its acetate in oil of neroli and menthol and its acetate in oil of peppermint; (3) dicyclic terpene alcohols are particularly abundant in the Coniferae (e.g. sabinol and its acetate in *Juniperus sabina*). In the dicotyledons oil of rosemary contains borneol and its esters.

Monohydric aromatic alcohols

Benzyl alcohol, $C_6H_5CH_2OH$, and cinnamyl alcohol, $C_6H_5CH=CHCH_2OH$, occur both free and as esters of benzoic and cinnamic acids in balsams such as Tolu and Peru. The latter balsam is sometimes adulterated with cheap synthetic benzyl benzoate.

Included in this class is coniferyl alcohol, which forms an important component of the lignin molecule. Lignins are extremely complex phenylpropane polymers; they form an important strengthening material of plant cell walls and vary in composition according to their source, see Chapter 21, section on 'Lignans and Lignin'.

Dihydric alcohols

Dihydric alcohols or *glycols* are compounds containing two hydroxyl groups; they are found naturally in many structural classes of compounds. The bicyclic amino alcohol 3,6-dihydroxytropane occurs

free and as esters in a number of species of the Solanaceae and Erythroxylaceae, the dihydric alcohol panaxadiol is a component of some ginseng steroids, and oenanthotoxin, the poisonous constituent of the hemlock water dropworts (*Oenanthe* spp.), is a polyene diol.

Trihydric alcohols

As with the glycols these compounds occur in a range of structural types. An important example is glycerol (propan-1,2,3-triol), an essential component of fixed oils and fats which are discussed in more detail below.

Polyhydric aliphatic alcohols

The following are alcohols with either four or six hydroxyl groups. The *meso* form of erythritol, $CH_2OHCHOHCHOHCH_2OH$, is found in seaweeds and certain lichens both free and combined with lecanoric acid. The hexahydric sugar alcohols (e.g. sorbitol, mannitol and dulcitol) are formed in nature by the reduction of either an aldehyde group of an aldose or the keto groups of a ketose. Sorbitol is abundant in many rosaceous fruits, mannitol in manna and dulcitol in species of *Euonymus*.

ESTERS

Esters arise from the union of an alcohol and an acid with loss of water:

$$R^1CH_2OH + R^2COOH \rightleftharpoons R^1CH_2OCOR^2 + HOH$$

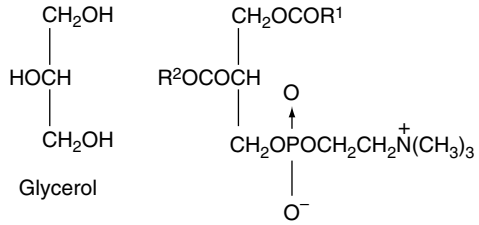
The reaction is reversible and in plants esterase enzymes control the reaction.

Many different types of esters are known, and those formed by an acetylation of an alcoholic group are very common and are found in many biosynthetic groups of metabolites including volatile oils, e.g. linalyl acetate in lavender. Esters which involve aromatic acids such as benzoic and cinnamic acids with corresponding alcohols are sometimes found associated with free acids, other volatile metabolites and resins, in such products as balsams (see drugs described at the end of this chapter). A number of alkaloids (e.g. atropine and reserpine) are esters.

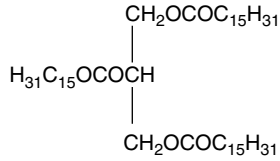
A particularly important group of esters from the pharmaceutical viewpoint is that comprising the lipids or fatty esters. These involve a long-chain fatty acid of the type described earlier and alcohols such as glycerol and the higher monohydric alcohols.

The term 'lipid' includes not only fixed oils, fats and waxes (simple lipids), but also phosphatides and lecithins (complex lipids), which may contain phosphorus and nitrogen in addition to carbon, hydrogen and oxygen. These substances are widely distributed in both the vegetable and animal kingdoms, and in plants they are particularly abundant in fruits and seeds. In animals the depot fats resemble those found in plants, while the complex lipids occur mainly in the more active tissues such as the brain and liver. The latter group plays an important role in the structure of cellular membranes, the hydrophobic nature of the fatty acids being all-important to their biological role.

The lecithins are esters of glycerophosphoric acid in which the two free hydroxyls of the glycerol are esterified with fatty acids while one of the two remaining groups of the phosphoric acid is esterified to an alcohol (choline, ethanolamine, serine, glycerol or inositol). Because plants have no mechanism for controlling their temperature, they must possess membrane lipids that remain mobile at relatively low temperatures. This property is conferred by the methylene-linked *cis* double bonds of the polyunsaturated acids bound as esters with the polar lipids. Conversely, in simple lipids, all three hydroxyl groups are esterified with fatty acids and these compounds have been traditionally referred to as triglycerides, although with current nomenclature triacylglycerols is preferred. The prefix *sn* is now employed to denote the *stereospecific numbering* of the molecule.



A complex lipid
(a 1,2-diacyl-*sn*-glycero-3-phosphorylcholine)



A simple lipid or triacylglycerol
(tripalmitin)

Fats and fixed oils

As agricultural crops, seeds used for the extraction of fixed oils rate in importance second only to cereals. Over the last 60 years the production of oils for the food industry has increased enormously, whereas consumption by industrial and other users has remained relatively static but, in the pharmaceutical industry at least, not without interesting developments. Fixed oils are also obtained from fruit pericarps and in some instances such as the palm, *Elaeis guineensis* (Palmae), two oils differing in properties and chemical composition are obtained—the pleasantly flavoured palm kernel oil from the endosperm and palm oil from the orange-yellow fleshy pericarp. Oil seed crops are particularly advantageous commercially as following the expression of the oil a valuable high protein cattle feed remains. Also, such crops have benefited from plant breeding both regarding the yield and nature of the oil produced, and the morphology of the plant itself (see Chapter 14).

A naturally occurring mixture of lipids such as olive oil or oil of theobroma may be either liquid or solid and the terms 'oil' and 'fat' have, therefore, no very precise significance. Coconut oil and chaulmoogra oil, for example, leave the tropics as an oil and arrive in Western Europe as a solid. Even an oil such as olive oil will largely solidify in cold weather. In general, acylglycerols involving saturated fatty acids are solid and those of unsaturated acids are liquids. When both types are present, as in crude cod-liver oil, cooling results in the deposition of saturated acylglycerols such as stearin. In most medicinal cod-liver oils these solid materials are removed by freezing and filtration. Acylglycerols are represented by the general formula given below and can be hydrolysed by heating with caustic alkali to form soaps and glycerin.

If the fatty acids represented by R^1, R^2 and R^3 are the same, the triacylglycerol is known as a simple triacylglycerol—for example, tripalmitin on hydrolysis yields three molecules of palmatic acid. In nature, however, R^1, R^2 and R^3 are usually different and the ester is known as a mixed triacylglycerol. On hydrolysis they frequently yield

both saturated and unsaturated acids (Fig. 19.6); there is a strong tendency for unsaturated acids, particularly the C_{18} olefinic acids, to be linked to the secondary hydroxyl.

Biogenesis. Acylglycerols are formed from the fatty acyl-CoA or, more probably, the fatty acyl carrier protein (ACP) and L-α-glycerophosphate, as indicated in Fig. 19.7.

Extraction. Most commercial oils are derived from either seeds or fruits and nowadays are mostly extracted by the producing country and exported as the crude oil. Sophisticated derivatizations of oils are mainly carried out by the importing countries.

The initial treatment before extraction depends on the botanical structure—for example, American cotton seeds require delinting and castor seeds and ground nuts require decorticating. Special machines are available for these purposes. Removal of the oil may take the form of cold or hot expression, centrifuging or solvent extraction, again depending on the commodity. With seeds the remaining cake usually forms a valuable cattle feed and for this reason complete removal of the oil is not always necessary. The crude oil requires refining, however, as for example with olive oil, the first expressed oils, *extra virgin* and *virgin* constitute the premium grades and require no further purification. Cold-drawn oils usually require nothing further than filtration; castor oil requires steaming to inactivate lipase; the addition of a determined amount of alkali may be required to remove free acid; and washing and decolorization may be performed. An antioxidant may be added and its nature and concentration stated on the label. Specific points concerning preparation are mentioned under the individual oils described at the end of the chapter. Where appropriate, the *refined* pharmaceutical oils are suitable for use in the manufacture of parenteral dosage forms.

Quantitative tests. A number of quantitative tests are commonly used to evaluate fixed oils and fats. ***Acid value*** refers to the number of mg of potassium hydroxide required to neutralize the free acids in 1 g of the oil; high acid values arise in rancidified oils. Particularly low values are officially specified for those oils to be used in parenteral dosage forms; for refined wheat-germ oil, for example, the value is ⩾0.3, whereas for the refined oil for general use the value is ⩾0.9 and for the unrefined oil ⩾20.0. Similar figures apply to other fixed oils so used. ***Saponification value***: the hydrolysis reaction of lipids (above) can be used to determine the saponification value of the oil and is expressed as the number of mg of potassium hydroxide required to neutralize the free acids in, and to hydrolyse the esters in, 1 g of the substance. ***Ester value*** is the difference between the saponification and acid values. ***Iodine value*** (see 'Fatty acids') gives a measure of the unsaturation of the oil. Oils which partially resinify on exposure to air are known as semidrying or drying oils. Such oils (e.g. linseed oil) have high iodine values. In some cases, particularly for animal fats such as butter, the determination of ***volatile acidity*** is useful, since the lower fatty acids such as butyric acid are volatile in steam and this may be used for their separation and estimation. It is frequently useful to determine ***unsaponifiable matter***, which consists of compounds such as sterols which remain after saponification of the acylglycerols and removal of the glycerol and soaps by means of solvents. The content of brassicasterol in the sterol fraction of fixed oils is limited by the *Pharmacopoeia* for some oils, e.g. maximum 0.3% for evening primrose oil and borage oil.

The ***acetyl value*** is the number of milligrams of potassium hydroxide required to neutralize the acetic acid freed by the hydrolysis of the acetylated fat or other substance. The oil is first acetylated with acetic anhydride, which combines with any free hydroxyl groups present, and the product is then isolated after thorough removal of acid resulting from the reagent; its saponification value is determined together with that of the original oil. The acetyl value is calculated from the difference between these two figures.

The ***hydroxyl value*** of a substance depends on the number of free hydroxyl groups present. It is expressed as the number of milligrams of potassium hydroxide required to neutralize the acid combined by acylation of the sample. Most fixed oils have low values, which arise from small quantities of sterols present; castor oil is an exception (minimum value 150), arising from the high proportion of ricinoleic acid present.

Under unsuitable storage conditions, such as exposure to light and air, fixed oils undergo secondary oxidation to give peroxides that then generate aldehydes and ketones. Such deterioration is detected by measurement of the ***peroxide value*** and ***anisidine value***. The former is described by the *Pharmacopoeia* as 'the number that expresses in milliequivalents of oxygen the quantity of peroxide contained in 1000 g of the substance, as determined by the prescribed methods'. These methods involve the liberation of iodine from a potassium iodide solution by the peroxides present in the sample and titration with 0.01 M

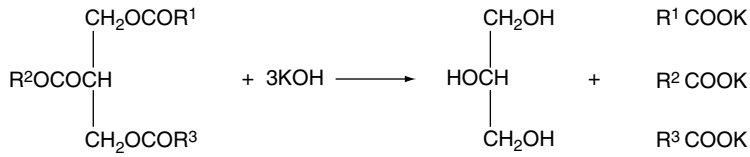


Fig. 19.6
Hydrolysis of a mixed triacylglycerol.

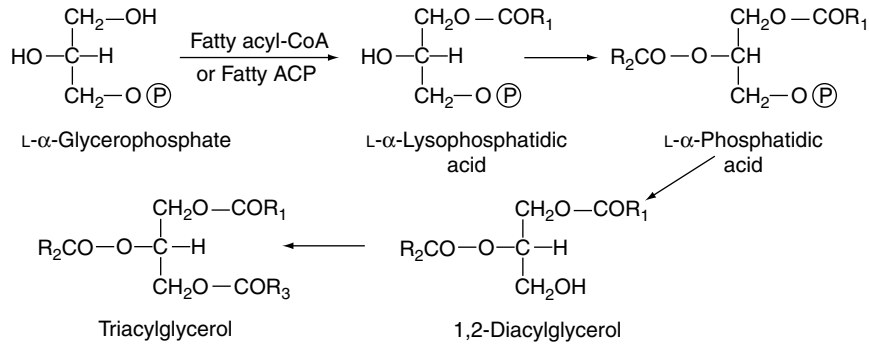


Fig. 19.7
Formation of acylglycerols; R^1, R^2 and R^3 may or may not be the same acyl groups.

sodium thiosulphate solution. For refined oils such as olive, borage, evening primrose and wheat-germ the typical value is 10; if these oils are to be used for parental dosage forms the maximum is 5. The maximum permissible value is higher for the virgin oils, e.g. olive = 20. Peroxide values are also used for fish oils, e.g. maximum value for farmed salmon oil, 5.0.

Anisidine values are determined photometrically (350 nm) and depend on the coloured complex produced by the interaction of *p*-anisidine (the methyl ether of *p*-aminophenol) with aldehydes and ketones. They are used principally for the evaluation of fish oils, including type-A cod-liver oil and farmed salmon oil (maximum 10).

Certain physical constants of fixed oils and fats are significant: specific gravity, melting point, refractive index and sometimes optical rotation (e.g. castor oil). Table 19.6 shows how chemical standards are related to chemical composition. The gas chromatographic separation and quantification of the acids produced by the hydrolysis of specific fixed oils is an official method for their identification and quality control; type chromatograms are included in the *BP*/*EP*. Such detailed analyses often eliminate the necessity of rountinely applying all the above quantitative standards. Some examples of this application are given for the oils in Table 16.4. Comments on the detection of adulterants in the more expensive oils can be found under individual headings.

Waxes

The term 'wax', although sometimes applied to the hydrocarbon mixture hard paraffin, is best confined to those natural mixtures containing appreciable quantities of esters derived from higher monohydric alcohols of the methyl alcohol series combined with fatty acids. In this series of alcohols the members change from liquids to solids, become less soluble in water and have higher melting points with increase in molecular weight. The first solid of the series is dodecyl alcohol, $C_{12}H_{25}OH$. Waxes include vegetable products such as carnauba wax and animal products such as spermaceti, beeswax and so-called 'wool-fat'.

Although waxes are abundant in nature (e.g. on epidermal surfaces), a limited number only are of commercial importance; some of the best-known and their chief alcohols are given at the end of the chapter.

An important practical difference between fats and waxes is that fats may be saponified by means of either aqueous or alcoholic alkali but waxes are only saponified by alcoholic alkali. This fact is used for the detection of fats when added as adulterants to waxes (e.g. for detecting the fat 'Japan wax' as an adulterant in beeswax). Saponification of the wax ester cetyl palmitate may be represented as:

$$\underset{\text{Cetyl palmitate}}{C_{15}H_{31}.COOC_{16}H_{33}} + \text{alcoholic KOH} \rightarrow \underset{\text{Cetylalcohol}}{C_{16}H_{33}OH} + \underset{\text{Potassium palmitate}}{C_{15}H_{13}.COOK}$$

While fats consist almost entirely of esters, waxes, in addition to esters of the cetyl palmitate type, often contain appreciable quantities of free acids, hydrocarbons, free alcohols and sterols. The hydrocarbons and sterols are unsaponifiable and both spermaceti and wool fat, which contain considerable quantities of these, have high saponification values. If analytical data for fats and waxes are compared, it will be noted that the acid values of waxes tend to be higher—for example, beeswax contains about 15% of free cerotic acid, $C_{26}H_{53}COOH$. In most waxes, iodine values are relatively low and unsaponifiable matter is high (Table 19.7).

Table 19.6 Numerical properties of fixed oils.

				Approximate fatty composition	
Fat or oil	Melting point, °C	Saponification value	Iodine value	Saturated (%)	Unsaturated (%)
Almond	–18	183–208	99–103	12	88 Ol. Ln.*
Castor	–18	175–183	84	0.3–2.5	88–94 Ricinoleic; 5–15 Ol. Ln.
Olive	†	185–196	79–88	7–20	75–93 Ol. Ln.
Arachis	–5 to +3	188–196	86–106	18	82 Ol. Ln.
Coconut	23–26	250–264	7–11	92	8 Ol.
Cottonseed	0 (Before winterizing)	190–198	109–116	27	73 Ln. Ol.
Palm	30	248	13.5	50	50 Ol. Ln.
Theobroma	31–34	193–195	33–42	59	41 Ol. Ln.
Lard	34–41	192–198	50–66	60	40 Ol.
Cod-liver	Clear at 0	180–190	155–180	Under 15	Over 85 with up to six unsaturated bonds

*Ol. = oleic; Ln. = linoleic; †deposits palmitin at 2°C.

Table 19.7 Chemical standards of waxes.

Wax	Acid value	Saponification value	Iodine value	Important constituents
Spermaceti	Below 1	120–136	Below 5	Cetyl palmitate and cetyl myristate
Beeswax	18–24	70–80 (ester value)	8–11	72% esters, mainly myricyl palmitate; free cerotic acid; steryl esters
Carnauba	4–7	79–95	10–14	
Wool-fat	Below 1	90–106	18–32	Steryl esters, esters of other aliphatic alcohols, fatty acids and hydrocarbons

DRUGS CONTAINING ACIDS, ALCOHOLS AND ESTERS

ROSELLE

The dried calyces and epicalyces of *Hibiscus sabdariffa* L., family Malvaceae, collected during the fruiting period, constitute the drug 'roselle'. As an ornamental, the plant is grown globally in subtropical areas and the leaves, stems and seeds also find use as colourants, flavourings and as a source of fibre (rosella hemp). Its common name, Jamaica sorrel, arose following its early introduction to that country. Commercial supplies of the drug come principally from S.E. Asia, Egypt and the Sudan.

Characters. The easily broken, crimson to violet drug consists of the flower portions comprising a pitcher-shaped calyx with five recurved teeth and, below, and attached to it, an epicalyx of up to about twelve obovate leaflets. The powder, examined microscopically, exhibits coloured parenchymatous cells containing cluster crystals of calcium oxalate, mucilage-containing cells, vascular tissue, epidermal cells, anisocytic stomata, covering trichomes, a few glandular trichomes and pollen grains with spiney exines.

Constituents. Roselle contains a considerable quantity of free acids including citric, tartaric and malic acids and the lactone of hydroxycitric acid. The *BP*/*EP* requires a minimum content of 13.5% of acids expressed as citric acid determined by potentiometric titration. Phenolic compounds include anthocyanins involving the glycosides of delphinidin and cyanidin for which the *Pharmacopoeia* specifies and absorbance of ⩽0.350 for the whole drug and ⩽0.250 for the cut drug when measured at 520 nm in a prescribed aqueous extract. A spectrophotometric assay for total anthocyanins has been reported (T. Sukwattanasinit *et al.*, *Planta Medica*, 2007, **73**, 1517). Various polysaccharides have been recorded; for a report on their stimulation of proliferation and differentiation of human keratinocytes see C. Brunold *et al.*, *Planta Med.*, 2004, **70**, 373.

Use. A colourant and flavouring component for herbal preparations. Traditionally, all parts of the plant have been employed as an astringent and cooling agent; it has a diuretic action. Antioxidant and hypocholesterolemic activities have been investigated (V. Hirunpanich *et al.*, *J. Ethnopharmacol*., 2006, **103**, 252).

Further reading

Vasudeva N, Sharma SK 2008 Biologically active compounds from the genus *Hibiscus*. Pharmaceutical Biology 46: 145–153. *A review with 68 references*

Tamarind pulp

The drug consists of the fruit of the tree *Tamarindus indica* (Leguminosae) deprived of the brittle, outer part of the pericarp and preserved with sugar. The fruits are about 5–15 cm long. They have a brittle epicarp, a pulpy mesocarp, through which run from the stalk about five to nine branched fibres, and a leathery endocarp. The latter forms from four to twelve chambers, in each of which is a single seed.

In the West Indies the fruits ripen in June, July and August. The epicarps are removed, the fruits are packed in layers in barrels, and boiling syrup is poured over them; alternatively, each layer of fruits is sprinkled with powdered sugar.

Tamarind pulp occurs as a reddish-brown, moist, sticky mass, in which the yellowish-brown fibres mentioned above are readily seen. Odour, pleasant and fruity; taste, sweet and acid.

The seeds, each enclosed in a leathery endocarp, are obscurely foursided or ovate and about 15 mm long. They have a rich brown testa marked with a large patch or oreole. Within the testa, which is very thick and hard, lies the embryo. The large cotyledons are composed very largely of hemicellulose which stains blue with iodine.

The pulp contains free organic acids (about 10% of tartaric, citric and malic), their salts (about 8% of potassium hydrogen tartrate), a little nicotinic acid and about 30–40% of invert sugar. It is reported that the tartaric acid is synthesized in the actively metabolizing leaves of the plant and then translocated to the fruits as they develop. The addition of sugar to the manufactured pulp, to act as a preservative, somewhat lowers the natural proportion of acids.

Flavonoid *C*-glycosides (vitexin, isovitexin, orientin and isoorientin) occur in the leaves. The fixed oil of the seeds contains a mixture of glycerides of saturated and unsaturated (oleic, linoleic) acids.

Tamarind pulp is a mild laxative and was formerly used in Confection of Senna; it has traditional medicinal uses in the W. Indies and in China and the leaves have been suggested as a commercial source of tartaric acid.

Manna

The name 'manna' is applied to a number of different plant products. The biblical manna was probably the lichen *Lecanora esculenta*, which can be carried long distances by wind. The only manna of commercial importance is ash manna, derived from *Fraxinus ornus* (Oleaceae). The drug is collected in Sicily. When the trees are about 10 years old, transverse cuts are made in the trunk. A sugary exudation takes place and when sufficiently dried is picked off (flake manna) or is collected on leaves or tiles.

Manna occurs in yellowish-white pieces up to 15 cm long and 2 cm wide or in agglutinated masses of broken flakes, with a pleasant odour and sweet taste. It contains about 55% of the hexahydric alcohol mannitol, relatively small amounts of hexose sugars but larger amounts of the more complex sugars mannotriose and mannotetrose (stachyose). The triose on hydrolysis yields glucose (1 mol) and galactose (2 mol), while the tetrose yields glucose (1 mol), fructose (1 mol) and galactose (2 mol). Manna has a mild laxative action.

SUMATRA BENZOIN

Two commercial varieties of benzoin—Sumatra benzoin and Siam benzoin—are included in the *BP*/*EP*. Sumatra benzoin (*Gum Benjamin*) is a balsamic resin obtained from the incised stem of *Styrax benzoin* Dryand, and *Styrax paralleloneurus* Perkins (Styracaceae). It is produced almost exclusively from cultivated trees grown in Sumatra, although the tree is also native to Java and Borneo.

History. The drug was noted by Ibn Batuta, who visited Sumatra in the fourteenth century, but was not regularly imported into Europe until the sixteenth century.

Collection and preparation. Sumatra benzoin is a purely pathological product and there is some evidence to show that its formation is brought about not only by the incisions made, but also by fungi (see 'Stress Compounds', Chapter 18). In Sumatra the seeds are sown in rice fields, the rice shading the young trees during their first year. After the harvesting of the rice the trees are allowed to grow until they are about 7 years old.

Tapping. The rather complicated process consists of making in each trunk three lines of incisions which are gradually lengthened.

The first triangular wounds are made in a vertical row about 40 cm apart, the bark between the wounds being then scraped smooth. The first secretion is very sticky and is rejected. After making further cuts, each about 4 cm above the preceding ones, a harder secretion is obtained. Further incisions are made at 3-monthly intervals and the secretion instead of being amorphous becomes crystalline. About 6 weeks after each fresh tapping the product is scraped off, the outer layer (finest quality) being kept separate from the next layer (intermediate quality). About 2 weeks later the strip is scraped again, giving a lower quality darker in colour and containing fragments of bark. Fresh incisions are then made and the above process is repeated. After a time the line of incisions is continued further up the trunk.

Grades. The above three qualities are not sold as such but are blended in Palembang to give the benzoin grades of commerce. The best grade contains the most 'almonds' and the worst contains a few almonds but abundant resinous matrix. The blending is done by breaking up the drug, mixing different proportions of the three qualities and softening in the sun. It was formerly exported after stamping into tins but now the commercial drug arrives in plaited containers with a plastic wrapping.

Characters. *Sumatra benzoin* occurs in brittle masses consisting of opaque, whitish or reddish tears embedded in a translucent, reddish-brown or greyish-brown, resinous matrix. Odour, agreeable and balsamic but not very marked; taste, slightly acrid. *Siamese benzoin* occurs in tears or in blocks. The tears are of variable size and flattened; they are yellowish-brown or reddish-brown externally, but milky-white and opaque internally. The block form consists of small tears embedded in a somewhat glassy, reddish-brown, resinous matrix. It has a vanilla-like odour and a balsamic taste.

When gradually heated, benzoin evolves white fumes of cinnamic and benzoic acids which readily condense on a cool surface as a crystalline sublimate. On warming a little powdered benzoin with solution of potassium permanganate, a faint odour of benzaldehyde is noted with Sumatra benzoin but not with the Siamese. When an alcoholic solution of ferric chloride is added to an alcoholic extract of Siamese benzoin, a green colour is produced. Sumatra benzoin does not give this test. The *BP*/*EP* includes a TLC test for the absence of Dammar gum, a copal resin, used in the manufacture of varnishes and apparently derived from species of *Hopea*, *Shorea* and *Vateria* family Dipterocarpaceae.

Constituents. Sumatra benzoin contains free balsamic acids (cinnamic and benzoic) and esters derived from them. Also present are triterpenoid acids such as siaresinolic acid (19-hydroxyoleanolic acid) and sumaresinolic acid (6-hydroxyoleanolic acid). For the formula of oleanolic acid see under 'Triterpenoid Saponins'. The content of total balsamic acids (calculated as cinnamic acid) is at least 20%, and the amount of cinnamic acid is usually about double that of benzoic acid. Up to about 20% of free acids may be present. High-grade material from *S. paralleloneurum* contains benzoic acid 3%, vanillin 0.5% and cinnamic acid 20–30%.

Allied drug. *Palembang benzoin*, an inferior variety produced in Sumatra, may be collected from isolated trees from which the resin has not been stripped for some time. It is easily distinguished, being very light in weight and breaking with an irregular porous fracture. It consists almost entirely of reddish-brown resin, with only a few very small tears embedded in it. Palembang benzoin is used as a source of natural benzoic acid.

Uses. Benzoin, when taken internally, acts as an expectorant and antiseptic. It is mainly used as an ingredient of friar's balsam, or as a cosmetic lotion prepared from a simple tincture. It finds considerable use world-wide in the food, drinks, perfumery and toiletry industries; it is a component of incense.

SIAM BENZOIN

Siam benzoin *BP*/*EP* is obtained by incision of the trunk of *Styrax tonkinensis* (Pierre) Craib ex Hartwich; it contains 45.0–55.0% of total acids calculated as benzoic acid (dried drug). It is the traditional source of benzoin for a number of European pharmacopoeias.

The drug is produced in relatively small areas in the Thai province of Luang Prabang, northern Laos and northern Vietnam from trees growing in the wild at an altitude of between 600 and 2500 m. It seems that this height is necessary for resin production; not all trees are productive.

The method of collection appears similar to that for Sumatra benzoin, the resin being produced at the interface of the bark and wood layers only after injury. The collected tears are sorted into grades based on size and colour, the most esteemed being the largest and palest. Length of tears commonly varies from a few millimetres to 3 cm, flattened or sometimes, if large, concavo-convex as would be expected if the resin collected between the bark and the wood of the tree. They are yellowish-white to reddish on the outer surface, often in the commercial drug cemented together by the brownish resin, which increases due to oxidation as the material is stored. The fracture of the tears is waxy-white with an agreeable odour resembling vanilla.

Constituents. The combined acid content of Siam benzoin consists principally of coniferyl benzoate and a very small amount of coniferyl cinnamate (coniferyl alcohol, see Fig. 21.2, is found in the cambial sap of both gymnosperms and angiosperms). Free benzoic acid amounts to some 10% of the drug. Other constituents are triterpenoid acids and esters, and vanillin.

Tests. Compared with Sumatra benzoin, Siam benzoin is expensive and is liable to adulteration with the former, which can be detected by the *BP*/*EP* TLC test; this indicates an absence of cinnamic acid in the genuine drug. This absence also means that no odour of benzaldehyde is produced when the powdered drug is warmed with a solution of potassium permanganate *cf.* Sumatra benzoin. Also, Siam benzoin in ethanol, on treatment with ferric chloride solution, gives a green, not yellow, colour. The pharmacopoeial assay for total acids involves the back-titration with hydrochloric acid of a hydrolysed solution of the drug in alcoholic potassium hydroxide solution.

Uses. Similar to those of Sumatra benzoin.

ASH LEAF

Ash leaf *BP*/*EP* consists of the dried leaves of *Fraxinus excelsior* L., the common ash, or *Fraxinus oxyphylla* M. Bieb., family Oleaceae, having a minimum content of 2.5% total hydroxycinnamic acid derivatives expressed as chlorogenic acid.

The common ash is found throughout temperate Europe, in Western Asia and extending northwards into Scandanavia; it is common in Britain. The leaves are up to 30 cm in length, opposite, pinnate and consisting of a rachis bearing 9–13 leaflets. It is the latter that constitute the official drug; they are about 7 cm long with short petiolules, lanceolate to ovate, apex apiculate to acuminate and a sharp, shallow forward-toothed margin. Colour dark green on the upper surface, lighter below.

Elements of the powdered drug include: upper epidermis with some striated cuticle and a lower surface with anomocytic stomata, occasional covering trichomes and, rarely, glandular trichomes.

Constituents of the leaf embrace the coumarin glycoside fraxin (formula shown in Table 21.2), various hydroxycinnamic acid derivatives, e.g. chlorogenic acid (see Fig. 19.5), tannins, the sugar alcohol mannite and bitter principles. The *BP* assay is based on the colour reaction of an acidified methanolic extract of the sample with solutions of sodium nitrite and sodium molybdate followed by alkali. Absorbance is measured at 525 nm.

Ash leaf has a mild laxative and diuretic action.

Allied species. *Fraxinus ornus* is a commercial source of manna (q.v.) and its leaflets can be substituted for the above. It may be distinguished by not affording the characteristic zones of acteoside, chlorogenic acid and rutin (see Fig. 21.15) when subjected to TLC examination. Hydroxycoumarins, secoiridoid glycosides, phenylethanoids and flavonoids have been reported in the plant. For a review, including biological activities, see I. Kostova, *Fitoterapia*, 2001, **72**, 471; similarly for the genus (39–63 spp., depending on the classification) and featuring over 150 compounds, biological activities and 150 references, see I. Kostova and T. Iossifova, *Fitoterapia*, 2007, **78**, 85–106.

ARTICHOKE LEAF

The leaves of the globe artichoke, *Cynara scolymus* L., family Asteraceae/Compositae, have been long-used in traditional medicine and are now included in the *BP*/*EP*, the *BHP* and the *Complete German Commission E Monographs*. The plant is native to the Mediterranean region and northern Africa and probably evolved from *C. cardunculus* at an early date (D. Bown, *Encyclopedia of Herbs*, 1995, Dorling Kindersley, London).

Leaves, up to *ca* 70 cm long and 30 cm wide, are collected and dried just before the flowering stage. The leaf lamina is deeply lobed, reaching to 1–2 cm of the midrib but becoming pinnatifid towards the petiole. The margin is coarsely toothed and trichomes cover both surfaces, being particularly dense and twisted on the lower surface. Hairs also cover the petioles, which, together with the main veins, are flat on the upper surfaces and raised and ridged on the lower.

The greyish-green to brown powder exhibits epidermi of straight- or sinuous-walled cells and many anomocytic stomata. Covering, multicellular, uniseriate trichomes occur scattered or in felted masses together with fewer glandular trichomes having brown contents in a monoseriate or biseriate head. Groups of lignified fibres and reticulately thickened vessels arise from veins of the petiole and midrib. The drug has a slight odour and a salty taste followed by bitterness.

Phenolic acids are important constituents and include chlorogenic acid, caffeic acid and cynarin (1, 5-di-*O*-caffeoylquinic acid) (see Fig. 19.5). The *BP* specifies a minimum requirement for chlorogenic acid of 0.8%, which is determined by liquid chromatography using chlorogenic acid reference solution for peak area comparison. Flavonoids include luteolin-7β-D-glucoside and the 7β-D-rutionoside. The former, together with chlorogenic acid, are used in the official TLC test for identity. Other constituents include volatile oil, sesquiperpene lactones, e.g. cynaropicrin, inulin, tannins and phytosterols.

Artichoke leaf is used for the treatment of indigestion and dyspepsia; for its use as a hepatic, see Chapter 29.

NETTLE LEAF

All parts of the nettle are used medicinally, the dried roots and herb in the *BHP* and the dried leaves in the *BP*/*EP*. The latter specifies two species—*Urtica dioica* L., the stinging nettle, and *U. urens* L., the small nettle. Both species are common throughout North temperate regions.

U. urens is an annual herb with the lower leaves shorter than their petioles, whereas *U. dioica* is a coarse hispid perennial with the lower leaves longer than their petioles.

The green powdered drug has a slight odour and bitter taste. Cells of both epidermises have sinuous anticlinal walls and the lower epidermis includes numerous anomocytic stomata. Cystoliths containing large calcium carbonate masses are present in the epidermal layers (see Fig. 42.1A). Clothing trichomes, stinging hairs and glandular trichomes are numerous. In the powder, the upper cells of the stinging trichomes are usually broken off.

The constituents of nettle have been extensively researched. They include a number of acids such as chlorogenic, caffeoylmalic, caffeic, malic and fumaric. Flavonoids include quercetin and its glycosides isoquercitrin and rutin (see Fig. 21.15). 5-Hydroxytryptamine (serotonin) is a component of the stinging hairs. Other metabolites include lignans, scopoletin (see Table 21.2) and choline acetyltransferase.

Scopoletin and chlorogenic acid are used as reference compounds in the *BP* TLC identification test. The assay is by liquid chromatography, requiring a minimum of 0.3% for the sum of the caffeoylmalic acid and chlorogenic acid content, expressed as chlorogenic acid. The high total ash limit for the drug (20.0%) is dictated by the considerable natural inorganic content, particularly silicic acid and calcium and potassium salts, which are present in the leaves.

Nettle has been used traditionally to treat many disorders. It is a diuretic and is employed in various rheumatic conditions and to assist micturition in cases of benign prostatic hyperplasia.

Further reading

Kavalali GM (ed), Hardman R (series ed) 2003 Urtica: therapeutic and nutritional aspects of stinging nettles. CRC Press UK, London

ECHINACEA SPP.

Echinacea species (coneflowers), are perennial herbs of the Compositae/Asteraceae native to the prairie regions of Ohio where they were used by the Plains tribes to treat a variety of conditions, particularly wounds. Three species are currently important. Roots of *Echinacea augustifolia* DC., the narrow-leaved coneflower, and *E. pallida* Nutt., the pale cornflower, are included in the *BP*/*EP*. The whole plant of *E. purpurpea* (the purple coneflower) is much used for the commercial preparation of herbal medicaments, it being the largest of the three species and easy to cultivate. All continue to receive research attention in connection with their phytochemical, pharmacological and clinical attributes.

Particular groups of compounds can be found in all three species but variations occur concerning specific metabolites. Some aspects of earlier research must also be treated with caution because it is now known that some commercial batches of so-called *E. augustifolia* grown in Europe were in fact *E. pallida* (R. Bauer *et al.*, *Planta Medica*, 1988, **54**, 426; *Sci. Pharm.*, 1987, **55**, 159; P. R. Bradley, *Brit. Herb. Comp.*, 1992, **1**, 81).

The roots of the two official drugs are not easily differentiated by their morphological and microscopical characteristics; in the powders, numerous sclereids and phytomelanin deposits occur in both. However, TLC can be used for the identification and also for the detection of *E. purpurea* in adulterated *E. augustifolia*, and to detect other *Echinacea* spp. and *Parthenium integrifolium* in *E. pallida*. A recent DNA study [sequence characterized amplified region (SCAR) analysis] has demonstrated the distinction of *E. purpurea* from the other two species (B. Adinolfi *et al*., *Fitoterapia*, 2007, **78**, 43).

Constituents. A caffeic acid derivative, echinacoside, is present in the roots of both *E. augustifolia* and *E. pallida*. Cynarin, a quinic acid derivative, occurs only in the former. Esters involving tartaric acid, such as caftaric acid and cichoric acid, occur in small amounts in both species. Other constituents include high-molecular-weight polysaccharides, alkylamides, acetylenes, volatiles including humulene (see Fig. 21.4) and traces of pyrrolizidine alkaloids (F. Pellati *et al*., *Phytochemistry*, 2006, **67**, 1359 and references cited therein).

The *BP*/*EP* requires minimum contents of echinacoside for *E. augustifolia* root (0.5%) and *E. pallida* root (0.2%) determined by liquid chromatography with spectrometric detection at 330 nm.

Actions. Echinacea is considered to have immunostimulant properties based on its alkylamide, polysaccharide and cichoric acid components. Preparations of the drug have become popular for the prevention and treatment of the common cold and other respiratory complaints. A recent meta-analysis of fourteen studies (S. A. Saah *et al*., *The Lancet Infectious Diseases*, 2007, **7**, 473) indicated that Echinacea reduced both the incidence (by 58.0%) and the duration (by 1.4 days) of the common cold. For a mini-review on the role of alkamides as an active principle, see K. Woelkart and R. Bauer, *Planta Medica*, 2007, **73**, 615; for a report on this controversial aspect, see B. Barrett, *HerbalGram*, 2006, **70**, 36. Other traditional uses involve its anti-inflammatory and antibacterial properties.

Further reading

Miller SC (ed), Hardman R (series ed) 2004 Medicinal and aromatic plants – industrial profiles. Vol. 39, Echinacea: the genus *Echinacea*. CRC Press, Taylor and Francis Group, London

PYGEUM BARK

Pygeum bark is obtained from the stems and branches of *Prunus africana* (Hook f.) Kalkm. syn. *Pygeum africanum* Hook f., family Rosaceae, a tree indigenous to the rain-forests of Africa. Cameroon is the principal exporter. The increased demand for the drug from Europe and the US has led, as with other trees not easily cultivated commercially, to the danger of over-collection.

The whole or cut bark is now included in the *BP*/*EP* and consists of dark- to reddish-brown pieces with an outer wrinkled cork with some lichens attached and an inner longitudinally striated surface. The powder exhibits typical bark characteristics: cork cells, sclereids in groups or singly, fibres principally in groups, pigmented cells, small starch grains and cluster crystals of calcium oxalate.

Constituents. Identified constituents include aliphatic alcohols occurring as ferulic acid esters, phytosterols, triterpenoid pentacyclic acids and a lipid fraction involving C_{12}–C_{24} fatty acids. Pharmacopoeial tests include a minimum extractive value of 0.5% (continuous Soxhlet-type extraction with methylene chloride as solvent) and a TLC separation to show characteristic bands including those of β-sitosterol and ursolic acid.

Uses. Traditionally for the symptomatic treatment of benign prostatic hyperplasia. The activity appears to be comparable to that of *Serenoa repens* fruit extracts.

PERU BALSAM

Balsam of Peru is obtained from the trunk of *Myroxylon balsamum* var. *pereirae* (Leguminosae), after it has been beaten and scorched. The drug is produced in Central America (San Salvador, Honduras and Guatemala) and is now included in the *European Pharmacopoeia* and the *BP* (2000).

History. The drug derives its name from the fact that when first imported into Spain it came via Callao in Peru. It was known to Monardes and the method of preparation was described as early as 1576, although afterwards forgotten. In 1860 the collection was described and illustrated by Dorat.

Collection and preparation. In November or December strips of bark, measuring about 30 × 15 cm, are beaten with the back of an axe or other blunt instrument. The bark soon cracks and may be pulled off after 2 weeks. As in the case of Tolu balsam, the secretion is purely pathological in origin and very little balsam can be obtained from the bark unless it is charred with a torch about 1 week after the beating. The balsam produced in the bark is obtained by boiling the bark in water and is known as *tacuasonte* (prepared without fire) or *balsamo de cascara* (balsam of the bark).

The greater part of the balsam, however, is prepared, after the removal of the bark, by the second method. The balsam which exudes is soaked up with rags, which, after some days, are cleaned by gently boiling with water and squeezing in a rope press. The balsam sinks to the bottom and, the water having been decanted, the balsam (*balsamo de trapo*) is poured off and strained.

Less destructive methods of preparation have been investigated and include the removal of narrow strips of bark and the replacement of scorching with the use of a hot iron. With this treatment the tree recovers in 6 months, compared with 8 years after the drastic traditional method. The drug is chiefly exported from Acajutla (San Salvador) and Belize (British Honduras) in tin canisters holding about 27 kg.

Characters. Balsam of Peru is a viscid liquid of a somewhat oily nature, but free from stickiness and stringiness. When seen in bulk, it is dark brown or nearly black in colour, but in thin layers it is reddish-brown and transparent. The original containers have a whitish scum on the surface. The balsam has a pleasant, somewhat vanilla-like odour and an acrid, slightly bitter taste.

The drug is almost insoluble in water. It is soluble in one volume of alcohol (90%), but the solution becomes turbid on the addition of further solvent. The relative density, 1.14–1.17, is a good indication of purity, and if abnormal indicates adulteration with fixed oils, alcohol, kerosene, etc. The *BP*/*EP* includes tests for the absence of artificial balsams (solubility characteristics in petroleum spirit), fixed oils (solubility in chloral hydrate solution) and turpentine (odour test).

Constituents. The official drug is required to contain not less than 45.0% w/w and not more than 70% w/w of esters, assayed gravimetrically. The chief balsamic esters present are benzyl cinnamate (cinnamein) $C_6H_5CH{=}CHCOOCH_2C_6H_5$ (sap. value 234), benzyl benzoate (sap. value 264.3) and cinamyl cinnamate (styracin). The drug also contains about 28% of resin, which is said to consist of peruresinotannol combined with cinnamic and benzoic acids, alcohols (nerolidol, farnesol and benzyl alcohol) and small quantities of vanillin and free cinnamic acid.

Work on the isoflavonoids contained in the trunk-wood has indicated that considerable chemical differences characterize the various forms or species of the *M. balsamum* group.

Uses. Balsam of Peru is used as an antiseptic dressing for wounds and as a parasiticide. Now that it is no longer used in Tulle gras

dressings, it is of less current interest in Western medicine. Taken internally it is used to treat catarrh and diarrhoea. Allergic responses are possible.

Prepared storax

Prepared storax is a balsam obtained from the wounded trunk of *Liquidambar orientalis* (Hamamelidaceae) and subsequently purified. This is known as Levant storax and is obtained from a small tree found in the south-west of Turkey.

Collection and preparation. In the early summer the bark is injured by bruising or by making incisions. After a time the outer bark may be pared off, or the whole bark may be left until the autumn, when it is removed. The pieces of bark are pressed in horse-hair bags, first in the cold and again after steeping in hot water. Sometimes the bark is boiled with water and again pressed. The exhausted bark is used in the East for fumigation. The crude or liquid storax is exported in casks from Izmir.

Storax is obtained by dissolving the crude balsam in alcohol, filtering and recovering the solvent at as low a temperature as possible so as not to lose any of the volatile constituents. The alcohol-insoluble matter consists of vegetable debris and a resin.

Characters. *Crude storax* is a greyish, viscous liquid with a pleasant odour and bitter taste. It usually contains about 20–30% of water. About 82–87% is alcohol soluble.

Purified storax forms a brown, viscous, semisolid mass which loses not more than 5% of its weight when dried on a water-bath for 1 h. It is completely soluble in alcohol and partially in either. It has a characteristic balsamic odour and taste.

Constituents. Storax is very rich in free and combined cinnamic acid. After purification it yields 30–47% of total balsamic acids.

By steam distillation storax yields an oily liquid containing phenylethylene (styrene), $C_6H_5CH{=}CH_2$, cinnamic esters, vanillin and free cinnamic acid. The resinous portion of the drug consists of resin alcohols present both free and combined with cinnamic acid. The presence of cinnamic acid in the drug is shown by the odour of benzaldehyde which is produced when the drug is mixed with sand and warmed with a solution of potassium permanganate.

Recent research carried out in Turkey has shown the presence of many compounds not previously reported; these include monoterpenes, phenylpropanes and aliphatic acids.

Allied drug. American storax obtained from *L. styraciflua*, a large tree found near the Atlantic coast from Central America to Connecticut, is also used in the USA. This balsam resembles the Levant storax in constituents. Thirty-six compounds have been identified in the leaf-oil of the plant and tannins and related phenolics obtained from cell cultures. American researchers have reported the fruits as a rich source of shikimic acid, starting material for the synthesis of the antiviral drug oseltamivir. The yield is greatly superior to that from star-anise fruits, the accepted source material.

Uses. Storax is chiefly used in the preparation of friars' balsam and benzoin inhalation.

TOLU BALSAM

Tolu Balsam is obtained by incision from the trunk of *Myroxylon balsamum* (L.) Harms. var. *balsamum* (Leguminosae), a large tree that differs but little from that yielding balsam of Peru. Wild trees occur in Colombia and Venezuela and in the former country large quantities of balsam were produced in the neighbourhood of the Magdalena and Cauca rivers. The trees are cultivated in the West Indies, particularly in Cuba.

History. Balsam of Tolu was described by Monardes in 1574 and its collection was observed by Weir in 1863.

Collection. The drug is collected by making V-shaped incisions in the bark, the secretion being received in a calabash placed in the angle of the V. Many such receivers are fixed on each tree, the yield per tree being 8–10 kg. Periodically, the balsam is transferred to larger containers. It is exported in tins from Cartagena, Sabanilla and Sta. Marta.

Characters. When freshly imported, tolu is a soft, yellow semisolid. On keeping it turns to a brown, brittle solid. It softens on warming, and if a little is then pressed between two glass slides, microscopical examination shows crystals of cinnamic acid, amorphous resin and vegetable debris. Odour is aromatic and fragrant; taste, aromatic; the drug forms a plastic mass when chewed.

It is almost entirely soluble in alcohol, the solution being acid to litmus, and giving a green colour with ferric chloride (the latter possibly owing to the presence of resinotannol). Like other drugs containing cinnamic acid, it yields an odour of benzaldehyde when a filtered decoction is oxidized with potassium permanganate solution.

Constituents. Tolu contains about 80% of resin derived from resin alcohols combined with cinnamic and benzoic acids. The drug is rich in free aromatic acids and contains about 12–15% of free cinnamic and about 8% of free benzoic acid (acid value from 100–160). Other constituents are esters such as benzyl benzoate and benzyl cinnamate and a little vanillin. Recent investigations have shown the presence of other esters, styrene, eugenol, vanillin, ferulic acid, 1,2-diphenylethane, mono- and sesquiterpene hydrocarbons and alcohols. The balsam also contains numerous triterpenoids. Total balsamic acids (*BP*/*EP*, 25–50%) are determined by titration following hydrolysis of the esters.

Uses. Balsam of Tolu has antiseptic and flavouring properties and is commonly added to cough mixtures in the form of a syrup of tincture.

PHARMACEUTICAL FIXED OILS AND FATS

ALMOND OIL

Almond oil is a fixed oil obtained by expression from the seeds of *Prunus dulcis* (Miller) E. A. Webb (Rosaceae) var. *dulcis* (sweet almond) or *P. dulcis* (Miller) D. A. Webb var. *amara* (D.C.) Buchheim (bitter almond) or a mixture of both varieties.

The oil is mainly produced from almonds grown in the countries bordering the Mediterranean (Italy, France, Spain and North Africa).

Characters of plants and seeds. Almond trees are about 5 m in height and the varieties, except for differences in the seeds, are almost indistinguishable. The young fruits have a soft, felt-like pericarp, the inner part of which gradually becomes sclerenchymatous as the fruit ripens to form a pitted endocarp or shell. The shells, consisting mainly of sclerenchymatous cells, are sometimes ground and used to adulterate powdered drugs.

The sweet almond is 2–3 cm in length, rounded at one end and pointed at the other. The bitter almond is 1.5–2 cm in length but of similar breadth to the sweet almond. Both varieties have a thin, cinnamon-brown testa which is easily removed after soaking in warm water, a process which is known as blanching. The oily kernel consists of two large, oily planoconvex cotyledons, and a small plumule and radicle, the latter lying at the pointed end of the seed. Some almonds have cotyledons of unequal sizes and are irregularly folded. Bitter almonds are sometimes found in samples of sweet almonds, particularly those of African origin; their presence may be detected by the sodium picrate test for cyanogenetic glycosides.

Constituents. Both varieties of almond contain 40–55% of fixed oil, about 20% of proteins, mucilage and emulsin. The bitter almonds contain in addition 2.5–4.0% of the colourless, crystalline, cyanogenetic glycoside amygdalin (see Chapter 25).

Refined almond oil is obtained by grinding the seeds and expressing them in canvas bags between slightly heated iron plates. They are sometimes blanched before grinding, but this does not appear to be of any particular advantage. The oil is clarified by subsidence and filtration. It is a pale yellow liquid with a slight odour and bland, nutty taste. The *BP*/*EP* specifies oleic acid 62–86%, linoleic acid 20–30%, palmitic acid 4–9% together with lesser amounts of other acids as produced by the hydrolysis of the oil using GLC analysis. There are tests for the absence of other oils and sterols; the sterol fraction of the genuine oil consists principally of β-sitosterol (73–87%).

Virgin almond oil BP/EP conforms to similar tests as above but is not refined.

Hydrogenated almond oil is also included in the *BP*/*EP*.

Essential or volatile oil of almonds is obtained from the cake left after expressing bitter almonds. This is macerated with water for some hours to allow hydrolysis of the amygdalin to take place. The benzaldehyde and hydrocyanic acid are then separated by steam distillation.

Bitter almond oil contains benzaldehyde and 2–4% of hydrocyanic acid. Purified volatile oil of bitter almonds has had all its hydrocyanic acid removed and therefore consists mainly of benzaldehyde.

Uses. Almond oil is used in the preparation of many toilet articles and as a vehicle for oily injections. When taken internally, it has a mild, laxative action. The volatile almond oils are used as flavouring agents.

ARACHIS OIL

Arachis oil is obtained by expression from the seeds of *Arachis hypogaea* L. (Leguminosae) (*earth-nut*, *ground-nut*, *peanut*) a small annual plant cultivated throughout tropical Africa and in India, Brazil, southern USA and Australia. Various genotypes exist which show differences in the relative amounts of fatty acids contained in the oil. Enormous quantities of the fruits and seeds are shipped to Marseilles and other European ports for expression. Ground-nuts are the world's fourth largest source of fixed oil.

Preparation. During ripening the fruits bury themselves in the sandy soil in which the plants grow. Each fruit contains from one to three reddish-brown seeds. The fruits are shelled by a machine. The kernels contain 40–50% of oil. Owing to the high oil content the seeds, when crushed, are somewhat difficult to express. After the initial 'cooking', part of the oil is removed in a low-pressure expeller and the cake is solvent extracted. The two oil fractions are then mixed before purification. The press cake forms an excellent cattle food. The ground pericarps have been used as an adulterant of powdered drugs.

Constituents. Arachis oil consists of the glycerides of oleic, linoleic, palmitic, arachidic, stearic, lignoceric and other acids. When saponified with alcoholic potassium hydroxide, crystals of impure potassium arachidate separate on standing. Arachis oil is one of the most likely adulterants of other fixed oils (e.g. olive oil). The *BP* examination of the oil is similar to that mentioned under 'Olive Oil' below; the temperature at which the cooling, hydrolysed oil becomes cloudy should not be below 36°C. As with olive oil more stringent standards are required for oil to be used parenterally.

Uses. Arachis oil has similar properties to olive oil. It is an ingredient of camphorated oil but is used mainly in the production of margarine, cooking fats, etc.

Hydrogenated arachis oil is produced by refining, bleaching, hydrogenating and deodorizing the above. As with other hydrogenated oils it is much thicker than the parent oil, constituting a soft, off-white mass. There are various types of the hydrogenated oil with so-called nominal drop points determined as prescribed in the *Pharmacopoeia*; these fall within the range 32–42°C and, within this range, the drop point should not differ by more than 3°C from the nominal value. Again, as with other hydrogenated oils there is a limit for nickel (⩾1 ppm) determined by atomic absorption spectrometry. The peroxide value should not exceed 5.0%.

COCONUT OIL

The expressed oil of the dried solid part of the endosperm of the coconut, *Cocos nucifera* L. (Palmae) is a semisolid, melting at about 24°C and consisting of the triglycerides of mainly lauric and myristic acids, together with smaller quantities of caproic, caprylic, oleic, palmitic and stearic acids. This constitution gives it a very low iodine value (7.0–11.0) and a high saponification value.

The particularly high proportion of medium chain-length acids means that the oil is easily absorbed from the gastrointestinal tract, which makes it of value to patients with fat absorption problems.

Fractionated coconut oil. Fractionated and purified endosperm oil of the coconut *C. nucifera*, or Thin Vegetable Oil of the *BPC,* contains triglycerides containing only the short and medium chain-length fatty acids (e.g. octanoic, decanoic; see Table 19.2). It maintains its low viscosity until near the solidification point (about 0°C) and is a useful non-aqueous medium for the oral administration of some medicaments.

Medium-chain Triglycerides BP/EP, synonymous with the above, may also be obtained from the dried endosperm of *Elaeis guineensis* (Palmae). The fatty acid composition of the hydrolysed oil, determined by GC, has the following specifications: caproic acid ⩾2%, caprylic acid 50–80%, capric acid 20–50%, lauric acid ⩾3.0%, myristic acid ⩾1.0%.

COTTONSEED OIL

Cottonseed oil is expressed from the seeds of various species of *Gossypium* (Malvaceae) in America and Europe. In the UK, Egyptian and Indian cottonseed, which do not require delinting on arrival, are largely used. See under 'Cotton'.

The preparation of cottonseed oil is one of hot expression and a pressure of about 10 000 kPa is used. The crude oil is thick and turbid and is refined in various ways, that known as 'winter bleached' being the best of the refined grades. Cottonseed oil is a semi-drying oil and has a fairly high iodine value. When used to adulterate other oils its presence may be detected by the test for semidrying oils described in the *BP* monograph for Arachis Oil.

The hydrogenated oil, only, is official.

LINSEED AND LINSEED OIL

Linseed (*flaxseed*) is the dried ripe seed of *Linum usitatissimum* L. (Linaceae), an annual herb about 0.7 m high with blue flowers and a globular capsule. The flax has long been cultivated for its pericyclic fibres and seeds. Supplies of the latter are derived from South America, India, the USA and Canada. Large quantities of oil are expressed in England, particularly at Hull, and on the Continent.

Macroscopical characters. The seeds are ovate, flattened and obliquely pointed at one end; about 4–6 mm long and 2–2.5 mm broad. The testa is brown, glossy and finely pitted. Odourless; taste, mucilaginous and oily. If cruciferous seeds are present, a pungent odour and taste may develop on crushing and moistening. A transverse section shows a narrow endosperm and two large, planoconvex cotyledons.

Microscopical characters. Microscopical examination of the testa shows a mucilage-containing outer epidermis; one or two layers of collenchyma or 'round cells'; a single layer of longitudinally directed elongated sclerenchyma; the hyaline layers or 'cross-cells' composed in the ripe seed of partially or completely obliterated parenchymatous cells with their long axis at right angles to those of the sclerenchymatous layer; and an innermost layer of pigment cells. The outer epidermis is composed of cells, rectangular or five-sided in surface view, which swell up in water and become mucilaginous. The outer cell walls, when swollen in water, show an outer solid stratified layer and an inner part yielding mucilage, itself faintly stratified. The radial layers or 'round cells' are cylindrical in shape and show distinct triangular intercellular air spaces. The sclerenchymatous layer is composed of elongated cells, up to 250 μm in length, with lignified pitted walls. The hyaline layers often remain attached to portions of the sclerenchymatous layer in the powdered drug (see Fig. 41.7I). The pigment layer is composed of cells with thickened pitted walls and containing amorphous reddish-brown contents (Fig. 41.7H). The cells of the endosperm and cotyledons are polygonal with somewhat thickened walls, and contain numerous aleurone grains and globules of fixed oil. Starch is present in unripe seeds only.

Constituents. Linseed contains about 30–40% of fixed oil, 6% of mucilage (*BP* swelling index for whole seeds ≮4.0), 25% of protein and small quantities of the cyanogenetic glucosides linamarin and lotaustralin. Other constituents are phenylpropanoid glycosides (L. Luyengi *et al*., *J. Nat. Prod*., 1993, **56**, 2012), flavonoids, the lignan (–)-pinoresinol diglucoside (a tetrahydrofurofuran-type lignan—see Table 21.7) and the cancer chemoprotective mammalian lignan precursor secoisolariciresinol diglucoside (S.-X. Qiu *et al*., *Pharm. Biol.*, 1999, **37**, 1). Recently, 22 different lignans, mainly of the aryltetralin type, have been identified from Bulgarian species of *Linum*, section Syllinum (N. Vasilev *et al.*, *Planta Medica*, 2008, **74**, 273).

Cell cultures of *Linum album* are able to synthesize and accumulate the lignans podophyllotoxin and 5-methylpodophyllotoxin (T. Smollny *et al*., *Phytochemistry*, 1998, **48**, 975).

Linseed oil. The extraction of linseed oil is one of hot expression of a linseed meal and the press is adjusted to leave sufficient oil in the cake to make it suitable as a cattle food.

Linseed oil of *BP* quality is a yellowish-brown drying oil with a characteristic odour and bland taste; much commercial oil has a marked odour and acrid taste. On exposure to air it gradually thickens and forms a hard varnish. It has a high iodine value (≮175) as it contains considerable quantities of the glycosides of unsaturated acids. Analyses show α-linolenic acid, $C_{17}H_{29}COOH$ (36–50%), linoleic acid $C_{17}H_{31}COOH$ (23–24%), oleic acid $C_{17}H_{33}COOH$ (10–18%), together with some saturated acids—myristic, stearic and palmitic (5–11%). For the formation of the unsaturated acids, see Figs. 19.1 and 19.2.

For use in paint, linseed oil was boiled with 'driers' such as litharge or manganese resinate which, by forming metallic salts, caused the oil to dry more rapidly. Such 'boiled oils' must not be used for medicinal purposes.

Uses. Crushed linseed is used in the form of a poultice and whole seeds are employed to make demulcent preparations. The oil is used in liniments, and research has suggested that hydrolysed linseed oil has potentially useful antibacterial properties as a topical preparation in that it is effective against *Staphylococcus aureus* strains resistant to antibiotics. Linseed cake is a valuable cattle food.

OLIVE OIL

Olive oil (*salad oil, sweet oil*) is a fixed oil which is expressed from the ripe fruits of *Olea europoea* L. (Oleaceae). The olive is an evergreen tree, which lives to a great age but seldom exceeds 12 m in height. It produces drupaceous fruits about 2–3 cm in length. The var. *latifolia* bears larger fruits than the var. *longifolia*, but the latter is said to yield the best oil. The oil is expressed in all the Mediterranean countries and in California. Italy, Spain, France, Greece and Tunisia produce 90% of the world's production.

Olive oil has been ranked sixth in the world's production of vegetable oils (F. D. Gunstone *et al*., 1994, *The Lipid Handbook*, Chapman and Hall).

History. The olive appears to be a native of Palestine. It was known in Egypt in the seventeenth century BC, and was introduced into Spain at an early period.

Collection and preparation. The methods used for the preparation of the oil naturally vary somewhat according to local conditions. In the modern factories hydraulic presses are widely used but in the more remote districts the procedure is essentially that which has been followed for hundreds of years and is described in earlier editions of this book. The first oil to be expressed from the ground fruits is known as virgin oil; subsequently the marc may be solvent extracted to obtain the lower quality oil. The superior grades of oil are extra virgin, virgin and pure or refined. The *Pharmacopoeia* includes monographs for both the virgin oil and refined oil.

Characters. Olive oil is a pale yellow liquid, which sometimes has a greenish tint. The amount of colouring matter present, whether chlorophyll or carotene, appears to determine the natural fluorescence of the oil in ultraviolet light.

The oil has a slight odour and a bland taste. If the fruits used have been allowed to ferment, the acid value of the oil will be higher than is officially permitted. It should comply with the tests for absence of arachis oil, cotton-seed oil, sesame oil and tea-seed oil. The latter oil, which is obtained from China, is not from the ordinary tea plant but from a tree, *Camellia sasanqua*.

Constituents. Olive oils from different sources differ somewhat in composition. This may be due either to the use of the different varieties of olive or to climatic differences. Two types of oil

may be distinguished: (a) that produced in Italy, Spain, Asia Minor and California, which contains more olein and less linolein than type; and (b), produced in the Dodecanese and Tunisia. Typical analyses of these types are:

	Type (a)	Type (b)	BP limits
	%	%	%
Oleic acid	78–86	65–70	56–85
Linoleic acid	0–7	10–15	3.5–20.0
Palmitic acid	9–12	15	7.5–20.0
Stearic acid			0.5–5.0

The characteristic odour of olive oil, particularly the virgin oils, arises from the presence of volatile C_6 alcohols (hexanol, *E*-2-hexenol, Z-3-hexenol), C_6 aldehydes and acetylated esters. Oils arising from different chemotypes can be distinguished by headspace gas–liquid chromatography (M. Williams *et al*., *Phytochemistry*, 1998, **47**, 1253). The dehydrogenases which produce the unsaturated alcohols have been studied in the pulp of developing olive pericarps (J. J. Salas and J. Sánchez, *Phytochemistry*, 1998, **48**, 35).

The *BP*/*EP* examination of the oil includes a TLC test for identity and, to detect foreign oils, the GC determination of the individual methyl esters of the acids produced by hydrolysis. Limits of the principal acids are given above: other percentage limits, which exclude foreign oils, include saturated fatty acids of chain length less than C_{16}(⩾0.1), linolenic (⩾1.2), arachidic (⩾0.7), behenic (⩾0.2). There are also limits for a number of sterols in this fraction of the oil including campestrol ⩾4.0%, cholesterol ⩾0.5%, Δ^7-stigmasterol ⩾0.5% and $\Delta^{5,24}$-stigmastadienol.

For the biosynthesis of the acids, see the introduction to this chapter.

The saturated glycerides tend to separate from the oil in cold weather; at 10°C the oil begins to become cloudy and at about 0°C forms a soft mass.

Uses. Olive oil is used in the preparation of soaps, plasters, etc., and is widely employed as a salad oil. Oil for use in the manufacture of parenteral preparations is required to have a lower acid value and peroxide value than that normally required, and to be almost free of water (0.1%) as determined by the specified Karl Fischer method.

Recent research has suggested that olive oil may protect against colonic carcinogenesis by virtue of its action on prostaglandins: rats fed on a diet containing olive oil, as distinct from those receiving safflower oil, were protected (R. Bartoli *et al*., *Gut*, 2000, **46**, 191).

Both fruits and oil are now widely promoted in health-food stores on account of their α-linolenic acid content.

PALM OIL AND PALM KERNEL OIL

Palm oil. The oil is obtained by steaming and expression of the mesocarp of the fruits of *Elaeis guineënsis* Jacq. (Palmae). This palm occurs throughout tropical Africa but over half of the world production of oil originates from Malaysia. In terms of world production palm oil had by 2003 overtaken that of sunflower and rapeseed oils (Table 19.8). It will be noted that for the oils listed, palm oil is superior regarding land use and productivity.

Palm oil is yellowish-brown in colour, of a buttery consistency (m.p. 30°C) and of agreeable odour. Palmitic and oleic acids are the principal esterifying acids.

Table 19.8 World production of some fixed oils (2003).

Crop	Production (10^6 ton)	Oil yield ton/ha/year	Area of plantations (10^6 ha)
Soybean	31.4	0.46	88.4
Sunflower seed	9.0	0.66	22.6
Rapeseed	12.5	1.33	25.2
Palm oil	28.7	3.30	8.5

Source: Malaysian Oil Palm Association.

Palm kernel oil. Palm kernel oil is obtained by heating the separated seeds for 4–6 hours to shrink the shell, which is then cracked and the kernels removed whole. The oil is then obtained by expression. It differs chemically from palm oil (above) in containing a high proportion (50%) of the triglycerides of lauric acid, a saturated, medium chain-length fatty acid (Table 19.2). The mixture of other acids resembles that found in coconut oil.

Fractionated Palm Kernel Oil *BP* is palm oil which has undergone selective solvent fractionation and hydrogenation. It is a white, brittle solid, odourless or almost so, with m.p. 31°–36°C making it suitable for use as a suppository base.

RAPESEED OIL

Refined Rapeseed Oil *BP*/*EP* is obtained by mechanical expression or by extraction from the seeds of *Brassica napus* L. and *B. campestris* L.

The crop is now extensively cultivated in Europe and oils with various properties depending on glyceride composition are commercially available. As was indicated in Chapter 14 not all varieties yield an oil suitable for medicinal purposes so that the specified pharmacopoeial limits of the various esterifying acids are important standards as are relative density (*c*. 0.917) and refractive index (*c*. 1.473). Oleic, linoleic and linolenic acids are the principal esterifying acids, with eicosenoic acid and erucic acid limited to 5.0 and 2.0% of the acid fraction, respectively.

As with some other oils, the addition of a suitable antioxidant is permitted, the name and concentration of which must be stated on the label together with a statement as to whether the oil was obtained by mechanical expression or by extraction.

SAFFLOWER OIL

Safflower oil is obtained by expression or extraction from the seeds (achenes) of *Carthamus tinctorius* L. (Compositae) or hybrids of this species. Commonly known as safflower, false saffron, saffron thistle, the plant is native to Mediterranean countries and Asia and has been used for colouring and medicinal purposes from Ancient Egyptian and Chinese times. The pigment from the flowers (carthamin) is yellow in water and red in alcohol and was the traditional dye for the robes of Buddhist monks. It is now cultivated largely for the seed oil in its countries of origin, as well as in the US, Australia, Africa and S.E. Asia.

As produced commercially, safflower oil is of two types—type 1 coming from the original species and type 2 from hybrid forms producing an oil rich in oleic acid; the acid fraction of the type 1 oil has oleic acid limits of 8–12% and the type 2 oil 70–84%. Other acids are palmitic, stearic and linoleic acids generally proportionately higher for the type 1 oil.

The pharmacopoeial oil is refined and may have an added antioxidant; it should comply with the usual tests for fixed oils with brassicasterol limited to 0.3% of the sterol fraction.

Safflower oil, like linseed oil, is a drying oil and has found commercial use in paints and varnishes. It is used in the food industry and is recommended for its cholesterol-lowering properties.

SESAME OIL

Sesame oil (*Gingelly oil, Teel oil*) is obtained by refining the expressed or extracted oil from the seeds of *Sesamum indicum* L. (Pedaliaceae), a herb which is widely cultivated in India, China, Japan and many tropical countries. The oil is official in the *EP* and *BP*.

The seeds contain about 50% of fixed oil which closely resembles olive oil in its properties and which it has, in some measure, replaced. It is a pale yellow, bland oil which on cooling to about −4°C solidifies to a buttery mass; it has a saponification value the same as that for olive oil and a somewhat higher iodine value (104–120).

Principal components of the oil are the glycerides of oleic and linoleic acids with small proportions of palmitic, stearic and arachidic acids. It also contains about 1% of the lignan sesamin, and the related sesamolin. The characteristic phenolic component is the basis of the *BP* test for identity and also the test for the detection of sesame oil in other oils. The original test involved the production of a pink colour when the oil was shaken with half its volume of concentrated hydrochloric acid containing 1% of sucrose (Baudouin's test). However, some commercially refined oils may not give a positive Baudouin's test. With the current *BP* test for the absence of sesame oil in other oils, e.g. olive oil, the reagents are acetic anhydride, a solution of furfuraldehyde and sulphuric acid; a bluish-green colour is a positive result. The composition of triglycerides in sesame oil is determined by liquid chromatography, those triglycerides having as acid radicals oleic 1 part and linoleic 2 parts, and those having oleic 2 parts and linoleic 1 part being among the most predominant.

As stated above, sesame seeds and oil also contain lignans; these are antioxidants of the tetrahydrofurofuran-type (Table 21.7) and include sesamin, sesamolinol and sesamolin. Another lignan, sesaminol, is formed during industrial bleaching of the oil. The biological activities of these compounds include reduction in serum cholesterol levels and increased vitamin E activities. For the biogenesis of such lignans see M. J. Kato *et al*., *Phytochemistry*, 1998, **47**, 583.

SOYA OIL

Soya oil is obtained from the seeds of *Glycine soja* Sieb. and Zucc. and *Glycine max* (L.) Merr (*G. hispida* (Moench) Maxim).

Refined soya oil. Processing involves deodorization and clarification by filtration at about 0°C. It should remain bright when kept at 0°C for 16 h. The principal esterifying fatty acids are linoleic (48–58%), oleic (17–30%), linolenic (5–11%) and stearic (3–5%). There is an official limit test of not more than 0.5% for the brassicasterol content of the sterol fraction of the oil.

Hydrogenated soya oil occurs as a white mass or powder; it consists principally of the triglycerides of palmitic and stearic acid.

SUNFLOWER OIL

Sunflower oil is obtained from the seeds of *Helianthus annuus* L. by mechanical expression or by extraction; it is then refined. Principal components of the fatty-acid mixture produced by hydrolysis of the oil are linoleic acid (48–74%), oleic acid (14–40%), palmitic acid (4–9%) and stearic acid (1–7%).

The oil can be identified (*BP*/*EP* test) by the comparison of a thin-layer chromatogram of the sample with that of the pharmacopoeial illustration of the typical oil. Other tests include relative density (*c.* 0.921) and refractive index (*c.* 1.474).

THEOBROMA OIL

Oil of theobroma or cocoa butter may be obtained from the ground kernels of *Theobroma cacao* (Sterculiaceae) by hot expression. The oil is filtered and allowed to set in moulds. Much is refined in Holland. Cocoa butter, as it is commonly termed, consists of the glycerides of stearic, palmitic, arachidic, oleic and other acids. These acids are combined with glycerol partly in the usual way as triglycerides and partly as mixed glycerides in which the glycerol is attached to more than one of the acids. It is the most expensive of the commercial fixed oils and may be adulterated with waxes, stearin (e.g. coconut stearin), animal tallows or vegetable tallows (e.g. from seeds of *Bassia longifolia* and *Stillingia sebifera*). For the character and tests for purity of oil of theobroma, see the pharmacopoeias. Its melting point (31–34°) makes it ideal for the preparation of suppositories.

GERM OILS

The *refined* and *virgin* wheat-germ oils are described in the *BP*/*EP* and are obtained by cold expression or by other suitable mechanical means from the germ of the wheat grain, *Triticum aestivum* L. The principal fatty acids involved are linoleic (52–59%), palmitic (14–19%), oleic (12–23%) and linolenic (3–10%) with limits for ecosenoic (⩾2.0%) and stearic (⩾2.0%). Brassicasterol is limited to 0.3% (max) in the sterol fraction. The acid value requirement for the refined oil is low (⩾0.9%, or ⩾0.3% if the oil is to be used for parenteral purposes) but much higher (⩾20.0%) for the virgin oil.

CASTOR OIL (VIRGIN CASTOR OIL)

Castor oil (*cold-drawn castor oil*) is a fixed oil obtained from the seeds of *Ricinus communis* (Euphorbiaceae). The fruit is a three-celled thorny capsule. The castor is a native of India; the principal producing countries are Brazil, India, China, the former Soviet Union and Thailand. There are about 17 varieties, which may be roughly grouped into shrubs and trees producing large seeds, and annual herbs producing smaller seeds. It is mainly the smaller varieties that are now cultivated and these have been developed, by breeding, to give high-yielding seed plants. Mechanical harvesting is now replacing hand-picking.

Characters of seeds. The seeds show considerable differences in size and colour. They are oval, somewhat compressed, 8–18 mm long and 4–12 mm broad. The testa is very smooth, thin and brittle. The colour may be a more or less uniform grey, brown or black, or may be variously mottled with brown or black. A small, often yellowish, caruncle is usually present at one end, from which runs the raphe to terminate in a slightly raised chalaza at the opposite end of the seed. The testa is easily removed to disclose the papery remains of the nucellus surrounding a large oily endosperm. Within the latter lies the embryo, with two thin, flat cotyledons and a radicle directed towards the caruncle. Castor seeds, if in good condition, have very little odour; taste, somewhat acrid. If the testas are broken, rancidity will develop.

Preparation and characters of oil. Ninety per cent of the world's castor oil is extracted in Brazil and India. Relatively small amounts of the whole seeds are now exported. The various processes involved in the preparation of castor oil are described in the 8th edition of this

book. Briefly, the seeds are deprived of their testas and the kernels cold-expressed in suitable hydraulic presses. The oil is refined by steaming, filtration and bleaching. Cold expression yields about 33% of medicinal oil and further quantities of oil of lower quality may be obtained by other methods.

Medicinal castor oil (virgin castor oil) is a colourless or pale yellow liquid, with a slight odour and faintly acrid taste. For its chemical and physical constants, see the pharmacopoeias. The acid value increases somewhat with age and an initially high value indicates the use of damaged seeds or careless extraction or storage. Castor oil has an extremely high viscosity.

Constituents. Castor seeds contain 46–53% of fixed oil, which consists of the glycosides of ricinoleic, isoricinoleic, stearic and dihydroxystearic acids. The purgative action of the oil is said to be due to free ricinoleic acid and its stereoisomer, which are produced by hydrolysis in the duodenum. These acids have the formula,

$$CH_3[CH_2]_5CH(OH)CH_2CH{=}CH[CH_2]_7COOH$$

For the biogenesis of ricinoleic acid, see Fig. 19.3. Castor oil and the oil from *Ricinus zanzibarinus* are remarkable for their high ricinoleic acid content, which is about 88% and 92% respectively.

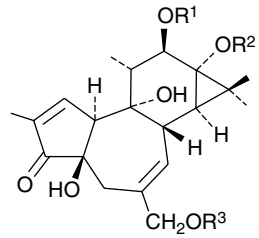
The cake left after expression contains extremely poisonous toxins known as ricins, which make it unfit for use as a cattle food. In the body they produce an antitoxin (antiricin). Ricin D is a sugar protein with a strong lethal toxicity; it contains 493 amino acids and 23 sugars. Two other ricins, acidic ricin and basic ricin, have similar properties. Ricin and abrin (see 'Abrus Seeds' below) exhibit antitumour properties. The seeds also contain lipases and a crystalline alkaloid, ricinine, which is not markedly toxic and is structurally related to nicotinamide.

Uses. Castor oil, once widely used as a domestic purgative, is now more restricted to hospital use for administration after food poisoning and as a preliminary to intestinal examination. Owing to the presence of ricin, the seeds have a much more violent action than the oil and are not used as a purgative in the West. Non-ionic surfactants (polyethoxylated castor oils) of variable composition are produced by the reaction of castor oil with ethylene oxide and are used in certain intravenous preparations which contain drugs with low aqueous solubility. The oil and its derivatives find many non-pharmaceutical uses including the manufacture of Turkey Red Oil, soaps, paints, varnishes, plasticizers and lubricants.

The following castor oil derivatives, described as pharmaceutical aids are included in the *BP*/*EP*:

- *Hydrogenated castor oil*. A fine, almost white to pale yellow powder, practically insoluble in water, m.p. 83–88°C. It contains principally 12-hydroxystearic acid.
- *Polyoxyl castor oil*. A clear yellow viscous liquid or semi-solid, freely soluble in water. Prepared by the reaction of castor oil with ethylene oxide.
- *Hydrogenated polyoxyl castor oil*. Of variable consistency depending on the amount of ethylene oxide/mol.

Allied drugs. *Croton seeds* are obtained from *Croton tiglium* (Euphorbiaceae), a small tree producing similar capsules to those of castor but devoid of spines. The seeds resemble castor seeds in size and shape but have a dull, cinnamon-brown colour and readily lose their caruncles. They contain about 50% of fixed oil which contains croton resin; also 'crotin', a mixture of croton-globulin and croton-albumin comparable with ricin. The oil also contains diesters of the tetracyclic diterpene phorbol (esterifying acid at R^1 and R^2, R^3 = H in the formula below); acids involved are acetic as a short-chain acid, and capric, lauric and palmitic as long-chain acids. These compounds are cocarcinogens and also possess inflammatory and vesicant properties (see 'Diterpenes'). Also present are phorbol-12,13,20-triesters (R^1, R^2 and R^3 are all acyl groups in the formula shown). These are 'cryptic irritants', so called because they are not biologically active as such but become so by removal of the C-20 acyl group by hydrolysis. Rotation locular counter-current chromatography (q.v.) has been used to separate these two groups of esters. A number of the phorbol esters have been tested for anti-HIV-1 activity (S. El-Mekkawy *et al.*, *Phytochemistry*, 2000, **53**, 457). The plant also contains alkaloids. Croton oil should be handled with extreme caution; it is not used in Western medicine, but if taken internally, it acts as a violent cathartic.



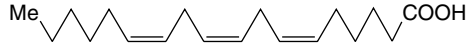
Phorbol esters of croton oil

Physic nuts or *Purging nuts* are the seeds of *Jatropha curcas*, another member of the Euphorbiaceae. The seeds are black, oval and 15–20 mm in length. They contain about 40% of fixed oil and a substance comparable with ricin, called curcin. Both seeds and oil are powerful purgatives.

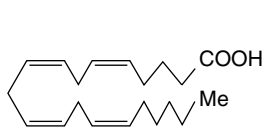
Abrus seeds (prayer beads) are the attractive red and black, but poisonous, seeds of *Abrus precatorius* (Leguminosae). They contain a toxic glycoprotein (abrin) resembling ricin together with another non-toxic peptide having haemagglutinating properties. Various alkaloids (abrine, hyaphorine, precatorine) of the indole type have been reported, also various sterols and lectins. The seeds have been used in folklore medicine in Asia, Africa and S. America to treat many ailments, also to procure abortion and to hasten labour. In India they are employed as an oral contraceptive and as they are remarkably uniform, and each weighs about 1 carat (*c*. 200 mg), have been used traditionally as weights.

EVENING PRIMROSE OIL

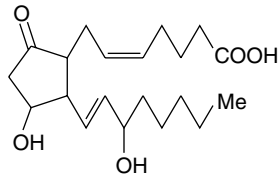
The fixed oil obtained by extraction and/or expression from the seeds of *Oenothera* spp. (*O. biennis* L., *O. lamarkiana* L.) Onagraceae contains substantial mounts of esterified γ-linolenic acid (GLA), a C_{18} 6,9,12-triene.



γ-Linolenic acid (GLA)



Arachidonic acid



Prostaglandin E_2 (PGE_2)

The principal species cultivated in the UK is *O. biennis* which yields an oil containing 7–9% GLA, although more recent work shows higher yields for the oils of some other species, namely *O. acerviphilla nova* (15.68%), *O. paradoxa* (14.41%) and an ecotype of *O. rubricaulis* (13.75%). Research has involved breeding new varieties for high yields of oil and reducing the lifecycle of the plant from 14 to 7 months (*Pharm. J.*, 1994, **252**, 189).

The sequence for the formation of such acids in the plant via *cis*-linoleic acid has already been indicated in Fig. 19.1. In animal tissues it appears that the prostaglandins are formed from dietary linoleic acid by conversion to GLA which undergoes C_2 addition and further desaturation to give acids such as arachidonic acid, an immediate precursor of some prostaglandins.

The pharmacopoeial tests for the oil are similar to those quoted for borage oil, below.

Beneficial effects of evening primrose oil may well be related to affording a precursor of the prostaglandins for those individuals whose enzymic conversion of linoleic acid to GLA is deficient. The oil is now widely marketed as a dietary supplement, for cosmetic purposes, and more specifically for the treatment of atopic eczema and premenstrual syndrome (prostaglandin E may be depleted in this condition). Further possibilities include its use in diabetic neuropathy and rheumatoid arthritis.

BORAGE OIL

The refined fixed oil expressed from the seeds of *Borago officinalis* L. (starflower), family Boraginaceae contains a higher content of γ-linolenic (GLA) and somewhat less linoleic acid than evening primrose oil. Cultivation of the crop in the UK started some years ago and details of the commercial production of the seed, plant-breeding programme and husbandry are given in a general article by A. Fieldsend, *Biologist*, 1995, **42**, 203.

Principal fatty acids involved in the oil, with *BP*/*EP* limits, are linoleic (30–41%), γ-linolenic (17–27%), oleic (12–22%), palmitic (9–12%) and smaller percentages of others. The *Pharmacopoeia* also states maximum limits for acid value, peroxide value, non-saponifiable matter, alkaline impurities and brassicasterol.

The oil is used for the same medicinal purposes as evening primrose oil. However, concerning its use for the treatment of atopic eczema, a clinical trial (A. Takwale *et al.*, *Br. Med. J.*, 2003, **327**, 1385) failed to establish any benefit from the high levels of GLA contained in the oil.

Other sources of GLA. A number of other seed oils contain appreciable quantities of GLA, including those of *Ribes nigrum* and *R. rubrum* (the black and red currant) and *Symphytum officinale* (comfrey).

SAW PALMETTO FRUIT

Saw palmetto fruit *BP*/*EP*, *BHP* is obtained from the palm *Serenoa repens* (Bartram) Small, [*Sabal serrulata* (Michaux) Nichols], family Arecaceae/Palmae, collected when ripe and dried. It is a low, shrubby palm with simple or branched stems usually up to about 3 m in length and bearing palmate leaves divided into many segments and having a petiole edged with sharp needles; hence the common name. The small flowers give rise to globose, one-seeded drupes turning bluish-black when ripe. It is indigenous to the sandy coastal regions of the S.E. states of the US, where it colonizes large areas; commercial supplies come mainly from Florida. The seeds were utilized both as a food and medicinally by the Native Americans.

The dried, dark brown to black fruit is semi-spherical to ovoid, up to 25 mm long, 15 mm wide with a rugged surface formed by shrinkage on drying. The remains of the three-toothed tubular calyx and style may be seen at the distal end, and the scar of the pedicel at the lower end. The hard seed is oval to spherical, measuring some 12 × 8 mm and comprising a thin testa, small perisperm and a larger, horny, pale endosperm and embryo.

A microscopical examination of the powder shows cuticularized, reddish-brown polygonal cells of the epicarp; mesocarpic parenchymatous cells, which are often oil filled or containing crystals of silica; scattered or small groups of sclereids; sclerenchymatous endocarp; and seed characteristics that include pigmented cells of the testa, thickened pitted cells of the endosperm containing fixed oil and aleurone grains.

The drug has an aromatic odour reminiscent of vanilla and a soapy, somewhat acrid, although sweetish, taste.

Constituents. Fatty acids constitute an important feature of the drug. In the mesocarp, originally the fleshy part of the fruit, they occur in the free state, and in the seeds as triacylglycerols. The principal free acids are oleic, lauric, myristic and palmitic acids, with lesser amounts of caproic, caprylic, capric, stearic, linoleic and linolenic acids; for formulae see Tables 19.1, 19.2 and 19.3. The same acids are involved in the fats of the seed endosperm, with oleic and lauric acids most commonly occurring as mixed triacylglycerols (see Fig. 19.7). Fatty acid esters involving propyl and ethyl alcohol (pricipally propyl laurate) are present in small amounts in various extracts.

Other constituents include: flavonoids such as rutin, isoquercitrin (see Fig. 21.15) and others; phytosterols—sitosterol (see Fig. 23.5), its glucosides and esters, and stigmasterol (see Fig. 23.5); immunostimulant polysaccharides involving galactose, arabinose, mannose, rhamnose, glucuronic acid and others; carotenoids; volatile oil.

The *BP*/*EP* gives a TLC identification test for the powdered drug using β-amyrin and β-sitosterol as reference substances. An assay for total fatty acids, minimum requirement for the dried drug 11.0%, employs gas chromatography of a dimethylformamide extract of the powder; the individual acids separated on the chromatogram are identified by comparison with the chromatogram of a reference solution of the acids mentioned above.

Actions. Most clinical and pharmacological studies on saw palmetto fruits have involved the use of hexane, supercritical CO_2 (see Chapter 17) and ethanol extracts of the drug. Among the activities observed is that of the inhibition of 5α-reductase thus impeding the conversion of testosterone to dehydrotestosterone. The principal use of the drug is for the symptomatic treatment of benign prostatic hyperplasia.

For extended accounts and bibliography on the drug, see E. Bombardelli and P. Morazzoni, *Fitoterapia*, 1997, **68**, 99–113 (58 refs); P. Bradley, *British Herbal Compendium*, 2006, pp. 345–352 (62 refs).

Hydnocarpus oil

This is the fixed oil obtained by cold expression from the fresh ripe seeds of *Hydnocarpus wightiana, H. anthelmintica, H. heterophylla* and other species of *Hydnocarpus*, and also of *Taraktogenos kurzii*. These plants are found in India, Burma, Siam and Indo-China and belong to the Flacourtiaceae.

The oil of *H. wightiana* contains hydnocarpic acid (about 48%), chaulmoogric acid (about 27%), gorlic and other acids (formulae, see Table 19.4); the structures of several new cyclopentenyl fatty acids have recently been elucidated. These acids do not appear to be formed from straight-chain acids and they accumulate during the last 3–4 months of maturation of the fruit. They are strongly bactericidal towards the leprosy micrococcus, but the oil has now to a large extent been replaced by the ethyl esters and salts of hydnocarpic and chaulmoogric acid. The esterified oil of *H. wightiana* is preferable to that of other species, in that it yields when fractionated almost pure ethyl hydnocarpate. This was included in the *BPC* (1965) but has now been deleted, as more effective remedies are available.

WOOL FAT

Wool fat (*anhydrous lanolin*) is a purified fat-like substance prepared from the wool of the sheep, *Ovis aries* (Bovidae).

Raw wool contains considerable quantities of 'wool grease' or crude lanolin, the potassium salts of fatty acids and earthy matter. Raw lanolin is separated by 'cracking' with sulphuric acid from the washings of the scouring process and purified to fit it for medicinal use. Purification may be done by centrifuging with water and by bleaching.

Wool fat is a pale yellow, tenacious substance with a faint but characteristic odour. It is insoluble in water and a high proportion of water may be incorporated with it by melting (m.p. 36–42°C) and stirring. Soluble in ether and chloroform. Like other waxes, it is not readily saponified by aqueous alkali, but an alcoholic solution of alkali causes saponification. Saponification value 90–105; iodine value 18–32; acid value not more than 1. Hydrous wool fat or lanolin contains 25% water.

The chief constituents of wool fat are cholesterol and isocholesterol, unsaturated monohydric alcohols of the formula $C_{27}H_{45}OH$, both free and combined with lanoceric, lanopalmitic, carnaubic and other fatty acids. Wool fat also contains aliphatic alcohols such as cetyl, ceryl and carnaubyl alcohols. Butylated hydroxytoluene, up to 200 ppm, may be added as an antioxidant.

The pharmacopoeial test for pesticide residues is complex and involves their isolation and subsequent identification. A maximum of 0.05 ppm is permitted for each organochlorine pesticide, 0.5 ppm for each other pesticide and 1ppm for the sum of all pesticides.

Wool Alcohols BP/EP are prepared by the saponification of crude lanolin and the separation of the alcohol fraction. The product consists of steroid and triterpene alcohols, including cholesterol (not less than 30%) and isocholesterol. As for wool fat, an antioxidant may be added.

To test for cholesterol dissolve 0.5 g in 5 ml of chloroform, add 1 ml acetic anhydride and two drops of sulphuric acid; a deep-green colour is produced.

Hydrogenated Wool Fat BP/EP is obtained by the high pressure/high temperature hydrogenation of anhydrous wool fat. It contains a mixture of higher aliphatic alcohols and sterols.

Wool fat is used as an emollient base for creams and ointments.

LARD

Lard (*prepared lard*) is the purified internal fat of the hog, *Sus scrofa* (order Ungulata, Suidae).

For medicinal purposes lard is prepared from the abdominal fat known as 'flare', from which it is obtained by treatment with hot water at a temperature not exceeding 57°C.

Lard is a soft, white fat with a non-rancid odour. Acid value not more than 1.2. Lard has a lower melting point (34–41°C) and a higher iodine value (52–66) than suet. Saponification value 192–198. It should be free from moisture, beef-fat, sesame-seed and cotton-seed oils, alkalis and chlorides.

Lard contains approximately 40% of solid glycerides such as myristin, stearin and palmitin, and 60% of mixed liquid glycerides such as olein. These fractions are somewhat separated by pressure at 0°C and sold as 'stearin' and 'lard oil' respectively. Lard is used as an ointment base but is no longer official in Britain. It is somewhat liable to become rancid, but this may be retarded by benzoination, Siamese benzoin being more effective than the Sumatra variety.

Suet. Suet is the purified internal fat of the abdomen of the sheep, *Ovis aries*. It contains about 50–60% of solid glycerides and melts at about 45°C. It is used as an ointment base in tropical and subtropical countries.

WAXES

YELLOW BEESWAX, WHITE BEESWAX

Beeswax is obtained by melting and purifying the honeycomb of *Apis mellifica* and other bees. The wax is imported from the West Indies, California, Chile, Africa, Madagascar and India. The *EP* and *BP* include separate monographs for the yellow and the white wax.

Preparation. Wax is secreted by worker bees in cells on the ventral surface of the last four segments of their abdomen. The wax passes out through pores in the chitinous plates of the sternum and is used, particularly by the young workers, to form the comb.

Yellow beeswax is prepared, after removal of the honey, by melting the comb under water (residual honey dissolving in the water and solid impurities sinking), straining, and allowing the wax to solidify in suitable moulds.

White beeswax is prepared from the above by treatment with charcoal, potassium permanganate, chromic acid, chlorine, etc., or by the slow bleaching action of light, air and moisture. In the latter method the melted wax is allowed to fall on a revolving cylinder which is kept moist. Ribbon-like strips of wax are thus formed which are exposed on cloths to the action of light and air, being moistened and turned at intervals until the outer surface is bleached. The whole process is repeated at least once, and the wax is finally cast into circular cakes.

Characters. Beeswax is a yellowish-brown or yellowish-white solid. It breaks with a granular fracture and has a characteristic odour. It is insoluble in water and sparingly soluble in cold alcohol, but dissolves in chloroform and in warm fixed and volatile oils (e.g. oil of turpentine).

Constituents. Beeswax is a true wax, consisting of about 80% of myricyl palmitate (myricin), $C_{15}H_{31}COOC_{30}H_{61}$, with possibly a little myricyl stearate. It also contains about 15% of free cerotic acid, $C_{26}H_{53}COOH$, an aromatic substance cerolein, hydrocarbons, lactones, cholesteryl esters and pollen pigments.

Standards. These include a 'drop point' of 61–65° for both the white and yellow wax, acid value, ester value, saponification value and tests for ceresin, paraffin and certain other waxes, and various phenols.

Uses. Beeswax is used in the preparation of plasters, ointments and polishes.

CARNAUBA WAX

Carnauba wax, included in the *BP/EP* (2000) and *USP/NF* (1995), is derived from the leaves of *Copernicia cerifera* (Palmae). It is removed from the leaves by shaking and purified to remove foreign matter.

The wax is hard, light brown to pale yellow in colour and is supplied as a moderately coarse powder, as flakes or irregular lumps; it is usually tasteless with a slight characteristic odour free from rancidity. Esters, chiefly myricyl cerotate, are the principal components, with some free alcohols and other minor constituents. The acid value is low (*BP*, *USP/NF*, 2–7), the saponification value 78–95. It has an iodine value of 7–14. The *BP/EP* includes a TLC test for identity. Carnauba wax is used in pharmacy as a tablet-coating agent and in other industries for the manufacture of candles and leather polish. It has been suggested as a replacement for beeswax in the preparation of phytocosmetics.

20

Carbohydrates

As the name implies, carbohydrates consist of carbon, hydrogen and oxygen with the last two elements usually present in the same proportions as in water. As we have previously noted, carbohydrates are among the first products to arise as a result of photosynthesis. They constitute a large proportion of the plant biomass and are responsible, as cellulose, for the rigid cellular framework and, as starch, for providing an important food reserve. Of special pharmacognostical importance is the fact that sugars unite with a wide variety of other compounds to form glycosides (Chapters 21–25). Mucilages, as found in marshmallow root and psyllium seeds, act as water-retaining vehicles, whereas gums, which are similar in composition and properties, are formed in the plant by injury or stress and usually appear as solidified exudates; both are typically composed of uronic acid and sugar units. The cell walls of the brown seaweeds and the middle lamellae of higher plant tissues contain polysaccharides consisting almost entirely of uronic acid components. All these groups are discussed more fully below, and the drugs and pharmaceutical necessities containing them are listed at the end of the chapter.

SUGARS (SACCHARIDES)

Monosaccharides

These sugars contain from three to nine carbon atoms, but those with five and six carbon atoms (pentoses, $C_5H_{10}O_5$, and hexoses, $C_6H_{12}O_6$) are accumulated in plants in greatest quantity.

The formulae of sugars and other carbohydrates are written in a number of different ways. The structure of glucose as a straight-chain pentahydroxy aldehyde was established by Kiliani in 1886. Emil Fischer, from 1884 onwards, was the most important of the early workers in this field. Their straight-chain formulae are still useful for illustrating the isomerism and stereochemical relationships and, as shown below, can be written in very abbreviated form. Many of the important biological properties of carbohydrates can, however, best be illustrated by ring formulae which show that the same sugar may exist either as a five-membered ring (furanose) or a six-membered ring (pyranose). Glucose has an aldehyde group and is therefore called an aldose or 'aldo' sugar; fructose has a ketone group and is therefore called a ketose. Terms such as 'aldopentose' and 'ketohexose' are self-explanatory. The formulae (Figs. 20.1, 20.2) illustrate these points.

The furanose structure is comparatively unstable but may be stabilized on glycoside formation. The fructose phosphate of the furanose form illustrated in Fig. 20.1 is an intermediate in glycolysis, the anaerobic degradation of hexoses which provides energy for metabolism (see Fig. 18.5). Fructose in nature is always in the furanose form, but when isolated in crystalline form, it has a pyranose structure.

Uronic acids are produced by oxidation of the terminal groups to –COOH (e.g. glucuronic acid from glucose and galacturonic acid from galactose).

Biosynthesis of monosaccharides. Various monosaccharides arise from the photosynthetic cycle (q.v.). D-Fructose-6-phosphate and D-glucose-6-phosphate are universal in their occurrence. Free sugars may accumulate as a result of hydrolysis of the phosphorylated sugars or the latter may be utilized in respiration, converted to sugar nucleotides (e.g. uridine-diphosphoglucose—UDPG) or, by the action of various epimerases, give rise to other monosaccharides (e.g. galactose).

Di-, tri- and tetrasaccharides

These sugars may also be called bioses, trioses and tetroses. They are theoretically derived from two, three or four monosaccharide molecules,

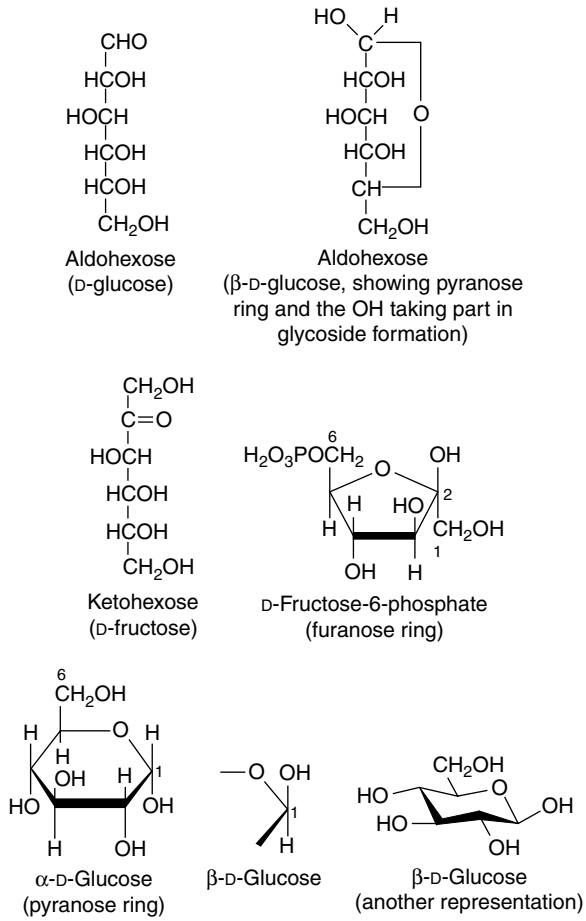


Fig. 20.1
Hexose structures and representation.

respectively, with the elimination of one, two or three molecules of water (Table 20.1). One of the commonest plant disaccharides is sucrose; it is formed in photosynthesis by the reaction of UDPG with fructose-6-phosphate (Fig. 20.3). Control mechanisms for the build-up of sucrose in leaves, and its breakdown for transport to storage organs, are achieved by metabolite effector control of the appropriate enzymes.

The reverse process, hydrolysis, is brought about by suitable enzymes or by boiling with dilute acid. The same sugars may be linked to one another in various ways. Thus, the disaccharides maltose, cellobiose, sophorose and trehalose are all composed of two molecules of glucose joined by α-1,4-, β-1,4-, β-1,2- and α,α-1,1-(non-reducing) linkages, respectively.

POLYSACCHARIDES

By condensation involving sugar phosphates and sugar nucleotides, polysaccharides are derived from monosaccharides in an exactly similar manner to the formation of di-, tri-and tetrasaccharides. The name 'oligosaccharide' (Greek *oligo*, few) is often applied to saccharides containing from two to 10 units. In polysaccharides the number of sugar units is much larger and the number forming the molecule is often only approximately known. The hydrolysis of polysaccharides, by enzymes or reagents, often results in a succession of cleavages, but the final products are hexoses or pentoses or their derivatives. The term 'polysaccharide' may usefully be taken to include polysaccharide complexes which yield in addition to monosaccharides their sulphate esters, uronic acids or amino sugars.

Table 20.2 indicates the character of some of the polysaccharides.

In addition to the well-established polysaccharide-containing pharmaceutical materials described later in this chapter there is now considerable interest in a number of polysaccharides with other pharmacological activities. These include immuno-modulating, antitumour, anti-inflammatory, anticoagulant, hypoglycaemic and antiviral properties. Specific examples are the glycyrrhizans of *Glycyrrhiza uralensis* and *G. glabra* and the glycans of ginseng and *Eleutherococcus* (q.v.). In general polysaccharides from fungi exhibit antitumour activity,

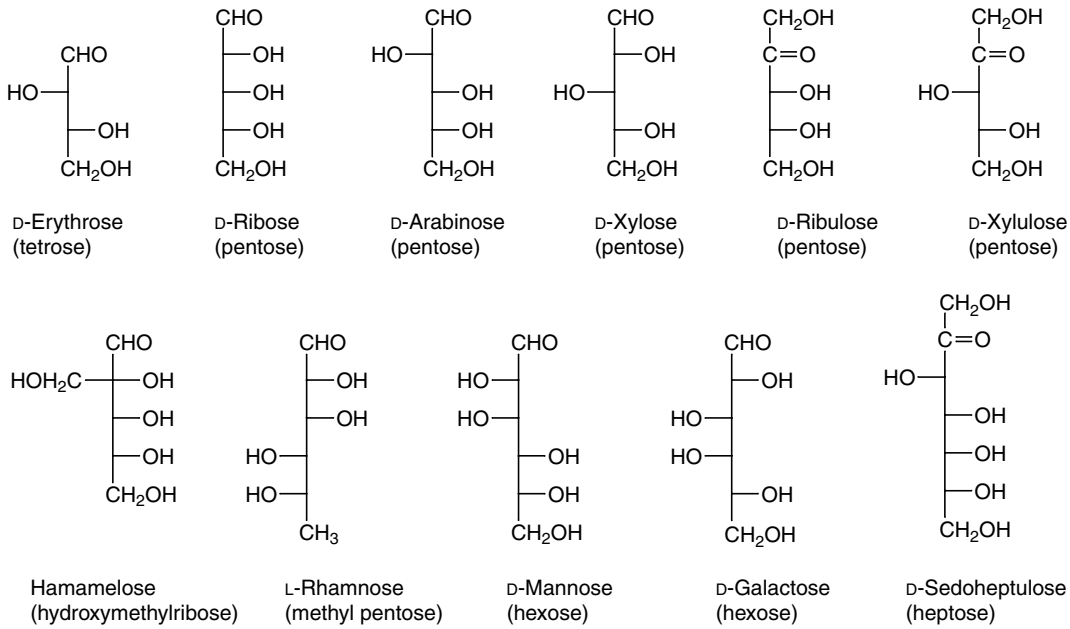


Fig. 20.2
Examples of C_4 to C_7 monosaccharides (Fischer structures).

Table 20.1 Some di-, tri- and tetrasaccharides.

Type	Name	Products of hydrolysis	Occurrence
Di-	Sucrose	Glucose, fructose	Sugar cane, sugar beet, etc.
	Maltose	Glucose, glucose	Enzymic hydrolysis of starch
	Lactose	Glucose, galactose	Milk
	Cellobiose	Glucose, glucose	Enzymic breakdown of cellulose
	Trehalose	Glucose, glucose	Ergot, Rhodophyceae, yeasts
	Sophorose	Glucose, glucose	*Sophora japonica*, hydrolysis of stevioside
	Primeverose	Glucose, xylose	*Filipendula ulmaria*, hydrolysis of spiraein
Tri-	Gentianose	Glucose, glucose, fructose	*Gentiana* spp.
	Melezitose	Glucose, fructose, glucose	Manna from *Larix*
	Planteose	Glucose, fructose, galactose	Seeds of *Psyllium* spp.
	Raffinose	Galactose, glucose, fructose	Many seeds (e.g. cotton-seed)
	Manneotriose	Galactose, galactose, glucose	Manna of ash, *Fraxinus ornus*
	Rhamninose	Rhamnose, rhamnose, galactose	*Rhamnus infectoria*
	Scillatriose	Rhamnose, glucose, glucose	Glycoside of squill
	Other examples of trisaccharides are among the glycosides of *Digitalis* and *Strophanthus* (q.v.)		
Tetra-	Stachyose or manneotetrose	Galactose, galactose, glucose, fructose	Tubers of *Stachys japonica* and manna of *Fraxinus ornus*
	Other examples of tetrasaccharides are among the glycosides of *Digitalis* (q.v.)		

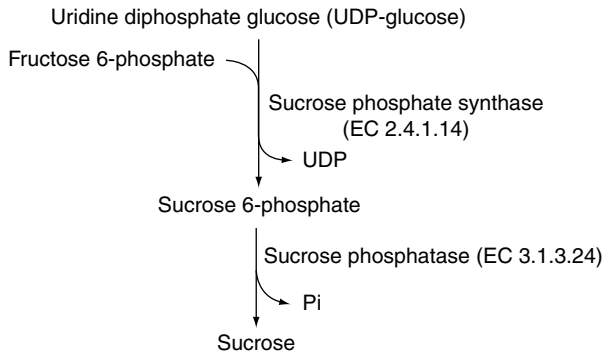


Fig. 20.3
Biosynthesis of sucrose.

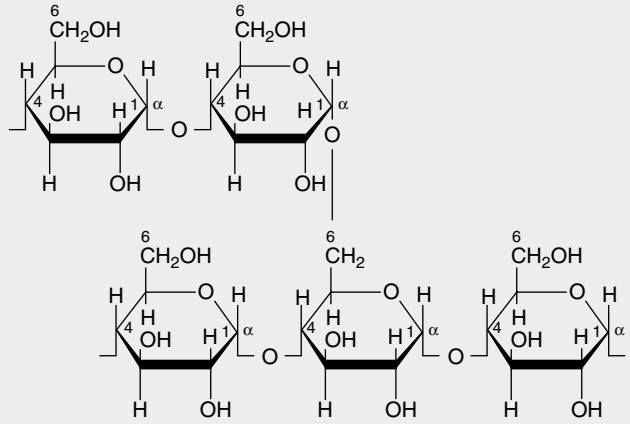
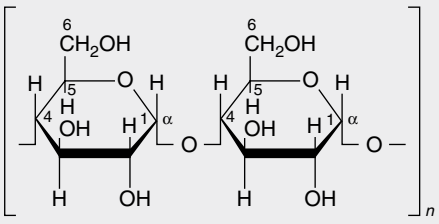
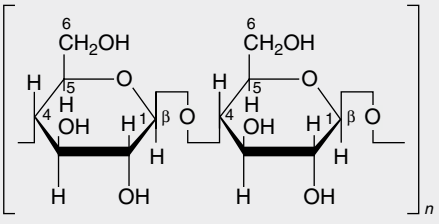
those from higher plants are immunostimulatory and the algal polysaccharides, which often contain sulphate, are good anticoagulants.

Tests for carbohydrates

The following are some of the more useful tests for sugars and other carbohydrates.

1. *Reduction of Fehling's solution*. To a heated solution of the substance add drop by drop a mixture of equal parts of Fehling's solution No. 1 and No. 2. In certain cases reduction takes place near the boiling point and is shown by a brick-red precipitate of cuprous oxide. Reducing sugars include all monosaccharides, many disaccharides (e.g. lactose, maltose, cellobiose and gentiobiose). Non-reducing substances include some disaccharides (sucrose and trehalose, the latter a sugar found in some fungi) and polysaccharides. Non-reducing carbohydrates will on boiling with acids be converted into reducing sugars, but students are reminded to neutralize any acid used for hydrolysis before testing with Fehling's solution, or cuprous oxide will fail to precipitate.
2. *Molisch's test*. All carbohydrates give a purple colour when treated with α-naphthol and concentrated sulphuric acid. With a soluble carbohydrate this appears as a ring if the sulphuric acid is gently poured in to form a layer below the aqueous solution. With an insoluble carbohydrate such as cotton-wool (cellulose) the colour will not appear until the acid layer is shaken to bring it in contact with the material.
3. *Osazone formation*. Osazones are sugar derivatives formed by heating a sugar solution with phenylhydrazine hydrochloride, sodium acetate and acetic acid. If the yellow crystals which form are examined under the microscope they are sufficiently characteristic for certain sugars to be identified. It should be noted that glucose and fructose form the same osazone (glucosazone, m.p. 205°C). Before melting points are taken, osazones should be purified by recrystallization from alcohol. Sucrose does not form an osazone, but under the conditions of the above test sufficient hydrolysis takes place for the production of glucosazone.
4. *Resorcinol test for ketones*. This is known as Selivanoff's test. A crystal of resorcinol is added to the solution and warmed on a water-bath with an equal volume of concentrated hydrochloric acid. A rose colour is produced if a ketone is present (e.g. fructose, honey or hydrolysed inulin).
5. *Test for pentoses*. Heat a solution of the substance in a test-tube with an equal volume of hydrochloric acid containing a little phloroglucinol. Formation of a red colour indicates pentoses.
6. *Keller–Kiliani test for deoxysugars*. Deoxysugars are found in cardiac glycosides such as those of *Digitalis* and *Strophanthus* spp. (see Chapter 23). The sugar is dissolved in acetic acid containing a trace of ferric chloride and transferred to the surface of concentrated sulphuric acid. At the junction of the liquids a reddish-brown colour is produced which gradually becomes blue.
7. *Enzyme reactions*. Since certain carbohydrate reactions are only brought about by certain specific enzymes, such enzymes may be used for identification.
8. *Chromatography*. Chromatographic methods are particularly suited to the examination of drug extracts, which may contain a number of carbohydrates often in very small amounts. Not only are they applicable to carbohydrates originally present in the sample (see pharmacopoeial TLC test for honey), but also they may be used to study the products of hydrolysis of polysaccharide complexes such as gums and mucilages. As standards for comparison many pure sugars, uronic acids and other sugar derivatives are commercially available.

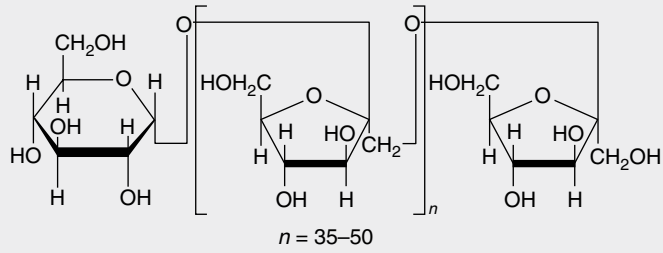
Table 20.2 The character of some polysaccharides.

Name	Occurrence and nature
Containing only monosaccharide units	
1. Amylopectin or α-amylose	The main constituent of most starches (over 80%). The molecule has branched chains each consisting of 20–26 α-1,4-linked glucose residues. Several hundred of these chains are linked by α-1,6 glycosidic bonds to neighbouring chains giving a molecule containing some 50 000 glycosyl units. The branching pattern throughout the molecule is not uniform, resulting in some areas that are apparently amorphous (high degree of branching) and others probably crystalline (linear chains predominate with little branching)
	
2. Amylose or β-amylose	Most starches contain up to 20%, but sometimes absent. Consists essentially of linear chains of α-1,4-linked glucose residues. Several thousand glucose units constitute a chain. It is now recognized that there is a very limited branching (α-1,6-linkages) to the extent of 2–8 branches per molecule
	
3. Glycogen or animal starch	Important reserve carbohydrate of animal tissues. Molecule resembles that of amylopectin
4. Cellulose	Chief polysaccharide of plant cell walls. Linear chains of β-1,4-linked glucose residues
	

(Continued)

Table 20.2 The character of some polysaccharides. (Cont'd)

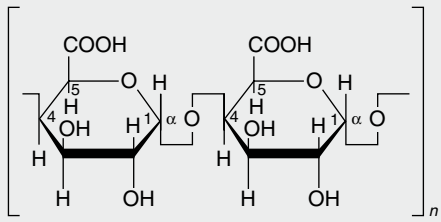
5. Inulin	A reserve carbohydrate particularly abundant in the Compositae. Linear chains of up to 50 β-1,2-linked fructofuranose units terminated by a single glucose unit



6. Xylans, mannans and galactans	These are often associated with one another and with cellulose. They are difficult to isolate in a pure form. On hydrolysis they yield xylose, mannose and galactose, respectively
7. Hemicelluloses	These polysaccharides occur in the cell wall with cellulose and pectic substances. The nomenclature, dating from 1891, is deceptive because hemicelluloses are not components of cellulose but are formed mainly from hexose and pentose units. Hemicelluloses vary according to source and can be classified as xylans, mannans and galactans according to their principal components
8. Lichenin or lichen starch	A polysaccharide found in lichens. Resembles cellulose but molecule contains about 25% of β-1,3 glucosidic linkages

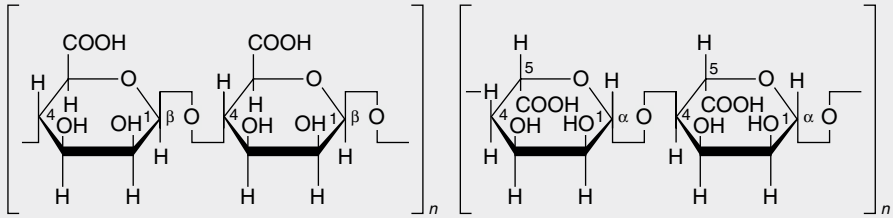
Polysaccharide complexes containing uronic acid or other units

1. Pectins	These occur in the middle lamellae of cell walls and are abundant in fruits (e.g. apples, oranges) and roots (beets and gentian). The parent substance protopectin is insoluble but is easily converted by restricted hydrolysis into pectinic acids (pectins). Pectins from different sources vary in their complex constitution, the principal components being blocks of D-galacturonic acid residues linked by α-1,4- glycosidic linkages and interspersed with rhamnose units; some of the carboxyl groups are methylated. These molecules are accompanied by small amounts of neutral arabinans (branched polymers of α-1,5-linked L-arabofuranose units) and galactans (largely linear chains of β-1,4- linked D-galactopyranose units)



Repeating units of D-galacturonic acid

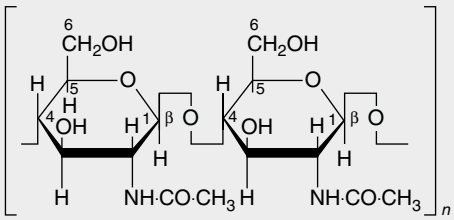
2. Algin or alginic acid	Alginic acid is the principal constituent of the cell walls of the brown algae. It was discovered by Stanford in 1880 and is now widely used for the manufacture of alginate salts and fibres (q.v.). The composition varies according to the biological source, thus providing a range of properties which are exploited commercially. It is a heteropolyuronide consisting of chains of β-1,4-linked D-mannuronic acid units interspersed with lengths of α-1,4-linked L-guluronic acid units together with sections in which the two monouronide units are regularly interspersed. In alginic acids from different sources the ratios of the two uronic acids vary from 2:1 to 1:2. The chain length varies with the method of preparation and molecular weight, and viscosity measurements suggest molecules of from 220 to 860 units



β-1,4-linked-D-mannuronic acid units α-1,4-linked L-guluronic acid units

(Continued)

Table 20.2 The character of some polysaccharides. (Cont'd)

3. Polysaccharides with sulphuric acid esters	Certain algae, including those yielding agar and carrageen, contain a mixture of polysaccharides. Agar, for example, contains a biose formed from D- and L-galactose but also a more complex agaropectin formed from galactose and uronic acid units partly esterified with sulphuric acid. Carrageen has a similar composition
4. Chitin	This is found in some of the lower plants, in insects and in crustaceans. The molecule consists of linear chains of β-1,4-linked *N*-acetyl-D-glycosamine residues. Its inclusion in the microfibrillar component of the fungal cell wall is analogous to that of the cellulose microfibril
	
5. Gums and mucilages	Gums such as acacia and tragacanth and mucilages, such as those found in linseed, psyllium seeds and marshmallow root, are found in many plants, where they are usually formed from the cell wall or deposited on it in layers. They are essentially polyuronides consisting of sugar and uronic acid units. Some gums have methoxyl groups (e.g. tragacanth); in others the acidic complex is united with metals (e.g. acacia)

Experimental details and R_f values of sugars in different systems are to be found in standard books on chromatography. The carbohydrate spots obtained after separation are identified by their positions and by reagents. It may be useful to examine them in ultraviolet light. A non-specific reagent for reducing sugars is a freshly prepared ammoniacal silver nitrate solution. More specific reagents giving coloured spots with different sugars include aniline hydrogen phthalate (in water-saturated *n*-butanol) and naphthoresorcinol (in acetone, water and phosphoric acid). These are applied to the chromatogram with a spray. Although sugars are non-volatile, it is possible by suitable treatment to render them satisfactory for gas chromatography (q.v.)

COMMERCIAL PLANT-DERIVED FIBRES AND PRODUCTS

The biological origin and the structure of plant fibres is discussed in Chapter 42; many have important commercial uses and for a review on their botany, chemistry and processing see McDougall *et al.*, *J. Sci. Food, Agric.*, 1993, **62**, 1.

A number of vegetable fibres have importance in pharmacy, particularly as components of surgical dressings and for the manufacture of artificial fibres and haemostatic dressings. The subject of surgical dressings was, and in many Schools still is, regarded as pharmacognosy-related. However with the more recent advances in the management and concept of wound-healing, many materials of non-vegetable origin are now used which, for an in-depth coverage, bring the topic outside the scope of this book. Described below are the more important primary carbohydrate materials involved.

COTTON, RAW COTTON

Cotton consists of the epidermal trichomes of the seeds of *Gossypium herbaceum* and other cultivated species of *Gossypium* (Malvaceae). The plants are shrubs or small trees which produce three- to five-celled capsules containing numerous seeds. The USA produces about half of the world's cotton, other important sources being Egypt, India and South America. The chief American cottons are derived from *G. barbadense* (Sea Island cotton) and *G. herbaceum* (Upland, Texas or New Orleans cotton).

The hairs of the different species vary in length or '*staple*'. The staples of important commercial varieties of cotton are as follows: (1) Sea Island, up to 54.5 mm; (2) Egyptian, 31–38 mm; (3) Brazilian and Peruvian, 29–30 mm; (4) American Upland, about 25.9 mm; (5) Indian, 21.4–29.2 mm.

Preparation. When ripe the bolls are collected, dried and subjected to a ginning process to separate the hairs from the seed. The gin, which may be of a roller or a pneumatic type, is designed to pull the hairs through a narrow space which is too small to allow the seed to pass. In ordinary American or Upland cotton the gin leaves the seeds with a coating of short hairs which have to be removed by a second type of gin known as a 'linter'. These short hairs are used for making the lower grades of cotton wool and rayons. The seeds are used for the preparation of cottonseed oil (q.v.) and cattle cake. Raw cotton contains various impurities, such as immature and broken seeds, fragments of leaf, etc., most of which are removed during the manufacture of yarn.

For spinning very fine yarns Sea Island cotton is used, but for coarser yarns it is possible to use shorter staple cottons. Different machines are used for these two types of yarn, which are known as *combed* and *carded*, respectively. The cotton-combing machine separates all the shorter fibres and a thread is spun consisting of long, well-paralleled, uniform fibres. The short fibres of *comber waste* are used for making the best grades of cotton wool. The carding machine uses fibres which are shorter and less uniform in length, and the absence of combing is shown in the yarn by the irregular arrangement of the fibres, the ends of which often project from the surface.

Microscopy of unbleached cotton. Cotton consists of unicellular hairs the appearance of which has been likened to that of empty, twisted fire-hoses. Their length is up to about 5 cm, diameter 9–24 μm, and the number of twists varies from about 75 cm^{-1} in the Indian to 150 cm^{-1}

in the Sea Island. Pieces of 'shell' or seed coat, which can often be picked from samples of raw cotton, show hair bases fitting between the thick-walled epidermal cells. The apex is rounded and solid. The cotton hair is cylindrical when young but becomes flattened and twisted as it matures, the large lumen, which contains the remains of protoplasm being much elongated in transverse section. The cellulose wall of the hair is covered with a waxy cuticle which renders it non-absorbent. The cuticle may be stained with ruthenium red. Bleached cotton yarn and absorbent cotton wool (see below) are readily wetted by water.

Tests. The following tests are applicable to cotton.

1. On ignition, which should be done both by advancing the fibre towards a flame and by heating on porcelain, cotton burns with a flame, gives very little odour or fumes, does not produce a bead, and leaves a small white ash; distinction from acetate rayon, alginate yarn (also wool, silk, nylon).
2. Moisten with N/50 iodine and, when nearly dry, add 80% w/w sulphuric acid. A blue colour is produced; distinction from acetate rayon, alginate yarn, jute, hemp (also wool, silk, nylon).
3. With ammoniacal copper oxide solution, raw cotton dissolves with ballooning, leaving a few fragments of cuticle; absorbent cotton dissolves completely with uniform swelling; distinction from acetate rayon, jute (also wool, nylon).
4. In cold sulphuric acid 80% w/w cotton dissolves; distinction from oxidized cellulose, jute, hemp (also wool).
5. In cold sulphuric acid 60% w/w insoluble; distinction from cellulose wadding and rayons.
6. In warm (40°C) hydrochloric acid *BP* insoluble; distinction from acetate rayon (also silk, nylon).
7. Insoluble in 5% potassium hydroxide solution; distinction from oxidized cellulose (also wool, silk).
8. Treat with cold Shirlastain A for 1 min and wash out; gives shades of blue, lilac or purple; distinction from viscose and acetate rayons, alginate yarn (also wool, silk, nylon).
9. Treat with cold Shirlastain C for 5 min and wash out; raw cotton gives a mauve to reddish-brown colour and absorbent cotton a pink one; distinction from flax, jute, hemp. The Shirlastains may be usefully applied to a small piece of the whole fabric under investigation to indicate the distribution of more than one type of yarn.
10. Gives no red stain with phloroglucinol and hydrochloric acid; distinction from jute, hemp and kapok.
11. Insoluble in formic acid 90% or phenol 90% (w/w); distinction from acetate rayon (also nylon).
12. Insoluble in acetone (distinction from acetate rayon).

ABSORBENT COTTON WOOL, ABSORBENT WOOL

Cotton wool is mainly prepared from linters, card strips, card fly and comber waste. Bales of these short-fibred cotton wastes pass from the yarn manufacturers to the makers of cotton wool. For best-quality cotton wool the comber waste of American and Egyptian cottons is preferred. In this the fibres are reasonably long and twisted and thus suitable for producing a cotton wool having an average staple that will offer appreciable resistance when pulled and not shed a significant quality of dust when shaken gently.

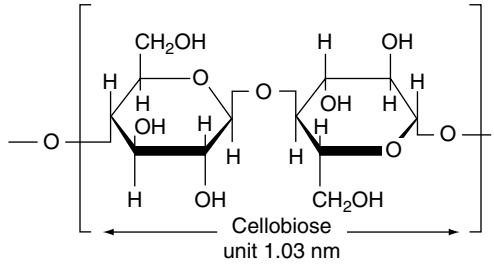
The preparation may be outlined as follows. The comber waste (which arrives in bales) is loosened by machinery and then heated with dilute caustic soda and soda ash solution at a pressure of 1–3 atmospheres for 10–15 h. This removes much of the fatty cuticle and renders the trichome wall absorbent. It is then well washed with water, bleached with dilute sodium hypochlorite solution and treated with very dilute hydrochloric acid. After washing and drying it is in a matted condition and is therefore opened up by machines and then 'scutched'; that is, it is converted into a continuous sheet of fairly even thickness with the fibres loosened ready for the carding machine. The carding machine effects a combing operation and forms a thin continuous film of cotton wool. Several such films are superimposed on one another, interleaved with paper and packaged in rolls.

Tests. For tests on cotton wool, see under 'Cotton, Raw Cotton'.

Structure of fibre. Like the cell wall in general, that of cotton consists of a primary and secondary wall. The formation of the former is modified to embrace the enormous longitudinal development of the cell, some thousands of times greater than the width. The microfibrils themselves are unexpandable and are initially laid down in hoops around the fibre, restricting its lateral growth. However, because of their different orientation at the fibre end, longitudinal extension can take place. Owing to the build-up of pressure from successive layers of cellulose the original orientation becomes lost as the fibre matures. Matrix polysaccharides and proteins derived from the Golgi apparatus are also present in the primary wall. The secondary wall, some 5–10 μm thick and consisting almost entirely of pure cellulose, constitutes the main bulk of the mature cotton fibre.

Chemical nature. Raw cotton consists of cellulose approximately 90% and moisture 7%, the remainder being wax, fat, remains of protoplasm and ash.

Absorbent cotton is a very pure form of cellulose and its chemical and physical properties have been extensively studied. The cellulose molecule is built up of glucose residues united by 1,4-β-glucosidic links (contrast starch). The wall of the cotton fibre, like that of plant cells in general, shows anisotropic properties. When swollen in water, the swelling is in a direction at right angles to the long axis. In the direction of the long axis it shows considerable tensile strength. Examined in polarized light, it shows birefringence, the value of the double refraction depending on the liquid in which the fibre is immersed. This phenomenon, characteristic of mixed bodies with rod-like structural elements, has been termed rodlet-double refraction. Stained with chlor-zinc-iodine and examined microscopically in polarized light (analyser removed), the fibre shows greater absorption when orientated with its long axis parallel to the plane of polarization than when orientated with the long axis at right angles (dichroism). These physical properties suggest that the fibre wall is built up of elongated structural units orientated in some definite manner. The study of the cotton fibre by X-ray analysis has confirmed this and has shown that its cell wall is composed of elongated chain-like molecules (built up of repeating units 1.03 nm long) and orientated in a spiral manner, the spiral making an angle of 30° with the long axis of the fibre. The length of the repeating unit of structure corresponds to that of two glucose residues fully extended. This unit is the 'cellobiose unit', many of which are united in the polysaccharide molecule of cellulose (Table 20.2).



The biosynthesis of cellulose in the cotton trichome would appear to involve UDP-glucose originating from sucrose.

Jute

Jute consists of the strands of phloem fibres from the stem bark of *Corchorus capsularis, C. olitorius* and other species of *Corchorus* (Tiliaceae). These are annual plants about 3–4 m high which are cultivated in Bengal, in the delta region of the Ganges and Brahmaputra rivers, and in Assam, Bihar and Orissa.

The fibres are separated from the other plant material by retting and then spun into yarn which can be made up into hessian and sacking. Short fibres left over from the preparation of the yarns and ropes constitute *tow* and in pharmacy the term 'tow' refers to jute, although it can also be applied to hemp and flax. The commercial strands are 1–3 m long and about 30–140 μm in diameter. Each consists of a bundle of phloem fibres composed of lignocellulose. The heavily lignified middle lamella is destroyed by oxidizing agents; a mixture of nitric acid and potassium chlorate may therefore be used to disintegrate the bundles, the individual fibres being then teased out and sketched. Prepared transverse sections should also be examined and compared with those of hemp and flax.

Flax

Flax is prepared from the pericyclic fibres of the stem of *Linum usitatissimum* (Linaceae).

The commercial fibres show fine transverse injuries received during the preparation. Good-quality flax fibre is non-lignified except for the middle lamella. Lignification of the secondary wall, however, takes place as the stem matures, starting at the base, and if the stems are too old before retting, the fibre is coarse and harsh in texture.

Hemp

Hemp is prepared from the pericyclic fibres of the stem of *Cannabis sativa* (Cannabinaceae). The fibre is composed chiefly of cellulose, but some lignification has usually taken place and the percentage of cellulose is lower than in flax.

The fibre ends, in contrast to those of flax, are bluntly rounded. Some of the fibre ends are forked, this bifurcation arising from injuries to the stem. The lumen of the hemp fibre is flattened or oval, in contrast to the small round lumen of flax. Transverse striations seen in commercial fibres arise from beating, the fibres being prepared by partial retting.

Jute, hemp and flax fibres are compared in Table 20.3.

REGENERATED CARBOHYDRATE MATERIAL AND CHEMICALLY MODIFIED FIBRES

Regenerated fibres are those produced from naturally occurring, long-chain molecules which have been isolated, controlled and, if necessary, modified to give a suitable fibre form. The term 'rayon', as in viscose, acetate and cuprammonium rayon, is applied to those derived from the polysaccharide cellulose. The term 'artificial silk' is now out of date. Also in this class is alginate fibre, derived from alginic acid (q.v.).

Viscose (regenerated cellulose, rayon)

This has been developed from a process introduced by three British chemists (Beadle, Bevan and Cross) in 1892, and accounts for the bulk of the world rayon output today. It is also the principal type used in surgical dressings.

The starting material is a cellulose prepared either from coniferous wood, particularly spruce, or scoured and bleached cotton linters. The wood is usually delignified at source (Canada, Scandinavia, etc.) by a process similar to that used for cellulose wadding. It reaches the rayon manufacturers as boards of white pulp, containing 80–90% of cellulose and some hemicellulose (mainly pentosans). The latter, being alkali-soluble, are removed in the first stage of the process, which consists of steeping in sodium hydroxide solution. After most of the excess alkaline liquor has been pressed out, alkali-cellulose (sodium cellulosate) remains. This is dissolved by treatment with carbon disulphide and sodium hydroxide solution to give a viscous (whence the name 'viscose') solution of sodium cellulose xanthate. After 'ripening' and filtering, the solution is forced through a spinneret, a jet with fine nozzles, immersed in a bath which includes dilute sulphuric acid and sodium sulphate, when the cellulose is regenerated as continuous filaments. These are drawn together as a yarn, which is twisted for strength, desulphurized by removing free sulphur with sodium sulphide, bleached, washed, dried and conditioned to a moisture content of 10%.

The viscose yarn may be left as such (i.e. *continuous filament rayon*) for use in such things as blouse fabrics, or it may be cut up to give *staple rayon* ('Fibro') of fixed length from 1 to 8 in. That used in surgical dressings and many other fabrics is made to resemble cotton in dimensions. Suitable spinnerets are used to give a diameter of 15–20 μm and the fibre is cut into lengths usually of 4.8 cm. This staple can be processed on types of spinning and weaving machines used for cotton dressings or it may be left in a loose fibre form as *viscose rayon absorbent wool*.

Table 20.3 Characters of jute, hemp and flax fibres.

	Jute (*Corchorus* spp.)	Hemp (*Cannabis sativa*)	Flax (*Linum usitatissimum*)
Apex	Bluntly pointed or rounded	Mostly blunt and sometimes forked	Sharply pointed
Wall	Without markings; lumen varying in size	Marked striations, cross fissures, and swellings; lumen large and uniform	Thick wall with fine cross lines some intersecting; lumen narrow
Transverse section	Polygonal, sharp angles; lumen oval or circular	Roughly 3- to 6-sided with rounded corners; lumen cleft or branched	5 or 6 straight sides; point-like lumen
Length	0.8–5 mm	35–40 mm	25–30 mm average, but up to 120 mm
Diameter	10–25 μm	16–50 μm	12–30 μm
Phloroglucinol test	Deep red	Slightly red	Colourless or slight pink
Iodine and sulphuric acid	Yellow throughout	Inner wall blue; middle lamella yellow	Blue or violet
Chlor-zinc-iodine	Yellow	Purple to yellow	Purple to yellow

Viscose rayon is a very pure form of cellulose. It yields a trace of ash which contains sulphur. The cellulose molecules of the original natural material, whether wood or cotton, become more separated from one another in the viscose solution than in the vegetable material and in the regenerated fibre are still less closely packed. Radiography has shown that the side-to-side aggregation of the long-chain molecules is different from that in natural celluloses. The size of the molecules is also reduced, wood cellulose having molecules of the order of 9000 glucose residue units, while those of viscose rayon have only about 450.

Viscose rayon gauze and other rayon dressings have the advantage over cotton dressings in that they show no loss of absorbency on storage.

Macroscopical characters. As normally produced, this rayon is a white, highly lustrous fibre (*natural* or *glossy viscose*). Its tensile strength varies from two-thirds to one-and-a-half times that of cotton. When wetted it loses about 60% of its tensile strength, a proportionately greater loss than is found with cotton. Where more than a certain amount of rayon is used in a dressing, the fabric may be required to be rendered water-repellent (e.g. cotton crêpe bandage).

Microscopical characters. The fibres are solid and transparent and 15–20 μm in diameter. They have a slight twist, and show grooves along their length which are principally caused by the spinnerets being immersed in the regenerating solution (compare nylon). The grooves give a characteristic appearance to the transverse section. The ends of the fibres are abrupt and characteristic. The fibres are clearly seen in chloral hydrate solution or in lactophenol, but are almost invisible in cresol (having the same refractive index of 1.53). They appear bright in polarized light with crossed Nicols.

Chemical tests

1. The fibres give the general tests for vegetable and regenerated carbohydrate fibres.
2. On ignition they behave like cotton; distinction from acetate rayon and alginate yarn (also wool, silk, nylon and glass).
3. With N/50 iodine and sulphuric acid, 80%, they give a blue colour similar to that given by cotton; distinction from acetate rayon, alginate yarn, jute, hemp (also wool, silk, nylon).
4. With ammoniacal copper oxide they behave like absorbent cotton; distinction from acetate rayon, jute (also wool, nylon).
5. Cold sulphuric acid, 60% w/w, dissolves the fibre; distinction from cotton, oxidized cellulose, alginate yarn, flax, jute, hemp (also wool).
6. Warm (40°C) Hydrochloric Acid BP does not cause solution; distinction from acetate rayon (also silk, nylon).
7. Boiling potassium hydroxide solution, 5%, insoluble; distinction from oxidized cellulose (also wool, silk).
8. Shirlastain A produces a bright pink; distinction from cotton, oxidized cellulose, acetate, rayon (also wool, silk, nylon).
9. Phloroglucinol and hydrochloric acid produce no red stain; distinction from jute, hemp and kapok.
10. The fibres, like cotton, are insoluble in acetone, formic acid 90% or phenol 90%; distinction from acetate rayon (also nylon).

Delustring and dyeing of fibres. Rayon and other artificial fibres with a natural lustrous appearance may be delustred by addition of the white pigment titanium oxide to the solution (e.g. viscose) or to the melt (e.g. nylon) before extrusion of the filaments. In this way the pigment is evenly distributed inside each filament and delustring is permanent. These fibres may be similarly 'spun-dyed' by addition of an appropriate dye instead of the titanium oxide. The method results in an exceptional degree of colour fastness.

Matt Viscose (delustrated viscose rayon) is the form normally used in the manufacture of surgical dressings; hence, in general appearance these are very similar to those manufactured from cotton. The individual filaments have the appearance already described, except for the matt white colour and on microscopical examination the pigment particles, which appear black by transmitted light and are scattered throughout the filament. The amount of pigment is controlled by the ash value. Titanium is detected in the ash by dissolving in sulphuric acid, diluting and adding hydrogen peroxide, 3%, when a yellow colour is produced.

Cellulose ethers. These are prepared from purified alkali cellulose derived from cotton linters or delignified wood pulp by the action of caustic soda, as in the initial stages of the production of viscose rayon.

Methylcellulose BP/EP is a whitish, fibrous powder prepared by the action of methyl chloride under pressure on an alkali cellulose, when hydroxyl groups become methylated. A useful grade is that in which two of the three hydroxyl groups of the glucose residue units of the cellulose chain are methylated, and this has the optimum solubility in water. In pharmacy a grade giving a low viscosity is used both to increase the viscosity and to stabilize lotions, suspensions, pastes and some ointments and ophthalmic preparations; one giving a high viscosity is used as a tablet disintegrant. In medicine it is used as a hydrophilic colloid laxative in chronic constipation and can be used in obese persons to curb the appetite, because it gives a feeling of fullness. *Ethylcellulose* is similarly prepared and has like applications.

Carmellose Sodium EP/BP (*sodium carboxymethylcellulose*) is an odourless and tasteless white hygroscopic powder or granules prepared by the action of monochloroacetic acid on alkali cellulose and removal of the byproduct salts. Substitution of hydroxyl groups by carboxymethyl groups occurs over a range depending on the conditions and the cellulose used; there are prescribed limits for the sodium content. It is water-soluble, and a grade giving a medium viscosity contains 0.7 carboxymethyl groups per glucose residue unit. It is insoluble in organic solvents. Its pharmaceutical and medical uses are similar to those of methylcellulose, but as well as being used as a laxative it is a useful antacid.

Carmellose calcium is also official.

Pyroxylin BP (Cellulose nitrate)

Pyroxylin is prepared by the action of nitric and sulphuric acids on wood pulp or cotton linters that have been freed from fatty materials. When dry it is explosive and must be carefully stored, dampened with not less than 25% its weight of isopropyl alcohol or industrial methylated spirits. It is used for making Flexible Collodion BP.

Absorbable haemostatic dressings

The control of bleeding is of vital concern in surgery, and the great disadvantage of the old-type dressing such as a cotton gauze plug is that it has to be removed after bleeding has been checked with a consequent danger of a recurrence of the haemorrhage. Gelatin sponge, oxidized cellulose and alginate dressings overcome this, in that there is no need to remove them after the bleeding has been checked, since they are absorbed by the tissues.

Oxidized cellulose

Oxidized cellulose originated in the USA as a result of the work published by Yackel and Kenyon in 1942. Cotton wool or gauze is treated with nitrogen dioxide until the number of carboxyl groups formed by

the oxidation of the primary alcohol groups of the glucose residue units of the cellulose molecules reaches 16–22%. The original cellulose now has glucuronic acid residue units (compare alginic acid) as well as some glucose residue units.

Appearance. Gauze, lint or knitted material, very similar to normal cotton but with an off-white colour, a harsher texture, charred odour and an acid taste. It does not go pasty on chewing. The wool tends to disintegrate on handling. In microscopical appearance the fibres are very similar to those of absorbent cotton.

Tests

1. Does not give the tests for animal fibres and animal source-haemostatics.
2. On ignition it behaves like normal cotton.
3. With iodine and sulphuric acid or ammoniacal copper oxide solution it behaves like absorbent cotton.
4. Slowly soluble in 80% sulphuric acid.
5. Insoluble in warm hydrochloric acid BP.
6. Soluble in the cold in 5% potassium hydroxide solution.
   Complete solubility in aqueous alkali is made the basis of a test for absence of unchanged cotton and foreign particles. The solution in alkali gives with excess acid a white flocculent precipitate (former *BP* test for identity).
7. It reduces Fehling's solution.
8. Shirlastain A gives a pale blue to mauve colour.
9. Shirlastain C gives a brown to olive green.

Uses. It is used as an absorbable haemostatic in many types of surgery, but is incompatible with pencillin, delays bone repair and cannot be sterilized by heat. It has found some application in chromatography.

Alginate fibres

These originated about 1938 in Britain and were further developed during World War II.

The fibres are prepared by a process similar to that for viscose rayon. An aqueous solution of sodium alginate (see this chapter) is pumped through a spinneret immersed in a bath of calcium chloride solution (acidified with hydrochloric acid), when water-insoluble calcium alignate is precipitated as continuous filaments. These are collected, washed and dried. For use in surgical dressings and bacteriological swabs they are reduced to a staple form which may then be processed to a *calcium alginate wool* or a fabric (e.g. *gauze*) in the same manner as used for viscose staple or cotton.

As indicated in Table 20.2, alginic acid is composed of polymers of both mannuronic and guluronic acids. The properties of the two are variable and alginates of different origin have different compositions and properties. This is illustrated by the two commercial haemostatic dressings—Kalostat (BritCair Limited) and Sorbsan (Steriseal—Pharmaplast Limited). The former is derived from the seaweed *Laminaria hyperborea* collected off the Norwegian coast and yields an alginate with a guluronic:mannuronic ratio of 2:1; the latter is prepared from *Laminaria* and *Ascophyllum* species collected off the west coast of Scotland and gives an alginate with a guluronic:mannuronic acid ratio of about 1:2. On a wound surface the α-linkages of the guluronic acid polymer are not easily broken so that fibre strength is retained and a strong gel is formed on contact with the wound exudate. A high ratio of mannuronic acid polymer (β-linkages) yields a product giving a weaker gel and less retention of fibre strength. In practice this means that the Kalostat dressing can be removed from the wound with forceps and Sorbsan is removed by irrigation with, for example, sodium citrate solution.

Calcium alginate fibres of commerce contain substantial traces of substances used to inhibit mould and bacterial growth in the sodium alginate spinning solution. Spinning lubricants such as lauryl or cetyl pyridinium bromide (antibacterial) are also applied to the filaments. These substances must not be used or must be removed in the case of calcium alginate staple for use in, for example, bacteriological swabs.

Before use as an absorbable haemostatic dressing some calcium alginate dressings must be immersed in sodium chloride to give a fibre of the calcium alginate covered by sodium alginate. The degree of conversion is conditioned to give the desired rate of absorption when in use; the greater the proportion of sodium alginate the faster the absorption rate.

Alginate filaments are composed of salts of the long-chain molecules of alginic acid (see Table 20.2) and there is little cross-linking between the chains in the fibre.

Appearance. Fairly lustrous, pale cream-coloured fibres which in microscopical appearance are very similar to those of viscose rayon, being solid grooved rods. The haemostatic dressing ('Calgitex') is almost tasteless and odourless and rather harsh to touch. The gauze is usually a knitted fabric and has little sheen. That with a fast rate of absorption when chewed readily assumes a pasty form somewhat like that of mashed potato. That with a slow rate of absorption remains smoothly coarser in the same time. They do not disintegrate easily on handling. These points and the tests below will serve to distinguish alginate haemostatic dressings from those of oxidized cellulose. First-aid dressings frequently embody an alginate gauze impregnated with a local anaesthetic.

Tests. These refer to calcium alginate fibre or the mixed sodium and calcium salt fibre. They give the general tests for vegetable and regenerated carbohydrate fibres. For distinctions from rayons and oxidized cellulose see earlier.

1. Smoulders in a flame and goes out when removed from flame.
2. With (N/50) iodine and sulphuric acid, a brownish-red colour is produced, the filaments swell and dissolve to leave a strand of insoluble alginic acid.
3. In ammoniacal copper nitrate solution they swell and dissolve.
4. Insoluble in 60% w/w sulphuric acid.
5. Insoluble in warm (40°C) hydrochloric acid BP.
6. Insoluble in boiling 5% KOH (swell and acquire a yellow tint).
7. Soluble in 5% sodium citrate solution.
8. Fibre, 0.1 g, boiled with 5 ml of water remains insoluble but dissolves when 1 ml 20% w/v sodium carbonate solution is added and boiled for 1 min. A white precipitate of calcium carbonate is formed, depending on the proportion of original calcium alginate present. When centrifuged and the clear supernatant acidified, a gelatinous precipitate of alginic acid is produced. The precipitate will give a purple colour after solution in NaOH and addition of an acid solution of ferric sulphate.
9. Shirlastain A gives a reddish-brown colour.
10. Alginate haemostatic fibres are invisible in polarized light with crossed Nicols.

Uses. The alginate absorbable haemostatic dressings are non-toxic and non-irritant. They have advantages over oxidized cellulose, which include selective rate of absorption, sterilization (and resterilization) by autoclaving or dry heat and compatibility with antibiotics such as penicillin. They may be used internally in neurosurgery, endaural and dental surgery to be subsequently absorbed. Externally, they may be used (e.g. for burns or sites from which skin grafts have been taken)

to arrest bleeding and form a protective dressing which may be left or later removed in a manner appropriate to the type of dressing employed (see above). Protective films of calcium alginate may also be used by painting the injured surface with sodium alginate solution and then spraying it with calcium chloride solution.

Calcium alginate wool as a swab for pathological work or bacterial examination of such things as food processing equipment and tableware has the great advantage over cotton wool in that it permits release of all the organisms by disintegration and solution of the swab in, for example, Ringer's solution containing sodium hexametaphosphate.

In fabrics the calcium alginate fibres would disintegrate in alkaline solutions (laundering), but this advantage is turned to a commercial virtue by the use of the yarn as a scaffolding thread to support yarns normally too fine to survive the weaving process. The scaffold is removed by an alkaline bath to leave a lightweight fabric.

Cellulose wadding

Cellulose wadding was official in the *BP* 1989. It is prepared from high-grade bleached sulphite wood pulp which is received by the manufacturer in the form of boards about 0.75 m square and 1 mm thick. These are packed in bales containing about 180 kg pulp. The pulp is put in a 'beater', where it is mixed with about 20 times its weight of water and the mixture circulates between a power-driven roll and the bed-plate of the 'beater'. The effect of this is to break up the pulp into separate fibres. When this process is complete, the contents of the beater are mixed with a further quantity of water and then allowed to run in a steady flow on to the 'wire' of the paper machines. This 'wire' is a very fine wire gauze through which water runs, leaving a fine web of fibres on top of the 'wire'. This web is then dried and crêped to give a thin, soft, absorbent sheet. About 30 of these thin sheets are laid together to form cellulose wadding.

When examined microscopically, chemical wood pulps or cellulose wadding show characteristic woody elements, which, however, give no lignin reaction (distinction from mechanical wood pulp). Tracheids with bordered pits and characteristic medullary ray cells are usually observed. The cellulose nature of the walls is shown by the blue colour obtained with iodine followed by 80% sulphuric acid and by their solubility in an ammoniacal solution of copper oxide.

STARCHES

Starch constitutes the principal form of carbohydrate reserve in the green plant and is to be found especially in seeds and underground organs. The green parts of plants exposed to sunlight contain small granules of transitional starch which arise from photosynthesis. During the hours of darkness these are removed to the storage organs. Starch occurs in the form of granules (starch grains) the shape and size of which are characteristic of the species as is also the ratio of the content of the principal constituents, amylose and amylopectin.

A number of starches are recognized for pharmaceutical use. They include maize (*Zea mays* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and potato (*Solanum tuberosum* L.). Tapioca or cassava starch (*Manihot utilissima*) may be used in place of the above in tropical and subtropical countries.

The more important commercial starches are listed in Table 20.4.

Table 20.4 Commercial starches.

Family	Plant	Economic product
Cycadaceae	*Zamia floridana*	Florida arrowroot
Gramineae	*Zea mays*	Maize or corn
	Oryza sativa	Rice
	Triticum aestivum	Wheat
	Avena sativa	Oats
	Hordeum sp.	Barley
	Secale cereale	Rye
Palmae	*Metroxylon rumphii*	Sago
Musaceae	*Musa* spp.	Bananas and plantains
Zingiberaceae	*Zingiber officinale*	Ginger
	Curcuma spp.	East Indian arrowroot and turmeric
Cannaceae	*Canna edulis*	Queensland arrowroot or tous les mois
Polygonaceae	*Polygonum fagopyrum*	Buckwheat
Euphorbiaceae	*Manihot utilissima*	Manihot or cassava starch and tapioca
Leguminosae	*Phaseolus vulgaris*	Bean flour
	Ervum lens	Lentil flour
	Pisum sativum	Pea flour
Convolvulaceae	*Ipomoea batatas*	Sweet potato
Solanaceae	*Solanum tuberosum*	Potato

Preparation of starches

Commercial starches, the preparation of which is described below, are not chemically pure and contain small amounts of nitrogenous and inorganic matter.

Many patented processes are in use for particular starches, and the procedure adopted depends on the degree of purity desired and the nature of the compounds from which the starch has to be freed. Cereal starches, for example, have to be freed from cell debris, oil, soluble protein matter and the abundant insoluble proteins (glutelins and prolamins) known as 'gluten'. Potato starch, on the other hand, is associated with vegetable tissue, mineral salts and soluble proteins.

Wheat and similar starches were at one time prepared by kneading the ground material in a stream of water, the gluten remaining as a sticky mass, while the starch separated on standing from the milky washings. The following methods are now employed.

Preparation of maize starch. The grain is first softened by soaking at 50°C for about 2 days in a 0.2% solution of sulphurous acid. This assists disintegration, enabling the embryo or germ to be easily liberated intact and permitting the starch to be readily freed from fibre. During this time lactic acid bacteria are active and metabolize soluble sugars extracted from the maize. The grain, in water, is then distintegrated by attrition mills; these do not break the liberated oil-containing embryos, which, in the older process, were skimmed off. Nowadays the germs are continuously separated from the suspension by liquid cyclones (hydroclones) which operate in batteries of about 12. The germs are used for the preparation of germ oils, which are an important source of vitamins. The remainder of the grain is ground wet and the starch and gluten separated from fibrous material in rotating, slightly inclined stainless steel reels covered with perforated metal sheets. The retained fibre is washed and the total mixture of starch and protein (mill starch) is fractionated into gluten and starch by the use of special starch purification centrifuges; separation depends on the fact that gluten is lighter than starch. In older processes this separation was accomplished by repeated 'tabling', in which the suspension was allowed to flow very slowly through troughs about 40 m long and 0.7 m wide, when the heavier starch was deposited first. The starch suspension from the centrifuge is further purified in other centrifuges and hydroclones, which reduces the protein level.

The subsequent drying process may involve flash dryers or a moving-belt dryer; considerable flexibility in drying time is required to accommodate the various modified starches which are now produced.

Preparation of rice starch. Rice is soaked in successive quantities of 0.4% caustic soda until the material can be easily disintegrated. The softened grain is ground (the compound grains separating into their components), made into a dilute suspension which is repeatedly screened, and the starch separated by standing or by means of a centrifuge. The damp starch is next cut into blocks and dried at 50–60°C for 2 days. The brown outer layer which forms is then scraped from the blocks and drying is continued at a lower temperature for about 14 days, during which time the blocks gradually crack into irregular masses. For pharmaceutical use this 'crystal' starch is powdered.

Preparation of potato starch. The potatoes are washed and reduced to a fine pulp in a rasping machine or in a disintegrator of the hammer-mill type. Much of the cell debris is removed from the pulp by rotary sieves and the milky liquid which passes through the sieve contains starch, soluble proteins and salts, and some cell debris. On standing, the starch separates more rapidly than the other, insoluble, matter and in older processes was purified by techniques resembling the 'tabling' described above; again high-speed centrifugal separators, for use with potato starch (and cassava starch), are now employed for separation and washing. At two or three points during the isolation, sulphur dioxide is added to prevent discoloration of the product by the action of oxidative enzymes. The washed starch is collected, dried to contain about 18% moisture and packaged.

Macroscopical characters. Starch occurs in irregular, angular masses or as a white powder. It is insoluble in cold water but forms a colloidal solution on boiling with about 15 times its weight of water, the solution forming a translucent jelly on cooling. A starch mucilage is coloured deep blue with solution of iodine, the colour disappearing on heating to 93°C but reappearing on cooling. When starches are heated with water, the granules first swell and then undergo gelatinization. The temperatures at which these changes commence and are complete vary with different starches. Starch granules also undergo gelatinization when treated with caustic potash, concentrated solutions of calcium or zinc chlorides, or concentrated solution of chloral hydrate.

Maize starch is neutral, but other commercial starches frequently show an acid (wheat and potato) or alkaline (rice) reaction. The *USP* gives microbial limit tests for *Salmonella* spp. and for *Escherichia coli*.

Microscopical characters. Starches can be identified by microscopical examination. They should be mounted in water or Smith's starch reagent (equal parts of water, glycerin and 50% acetic acid). The size, shape and structure of the starch granules from any particular plant only vary within definite limits, so that it is possible to distinguish between the starches derived from different species. Starch granules may be simple or compound, and the description of a starch granule as 2-, 3-, 4- or 5-compound refers to the number of component granules present in the compound granule. In some cases the compound granule is formed by the aggregation of a large number of simple granules (e.g. rice and cardamoms).

The starting point of formation of the granule in the amyloplast is marked by the hilum, which may be central or eccentric. Granules with an eccentric hilum are usually longer than broad. On drying, fissures often appear in the granule and are seen to originate from the hilum. On microscopical examination, the hilum takes the form of a rounded dot or of a simple, curved or multiple cleft.

The starch granule is built up by the deposition of successive layers around the hilum, and concentric rings or striations are often clearly visible in larger granules, e.g. potato. The striations probably arise from the diurnal deposition of the starch giving variations in refractive index, density and crystallinity. The position and form of the hilum and the presence or absence of well-defined striations are of importance in the characterization of starches.

Some of the more important microscopic characters of the principal starches are set out in Table 20.5 and Figure 20.4.

Table 20.5 Microscopy of starches.

Variety	Form	Size (μm)			Hilum and striations
		Small	Medium	Large	
Maize	Granules from the outer horny endosperm muller-shaped	10	15–25	30	Hilum a central triangular or 2- to 5-stellate cleft. No striations
	Granules from the inner mealy endosperm polyhedral or subspherical	2	10–30	35	
	In commercial starch all the granules are simple				
Wheat	Larger granules lenticular, smaller ones globular. A few compound granules with 2–4 components, which, if separated, are polyhedral	2–9	30–40	45	Hilum a central point, seldom cleft. Concentric but rather faint striations
Rice	Compound granules with an angular outline and from 2 to about 150 components	2	4–6	10	Hilum a central point. No striations
	Component granules polyhedral, with sharp angles				
Potato	Mostly simple granules, hatchet-, wedge-, or mussel-shaped. A few compound granules of 2 or 3 components firmly fused together	2	45–65	110	Hilum in the form of a point; eccentric about 1/3 to 1/4. Concentric striations well-marked; some rings, however, more distinct than others
Tapioca	Mostly simple, subspherical, muller-shaped or round polyhedral	5–10		20–35	Hilum punctate or cleft. Concentric striations

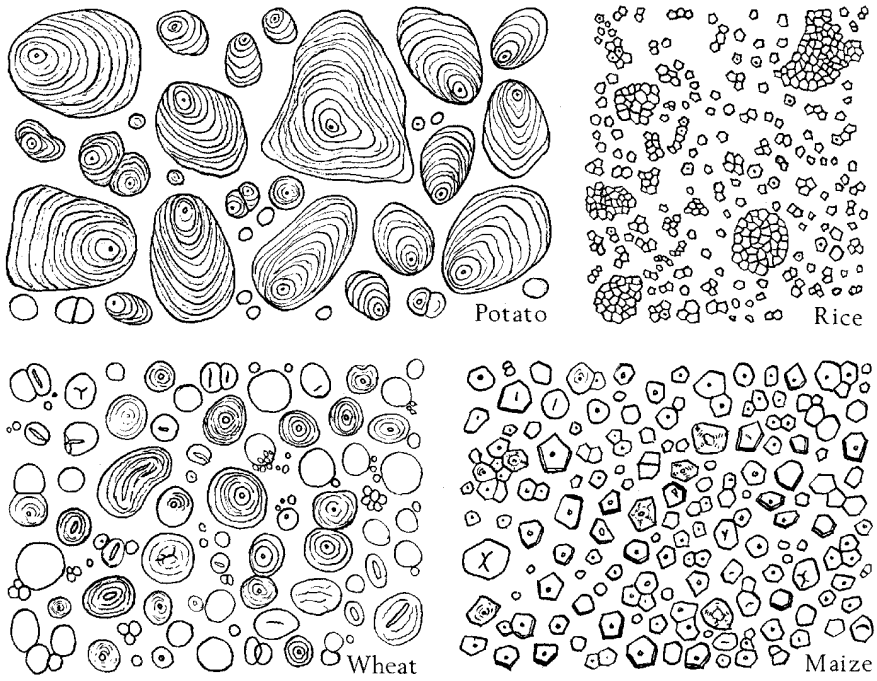


Fig. 20.4
Pharmacopoeial starches. All ×200.

Starch granules show double refraction when examined between crossed Nicols, the granules appearing in the dark field as illuminated objects marked by a dark cross, the bars of which intersect at the hilum.

Starch is required to comply with specified limits for viable microbial contamination including, specifically, *Escherichia coli*.

Chemical composition of starch

Starch granules usually contain two carbohydrates, amylopectin (α-amylose) and amylose (β-amylose); the former constitutes over 80% of most starches. Fractionation of the two components can be achieved by selective precipitation involving the formation of an insoluble complex of amylose with such polar organic substances as butanol or thymol. As indicated in Table 20.2, β-amylose consists essentially of linear chains; these have a helical arrangement with each turn comprising six glucosyl units and giving a diameter of 1.3 nm. Conversely, amylopectin has a branched structure; these differences give the two substances different properties and it is their variation in proportion that contributes towards the distinctive characteristics of a starch from a particular biological source.

Amylose, although water-soluble, gives an unstable solution, which irreversibly precipitates. It is mainly responsible for the deep blue coloration (λ_{max} *c.*660 nm) given by starch and iodine in which the latter as I_5 becomes trapped as an inclusion complex in the amylose helix. The strong affinity of amylose for iodine means that it will take up to 19% of its weight of iodine and this figure can be used in the determination of amylose in starch. Dilute solutions in water or alkali have an appreciable vicosity and the molecule is extensively degraded by β-amylase to maltose. The course of the hydrolytic reaction may be followed: (1) by treating with iodine and observing the colour changes (starch giving a blue; dextrins purple to reddish-brown; maltose, and glucose, if acid hydrolysis, no colour); (2) by testing portions at intervals with Fehling's solution (the amount of reduction increases with the amounts of sugar formed); or (3) by successive measurements of viscosity (viscosity decreases as hydrolysis proceeds). On the other hand, solutions of amylopectin are relatively stable, the colour given with iodine is purple (λ_{max} *c.*540 nm), and the iodine-binding is low. β-Amylase can only attack the outer linear chains, not being able to bypass the 1–6 interchain links; as a consequence, amylopectin is hydrolysed to the extent of 50–60% only by the enzyme; complete hydrolysis is achieved by mineral acids and other enzymes.

A very small amount of covalently bound phosphate appears to be a normal component of starch; its exact location within the molecule is uncertain but may represent the phosphorylation of some 1 in 300 glucose molecules. Cereal starches also contain about 1% of lipid which occupies the same helix space as does added iodine.

Biosynthesis of starch. The final stages of the synthesis of starch are associated with amyloplasts—double membrane organelles which develop, like chloroplasts, from protoplasts. Sucrose appears to be the primary substrate which by the mediation of the reversible sucrose synthase and other enzymes is converted to fructose, glucose-1-phosphate and glucose-6-phosphate in the cytosol. The precise pathway involved and the specific substrate which passes into the amyloplast for the final stages of synthesis are a current area of study. One problem is the difficulty of isolating intact amyloplasts for biochemical study. α-Amylase activity in barley has been extensively studied; the endosperm of germinating maize seeds contains four isozymes of α-amylases (α-amylase-1 to -4) and one isozyme of β-amylase (K. B. Subbarao *et al*., *Phytochemistry*, 1998, **49**, 657). The probable final reactions are indicated in Fig. 20.5. (For a review with 81 refs. see A. M. Smith and K. Denyer, *New Phytologist*, 1992, **122**, 21.)

Mutant varieties. A number of mutant varieties of maize and other crops produce abnormal starch grains some of which have commercial use and possibilities. Thus 'waxy' maize starch contains principally amylopectin producing a tapioca-like starch. It derives its name from the shiny appearance of the broken endosperm. Another mutant, 'amylose extender' is deficient in one of the enzymes responsible for producing the branching of the amylopectin molecule. At least six specific enzyme deficiencies have been identified as associated with abnormal maize starch mutants.

Uses. Starch finds extensive use in dusting powders, in which its absorbent properties are important. In mucilage form it is used as a

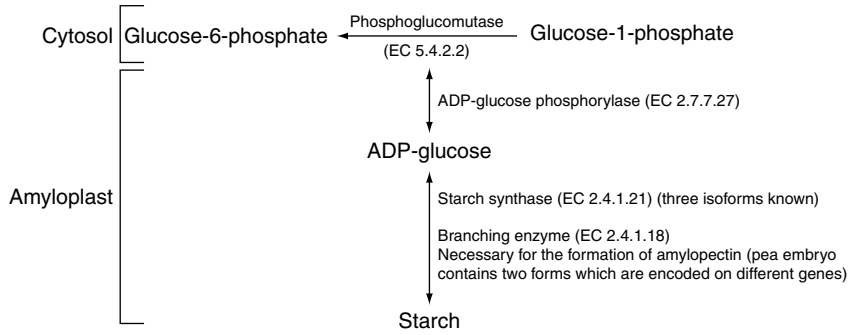


Fig. 20.5
Probable final stages in the biosynthesis of starch.

skin emollient, as a basis for some enemas and as an antidote in the treatment of iodine poisoning. Starch is also used as a tablet disintegrant. For the US food and drinks industry large quantities of maize starch are converted to high-fructose corn syrup by a process involving hydrolysis (glucose producing) and subsequent isomerization. Starch has also provided the plastics industry with a number of new products, including biodegradable polyvinylchloride and polyethylene plastics.

Sterilizable Maize Starch BP is used as a lubricant for surgeons' gloves; it is maize starch subjected to physical and chemical treatments so that it does not gelatinize on exposure to moisture of steam sterilization. Unlike talc, it is completely absorbed by body tissues.

Brazilian arrowroot. This is the starch obtained from the tubers of the sweet potato, *Ipomoea batatas* (Convolvulaceae). The granules are rounded, polyhedral or muller-shaped, the larger ones being 25–55 μm in diameter.

Portland arrowroot. The English hedgerow plant, *Arum maculatum* (Araceae) is known by a number of names, including Lords and Ladies, Cuckoo Pint and Wake Robin. The tuberous rootstock is rich in starch and was formerly extracted to give Portland arrowroot. Used for starching Elizabethan ruffs, it was a cause of dermatitis among laundrymaids.

Modified starches

As with cellulose, the starch molecule can be considerably modified by chemical treatments and some of the products have a use in the paper, food, textile, adhesive and other industries. Treatments include acetylation, hydroxyethylation, phosphorylation, inorganic esterification and cross-linking. For pharmaceutical purposes maranta starch (St Vincent Arrowroot), which is no longer commercially available, has been replaced by an ester starch of cassava which has similar properties.

Pregelatinized starch. This is prepared from maize, rice or potato starch by suitable mechanical rupturing of the grains in the presence of water either with or without heat followed by drying. There are no added materials but the product may be further manipulated to improve flow rate and compressibility properties. It is widely used as a tablet excipient.

Soluble starch. Soluble starch is prepared by treating commercial potato starch with hydrochloric acid until, after washing, it forms a limpid, almost clear solution in hot water. A soluble starch solution should show little reduction with Fehling's solution and gives a deep blue colour with iodine. On heating with 5% potassium hydroxide solution, it gives a canary-yellow colour; no colour is afforded by ordinary starch and the dextrins give a brown colour when similarly treated.

Commercial dextrins. High-grade dextrins are prepared by heating starch which has been moistened with a small quantity of dilute nitric acid and dried, at 110–115°C. The product is known as white dextrin. Inferior dextrins, which have a yellow or brown colour, are prepared by roasting starch at 150–250°C without the addition of acid.

White dextrins may contain up to 15% of soluble starch, the remainder consisting largely of erythrodextrin. Yellow dextrins are more completely hydrolysed and, unlike the white variety, contain appreciable quantities of maltose, which may be detected and estimated by means of Fehling's solution.

FRUCTANS

Fructans are D-fructose polymers each chain being terminated by a single D-glucosyl residue. They are found in nature as oligosaccharides with up to 10 units and as polysaccharides with up to 50 units. The best-known fructan, and the most important pharmaceutically, is inulin, a reserve carbohydrate found in many roots of members of the Compositae and Campanulaceae. The tubers of the Jerusalem artichoke (*Helianthus tuberosus*) and roots of chicory (*Cichorium intybus*) are particularly rich sources. Other fructans are the phleins found in grasses e.g. in *Phleum pratense*, agropyrene in couch grass (q.v.), and sinistrin a component of *Urginea maritima* (q.v.).

Unlike the biosynthesis of starch and cellulose, that of fructan does not originate by the conjugation of identical monosaccharide units but in all cases starts with a molecule of sucrose (glucose + fructose) to which is successively added further molecules of furanofructose. A further distinction from starch biosynthesis is that no monosaccharide nucleotide (cf. glucose adenine diphosphate) is involved in the addition of the fructose units. Various trisaccharides composed of one glucose and two fructose molecules occur in nature and the mode of the linkage of the second fructosyl unit to the sucrose and the extent of the addition of more fructosyl units to the trisaccharide determine the properties of the final polymer. Thus, inulin biosynthesis proceeds via the enzymatic transfer of a fructosyl group from sucrose to another molecule of sucrose giving the trisaccharide 1-ketose and free glucose. A second enzyme (a fructan fructosyl transferase) then mediates the addition of further fructosyl units from other oligomeric fructans. Thus, the final molecule is terminated at one end by a glucosyl unit. However, for the formation of some other fructans different fructosylsucrose trisaccharides are involved and elongation of the polymer chain may occur at either end of the trisaccharide so that the final fructan molecule has a glucosyl residue situated towards the middle of the chain.

INULIN

Inulin *BP*/*EP* is obtained from the tubers of *Dahlia variabilis, Helianthus tuberosus* and other genera of the Compositae; it derives its name from the dahlia, *Inula helenium*, from which it was first isolated in the 19th century. It occurs either in solution in the cell sap (cf. starch

granules which are formed in plastids) or in alcohol-preserved material as sphaerocrystalline masses (Fig. 42.1F). It is sparingly soluble in cold water but readily dissolves at around 70°C without gelatinizing. It is neither stained by iodine solution nor hydrolysed by mammalian enzymes.

Chemically inulin consists of a chain of 35–50 1,2-linked fructofuranose units terminated by one glucose unit. The furanose ring systems render the molecules much less rigid than either those of cellulose or starch. In any sample of inulin there is a mixture of molecular species the smaller molecules being probably intermediates in the polymerizing chain.

BP/*EP* tests include a thin-layer chromatography examination; clarity, colour and specific optical rotation (−36.5° to −40.5°, 2% solution) of solutions and limits for acidity, sulphated ash (0.1%), heavy metals, oxalate etc.

Inulin is not metabolized by the body and is excreted unchanged. As Inulin Injection it is used for the measurement of glomerular filtration rate.

Dandelion root

The root of the dandelion (*Taraxacum officinale*) is an important drug of herbal medicine. Among other constituents it contains up to 40% of carbohydrates, particularly inulin, in the autumn and about 2% inulin in the spring. The fructose content reaches about 18% in the spring. The drug is described in Chapter 29.

ALGAL GELLING AGENTS

The two most important pharmaceutical products in this class are the alginates and agar.

ALGINIC ACID

Large quantities of brown seaweeds are collected from many of the colder waters of the world. Principal producers, approximately in order of quantity, are the USA (California), Norway, Chile, China, Canada (Nova Scotia), Irish Republic, Australia (Tasmania), Iceland, UK (Scotland), South Africa. Some years ago it was reported that the Chinese had developed strains of brown seaweed which would flourish in the warmer East and South China Seas.

The North Atlantic rockweeds (littoral types) (e.g. *Ascophyllum nodosum*) are cut either by hand with sickles or by means of various designs of floating 'combine harvesters'. The remaining world total is mainly storm-cast. After collection, the raw, wet seaweed may either go immediately for processing or be fuel- or sun-dried to 12–17% moisture content, in which form it has an indefinite storage life.

Alginic acid, a hard, horny polysaccharide, was first isolated by the English chemist Stanford (1883) and in Britain was first marketed in 1910; the 1976 estimated world production of alginate was 19507 tonnes. New methods of extraction are continually being patented but the pattern of Stanford's process is still much followed. The dried milled seaweed is macerated with dilute sodium carbonate solution and the resulting pasty mass diluted with sufficient soft water to make practicable the separation of the insoluble matter by modern super-decanters or continuous-settling devices. Soft water is essential to avoid the precipitation of insoluble alginates. The resulting clear liquor, which contains most of the alginate originally present in the algae, may now be treated in one of two ways: (1) it is poured into dilute sulphuric acid or dilute calcium chloride solution, when the insoluble alginic acid or its salt, calcium alginate, is precipitated as a bulky, heavily hydrated gel, from which liquor is removed by roller- or expeller-presses. The product obtained looks and handles like wood pulp. By moving the calcium alginate with constant agitation against a stream of hydrochloric acid, the calcium is removed and the highly swollen pulp of alginic acid is roller-pressed and then neutralized with sodium carbonate to give sodium alginate. (2) The clear liquor can be made to precipitate sodium alginate of high purity by the addition of ethyl alcohol directly or after partial evaporation.

Alginic acid *BP*/*EP* (formula, Table 20.2) is composed of residues of D-mannuronic and L-guluronic acids; the chain length is long and varies (mol. wt. from 35 000 to 1.5×10^6) with the method of isolation and the source of the algae. The degree of polymerization can be varied to meet the properties required. A small proportion of the carbonyls may be neutralized, the pharmacopoeial material having not less than 19.0% and not more than 25.0% carbonyl groups calculated with reference to the dried material. The assay involves back-titration with standardized acid. There are also tests for chlorides, heavy metals, microbial contamination, loss on drying and sulphated ash.

Alginic acid is insoluble in cold water (but swells and absorbs many times its own weight) and slightly soluble in hot water. It is insoluble in most organic solvents. It liberates carbon dioxide from carbonates. With compounds containing ions of alkali metals, or ammonium or magnesium, it reacts to give salts (alginates) which are water-soluble and form viscous solutions typical of hydrophilic colloids. The salts of most other metals are water-insoluble.

The alginates, particularly the sodium salt, have, because of their greater chemical reactivity, certain advantages over agar, starch, pectin, vegetable gums and gelatin. Alginates find applications as stabilizing, thickening, emulsifying, deflocculating, gelling and film- and filament-forming agents in the rubber, paint, textile, dental, food (including ice-cream), cosmetic and pharmaceutical industries. The formulation of creams, ointments, pastes, jellies and tablets are examples in the last-named industry. Alginic acid is also used in tablet and liquid preparations for the control of gastro-oesophageal reflux. Alginate textile fibres and their uses, for example, as absorbable haemostatic dressings have been discussed earlier.

AGAR

Agar (*Japanese Isinglass*) is the dried colloidal concentrate from a decoction of various red algae, particularly species of *Gelidium, Pterocladia* (both Gelidaceae, order Gelidiales), and *Gracilaria* (Gracilariaceae, order Gigartinales). Agar is obtained from Japan (*Gelidium amansii*), Korea, South Africa, both Atlantic and Pacific Coasts of the USA, Chile, Spain and Portugal. Some 6500 tonnes are produced annually, of which about one-third originates from Japan. The genus *Gelidium* provides about 35% of the total source material.

Collection and preparation. On the Japanese coast the algae are largely cultivated in special areas, poles being planted in the sea to form supports on which they develop. From time to time the poles are withdrawn and the algae stripped off. Some are also collected from small boats by means of rakes or shovels, or even by diving. The algae are taken ashore and dried; beaten and shaken to remove sand and shells; bleached by watering and exposure to sunlight, the washing also serving to remove salt. They are then boiled with acidulated water for several hours (about 1 part of dry algae to 55 or 60 parts of water), and the mucilaginous decoction filtered, while hot, through linen. On cooling, a jelly is produced which is cut into bars, these being afterwards forced through wire netting to form strips. The manufacture of agar takes place only in winter (November to February), and moisture

is removed by successively freezing, thawing and drying at about 35°C. In Japan the algae are collected from May to October.

Characters. Agar occurs in two forms: (1) bundles of somewhat agglutinated, translucent, yellowish-white strips, these being about the thickness of leaf gelatin, 4 mm wide and about 60 cm long; (2) coarse powder or flakes. Agar swells in cold water but only a small fraction dissolves. A 1% solution may be made by boiling and a stiff jelly separates from this on cooling. When Japanese agar is not used, jellies of similar stiffness may be obtained by using 0.7% New Zealand, 1% South African or 2% Australian agar.

A nearly boiling 0.2% solution gives no precipitate with an aqueous solution of tannic acid (distinction from gelatin). Agar also differs from gelatin in that it contains no nitrogen and therefore gives no ammonia when heated with soda lime. When hydrolysed by boiling with dilute acid, galactose and sulphate ions are produced, the former reducing Fehling's solution and the latter precipitating with barium chloride. If agar is ashed and the residue, after treatment with dilute hydrochloric acid, examined microscopically, the silica skeletons of diatoms and sponge spicules will be found. More perfect diatoms can often be isolated by centrifuging a 0.5% solution. The large discoid diatom *Arachnoidiscus*, which is about 0.1–0.3 mm in diameter, species of *Grammatophora* and *Cocconeis*, and sponge spicules are readily discernible in the ash of Japanese agar (see Fig. 20.6). Powdered agar is distinguished from powdered acacia and tragacanth by giving a deep crimson to brown colour with 0.05 M iodine and by staining pink when mounted in a solution of ruthenium red.

Agar *BP*/*EP* is required to comply with tests for the absence of *Escherichia coli* and *Salmonella*, and general microbial contamination should not exceed a level of 10^3 microorganisms per g^{-1} as determined by a plate count. It has a swelling index (q.v.) of not less than 10 and the determined value must be quoted on the product label.

Constituents. Agar has long been known to yield on hydrolysis D- and L-galactose and sulphate ions. It is now known to be a heterogeneous polysaccharide the two principal constituents of which are agarose and agaropectin. Agarose is a neutral galactose polymer (free from sulphate) which is principally responsible for the gel strength of agar. It consists of alternate residues of 3,6-anhydro-L-galactose and -D-galactose (the disaccharide known as agarobiose). The structure of agaropectin, responsible for the viscosity of agar solutions, is less well established, but it appears to be a sulphonated polysaccharide in which galactose and uronic acid units are partly esterified with sulphuric acid. Pure agarose is commercially available and its gels are recommended for the electrophoresis of, for example, proteins.

Uses. Agar is used in the preparation of culture media, as an emulsifying agent and in the treatment of chronic constipation. Both agar and agarose find extensive use in affinity chromatography (q.v.).

Irish Moss

Chondrus (*Carrageen*) is obtained from the variable red alga *Chondrus crispus* and to some extent from *Gigartina stellata* (Gigartinaceae). Commercial supplies are derived from the north and north-west coast of Ireland, from Brittany and from the Massachusetts coast south of Boston.

Collection and preparation. The algae grow on rocks just below low-water mark, being covered by about 5 or 7 m of water at high tide. In Ireland collection takes place during the autumn; in America, during the summer. The collectors put out in small boats at about half-tide and, after detaching a load of algae from the rocks by means of long rakes, return with them at half-flood. Carrageen is bleached by spreading it on the shore and submitting it for some weeks to the action of sun and dew with about four or five soakings in seawater at suitable intervals. Bleaching by sulphur dioxide has not proved particularly satisfactory. After drying in sheds, the drug is packed in bales weighing up to 300 kg. World production of Irish moss is estimated to be around 20 000 tonnes.

Carrageenan *USP*/*NF* (1995) is obtained from red seaweeds by extraction with water or aqueous alkali and recovered by alcoholic precipitation, drum drying or by freezing.

Characters. Chondrus when fresh varies in colour from purplish-red to purplish-brown, but the bleached drug is yellowish-white, translucent and horny. It consists of complete, dichotomously branched thalli about 5–15 cm long and of very variable form, some thalli having broad fan-like segments, others having ribbon-like ones. Many samples of Chondrus contain large quantities of the related alga *Gigartina mamillosa*, the mixture being officially sanctioned in many pharmacopoeias. In some districts (e.g. south of Boston) almost pure *Chondrus crispus* may be collected, while in others (e.g. north of Boston) it is almost invariably closely associated with *G. mamillosa*. These algae may be distinguished from one another by the form of their large compound cystocarps, which contain carpospores. Chondrus has oval cystocarps about 2 mm long which are sunk in the thallus, while *G. mamillosa* has peg-like ones about 2–5 mm long, as also has *G. pistillata*. The latter species is rare round the coast of Britain, and its presence would indicate a drug of French origin.

Chondrus is sometimes covered with calcareous matter which effervesces with hydrochloric acid. The drug has a slight odour, and a mucilaginous and saline taste.

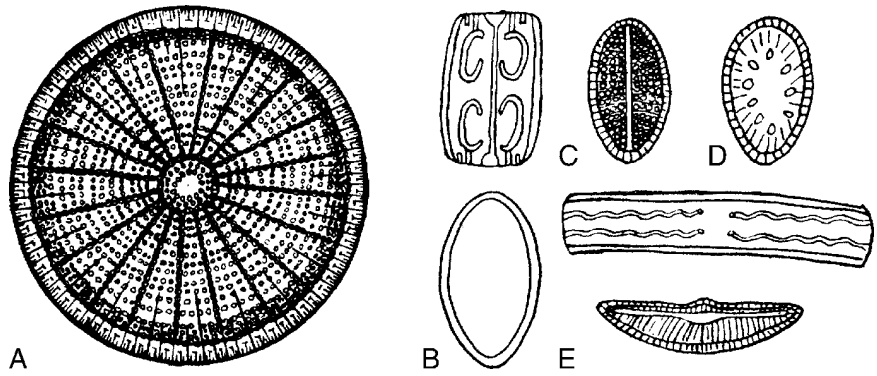


Fig. 20.6
Diatoms associated with Japanese agar. A, *Arachnoidiscus* (diameter 130 µm); B, species of *Grammatophora*; C, *Cocconeis*; D, *Campyloneis*; E, other diatoms.

Chondrus swells in cold water, about 47% slowly dissolving, while on boiling about 75% passes into solution. A 5% decoction forms a jelly on cooling. A cooled 0.3% solution gives no precipitate with solution of tannic acid (distinction from gelatin), and gives no blue colour with iodine (distinction from Iceland moss and absence of starch).

Constituents. The constituents of chondrus resemble those of agar. At least five galactans, known as carrageenans, are present. Three important ones are κ-, ι- and λ-carrageenans, which differ in the amount of 3,6-anhydro-D-galactose they contain and in the number and position of the ester sulphate groups. Like other members of the Gigartinaceae *C. crispus* produces different carrageenans in the two phases of its life cycle; κ-carrageenan predominates in the gametophyte generation and λ-carrageenan in the diploid tetrasporophyte generation (for research on the heterogeneity of both types of plants see B. Matsuhiro and C. C. Urzua, *Phytochemistry*, 1992, **31**, 531). The drug is rich in halogen salts, and, according to Schulzen (1964) the extract differs from that of agar in that it has a higher sulphate and ash content.

Uses. Chondrus is used as an emulsifying agent for cod-liver oil and other oils, as a gelling agent, and as a binder in toothpastes. Its many technical uses involve mainly the food industry.

GUMS AND MUCILAGES

Gums and mucilages have similar constitutions and on hydrolysis yield a mixture of sugars and uronic acids. Gums are considered to be pathological products formed upon injury of the plant or owing to unfavourable conditions, such as drought, by a breakdown of cell walls (extracellular formation; gummosis). Conversely, mucilages are generally normal products of metabolism formed within the cell (intracellular formation) and may represent storage material, a water-storage reservoir or a protection for germinating seeds. They are often found in quantity in the epidermal cells of leaves, e.g. senna, in seed coats (linseed, psyllium etc.), roots (marshmallow) and barks (slippery elm).

TRAGACANTH

The *BP*/*EP* defines Tragacanth as 'the air-hardened gummy exudate, flowing naturally or obtained by incision, from the trunk and branches of *Astragalus gummifer* Labillardière and certain other species of *Astragalus* from Western Asia'. The genus (Leguminosae) contains some 2000 species and those that yield gum are chiefly thorny shrubs found in the mountainous districts of Anatolia, Syria, Iraq, Iran and the former USSR. So-called Persian tragacanth has been traditionally employed in the UK, with Anatolian tragacanth finding a considerable market on the continent of Europe. The term Persian tragacanth is used by pharmacists to denote the better grades of tragacanth produced in Iran and Turkish Kurdistan.

Formation. The mode of formation of tragacanth is entirely different from that of acacia, the gum exuding immediately after injury and therefore being preformed in the plant, whereas acacia is slowly produced after injury. A section of a tragacanth stem shows that the cell walls of the pith and medullary rays are gradually transformed into gum, the change being termed 'gummosis'. The gum absorbs water and a considerable pressure is set up within the stem. Hanbury, having cut off branches of living plants, stated, 'there immediately exudes from the centre a stream of soft, solid tragacanth, pushing itself out like a worm, to the length of three-quarters of an inch, sometimes in the course of half an hour'.

Botanical sources. The requirement for precise botanical specifications and satisfactory analytical procedures for tragacanth, necessitated by the legal aspects covering its use as a food additive, has rendered the above *BP*/*EP* definition somewhat inadequate. A survey of the Turkish gum-producing species has indicated that *A. microcephalus* is the principal species employed with smaller amounts of *A. gummifer* and *A. kurdicus* being collected. Also, in an investigation of tragacanth production in Iran in 1957, Gentry (*Econ. Bot*., 1957, **11**, 40) reported *A. echidnaeformis, A. gossypinus* and *A. microcephalus* to be important species. The approximate distribution of a number of gum-producing species found in the areas where tragacanth is collected is shown in Table 20.6.

Collection. Most of the plants from which tragacanth is collected grow at an altitude of 1000–3000 m. The shrubs are very thorny; each of their compound leaves has a stout, sharply pointed rachis which persists after the fall of the leaflets. The mode of collection varies somewhat in different districts, but the following details of collection in the province of Fars are typical.

Gum can be obtained from the plants in their first year but is then said to be of poor quality and unfit for commercial use. The plants are therefore tapped in the second year. The earth is taken away from the base to a depth of 5 cm and the exposed part is incised with a sharp knife having a thin cutting edge. A wedge-shaped piece of wood is used by the collector to force open the incision so that the gum will exude more freely. The wedge is generally left in the cut for some 12–24 h before being withdrawn. The gum exudes and is collected 2 days after the incision. Some of the plants are burned at the top after having had the incision made. The plant then sickens and gives off a greater quantity of gum. However, this practice is not universal, as many plants cannot recover their strength and are killed by the burning. The gum obtained after burning is of lower quality than that obtained by incision only, and is reddish and dirty looking. The crop becomes available in August–September.

Grades. Tragacanth is graded into several qualities by the exporter or middle man. The best grades form the official drug, while the lower grades are used in the food, textile and other industries; their approximate relative values are listed in Table 20.7.

Tragacanth is an expensive commodity; not only has the supply situation increased the price, but also the extra treatment and tests required to meet the *BP*/*EP* microbial requirements have added to the cost.

Table 20.6 Distribution of gum-yielding *Astragalus* species.

Species	Geographical distribution
A. gummifer	Anatolia and Syria
A. kurdicus	Northern Iraq, Turkey and Syria
A. brachycalyx	Western and S.W. Iran
A. eriostylus	S.W. Iran
A. verus	Western Iran
A. leioclados	Western and central Iran
A. echidnaeformis	Isfahan region of Iran
A. gossypinus	Isfahan region of Iran
A. microcephalus (syn. *A. pycnocladus*)	Shiraz and Kerman regions of Iran, Turkey
A. adscendens	South western and southern Iran
A. strobiliferus	Eastern Iran
A. heratensis	Khorasan to Afghanistan

Table 20.7 Grades of tragacanth.

Grade		Description	Relative value
Ribbon	No. 1	Fine flat druggists' ribbon	100
	No. 2	White flat druggists' ribbon	93
	No. 3	Light-cream curly ribbon	82
	No. 4	Mid-cream flat ribbon	62
	No. 5	Pinkish mixed ribbon	41
Flake	No. 26	Mid-cream thin flake	38
	No. 27	Amber thick flake	34
	No. 28	Amber-brown thick flake	29
	No. 55	Reddish-brown mixed hoggy flake	26

The ribbon grades are stated to comply with the *BP/EP* requirements; it is principally the flake (26 and down) from Turkey that is available in quantity.

Characters. The official Persian tragacanth occurs in flattened ribbons up to 25 mm long and 12 mm wide. The surface shows a number of ridges which indicates the successive, temporary stoppages of flow from the incision. Fine furrows parallel to the margin of the flake are produced by the uneven edges of the incision. The gum is white or very pale yellowish-white in colour, translucent and horny. It breaks with a short fracture, is odourless and has little taste.

Tragacanth swells into a gelatinous mass when placed in water, but only a small portion dissolves. On the addition of a dilute solution of iodine to a fragment previously soaked in water, relatively few blue points are visible (distinction from Smyrna tragacanth, which contains more starch). With stronger iodine solution the gum acquires a greenish colour (cf. 'Agar').

Constituents. Tragacanth consists of a water-soluble fraction known as tragacanthin and a water-insoluble fraction known as bassorin; they have molecular weights of the order of 840 000. Both are insoluble in alcohol. Tragacanthin and bassorin may be separated by ordinary filtration of an extremely dilute mucilage and the tragacanthin may be estimated by the evaporation of an aliquot portion of the filtrate. Tests show that the best grades of gum contain the least tragacanthin. If the tragacanthin content and moisture content are known, the amount of bassorin may be calculated.

Like other gums, tragacanth is composed of sugar and uronic acid units. Among the products of hydrolysis galacturonic acid, D-galactopyranose, L-arabinofuranose and D-xylopyranose have been identified. C. A. Tischer *et al.*, have reported on the structure of the arabinogalactan fraction (*Carbohydr. Res.*, 2002, **337**, 1647). The *BP*/*EP* thin-layer chromatography test for identification is based on the products of acid hydrolysis of the gum. Von Fellenberg (1918) pointed out that in the tragacanthin fraction there are no methoxyl groups, but that the bassorin fraction contained about 5.38% methoxyl. Rowson (1937) showed that gums with high methoxyl contents and high bassorin contents gave the most viscous mucilages. Heating or fine powdering produces demethylation with loss of viscosity. Anderson and Bridgeman (*Phytochemistry*, 1985, **24**, 2301) showed that the gum-exudates of the three principal species of Turkish gum-producers are proteinaceous polysaccharides and represent a protein content of about 3–4%, involving 18 amino acids. The relative amino-acid proportions differed in the three gums.

The *BP*/*EP* includes a test for minimum apparent viscosity of the powdered gum, a *flow time* for gum to be used in the preparation of emulsions, and a microbial limit test, together with compliance tests for *Escherichia coli* and *Salmonella*. Peroxidase enzymes are usually considered to be absent, but their presence has been detected in commercial samples of genuine drug. The presence of peroxidase enzymes appears to be related to a high starch content; both disappear as gummosis proceeds.

Uses. Tragacanth is used in pharmacy as a suspending agent for insoluble powders, etc., or as a binding agent in pills and tablets. As substitutes become available, its use in the food industry is declining.

Allied drugs

Non-pharmaceutical grades of tragacanth. Large quantities of tragacanth of the lower grades are imported and used in the textile industry and pickle manufacture. The pieces vary in shape and are from a yellow ivory colour to almost black. The lower grades are much contaminated with earth, and their ashes give a strong reaction for iron. The viscosity of mucilages prepared from these grades of tragacanth falls rapidly from the No. 1 to the No. 55, the marked difference in price being fully justified. The lower grades of tragacanth are known as *hog gum* or *hog tragacanth*.

Acacia gum admixed with tragacanth can be detected by TLC of the hydrolysed sample—the presence of rhamnose indicates adulteration.

Chitral gum of Indian commerce is said to be obtained from *Astragalus strobiliferus*.

Sterculia gum (*Karaya gum*) is itself an important article of commerce and is described below. Its presence in tragacanth gum is detected by the gel formation in alcoholic solution and by an acidity test.

Insoluble Shiraz gum is a gum of doubtful botanical origin imported from Iran. When of good quality, it resembles a mixture of bleached and natural Kordofan acacia. It may be distinguished from tragacanth by the fact that it contains no starch and that it gives a reaction for oxidase enzyme.

STERCULIA GUM

Sterculia (*Karaya Gum, Indian Tragacanth, Bassora Tragacanth*) is the dried gummy exudate obtained from the tree *Sterculia urens* (Sterculiaceae). It is produced in India, Pakistan and, to a small extent, in Africa. The gum is of relatively recent introduction, being generally regarded during the early part of the last century as an adulterant and inferior substitute for tragacanth. Now, however, having been shown to be superior to other gums in certain respects, it constitutes an integral part of the gum industry and is official in the *BP*.

Collection and preparation. In central and northern India (Andhra Pradesh, Madhya Pradesh, Rajasthan and Uttar Pradesh) two collections are made each year, before and after the monsoon season: in April–June and in September, respectively. The first collection gives a gum affording the highest viscosity. Blazes, up to about one square foot in area, are made in the larger trees (smaller trees are tapped) and the gum immediately starts to exude; the flow is greatest during the first 24 h and continues for several days. The dried, irregular masses, weighing up to several pounds, are picked off and transported to village centres for purchase by Mumbai merchants. The Indian merchants remove excess bark and roughly sort the gum into two grades; it is further graded in Europe and the USA according to colour and presence of foreign organic matter (mainly bark). It is finally sold as a granulated (crystal), or finely powdered, product.

Unfortunately, in some areas over-production and destructive tapping methods have led to a serious decline in the natural tree population and have necessitated the introduction of 10-year bans on collection.

This has stimulated research on *in vitro* propagation using seedling explants, nodal explants and somatic embryogenesis (S. D. Purohit and A. Dave, *Plant Cell Reports*, 1996, **15**, 704; V. G. Sunnichan *et al*., *ibid*., 1998, **17**, 951).

Characters. Good-quality gum occurs in irregular almost colourless, translucent, striated masses weighing up to 25 g or more. Medium grades have a marked pinkish tinge, while the lower grades are very dark and contain a considerable amount of bark. Karaya gum has a marked odour of acetic acid, and when hydrolysed with 5% phosphoric acid, has a volatile acidity (*BP*) of not less than 14% (tragacanth, about 2–3%). According to Janot and Gounard (1938), sterculia has a methoxyl value of 0 (tragacanth 30–40). When boiled with solution of potash, it becomes slightly brownish (tragacanth, canary yellow). Karaya also differs from tragacanth, in that it contains no starch and stains pink with solution of ruthenium red.

In water, sterculia gum has low solubility but swells to many times its original volume. This means that the processing of the gum influences the final product—the coarser granulated grades give a discontinuous grainy dispersion, whereas the fine powder affords an apparently homogeneous dispersion.

Constituents. Partial acid hydrolysis of sterculia yields D-galactose, L-rhamnose, D-galacturonic acid, aldobiouronic acids, an acid trisaccharide and acetic acid; the galacturonic acid and rhamnose units are the branching points within the molecule. Uronic acid residues represent about 37% of the gum.

Uses. The granular grades are used as a bulk laxative, being second only to psyllium seed in use in this respect. The powdered gum is used in lozenges, pastes and denture fixative powders, and it has proved particularly useful as an adhesive for stoma appliances. As a bulk laxative and stimulant it is available, with frangula, as granules.

ACACIA GUM

Acacia (*Gum Arabic*) is a dried gum obtained from the stem and branches of *Acacia senegal* Wild, other species of *Acacia* of African origin and *Acacia seyal* Del. (Leguminosae). *A. senegal* is a tree about 6 m high, which is abundant in the Sudan, particularly in the province of Kordofan, in central Africa and in West Africa. The tree is known in Kordofan as *Hashab* and in Senegambia as *Verek*. The best gum is that produced in Kordofan from tapped trees, but some Senegal and Nigerian gum is of good quality. Apart from 'acacia gardens', wild, self-sown plants are the main source of the gum.

History. Gum was brought from the Gulf of Aden to Egypt in the seventeenth century BC, and in the works of Theophrastus it is spoken of as a product of Upper Egypt. The West African product was imported by the Portuguese in the fifteenth century. Previously, commerce in the Sudan was in the hands of a number of local merchants, but it is now entirely controlled by the 'Gum Arabic Company Ltd', a concessional company set up by the Sudanese Government.

Collection and preparation. Some gum exudes from trees as a result of the cracking of the bark, but the most esteemed, Kordofan variety, is obtained from trees, about 6 years old, tapped in February and March, or earlier, in September after the rains, when the leaves fall. The tapper, with a blow from a small axe, makes a transverse incision in a branch and so twists the axe that the bark is loosened, strips of it being then pulled off above and below the cut. The portion of branch so bared to the cambium measures about 0.5–1.0 m long and 5–7.5 cm wide. This cambium produces new phloem and in about 20–30 days the tears of gum which have formed on the surface may be picked off. The gum is collected in leather bags and is conveyed in sacks to El Obeid and other centres, mostly located along the railway. Here the gum is garbled to free it from sand and vegetable debris, and is sorted. Other acacia gums such as talka gum, the product obtained from *A. seyal* (the talka of the Arabs), are also separated. At one time some of the gum was 'ripened' by exposure to the sun, when it became bleached and dried, developing numerous cracks. During this process, which took 3–4 months, the gum lost about 30% of its weight. Subsequently, this bleached gum became unobtainable.

From El Obeid the drug is sent by rail to Port Sudan, whence large quantities are shipped to London, the USA and other countries. In the London Market Reports three grades are usually quoted, namely handpicked selected (h.p.s.) Kordofan, cleaned Kordofan and talka. Gum arabic is sold in one currency only ($US), so that currency changes also affect the price. The Senegal acacia gum is largely used for pharmaceutical purposes on the Continent and is shipped to Marseilles and Bordeaux. This also occurs in three grades, namely 'gomme du bas du fleuve', 'gomme du haut du fleuve' and 'gomme friable'.

For comments on the impact of the recent Sudan war on the gum arabic industry, see K. Purcell, *HerbalGram*, 2005, **65**, 25; K. Purcell and N. Dennis, *HerbalGram*, 2006, **71**, 24.

Spray-dried acacia, produced by the importers, is becoming of increasing importance and is included as a monograph in the *BP*. In addition to its general usefulness, it has the further advantage of a low viable bacterial count (see below).

Characters. Bleached Kordofan acacia, when available, occurs in rounded or ovoid tears up to about 3 cm diameter, or in angular fragments. The outer surface bears numerous fine cracks which form during the 'ripening' and make the tears opaque. The gum is white or very pale yellow in colour. The tears break rapidly with a somewhat glassy fracture, and much of the drug consists of small pieces. It is odourless and has a bland and mucilaginous taste.

Cleaned and h.p.s. Kordofan gum differs from the above in having fewer cracks which causes it to be more transparent, and in being more yellowish or pinkish in colour. The tears are usually of less uniform size, some being quite small, while others have a diameter of 4 cm or more. The better qualities of Senegal gum closely resemble the Kordofan, but some of the tears are vermiform in shape and, speaking generally, the gum is rather more yellowish in colour.

Tests. Acacia is almost completely soluble in an equal weight of water, solution taking place rather slowly. The solution is slightly acid and becomes more so on keeping, especially if hot water is used to make the solution. It is viscid, but not glairy, and when diluted does not deposit on standing.

A 10% aqueous solution is laevorotatory, gives no precipitate with dilute solution of lead acetate (distinction from tragacanth and agar), gives no colour with solution of iodine (absence of starch and dextrin), and, if of pharmacopoeial quality, gives no reaction for tannin with ferric chloride. A very weak solution precipitates with lead subacetate solution. The mucilage gives a blue colour when treated with solution of benzidine and a few drops of hydrogen peroxide, which indicates the presence of a peroxidase (possible distinction from tragacanth). Because benzidine has carcinogenetic properties, this test is no longer advocated; however, as some pharmaceutical grades of tragacanth have now been shown to contain a peroxidase enzyme system (q.v.), the test has probably less significance than was previously considered. A tincture of guaiacum can also be used to test for the enzyme.

The *BP*/*EP* thin-layer chromatography test involves fractionation of an acid hydrolysate of the gum and visualization of the separated sugars with anisaldehyde reagent followed by heating.

There are official tests for compliance with limits for microbial contamination (total viable aerobic count, *Escherichia coli* and in the *USP/NF, Salmonella* spp.).

Constituents. Acacia consists mainly of arabin, the calcium (with traces of magnesium and potassium) salt of arabic acid. Arabic acid may be prepared by acidifying a mucilage with hydrochloric acid and dialysing.

When hydrolysed with dilute sulphuric acid, it yields L-rhamnopyranose, D-galactopyranose, L-arabinofuranose and the aldobionic acid 6-β-D-glucuronosido-D-galactose. This major polysaccharide fraction consists of branched β-(1,3)-linked galactose units with side-chains of arabinose, rhamnose and uronic acids linked through the 1,6-positions. A second component of the gum is a hydroxyproline-rich glycoprotein of high molecular weight; it has a high amino acid composition with a repetitive polypeptide backbone. For further studies on this fraction, see L. J. Goodrum *et al.*, *Phytochemistry*, 2000, **54**, 99.

The glycan composition of the gum is variable, depending on source. Thus that derived from *A. senegal* contains a high proportion of D-galactose relative to L-arabinose, whereas the reverse holds for the gum obtained from *A. seyal*. Also, the latter contains significantly more 4-*O*-methyl-D-glucuronic acid but less L-rhamnose and unsubstituted D-glucuronic acid than does the gum from *A. senegal*.

Acacia also contains an oxidase enzyme and about 14% of water. It yields about 2.7–4% of ash.

The gum is formed in the cambial region of the plant with gum-containing cysts developing in the inner bark (J.-P. Joseleau and G. Ullmann, *Phytochemistry*, 1990, **29**, 3401).

Hairy root cultures. These produce a mucilage with a different polysaccharide composition to that of the parent plant.

Uses. As a general stabilizer in emulsions and as a pharmaceutical necessity in lozenges, etc. Its demulcent properties are employed in various cough, diarrhoea and throat preparations but it is incompatible with readily oxidized materials such as phenols, and the vitamin A of cod-liver oil. It has widespread use in the food, drinks and other industries.

Allied drugs. *Talka gum* is usually much broken and of very variable composition, some of the tears being almost colourless and others brown.

Ghatti or *Indian gum* is derived from *Anogeissus latifolia* (Combretaceae). It is produced in much the same localities as sterculia gum, and is harvested and prepared in a similar manner. It resembles talka in possessing tears of various colours. Some of the tears are vermiform in shape and their surface shows fewer cracks than even the natural acacia. Aqueous dispersions of the gum have a viscosity intermediate between those of acacia and sterculia gums.

West African Gum Combretum, obtained from *Combretum nigricans*, is not permitted as a food additive but is exploited as an adulterant of gum arabic. Unlike the latter in which the rhamnose and uronic acid units are chain terminal, in gum combretum these moieties are located within the polysaccharides chain. The leaves of this plant contain cytotoxic dammar-3-one pentacyclic triterpene derivatives (G. Simon *et al*., *Fitoterapia*, 2003, **74**, 339).

Many other gums of the acacia type are occasionally met with in commerce, and many gum exudates of the large genus *Acacia* have been given chemotaxonomic consideration.

GUAR GUM

Guar *BP*/*EP* is obtained from the ground endosperms of the leguminous plant *Cyamopsis tetragonolobus* (L.) Taub., a species cultivated in India as a fodder crop. The gum is a white or off-white powder which readily forms a mucilage with water. Examined under the microscope the powder, in a glycerol mountant, shows the thick-walled endosperm cells with granular contents.

The principal constituent of the gum is a galactomannan which on hydrolysis gives galactose and mannose; these sugars of the hydrolysate constitute the basis of the pharmacopoeial thin-layer chromatography test for the drug. Other tests refer to the absence of other gums, viscosity, loss on drying, ash and microbial contamination.

Fatty acids, both free and combined as esters, have been reported by GLC-MS analysis.

Guar is available as an oral hypoglycaemic drug; it produces changes in gastric emptying and in the gastrointestinal transition time, which can delay absorption of sugars and oligosaccharides from the gut. Guar also lowers cholesterol levels, possibly by binding bile salts in the gut. However its efficacy in the treatment of diabetes is not considered by all to be fully proven. The gum, with 5–6 times the thickening power of starch, is also used in the food industry.

Guar galactomannan. *BP*/*EP* consists of the ground endosperms of *Cyamopsis tetragonolobus* which have been subjected to partial hydrolysis. Tests for identity and purity are given in the *Pharmacopoeia*. It is classed as a pharmaceutical aid.

XANTHAN GUM

This gum is produced artificially by the pure culture fermentation of the bacterium *Xanthomonas campestris* on glucose. It, like cellulose, consists of 1,4-β-glycosidically linked chains of glucose with, additionally, trisaccharide side-chains on alternating anhydroglucose units. These side-chains are composed of two mannose units which encompass a glucuronic acid unit. Pyruvate groups are attached to most of the terminal units with acetyl groups at the C-6 of a number of the mannose moieties next to the glucose chain. The *BP*/*EP* assay is based on the amount of the pyruvic acid produced by the acid hydrolysis of the gum and should correspond to a content of $\prec$1.5%.

Other tests include a limit for 2-propanol (750 ppm as determined by gas chromatography), foreign polysaccharides, a limit of 10^3 bacteria and 10^2 fungi per gram, and a total-ash range of 6.5–16.0% consistent with xanthan gum occurring as the sodium, potassium or calcium salt.

Xanthan gum is used as a pharmaceutical aid, and also finds use in the food and cosmetics industries.

Dextran. This is another microbial product, produced by species of *Leuconostoc, Klebsiella, Acetobacter* and *Streptococcus*. It is an α-1,6-glucan and is used as a replacement for blood plasma and as an absorbent in biochemical analysis.

PSYLLIUM

(Flea Seed)

The dried, ripe seeds of *Plantago afra* (*P. psyllium*), *P. indica* (*P. arenaria*) and *P. ovata* (Plantaginaceae) are used in medicine. The *US National Formulary* includes all three species under the name 'Plantago Seed'. The *BP*/*EP* describes the seeds of the first two species under the title 'Psyllium' and the husks of seeds of *P. ovata* are included under 'Ispaghula Husk'. The latter consists of the epidermis and collapsed adjacent layers removed from the ripe seeds.

The seeds of *P. afra* and *P. indica* are known in commerce as Spanish or French psyllium, while those of *P. ovata* are known as blond psyllium, ispaghula, spogel seeds or Indian plantage seeds.

Characters. Some of the more important characters of these seeds are given in Table 20.8.

Constituents. All the seeds contain mucilage in the epidermis of the testa. The seeds may be evaluated by measuring the volume of mucilage produced after shaking the seeds with water and allowing to stand (see *swelling index*, Chapter 16).

Two fractions have been separated from the mucilage; one is soluble in cold water, and the other in hot water giving a highly viscous solution which gels on cooling. On hydrolysis fractions yield D-xylose, L-arabinose and aldobiuronic acid. The seeds also contain fixed oil, aucubin glycoside, various bases, sugars, sterols and protein. The aucubin content differs appreciably in different seed samples and species.

Allied drugs. Wild seeds of *P. ovata* and related species are reported to contain less mucilage than the cultivated variety. *P. asiatica*, a species used in Chinese medicine, contains mucilage the backbone chain of which is composed of β-1,4-linked D-xylopyranose residues having three kinds of branches.

Seeds of *P. major* are reportedly used as a medicinal substitute for *P. ovata*; the seed oil contains an unusual hydroxyolefinic acid. For a review of the traditional uses, chemical constitutents and biological activities of the *P. major* plant see A. B. Samuelsen, *J. Ethnopharm*., 2000, **71**, 1.

Uses. Plantago seeds are used as demulcents and in the treatment of chronic constipation. Ispaghula husk is used for similar purposes but has a higher swelling factor (40–90).

MARSHMALLOW LEAF

Marshmallow leaf *BP*/*EP* is the whole, cut or dried leaf of *Althaea officinalis* L. The alternate petiolate leaves, about 7–10 cm long, arise from tall, erect, velvety, stems; the lower leaves are roundish, 3–8 cm across, and the upper ones are narrower and ovate; both are slightly 3–5 lobed, the upper more deeply so, and folded, toothed and covered with a soft velvety down. Microscopically, the powdered drug exhibits numerous long, unicellular trichomes, a few glandular trichomes, anomocytic or paracytic stomata, cluster crystals of calcium oxalate and mucilage-containing parenchymatous cells staining red with ruthenium red solution.

As with marshmallow root, mucilage is the effective constituent, giving the leaves emollient, slightly laxative and antitussive properties. Traditionally, the leaves have been used as a poultice for abcesses. Medicinally, non-specific constituents include flavonoids, e.g. quercetin and kaempferol (see Table 21.5), coumarin and polyphenolic acids.

The leaf is characterized by the pharmacopoeial TLC test, which, with the genuine drug, gives a number of fluorescent zones when viewed in UV light at 365 nm. The swelling index, minimum 12, is higher than that for the root (10).

MARSHMALLOW ROOT

Marshmallow root is derived from *Althaea officinalis* (Malvaceae), a perennial herb which is found wild in moist situations in southern England and Europe. In general appearance it closely resembles the common hollyhock, *Althaea rosea*. The plant has a woody rootstock from which arise numerous roots up to 30 cm in length. The drug is chiefly collected on the Continent from cultivated plants at least 2 years old. The roots are dug up in the autumn, scraped free from cork and dried, either entire or after slicing. The *EP*/*BP* drug may be peeled or unpeeled, whole or sliced.

The drug occurs in whitish, fibrous pieces about 15–20 cm long and 1–2 cm in diameter, or in small transverse slices. Odour, slight; taste, sweetish and mucilaginous. A transverse section shows a bark about 1–2 mm thick which is separated by a greyish, sinuate cambium from the white, radiate wood. The section shows numerous mucilage cells, the contents of which are coloured a deep yellow by a solution of sodium hydroxide. Structures to be seen in the powdered drug include: variously thickened vessels; fibres; calcium oxalate as cluster crystals 20–25–30–35 μm in diameter; starch granules, mainly single, 3–25 μm in size; thin-walled cork cells.

Marshmallow root contains about 10% of mucilage, the amount being season-dependent; it contains a polysaccharide giving on hydrolysis galactose, rhamnose, galacturonic acid and glucuronic acid. Other components of the mucilage are glucans and an arabinan. The mucilage content of the drug is indicated by the swelling index which, for *BP* purposes should be not less than 10. The upper limit for the total ash of the peeled root is 6.0% and that for the unpeeled 8.0%. Starch, pectin and sugars, and about 2% of asparagine are also present. The latter, which is the amide of aspartic (amino-succinic) acid, is also found in asparagus, potatoes, liquorice, etc.

Marshmallow root, and also the leaves, are used as demulcents, particularly for irritable coughs and throat and gastric inflammation.

MULLEIN FLOWER

Mullein flower *BP*/*EP* is the dried flower, reduced to the corolla and androecium, of *Verbascum thapsus* L., *V. densiflorum* Bertol. (*V. thapsiforma Schrad*.) and/or *V. phlomoides L.* Family Scrophulariceae.

V. thapsus (great mullein, Aaron's rod, blanket leaf and a number of other synonyms) is common throughout Europe and has long been naturalized in the US from the Atlantic coast west to South Dakota and the south-western states. In general, it is found in waste places, roadsides and on sunny banks. Reaching a height of 2 m, it is a biennial

Table 20.8 Characters of *Psyllium* seeds.

	P. afra	*P. indica*	*P. ovata*
Origin	France, Spain, Cuba	Mediterranean Europe, Egypt	Pakistan, India
Colour	Glossy; deep brown	Dull; blackish-brown	Dull; pinkish grey–brown
Shape	Boat-shaped; outline elongated ovate	Boat-shaped; outline elliptical	Boat-shaped; outline ovate
Length	2.0–3.0 mm	2.0–3.0 mm	1.5–3.5 mm
Weight of 100 seeds	0.09–0.10 g	0.12–0.14 g	0.15–0.19 g
Swelling index	⩽10	⩽10	⩽9; husk, ⩽40

terminating in a dense cylindrical spike of yellow flowers. Oval to lanceolate shortly petiolate leaves alternate on the simple stem. The whole plant is blanketed with woolly hairs. *V. densiflorum* (large-flowered mullein), found throughout continental Europe but a rare casual in Britain, differs from the above species in having larger quite flat corollas and decurrent leaves. *V. phlomoides* (orange mullein) is native to central and southern Europe and West Asia. It is an occasional casual in Britain but is widely cultivated as an ornamental. The flowers are yellow to yellow–orange.

A microscopical examination of the yellow to brown or orange powder shows many branched clothing trichomes (see Fig. 42.3), yellow fragments of petals and numerous ovoid pollen grains with a finely pitted exine and three pores.

Constituents. The three species contain the same groups of chemical constituents but with varying proportions and nature of individual compounds. *V. densiflorum* and *V. phlomoides* have been the most intensively studied as these species are the most commonly used on continental Europe. Flavonoids include, among others, apigenin, luteolin, their 7-glucosides and verbascoside. Iridoids include aucubin, catapol and their 6-xylosides (Fig. 20.7). Polysaccharides constitute the important mucilage components of the flowers; a water-soluble acidic arabinogalactan, MW 70 000, has been isolated from commercial material. As a standard for the mucilage content of the drug the *BP* specifies a minimum swelling index of 9 determined on the powdered flowers. A number of saponins have been recorded, including verbascosaponin, the structure of which was finally elucidated in 1992. Other constituents include phenolic acids not specific to mullein.

The *Pharmacopoeia* includes a TLC comparison of an extract of the flowers against reference substances and a test for the presence of iridoids.

Uses. Traditionally for the treatment of bronchial conditions particularly bronchitis and catarrh in which the saponins and mucilage probably act as expectorants and demulcents.

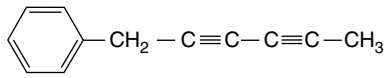
COUCH GRASS RHIZOME

Couch Grass Rhizome *BP/EP, BHP* consists of the washed and dried rhizomes of *Agropyron repens* (L.) Beauv. (*Elymus repens* (L.) Gould), family Gramineae, sliced or whole with most of the adventitious roots and leaves removed.

The plant, although an invasive and troublesome weed (twitch), has a long history of medicinal use; it was known to Dioscorides and has subsequently been included in many herbals and pharmacopoeias. In Britain it was last included in the *BP* 1914 but was, in 1999, returned as a result of its *EP* status. S.E. Europe (Hungary) supplies most of the commercial material.

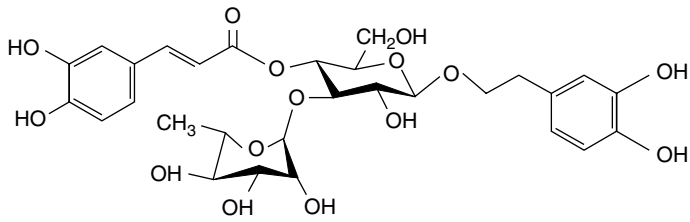
Characters. The dried, rigid rhizome is pale yellow, shiny, 2–3 mm thick and usually cut into pieces up to 6 mm in length. It is strongly furrowed longitudinally and, except at the nodes, hollow. The drug is odourless with a slightly sweet taste. Features of the powder include: epidermal cells of two types—one, narrow elongated with wavy and pitted, lignified walls—the second, alternating with the first type, somewhat rounded and unlignified; endodermal cells with U-shaped thickenings; fibres; pitted vessels with annular or spiral thickenings, pitted parenchyma. Calcium oxalate and starch are absent.

Constituents. The rhizomes contain about 10% mucilage and up to 8% of the fructosan polysaccharide triticin. Unlike the inulins (β, 2-1 linkages) the fructosans of the Gramineae, termed *levans*, are fructofuranose units linked by β,2-6 glycosidic linkages and, as with the inulins, terminated with a sucrose unit. Other constituents are 2–3% of sugar alcohols (mannitol, inositol), 0.01–0.05% volatile oil containing the polyacetylene agropyrene and other volatiles, vanillin and its monoglucoside, phenolic carboxylic acids and silicates.

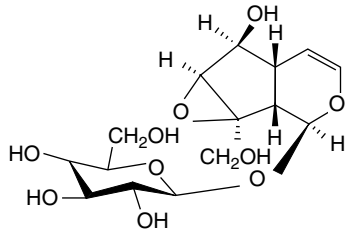


Agropyrene

Tests. The *BP/EP* specifies a water-soluble extractive of ⩽25%and foreign matter consisting of greyish-black pieces of rhizome ⩽15%. Tests, depending on the presence of starch and thickening of the endodermic cells are given to detect *Cynodon dactylon* (Bermuda grass), found on sandy soils in warmer temperate regions of the world including the shores of S.W. England, and *Imperata cylindrica*, a troublesome weed of subtropical regions.



Verbascoside
(Acteoside)



Catapol

Fig. 20.7
Constituents of mullein flowers.

Uses. Couchgrass is used as a demulcent and as a diuretic for bladder and kidney complaints, cystitis, gout, etc.

'Aloe vera' products

Aloe vera products are derived from the mucilage located in the parenchymatous cells of the *Aloe vera* leaf and should not be confused with aloes, described in Chapter 21, which originate from the aloetic juice of the pericyclic region. The mucilaginous gel has been used from early times for the treatment of numerous conditions but in recent years its use in the herbal and cosmetic industries has become very big business in the USA, Europe and elsewhere. Raw materials are obtained from plantations in the southern states of the USA, South America and elsewhere. Exaggerated claims have inevitably brought scepticism about the true usefulness of the products which feature as suntan lotions, tonics and food additives.

Research over the last 10 years has, however, largely upheld a number of the therapeutic properties ascribed to the gel. These include the anti-inflammatory properties (wound healing, burn healing, frostbite), gastrointestinal activity (peptic ulcer), antidiabetic activity, anticancer activity (principally animal tests), antifungal activity, antibacterial activity and radiobiological protection. Not all these properties have been unequivocally accepted.

The complex chemistry of the gel makes the attribution of the various activities to specific compounds difficult. Indeed beneficial clinical results for a particular condition may arise from more than one component. Thus wound-healing benefits may derive from the anti-inflammatory, fibroblast-stimulating, antibacterial and hydrophilic properties of the gel.

Some of the conflicting results of tests may be due, in some measure, to the different methods of collecting and subsequent processing of the gel. Thus anthraquinones have been reported as constituents of the gel but their presence may, or may not be, due to some admixture with the aloetic juice from the pericyclic region of the leaf. Also, variations in carbohydrate composition arise due to varietal differences within the species and to seasonal, climatic and soil factors.

Glucomannans constitute a principal component of the gel; some consist of glucose and mannose only or glucose, mannose and glucuronic acid, others are acetylated. Other polysaccharides are galactogalacturans (galactose + galacturonic acid) and galactoglucoarabinomannans. Molecular weights range from 200 000–450 000. An acetylated mannan is available commercially and has a range of reported biological activities. Glycoprotein fractions of the gel which may also influence the immune system have been shown to have proliferation-promoting activity on human and hamster cells *in vitro* (A. Yagi *et al*., *Planta Medica*, 1997, **63**, 18; 2000, **66**, 180). In a series of papers N. Okamura *et al*. (*Phytochemistry*, 1996, **43**, 495; 1997, **45**, 1511; 1998, **49**, 219) have reported on the isolation of some eleven new chromones. Pectic substances, the triterpenoid lupeol, plant sterols (cholesterol, campestrol, β-sitosterol), possible prostanoids and other organic and inorganic compounds have also been identified in the gel. Five phytosterols which, in animals, show long-term blood-glucose-level control, could be beneficial in the treatment of type 2 diabetes. These were identified as cycloartenol (Fig. 23.1), its 24-methylene derivate, lophenol and its 24-methyl and ethyl derivatives (M. Tanaka *et al.*, *Biol. Pharm. Bull.*, 2006, **29**, 1418).

The intense interest in aloe vera products both from the commercial and scientific viewpoints has made this a topic for very active research. For an extensive review update on the subject (over 300 refs) see T. Reynolds and A. C. Dweck, *J. Ethnopharmacology*, 1999, **68**, 3.

Gels from other species of *Aloe*, e.g. *A. arborescens, A. ferox*, have also been examined for their polysaccharide composition.

Quince seeds

Quince seeds are obtained from *Cydonia oblonga* Mill. (Rosaceae), a tree cultivated in South Africa, Central Europe and the Middle East. Iran supplies about 75% of the total world production.

The seeds are separated from the apple- or pear-shaped fruits and dried. They resemble apple pips and frequently adhere together in masses, owing to their surface coating of dried mucilage. The latter is derived from the outer epidermis of the testa. Quince seeds contain about 20% of mucilage, composed of units of arabinose, xylose and uronic acid derivatives, 15% of fixed oil and a small quantity of cyanogenetic glycoside and an enzyme which effects its hydrolysis. The seeds are used as a demulcent, as an emulsifying agent and in the preparation of hair-fixing lotions.

Slippery elm bark

Slippery elm bark is obtained from *Ulmus rubra* Muhlenberg (*Ulmus fulva* Michaux) (Ulmaceae), a tree 15–20 m in height which is widely distributed in the USA and in Canada. In the spring fairly old bark is stripped from the trees. The outer part of the bark is then removed, only the inner part, which forms the commercial drug, being dried. After this has been sawn into convenient lengths, it is bound into bundles with wire.

The drug occurs in broad, flat strips about 50–100 cm in length and from 1 to 4 mm in thickness. A few reddish-brown patches of the imperfectly removed rhytidome may be seen but the remainder consists only of secondary phloem. The outer surface is brownish-yellow and striated, the inner surface yellowish-white and finely ridged. The bark is easily identified by the characteristic, fenugreek-like odour, the strongly fibrous fracture and the fact that it yields mucilage when moistened.

The chief constituent of the bark is mucilage, which is a mixture of two or more polyuronides. The mucilage has demulcent, emollient and nutritive properties. A poultice of the powdered bark is sometimes used.

Other mucilage-containing medicinal plants

Coltsfoot *BHP* 1983 consists of the dried leaves of *Tussilago farfara*, Compositae. It is used (and also the flowers) as a herbal expectorant and contains up to 10% mucilage which on hydrolysis yields a number of sugars and uronic acids. The leaves also contain tannin and a small percentage of pyrrolizidine alkaloids, e.g. senkirkine, which can prove hepatotoxic in sufficient doses. Probably for this reason the herb is no longer included in recent editions of the *BHP*; the German Commission E sets limits for the daily dose of these compounds, as occurring in coltsfoot. Further information on the drug will be found in a review by M. Berry (*Pharm. J*., 1996, **256**, 234).

For other drugs containing appreciable mucilage see fenugreek seeds and linseed.

MISCELLANEOUS CARBOHYDRATE-CONTAINING DRUGS

HONEY

Honey is a saccharine substance deposited by the hive bee, *Apis mellifera* (order Hymenoptera, Apidae), and other species of *Apis*, in the cells of the honeycomb. Honey is produced in England, but the chief sources of supply are the West Indies, California, Chile, various parts of Africa, Australia and New Zealand.

Collection and preparation. The worker bees, by means of a long, hollow tube formed from the maxillae and labium, take nectar from the flowers they visit and pass it through the oesophagus into the honey-

sac or crop. The nectar, which consists largely of sucrose, is mixed with salivary secretion containing the enzyme invertase and while in the honey-sac is hydrolysed into invert sugar. On arrival at the hive the bee brings back the contents of the honey-sac and deposits them in a previously prepared cell of the honeycomb.

The best honey is that derived from flowers such as clover and heather, obtained from hives that have never swarmed, and separated from the cut comb either by draining or by means of a centrifuge. Honey obtained by expression is liable to be contaminated with the wax. The nectar of certain flowers (e.g. of species of *Eucalyptus* or *Banksia*) may give the honey a somewhat unpleasant odour and taste; nectar from *Datura stramonium*, ragworts and *Rhododendron* spp. are known to give poisonous honey.

Appropriate cultivation measures should be in force to prevent the build-up of undue levels of pesticide and herbicide residues in the honey.

Preparation of the honey may involve melting at a moderate temperature, skimming off any impurities that collect on standing and, if necessary, adjusting the water content using refractive index measurement.

Characters. Honey, when freshly prepared, is a clear, syrupy liquid of a pale yellow or reddish-brown colour. On keeping, it tends to crystallize and become opaque and granular. The odour and taste depend very largely on the flowers used in its preparation.

Constituents and tests. Honey consists mainly of invert sugar and water. It contains small quantities of sucrose, dextrin, formic acid, volatile oil, wax and pollen grains. Microscopical examination of the latter afford valuable evidence of the source. The most likely adulterants are artificial invert sugar, sucrose and commercial liquid glucose. The tests for purity of the *BP*/*EP* purified honey should be noted. The limit tests for chloride and sulphate are important, as starch and sucrose may be hydrolysed with acids to give commercial liquid glucose and artificial invert sugar, respectively. Artificial invert sugar contains furfural, which gives a red colour with resorcinol in hydrochloric acid, but this may be formed in genuine honey by prolonged heating or lengthy storage.

The pharmacopoeia limits 5-hydroxymethylfurfural to a maximum of 80 ppm, calculated on dry solids, determined by absorbance measurements at 284 μm; the TLC test identifies glucose and fructose and eliminates excess sucrose. Water content is limited to 20% determined by refractive index measurements (minimum value 1.487). Conductivity (maximum 800 μS. cm^{-1}) and optical rotation (maximum +0.6°) are further standards.

Uses. Honey is chiefly used in pharmacy as a component of linctuses and cough mixtures and for Oxymel and Squill Oxymel.

FIGS

Fig *BP* is the sun-dried succulent fruits of *Ficus carica* L. (Moraceae). It is widely produced in the Mediterranean countries, particularly Turkey (Smyrna figs), Greece and Spain.

The fruit is produced by the union of the cymose inflorescence to form a hollow, fleshy axis bearing the flowers on its inner surface. The young fruit is rich in latex, but when mature, no latex is found and the fleshy axis contains much sugar. Figs contain about 50% of sugars (chiefly glucose), appreciable quantities of vitamins A and C, smaller amounts of vitamins B and D, and enzymes (protease, lipase and diastase). There is an official requirement for a water-soluble extractive of not less than 60.0%. Figs are used by the *BP* and *BPC* in laxative preparations (e.g. Compound Fig Elixir).

For a review of *Ficus* spp. covering the ethnobotany and potential as anticancer and anti-inflammatory agents see E. P. Lansky *et al*., *J. Ethnobotany*, 2008, **119**, 195.

FUCUS

Fucus, or bladderwrack, consists of the dried thallus of *Fucus vesiculosus* L., *F. serratus* L. or *Ascophyllum nodosum* Le Jolis., family Fucaceae. The *BP*/*EP*, under the title Kelp, admits all three species, whereas the *BHP* (1990) monograph is restricted to *F. vesiculosus*. Although the name 'kelp' is often used in connection with *Fucus* spp. it more strictly applies to the larger brown seaweeds of the genera *Laminaria* and *Macrocystis* family, Laminariaceae.

The dried grey to black thallus, sometimes having a whitish coating, is hard and brittle but readily softens in water to become mucilaginous. Depending on the species, air vesicles may, or may not be present.

Bladderwrack contains mucilaginous polysaccharides such as alginic acid, for the extraction of which it may be used (q.v.). It is also rich in trace elements and iodine. The latter is in the form of inorganic salts, bound to protein, and as a constituent of iodo-amino acids such as di-iodotyrosine. Other constituents are polyphenols consisting of phloroglucinol units, sterols and complex lipids.

Note the official limit tests for heavy metals and arsenic, the high total ash value (⩾24.0%) (mainly soluble in dilute hydrochloric acid) and loss on drying (⩾15.0%). Other standards are the swelling index (⩽6.0) and total iodine content (0.03–0.2%) (sodium thiosulphate titration).

Bladderwrack has thyroactive properties and has, in the past, been employed in iodine therapy; however other preparations with a more consistent iodine content are now preferred. Again arising from its iodine content, bladderwrack has been promoted in teas as a slimming agent. In suitable doses it serves as a dietary supplement for trace elements and as a bulk laxative.

CETRARIA

Cetraria or Iceland moss, *Cetraria islandica* (L.) Acharius s.l. (Parmeliaceae), is a foliaceous lichen growing amidst moss and grass in central Europe, Siberia and North America, and on the lower mountain slopes of central Europe and Spain. For medicinal purposes it is usually collected in Scandinavia and central Europe.

The *BP*/*EP* drug consists of irregularly lobed, leafy thalli, about 5–10 cm long and about 0.5 mm thick. The upper surface is greenish-brown and sometimes covered with reddish points, while the lower surface is pale brown or greyish-green and marked with white, irregular spots. At frequent intervals along the margin of the thallus are minute projections. The dried drug is brittle but becomes cartilaginous on moistening with water. Odour, slight; taste, mucilaginous and bitter. A 5% decoction forms a jelly on cooling, which is stained blue by iodine (distinction from carrageen). It has an official swelling value of ⩽4.5 determined on the powdered drug. Sections of the drug reveal the dual nature of the plant, the small rounded cells of the unicellular alga *Cystococcus humicola* being enclosed by the more or less closely woven hyphae of the fungus.

The drug contains carbohydrates, known as lichenin and isolichenin. Lichenin is only soluble in hot water and is not coloured blue by iodine, while isolichenin is soluble in cold water and gives a blue colour with iodine. Both on hydrolysis give glucose. Cetraria also contains a very bitter depsidone, cetraric acid, and other acids such as lichestearic and usnic acids. The latter compound is an antibiotic which may not be unimportant in contributing to the efficacy of Iceland moss as a demulcent for the treatment of cough involving throat irritation. Iceland moss has been used as a bitter tonic and for disguising the taste of nauseous medicines.

Prunes

Prunes (Rosaceae) are dried plums derived from *Prunus domestica* L. Of the many cultivated forms, var. *Juliana de Candolle*, which is largely grown in France, produces those commonly used in European medicine. Prunes of excellent quality are also produced in California.

The appearance of prunes requires no description. The pulp contains about 44% of sugars (mainly glucose), malic acid and water. The kernel contains 45% of fixed oil, and small quantities of amygdalin and benzoic acid. Prunes are used in Confection of Senna.

Further reading

Weymouth-Wilson AC 1997 The role of carbohydrates in biologically active natural products. Natural Product Reports 14(2): 99–110

Whistler RL, BeMiller JN (eds) 1993 Industrial gums; polysaccharides and their derivatives, 3rd edn. Academic Press, London, UK

Whistler RL, BeMiller JN 1997 Carbohydrate chemistry for food scientists. Eagan Press, St Paul, Minnesota